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BRAIN-DERIVED NEUROTROPHIC FACTOR (BDNF) AND THE DENTATE GYRUS MOSSY FIBERS: IMPLICATIONS FOR EPILEPSY

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1. INTRODUCTION

In the last decade, one of the most avidly studied compounds in the central nervous system (CNS) has been the neurotrophin BDNF. Although historically it had been studied in the context of development, where it plays numerous critical roles, more recent studies have shown striking actions on a number of pathways and processes that are critical for normal function of the *adult* CNS. In addition, studies of neurological and psychiatric diseases indicate a potential role for BDNF in pathology.

Our interest in BDNF began with the demonstration that one of the regions where BDNF protein expression is among the highest in the adult rat brain is the dentate gyrus (Conner *et al.*, 1997; Yan *et al.*, 1997). Previous studies had not clearly defined where BDNF protein was expressed, although mRNA was definitely in hippocampal neurons, and many other adult brain regions. One of the interesting aspects of BDNF protein expression in the dentate gyrus that came to light after the development of specific antibodies was that its location was extremely specific. Thus, in the normal adult rat, hippocampal BDNF protein expression is much greater in the axons of the dentate granule cells, the mossy fibers, than anywhere else in hippocampus. This led us to study mossy fiber BDNF specifically, and the results showed that mossy fiber BDNF has potent functional effects. These studies, and their implications for epilepsy, are reviewed below.

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2. FUNDAMENTAL STUDIES OF BDNF IN THE CNS

Historically, BDNF has been studied as one of the critical mediators of CNS development. This is no surprise given it is one of the family of neurotrophins, which include nerve growth factor (NGF), BDNF, as well as other potent neurotrophic molecules such as neurotrophin-3 (NT-3) and neurotrophin-4/5 (NT-4/5; Figure 1). All neurotrophins have potent actions at tropomyosin receptor kinases (trk), as shown in Figure 1. BDNF binds with high specificity to trkB, which is also a ligand for NT-4/5, and possibly NT-3 (Figure 1). Neurotrophins also bind to what was originally termed the low affinity neurotrophin receptor (LNTR), now referred to as p75. Both trkB and p75 are coupled to a complex array of signal transduction pathways and mediate numerous actions in different systems (Bibel and Barde, 2000; Vicario-Abejon *et al.*, 2002; Chao, 2003). More recently, it has become clear that proneurotrophins may be functionally active, as well as the mature peptide (Brake *et al.*, 2001).

Our interest in this field began with the studies of (Lohof *et al.*, 1993)**,** who showed that BDNF exposure to xenopus cultures could increase synaptic transmission. Subsequently it became clear that this was a principle that could be generalized well beyond xenopus. Relevance to the hippocampus became evident when it was shown that exposure of adult rat hippocampal slices to recombinant BDNF led to a long lasting potentiation of synaptic transmission in area CA1 (Kang and Schuman, 1995). Further studies at a similar time also showed that BDNF contributes to a long-lasting potentiation, similar to long-term potentiation (Patterson *et al.*, 1996; Korte *et al.*, 1996), and it is now well accepted that BDNF has an important role in synaptic plasticity in area CA1.

Our interest to enter this field arose from the demonstration that BDNF protein is expressed much more strongly in the axons of granule cells, the mossy fibers, than in area CA1. Although it is not always the case that brain regions with relatively greater amounts of protein are associated with enhanced function, this relative difference piqued our interest in the potential functional effects of BDNF in the dentate gyrus and CA3 regions. An additional reason for this interest was the known role of the mossy fibers in the hippocampal trisynaptic circuit. The trisynaptic circuit refers to a sequence of glutamatergic synapses that mediates much of the signal processing from principal cells of the entorhinal cortex to the granule cells, pyramidal cells, and back to cortex. As a key

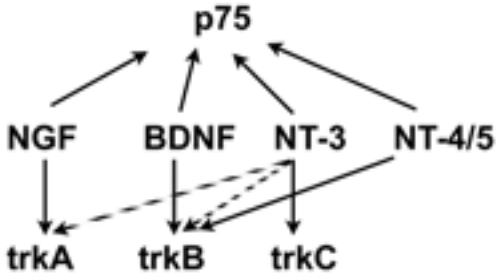

Figure 1. The neurotrophin family and its receptors. Nerve growth factor, NGF; Brain-derived neurotrophic factor, BDNF; Neurotrophin-3, NT-3; Neurotrophin-4/5, NT-4/5.

intermediary synapse in this circuit, i.e., the synapse from granule cells to CA3 pyramidal cells, it is likely that the relative strength of mossy fiber transmission plays an important role in information processing between cortex and hippocampus. Indeed, there are several control points for the trisynaptic circuit, and although much attention has focused on perforant path - to - granule cell synapse as a primary "gate" for entry into hippocampus, the mossy fibers may have an accessory gate function. One reason for this is that recent studies of the mossy fibers have illustrated that these axons primarily inhibit CA3 neurons because they activate GABAergic neurons so well, and the GABAergic neurons innervate the CA3 pyramidal cells (Acsady *et al.,* 1998**).** Yet the underlying excitation by glutamate released from mossy fibers directly onto pyramidal cells, which GABAergic inhibition normally masks, is extremely powerful; furthermore, it facilitates more strongly than many other hippocampal synapses (Scharfman *et al.*, 1990; von Kitzing *et al.*, 1994). It therefore is likely that normal inhibition of mossy fiber glutamatergic transmission plays a critical role in the control of information flow through the hippocampus.

3. EFFECTS OF BDNF ON MOSSY FIBER TRANSMISSION IN NORMAL ADULT RATS

Our first study was simply to examine the mossy fiber synapses and determine if exogenous BDNF application in adult rat hippocampal slices could influence this pathway. We also questioned whether BDNF might influence other aspects of granule cell function, perhaps by having a retrograde effect, given that there are many examples in the literature of retrograde effects of neurotrophins.

