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BDNF infusion and up-regulation rescue synaptic plasticity in middle-aged ovariectomized rats

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Abstract

Brain Derived Neurotrophic Factor (BDNF) has emerged as a possible broad-spectrum treatment for the plasticity losses found in rodent models of human conditions associated with memory and cognitive deficits. We have tested this strategy in the particular case of ovariectomy. The actin polymerization in spines normally found after patterned afferent stimulation was greatly reduced, along with the stabilization of long-term potentiation, in hippocampal slices prepared from middle-aged ovariectomized rats. Both effects were fully restored by a 60 min infusion of 2 nM BDNF. Comparable rescue results were obtained after elevating endogenous BDNF protein levels in hippocampus with four daily injections of a short half-life ampakine (positive modulator of AMPA-type glutamate receptors). These results provide the first evidence that minimally invasive, mechanism-based drug treatments can ameliorate defects in spine plasticity caused by depressed estrogen levels.

Keywords

hippocampus; spines; actin; LTP; theta bursts; ampakine; estrogen

1. Introduction

Appropriately patterned afferent activity causes rapid reorganization of the dendritic spine cytoskeleton, as evidenced by intense actin polymerization (Lin et al., 2005; Kramár et al., 2006; Okomoto, et al., 2004), along with marked changes in spine and synapse morphology in adult hippocampus (Yang et al., 2008; Matsuzaki et al., 2004; Honkura et al., 2008; Chen et al., 2007; Rex et al., 2009). Recent studies suggest that at least some of these effects occur

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during learning and are critical to the encoding of long-term memory (Fedulov et al., 2007). There are also reasons to suspect that defects in spine plasticity are important contributors to memory and cognitive problems in certain neuropsychiatric disorders (Kaufmann and Moser, 2000). Consonant with this idea, activity-driven polymerization of spine actin is disturbed in animal models of early-stage Huntington's Disease (HD) (Lynch et al., 2007), a condition associated with memory impairments of varying degrees of severity. Deficits in spine cytoskeletal reorganization are also observed in ovariectomized (OVX) rats not given estrogen replacement (Kramár et al., 2009b). A substantial body of evidence links rapid changes in spine actin networks to long-term potentiation (LTP) (Krucker et al., 2000; Fukazawa et al., 2003; Okamoto et al., 2004; Kramár et al., 2006; Bramham, 2008; Rex et al., 2009) and, as expected from this, the spine defects found in HD and OVX rodents are accompanied by a failure of LTP consolidation (Kramár et al., 2009b; Lynch et al., 2007).

The loss of plasticity in the OVX cases raised the possibility that estrogen engages actin signaling at synapses. We tested this idea and found that the steroid promotes rapid actin filament assembly within adult spines by stimulating the small GTPase RhoA, which then drives the phosphorylation, and thus inactivation, of the actin severing protein cofilin (Kramár et al., 2009b). Induction of LTP by theta pattern stimulation also phosphorylates cofilin via RhoA-dependent kinase (Rex et al., 2009), so it appears that estrogen regulates spine plasticity through actions on a learning-related enzyme cascade.

The above conclusion has significance with regard to the development of strategies for treating memory problems commonly reported to accompany the loss of estrogen (Phillips and Sherwin, 1992; Devi et al., 2005; Wegesin and Stern, 2007). Recent work indicates that the RhoA to cofilin signaling targeted by estrogen is also acutely modulated by factors released during the brief periods of repetitive afferent activity used to induce LTP (for review Kramár et al., 2009a). Thus, it is conceivable that manipulation of these other factors such as increasing TrkB signaling (Chen et al., 2009) can be used to offset the loss of estrogen's positive influence on spine cytoskeletal reorganization and LTP. We have explored this possibility by measuring the effects of BDNF, an established and potent positive modulator of LTP, on ovariectomy-induced defects in actin filament assembly and LTP consolidation. More directly relevant to the clinical problem of estrogen-related memory loss, we tested if elevating forebrain BDNF levels with the short-half life ampakine CX929 rescues spine plasticity in hippocampal slices from OVX rats. Ampakines, via positive modulation of central AMPA-receptors, enhance excitatory transmission in the forebrain and thereby increase BDNF expression (Lauterborn et al., 2003). CX929 was chosen for the study because of its short-half life (<15 minutes) and past work showing that it up-regulates BDNF and restores LTP in diverse rodent models (Rex et al., 2006; Simmons et al., 2009).

2. Methods

All animal procedures were conducted in accordance with the National Institutes of Health guide for the care and use of laboratory animals and with protocols approved by the Institutional Animal Care and Use Committee of the University of California at Irvine. This includes efforts to minimize animal suffering and numbers of rats used in the work described.

2.1 Slice preparation and recording

Studies employed Long Evans rats (Charles River) that were OVX at 9-10 mo old (retired breeders) and allowed to age for at least 6 months before testing. Methods were slightly modified from those described previously (Kramár et al., 2006). Briefly, acute hippocampal slices were prepared and maintained in an