We found striking effects of BDNF on mossy fiber transmission, similar to the studies of others in area CA1. Thus, exposure to 50-250 ng/ml recombinant BDNF (generously provided by Regeneron Pharmaceuticals) for over 30 min led to a potentiation of the extracellularly-recorded response of pyramidal cells to an electrical stimulus to the mossy fibers, i.e. a potentiation of the amplitude of the population spike (Figure 2). Importantly, the fiber volley (an index of mossy fiber action potentials) was unchanged, indicating non-specific excitatory effects could not explain the result. In addition, the effect maximized at approximately 60 min and outlasted BDNF exposure. In fact, we could not reverse the potentiation, even if drug-free buffer was continuously applied for several hours after BDNF had been removed. These robust effects were supported by control experiments showing that there was no effect of vehicle (bovine serum albumin, diluted to the same concentration as was used for BDNF application), or cytochrome C, which has similar physical chemistry as BDNF but does not bind trk receptors. Similarly, there was no effect of heat-inactivated BDNF (Scharfman, 1997). The trk antagonist K252a could block the effect, but the NMDA receptor antagonist D-APV did not. The latter was consistent with the known characteristics of normal mossy fiber transmission, and mossy fiber LTP, which have a very small NMDA receptor-mediated component.

Interestingly, other inputs to the CA3 neurons were not influenced by exposure to BDNF. Thus, stimuli to the fimbria or Schaffer collaterals evoked responses that were unchanged in the same slices with potentiated mossy fiber responses. This was important because it suggested that in fact we were able to stimulate mossy fibers selectively by electrical activation of the deep hilus, which is not trivial. Taken together, these studies

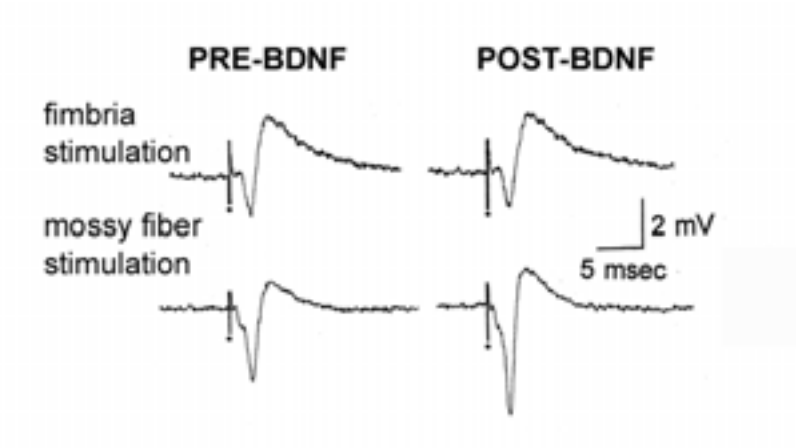

Figure 2. Selective potentiation of mossy fiber-evoked, but not fimbria-evoked, responses of CA3 neurons by BDNF. Simultaneous extracellular recordings in the CA3 cell layer to single stimuli applied to the fimbria or mossy fibers, before and after exposure to BDNF in a slice from an adult male rat (Scharfman, 1997).

suggested that BDNF exposure could lead to a long-lasting and specific potentiation of mossy fiber transmission, a homosynaptic mossy fiber - "LTP - like" effect. The fact that other inputs appeared unaffected suggested a presynaptic mechanism, or at least a mechanism highly restricted to the mossy fiber synapses. Given the large literature that has now accumulated to suggest that BDNF has presynaptic effects (Tyler *et al.*, 2002), and that trkB receptors have been shown on mossy fiber boutons (Drake *et al.*, 1999), we currently favor the hypothesis that BDNF's actions are presynaptic. We hypothesize that BDNF binds to presynaptic trkB receptors, leading to a change in release mechanisms. Indeed, there are data to suggest that BDNF acts to phosphorylate proteins critical to transmitter release (Jovanic *et al.*, 1996; Tartaglia *et al.*, 2001)**.** However, postsynaptic effects of BDNF have been documented in several systems (Black, 1999; Kovalchuk *et al.*, 2002; Tongiorgi *et al.*, 2004), and trkB receptors have been identified postsynaptically in CA3 (Drake *et al.*, 1999). Potential actions on GABAergic neurons and glia also can not be overlooked (Schinder *et al.*, 2000; Rose *et al.*, 2003). Therefore, we continued to examine effects of BDNF in the dentate gyrus and CA3 and indeed are still doing so. Some of these experiments, particularly those conducted towards defining the pre- vs. postsynaptic locus of BDNF's effects, are described below.

It is important to raise the point that the majority of slices, but not all slices, demonstrated effects of exogenous BDNF. This has been an issue that has been noticed by our colleagues also. In fact, some laboratories have found that BDNF exposure to area CA1 does not potentiate synaptic transmission. In our own work, we found no effects of BDNF obtained from some manufacturers, or if BDNF had been frozen and then thawed. Other factors may also contribute to variability, such as the fact that some slices might not preserve all constituents of the signaling cascade initiated by trkB. There is also a significant difficulty in perfusion of tissue by BDNF because it is a large molecule that does not readily diffuse through neuropil. Depending on the preparation, differential access of BDNF to its receptors may lead to variable effects. Another possibility was raised recently by a study which showed that BDNF levels can be

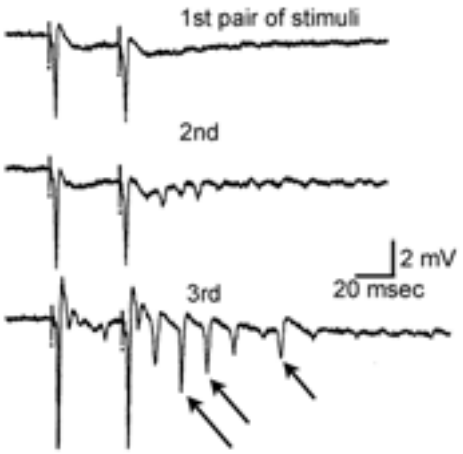

Figure 3. BDNF exposure leads to hyperexcitability in area CA3 upon mossy fiber stimulation. Extracellular recordings from a slice of an adult male rat demonstrate multiple population spikes (defined here as hyperexcitability; arrows) in response to 3 pairs of 1 Hz stimuli to the mossy fibers in the presence of BDNF are shown (Scharfman, 1997).

increased simply by slicing (Danzer *et al.*, 2004); if so, effects of exogenous BDNF thereafter might be occluded, or at the very least altered.

Besides examination of BDNF's influence on responses to single stimuli to the mossy fibers, we tested the effects of 1 Hz stimuli. The paradigm we developed was paired stimuli (40 msec apart, using 50% of the maximum population spike amplitude) at 1 Hz for 5-10 sec (total of 10-20 stimuli). Surprisingly, even after as few as 2-3 pairs of stimuli, BDNF-exposed slices demonstrated multiple population spikes (Figure 3). If stimulation continued at 1 Hz for the full 10 sec period, spreading depression often occurred. Yet even 20 pairs of stimuli at maximum intensity using other inputs to CA3, in the same slices, could not elicit multiple population spikes or spreading depression (Scharfman, 1997). This striking effect of 1 Hz paired stimuli has continued to be useful as a benchmark of BDNF's effects across numerous experimental preparations (see below). It appears to be unique among compounds that induce hyperexcitability, because no other convulsant we have tested has had the same type of effect. For example, we have tried to use very low (0.1 up to 10 μM) concentrations of the $GABA_A$ receptor antagonist bicuculline methiodide, because it is well known that $GABA_A$ receptor antagonism leads to multiple population spikes in response to stimulation in hippocampus. However, bicuculline never mimicked the effects of BDNF at any time during its administration or at the time it reached its final concentration. The effect of low doses of bicuculline (also observed in the initial minutes after adding a higher dose) was to elicit 2-3 population spikes per single stimulus. These occurred immediately after the stimulus, and were independent of stimulus site. If concentration was raised, spontaneous bursts developed. In contrast, BDNF exposure did not lead to multiple population spikes in response to one stimulus. Instead, the paired 1 Hz paradigm was required, and in that case the population spikes did not immediately follow the stimulus.

Instead, slower, longer duration trains of population spikes were evoked. Furthermore, they only were evoked in response to mossy fiber stimuli, and spontaneous bursts never developed. Thus, although hyperexcitability can be invoked by a number of convulsants using the slice preparation, or a number of manipulations of the perfusing buffer, BDNF appears to induce a qualitatively unique pattern of hyperexcitability.

The fact that hyperexcitability was present in area CA3 after exposure to BDNF was surprising to us, because it had been widely considered that BDNF was neuroprotective, critical to normal development, and important to learning and memory (Cirulli *et al.*, 2004; Koponen *et al.*, 2004; Monteggia *et al.*, 2004). However, upon further examination, there have been studies of CA3 neuronal damage after seizures that indicate that BDNF is exactly the opposite, i.e., it can exacerbate damage (Rudge *et al.*, 1998; Lahteinen *et al.*, 2003). It may be that a low level of BDNF is necessary and important to normal function, but excess BDNF (as would occur after administration of exogenous BDNF, or manipulations that induce its expression) leads to precisely the opposite, that BDNF in excess might not necessarily a "good thing" (Binder *et al.*, 2001). Indeed, BDNF overexpressing mice had cognitive deficits and deficits in LTP, as well as increased susceptibility to seizures (Croll *et al.*, 1999). Indeed, others have identified that BDNF has actions that were consistent with a pro-epileptogenic effect (Binder *et al.*, 1999), although others have not always concluded the same (Larmet *et al.*, 1995; Osehobo *et al.*, 1999). Whether or not BDNF is proconvulsant may be dependent on how BDNF is manipulated and the ultimate concentration, as well as other factors, such as compensatory changes. Indeed, prolonged BDNF exposure may downregulate trkB receptors (Xu *et al.*, 2004) and increase the expression of neuropeptide Y, which is anticonvulsant (Reibel *et al.*, 2000). In summary, it may be that hippocampal BDNF is critical to development, and the maintenance of dendritic and synaptic plasticity in the normal adult under most conditions. However, under abnormal conditions, it may be maladaptive for the hippocampus to have such a high concentration and contribute to hippocampal seizures susceptibility (see Figure 8).

To fully examine where BDNF might have effects in the dentate gyrus, we expanded our tests of synaptic and nonsynaptic function outside CA3. Thus, we examined antidromic transmission to granule cells. However, no effects on the antidromic population spike were ever detected, suggesting that axon conduction was an unlikely target. In addition, we tested orthodromic inputs to granule cells. Interestingly, extracellularly-evoked responses to stimulation of the outer molecular layer, to activate the perforant path input to the dentate granule cells, were unaffected by exogenous BDNF, using the same concentrations that had a robust influence on mossy fiber transmission (Scharfman, 1997). Furthermore, paired pulse inhibition of the population spike was unaffected, suggesting that BDNF did not act by depression of inhibition (Scharfman, 1997). However, other studies *in vivo* (Messaoudi *et al.*, 1998) subsequently identified that BDNF can potentiate the perforant path input. Furthermore, studies of granule cell inhibition in heterozygous knockouts suggests a role of BDNF at dentate gyrus inhibitory synapses (Olofsdotter *et al.*, 2000). This is unlikely to be due to alterations in GABAergic inhibition (due to developmental deficiency in the knockouts), because a scavenger of BDNF had similar effects. Others have identified that BDNF can depress IPSPs in area CA1 (Tanaka *et al.*, 1997; Frerking *et al.*, 1998), although the mechanism in the dentate gyrus appeared to be presynaptic, and in CA1 this was not necessarily the case. Additional effects of BDNF have also been reported that further complicate analysis of its actions: there appears to be a very rapid effect of BDNF to

depolarize neurons by an action on a specific subtype of sodium channel, Nav 1.9 (Kafitz *et al.*, 1999; Blum *et al.*, 2002).

These differences in effects of BDNF could be due to the different methods that were used, different sources/concentrations of BDNF (possibly due to contaminants of proneurotrophins, binding proteins, etc.), and also that some synapses/regions could simply be influenced differentially. They raise the important point that BDNF's effects may be highly sensitive to the preparation of the neurotrophin, experimental setting, and conditions of an experiment. They also could be explained by the fact that many synapses can be influenced by BDNF, but mossy fiber transmission might simply be one of the most important given protein expression there is highest. This leads naturally to a key question: are the robust effects on mossy fiber transmission reflective of physiological actions of BDNF? What is the physiological effect, in contrast to the pharmacological effect, of BDNF? In other words, what is the effect of endogenous BDNF?

4. BDNF OVEREXPRESSING MICE

To address this issue, we chose to take an approach that would not rely on exogenous application of recombinant BDNF at all, but to find a way to probe actions of BDNF that might occur *in vivo*. We were fortunate to have the opportunity to work with a transgenic mouse line that overexpressed BDNF, provided by Regeneron Pharmaceuticals. This mouse overexpresses BDNF by approximately 30%, and overexpression occurs in the areas of the CNS where the β-actin promoter is present (Croll *et al.*, 1999). Thus, in a blinded study, we examined mossy fiber transmission in slices of transgenics and wild type controls. Exogenous BDNF was not added at all. Our hypothesis was that, without the addition of BDNF, we would be able to examine any endogenous effects of BDNF simply by comparison to the wild type. A critical assumption was that transgenic overexpression would not lead to abnormal effects that would be confounding, but reflect effects of endogenous BDNF. Therefore, an important first step was an analysis of gross morphology and hippocampal structure, and no apparent abnormalities were present in the animals. Indeed, the animals appeared normal, were viable, and were fertile, although there of course could have been fine changes that more specific markers could have potentially detected.

We found that normal mossy fiber transmission was robust in both the transgenic and wild type mouse in response to a single stimulus. Although a comparison of population spike amplitude evoked by selective mossy fiber stimulation is difficult across slices, extensive differences in the amplitude of the population spike that could be evoked using standardized electrode locations were not noticed. However, there was a large difference when we used the paradigm described above that involved repeated stimuli. In the transgenics, we found an abnormality of mossy fiber transmission that was striking in its similarity to the rat slices that were exposed to exogenous BDNF: multiple population spikes and spreading depression could be evoked by pairs of mossy fiber stimuli at 1 Hz (Figure 4). This was not observed in response to other inputs to CA3, and no differences were detected in dentate gyrus recordings of the response to perforant path input, all reminiscent of the effects of exogenous BDNF in normal adult male rat slices.

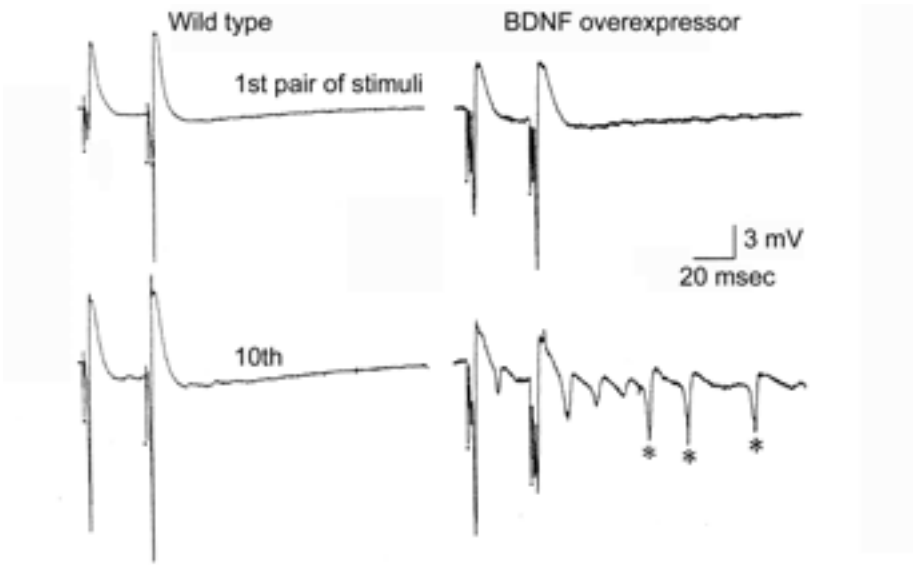

Figure 4. Hyperexcitability after mossy fiber stimuli in BDNF overexpressing mice. Extracellular recordings from the area CA3 cell layer in response to mossy fiber stimuli (paired stimuli at 1 Hz) are shown for a slice from an adult male wild type mouse and a slice from an adult male mouse that overexpressed BDNF. Asterisks mark abnormal population spikes after the 10th pair of stimuli in the slice from the transgenic mouse (Croll et al., 1999).

Interestingly, entorhinal cortex also appeared hyperexcitable in slices of transgenic mice (Croll *et al.*, 1999), as it did in slices of normal male rats exposed to BDNF (Scharfman, 1997). The effects in entorhinal cortex may actually be more robust, in a way, because multiple field potentials could be evoked even by single stimuli (Scharfman, 1997; Croll *et al.*, 1999). This is intriguing given that these areas (CA3 and the entorhinal cortex) are those that exhibit vulnerability in animal models of epilepsy and human temporal lobe epilepsy (discussed further below). It is also intriguing because there are numerous studies which have demonstrated that seizures increase BDNF (Gall, 1993) and in patients with temporal lobe epilepsy there is an elevation in BDNF (Mathern *et al.*, 1997; Murray *et al.*, 2000). Thus, BDNF may initiate a positive feedback loop that leads to its upregulation and further ability to potentiate synaptic transmission (Binder *et al.*, 2001). This could be a substrate for epileptogenesis, and a novel one at that, given that prevailing hypotheses have only just begun to consider BDNF in epileptogenesis (Lahteinen *et al.*, 2002; He *et al.*, 2004; Tongiorgi *et al.*, 2004).

5. EFFECTS OF BDNF IN AN ANIMAL MODEL OF EPILSPY WITH MOSSY FIBER SPROUTING

As mentioned above, we have continued to pursue how BDNF acts in CA3 and one of the key issues is whether it acts on mossy fiber boutons. Also mentioned above, was

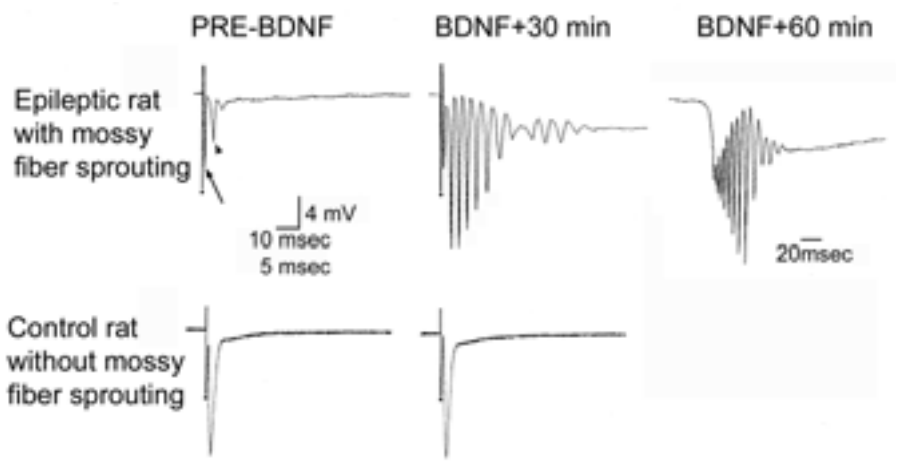

Figure 5. Effects of BDNF in an animal with chronic seizures (i.e., epileptic) and mossy fiber sprouting. Stimulation of the hilus to activate mossy fibers in an epileptic rat with mossy fiber sprouting (Top) evoked an antidromic population spike (arrow) followed by an orthodromic population spike (arrowhead). After BDNF was added to the buffer, the same stimulus evoked multiple population spikes (Top, center) and subsequently spontaneous population spike occur in bursts (Top, right). In contrast, only an antidromic spike was evoked by hilar stimulation in a slice from a control rat without sprouting (Bottom), even after supramaximal stimuli, and even after prolonged exposure to BDNF. No spontaneous activity occurred. Calibration for recordings from epileptic tissue, 10 msec; for control recordings, 5 msec (Scharfman *et al*., 1999).

our interest in the possible relevance of mossy fiber BDNF to epileptiform activity. To address these "two birds" with one stone, so to speak, we chose to examine effects of BDNF after an experimental manipulation that induces mossy fibers to form synapses in a new location, the inner molecular layer. We reasoned that if there was an effect of BDNF that was specific to the mossy fiber boutons, it would be present in the new location. If it were not, we would not be able to conclude much, but if it were, we would have a compelling reason to associate BDNF actions with mossy fiber boutons. The reason why this approach harkened the adage about killing two birds with one stone was due to the fact that the experimental manipulation was one that led to an epileptic state. Thus, the same approach would allow us to potentially test the hypothesis that in the epileptic brain, BDNF may trigger seizures.

The experimental manipulation that we used is one with a long history: the muscarinic agonist pilocarpine is administered at a dose that leads to status epilepticus (continuous severe seizures; Turski *et al.*, 1983). Our laboratory uses the anticonvulsant diazepam after 1 hr of status to truncate status (Scharfman *et al.*, 2000, 2001, 2002b), because if left undisturbed, status may continue for many hours and animals have severe hippocampal damage. However, if truncated, there is much less damage in the dentate gyrus and CA3 region. In fact, many of the hilar mossy cells, considered to be one of the most vulnerable cell types to seizure-induced neuronal damage, survive in our animals (Scharfman *et al.*, 2001). Nevertheless, there is some hilar cell loss, and these animals do demonstrate two consequences of status epilepticus that are associated with seizure-induced neuronal damage: 1) chronic spontaneous seizures develop and last the lifetime

of the rat (i.e., it develops epilepsy), and 2) the mossy fibers develop new collaterals that innervate an abnormal target region, the inner molecular layer (a phenomenon dubbed "mossy fiber sprouting"). The new mossy fibers in the inner molecular layer form synapses on both granule cells (Wenzel *et al.*, 2000; Buckmaster *et al.*, 2002) and interneurons (Sloviter, 1992; Kotti *et al.*, 1997). Synapses on granule cells are excitatory, but appear to have low probability of release (Scharfman *et al.*, 2003; Nadler, 2003). The net effect of mossy fiber sprouting on excitability is still a matter of controversy.

Using animals that had pilocarpine-induced status and subsequent repetitive spontaneous seizures, we made hippocampal slices and asked whether BDNF would 1) influence transmission from mossy fibers to granule cells, and 2) trigger spontaneous epileptiform events reflective of seizures (slices of course do not have seizures, but spontaneous epileptiform discharges recorded from slices, particularly long-lasting discharges, are often used to predict conditions that might relate to seizure activity *in vivo*). Indeed, this is what we found (Figure 5; Scharfman *et al.*, 1999). In animals with sprouting, hilar stimulation evoked an antidromic population spike in the granule cell layer that was followed by a second, orthodromic population spike (Figure 5). The second population spike was absent in animals without sprouting, or animals that had pilocarpine administered but failed to have status epilepticus (Figure 5). Presumably this second population spike reflects activation of recurrent collaterals on granule cells (Tauck and Nadler, 1985; Lynch and Sutula, 2000; Nadler, 2003). After BDNF exposure, the same stimulus that elicited one antidromic and one orthodromic population spike evoked an antidromic population spike followed by multiple, large amplitude, orthodromic population spikes (Figure 5). Indeed, after approximately 60 minutes, spontaneous bursts of population spikes were recorded from the granule cell layer. Intracellular recordings showed that these bursts reflected discharges of granule cells (Scharfman *et al.*, 1999), which was important to verify because volume transmission could theoretically have made it possible to record pyramidal cell discharges from the granule cell layer (Scharfman and Schwartzkroin, 1990). Other recordings indicated that the EPSPs underlying these bursts were generated from the inner molecular layer (Scharfman *et al.*, 1999). In extreme ventral slices, burst discharges were long lasting and particularly striking in the ventral (inferior) blade, where there appears to be greater sprouting and more BDNF (Scharfman *et al.*, 2002b).

6. EFFECTS OF BDNF INFUSION INTO THE DENTATE GYRUS: SEIZURES

These studies suggested that BDNF might be proconvulsant, particularly in the epileptic brain, but possibly in the normal brain as well. Indeed, sporadic spontaneous seizures had been witnessed in a few of the transgenic mice overexpressing BDNF. Perhaps more BDNF, applied directly to the mossy fiber area, would demonstrate more robust seizures. To test that hypothesis, we infused BDNF directly into the hilus using osmotic pumps. Interestingly, this did induce limbic seizures in approximately 1/3 of animals, witnessed by blinded investigators (Scharfman *et al.*, 2002a). They were not detected in animals infused with another large protein, albumin, at a similar concentration and infusion rate (Scharfman *et al.*, 2002a). We think that more BDNF-infused animals actually had seizures than the 1/3 that were witnessed, because animals were not examined 24 hr/day, so seizures could have been missed. In addition, we perfused the animals that had not been observed to have a seizure, and the hippocampi demonstrated

pathology similar to a hippocampus with chronic spontaneous seizures (Scharfman *et al.*, 2002a). However, control animals did not exhibit abnormalities. One tool used to examine whether animals had a history of seizures was increased mossy fiber expression of neuropeptide Y, which develops in animal models of chronic limbic seizures (Sperk *et al.*, 1996). However, mossy fiber sprouting was minimal or not present, suggesting that severe seizures and numerous repetitive seizures had not necessarily occurred. This might be due to the fact that chronic infusion downregulated trkB receptors (as mentioned above), or simply that BDNF is not as potent a convulsant as other drugs that induce status and lead to an epileptic phenotype.

The studies described above demonstrated that BDNF had a robust effect on mossy fiber transmission, probably by an action of trkB receptors on mossy fiber boutons. In both the normal and epileptic brain, it appears that this can bias the network to a hyperexcitable state. And in fact, seizures were triggered in the normal rat *in vivo* by BDNF, and seizure-like events were triggered by exogenous BDNF in slices of epileptic rats. But a key issue was the use of exogenous BDNF to trigger the seizures. This led us to question whether endogenous BDNF would be sufficient to trigger seizures. If this was true, a much stronger argument could be made for a role of BDNF in epilepsy.

It should be noted that recently two laboratories have provided evidence using trkB transgenics or conditional knockouts that endogenous BDNF is quite likely to play a role in epileptogenesis. Thus, overexpression of the truncated form of trkB altered epileptogenesis and the seizures that ultimately resulted (Lahteinen *et al.*, 2002). In addition, conditional deletion of hippocampal trkB blocked epileptogenesis using the kindling model (He *et al.*, 2004). Moreover, microarray analyses of epileptic tissue have shown that the BDNF gene is commonly represented (Lahteinen *et al.*, 2004). However, microarrays have shown that many genes besides BDNF are induced during epileptogenesis (Lahteinen *et al.*, 2004). Furthermore, overexpression of the full-length form of trkB did not necessarily alter epileptogenesis (although it did modify the acute response to convulsants; Lahteinen *et al.*, 2003), and conditional deletion of BDNF did not modify kindling (He *et al.*, 2004). The latter result can be explained by the potential for alternate neurotrophins to bind to trkB if BDNF is not present (He *et al.*, 2004). In summary, it is clear that much still needs to be learned about the relationship between BDNF, epileptogenesis, and epilepsy.

7. THE INDUCTION OF BDNF BY ESTROGEN AND ITS CONSEQUENCES

Although there are many ways BDNF expression is regulated, one that came to our attention that was particularly intriguing was the reproductive hormone estrogen. It had been identified that estrogen can induce BDNF expression by an estrogen-sensitive response element on the BDNF gene (Sohrabji *et al.*, 1995). We found this interesting because many women are known to have seizures at times of their menstrual cycle when estrogen transiently rises (i.e., at ovulation; Herzog *et al.*, 1997). Other women with limbic epilepsy clearly have seizures that are influenced by their levels of estradiol (Bauer, 2001). Thus, we hypothesized that perhaps the normal fluctuations in estrogen in females provided an example of an endogenous mechanism that can induce BDNF and could trigger increased seizures susceptibility. This might occur only in a fraction of individuals, of course, because all women do not have epilepsy. Therefore, we hypothesized that all women might demonstrate BDNF induction after elevated estradiol,

but only in those with an additional predisposition (such as abnormally high levels of BDNF induction by estradiol) would seizures occur. Other factors, that in themselves do not cause seizures but in addition to another factor such as high estradiol/BDNF would, could be genetic. Indeed, polymorphisms in the BDNF gene are linked to epilepsy (Kanemoto *et al.*, 2003), although not yet explored is the link to women with epilepsy. Structural abnormalities such as cortical dysplasias might also be a predisposing factor. In and of themselves they might not be sufficient to trigger seizures, but together with abnormally high BDNF they might lead to seizures.

What is the evidence that estradiol induces BDNF? After the identification of an estrogen response element on the BDNF gene, a number of studies examined the potential changes in BDNF expression after estradiol treatment. These studies demonstrated that in ovariectomized rats treated with estradiol, BDNF mRNA increases (Sohrabji *et al.*, 1995; Singh *et al.*, 1995). However, the functional effect of increased mRNA was not clear. Given that studies up to this time were conducted mostly in ovariectomized tissue, a setting that at best can be extrapolated only to the postmenopausal state, we chose to examine the intact female hippocampus. We focused on BDNF protein and potential functional implications.

To address potential changes due to elevated estradiol in the intact rat, we chose a comparison that is common to endocrinologists interested in dissecting the influence of estradiol from other reproductive steroids: a comparison of female brain on the mornings of proestrus, estrus and metestrus. For this approach, the standard 4-day estrous cycle was first established by daily vaginal cytologic exam (hormone levels were confirmed later by RIA; Scharfman *et al.*, 2003). The relative changes in hormones during the mornings of each day of the 4-day estrous cycle can be simplified as follows: 1) the morning of proestrus, when estradiol rises and is followed by a rise in progesterone in the afternoon, 2) the morning of estrus, when both estradiol and progesterone levels have returned to baseline over the course of the previous 12 -18 hrs, 3) the morning of metestrus (also referred to as diestrus 1), when both estradiol and progesterone have been low for the last 24 hrs, and 4) the morning of diestrus 2, when hormone levels are also low. The morning of proestrus as compared to the morning of metestrus is ideal to dissect the effects of estradiol, because the rise in progesterone, and other related hormones (LH, FSH) that occur at other times are not confounding.

We examined the expression of BDNF in animals at the cycle stages indicated above, as well as after ovariectomy, and in male rats for comparative purposes. We also examined mossy fiber transmission in these experimental groups. A summary of our findings are shown in Figure 6 (see also Scharfman *et al.*, 2003). First, BDNF protein expression does appear to rise on the morning of proestrus, consistent with a role of estradiol. Second, BDNF expression remains elevated through the morning of estrus, and appears to decline by the morning of metestrus. The persistent elevation is intriguing in light of the fact that mossy fiber transmission was abnormal on the morning of proestrus and estrus as well (Figure 6). This might mean that both estradiol and progesterone play roles, but it also may simply mean that after induction of mRNA, BDNF protein is maintained for up to 24 hours, regardless of the rapid decline in estradiol and changes in other reproductive steroids.

Remarkably, the slices from proestrous and estrous females behaved as if they were male rats with exogenous BDNF administered, or male mice with overexpression of BDNF. Thus, paired stimuli at 1 Hz to the mossy fibers, but no other CA3 input, evoked multiple population spikes. These were reversed by the trk inhibitor K252a, but not D-

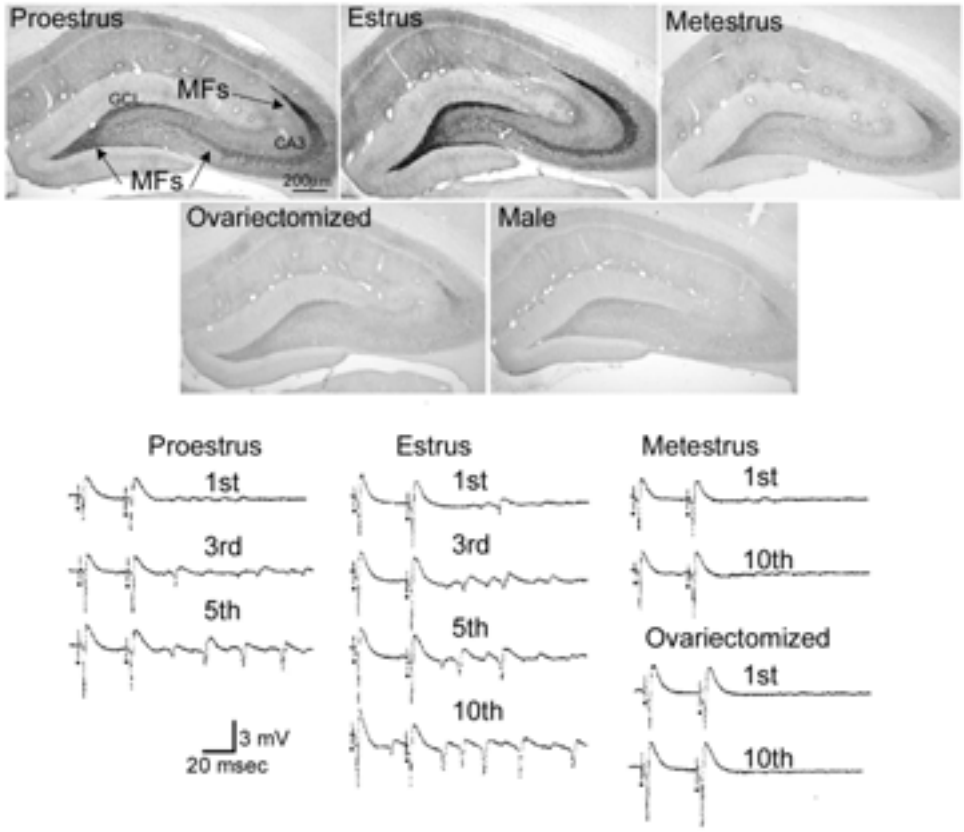

Figure 6. Effects of the estrous cycle on BDNF immunoreactivity in the mossy fibers and mossy fiber-evoked responses recorded extracellularly in the area CA3 cell layer. Top: Immunocytochemistry demonstrated an increase in mossy fiber BDNF on the mornings of proestrus and estrus relative to metestrus, ovariectomy, and BDNF expression in a male rat. Bottom: Mossy fiber-evoked responses recorded extracellularly in the area CA3 cell layer demonstrated that multiple population spikes could be evoked by pairs of mossy fiber stimuli at 1 Hz from slices prepared from rats on the morning of proestrus and estrus but not other cycle stages or after ovariectomy. Note that ovariectomized rats and male rats do express mossy fiber BDNF, but it is simply less than the proestrous or estrous rats; indeed, if the duration of incubation with reactants (DAB) is increased, mossy fiber BDNF in male rats is striking (Conner *et al.*, 1997). GCL= granule cell layer; MF = mossy fibers (Scharfman *et al.*, 2003).

APV (Scharfman *et al.*, 2003). Thus, BDNF protein, and physiological effects of BDNF, appear to fluctuate in concert across the estrous cycle of normal female rats, presumably stimulated by the proestrus surge in estradiol.

Although intriguing, the results raise several questions. One series of questions is how the results might be relevant to seizures in women with epilepsy. Although it will be a challenge to prove that studies in rats can be generalized to women, there are many possibilities. First, in women with hormone-sensitive seizures, is the hormone sensitivity due to induction of BDNF by natural fluctuations in estradiol? Second, at puberty, when epilepsy is often first diagnosed, might it be due to the induction of BDNF by estradiol? Third, does hormone replacement in postmenopausal women lead to increased BDNF in

hippocampus and altered seizure susceptibility? Fourth, what is the role of BDNF in the male- might testosterone play a role analogous to estradiol?

Other questions relate to underlying mechanisms. As raised by Blurton-Jones et al. (2004), there is a puzzling mismatch between estrogen receptor localization and BDNF expressing cells. In their studies, which were notably in male rats, the point was made that classic estrogen receptors (ER), both ERα and ERβ, were not evident in granule cells (Blurton-Jones *et al.*, 2004), yet this is where mossy fiber BDNF is synthesized. Interestingly, ERα was present in granule cell endosomes in proestrous female rats (Milner *et al.*, 2001), providing a potential resolution to the paradox. However, it is usually assumed that the classic estrogen receptor that interacts with the genome is not a membrane receptor. A possibility raised by (Blurton-Jones *et al.*, 2004) is that estradiol acts indirectly on granule cells to regulate BDNF expression. Activity might be the mediator in this case, because estrogen receptors were found on a subset of GABAergic neurons (Blurton-Jones *et al.*, 2004), which innervate granule cells. Another possibility is that there is local regulation of BDNF synthesis by membrane estrogen receptors that bypass the gene. Indeed, there is increasing evidence for local control points in the cell for BDNF synthesis (Schratt *et al.*, 2004).

Other possibilities to consider are additional hormonal effects on BDNF expression, and cortisol is a prime candidate given that it rises during the morning of proestrus, and granule cells are well endowed with corticosteroid receptors. Moreover, stress clearly influences BDNF expression. The problem with this hypothesis is that stress appears to decrease BDNF in most studies to date (Smith *et al.*, 1995; Schaaf *et al.*, 1998). A few have shown that a transient elevation can occur before BDNF expression declines (Marmigere *et al.*, 2003), but the decline occurs within hours and hence would not explain the protracted elevation in BDNF that continues from proestrus throughout the morning of estrus.

8. CELLULAR MECHANISMS

Regarding cellular mechanisms, intracellular recordings have provided insight into potential reasons for underlying changes in mossy fiber transmission that lead to multiple population spikes. Thus, in slices which demonstrate multiple population spikes in response to a 1 Hz train of paired pulses to the mossy fibers, the intracellular correlate reveals that the initial responses, which are dominated by IPSPs or afterhyperpolarizations, are transformed as stimulation progresses into long lasting depolarizations with superimposed action potentials. An example of this is shown for a CA3 pyramidal cell from a slice of a proestrous rat vs. a metestrous rat in Figure 7. Nothing like this can be evoked by other inputs to the same CA3 pyramidal cells. It is unlikely that there are changes in intrinsic properties that explain the effect, because, for example, intrinsic properties of CA3 neurons do not appear to change after exposure to BDNF (MacLusky *et al.*, 2003).

Thus, the next step will be to examine what underlies the long-lasting depolarization. Is it a reflection of increased glutamate release, possibly so much that concomitant afterhyperpolarizations and IPSPs are unable to control neuronal discharge? Perhaps a decrement in glutamate transport works in conjunction with increased release to produce a long-lasting and large EPSP. Indeed, estradiol can influence glutamate

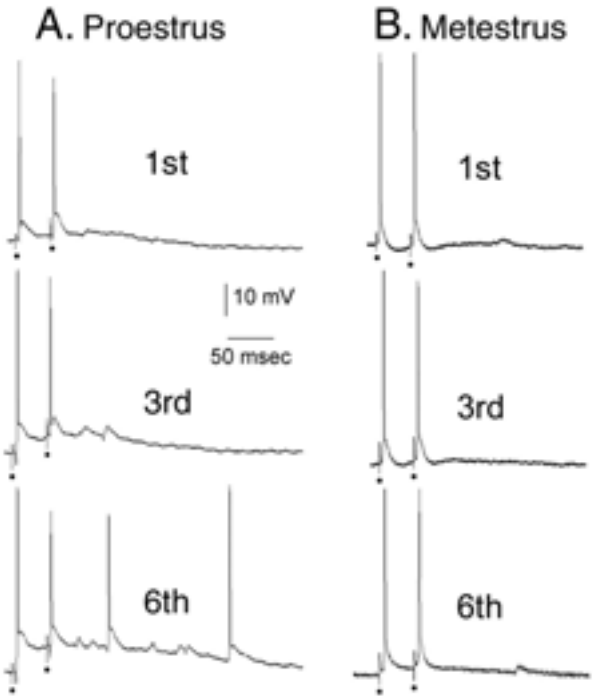

Figure 8. Recordings from CA3 pyramidal cells illustrate the intracellular correlate to extracellularly-recorded population spikes induced by repeated mossy fiber stimuli in the presence of increased BDNF. A. Responses to mossy fiber stimuli (paired pulses, 40 msec interstimulus interval, 1 Hz) are recorded from a CA3 pyramidal cell of a slice from a proestrous rat with elevated mossy fiber BDNF. Membrane potential, -72 mV. A long-lasting depolarization develops by the 3rd pair of stimuli and increases further by the 6th pair of stimuli. On this depolarization, action potentials are triggered that correspond to the multiple population spikes observed extracellularly, shown in Figure 6. B. Recordings from a CA3 pyramidal cell in a slice from a metestrous rat with relatively low mossy fiber BDNF. Membrane potential, -68 mV. No long-lasting depolarizations or extra action potentials are evoked. The cells from metestrous rats demonstrate similar responses as other CA3 pyramidal cells examined under other conditions when BDNF levels in mossy fibers are relatively low, such as male rats prior to exposure to exogenous BDNF.

transport, so this could be a mechanism that might be applicable to the actions of BDNF in female rats described above. But at least in cortical synaptosomes, high affinity transport of glutamate appears stronger at proestrus, not weaker (Mitrovic *et al.*, 1999), so this mechanism seems an unlikely explanation.

Another possibility is that IPSPs switch from hyperpolarizing to depolarizing, a possibility suggested by the fact that BDNF induces changes in KCC2 expression (Rivera *et al.*, 2004), and KCC2 is a key control of chloride entry during $GABA_A$ receptor-mediated IPSPs (Rivera *et al.*, 1999). In addition, there is a sex-related difference in KCC2 expression that has been documented outside the hippocampus (Galanopoulou *et al.*, 2003). However, this mechanism may not provide a good explanation, because IPSPs evoked by single stimuli to the mossy fiber across the estrous cycle had identical amplitude and reversal potentials (unpublished data). Nevertheless, a partial change in

KCC2 expression might make IPSPs in response to *single stimuli* relatively unaffected, but IPSPs evoked by *repetitive stimuli* much more labile. Indeed, the lability of IPSPs to repeated stimuli has long been known (Wong and Watkins, 1982; Deisz and Prince, 1989; Thompson and Gahwiler, 1989).

9. SUMMARY AND PERSPECTIVE

This chapter reviews studies of BDNF's effects on mossy fiber transmission that suggest a prominent role for this neurotrophin in regulation of this synapse. BDNF appears to increase the ability of mossy fibers to excite CA3 pyramidal neurons by an action on trkB receptors that leads to altered glutamate release. Under normal conditions, the result may be beneficial, because a slight increase in glutamate release may promote synaptogenesis during development, dendritic spine plasticity, as well as a long-lasting potentiation, analogous to what has been documented in CA1 (Figure 8).

However, too much BDNF may not be beneficial. Excess BDNF may arise following a variety of stimuli, insults, hormonal changes, or activity-induced upregulation. This may ultimately have negative consequences by increasing seizure susceptibility and leading to a chronic elevation in BDNF, similar to what has been detected in epileptic rats and human temporal lobe epilepsy (Figure 8). Under these conditions, any subsequent release of BDNF could trigger seizures theoretically. If correct, this hypothesis suggests that anticonvulsants that block BDNF upregulation or trkB may be a novel therapeutic strategy to treat epilepsy.

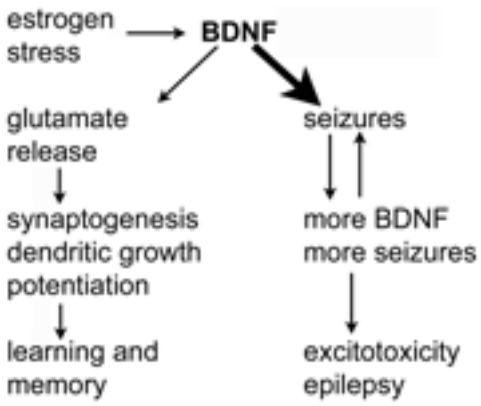

Figure 8. A working hypothesis for the role of BDNF in hippocampus. Several factors, such as estrogen and stress, can modulate BDNF levels in glutamatergic pathways such as the mossy fibers. Normal levels or small increases lead to enhanced glutamate release and potentiation, as well as normal development and plasticity of structure and function, such as learning. Excess levels lead to hyperexcitability and a positive feedback that ultimate raises BDNF levels further and promotes seizures and excitotoxicity. The two branchs of this schematic are not mutually exclusive, given that excess BDNF after an initial period of seizures may foster the changes in structure and function that underlie the epileptic state.

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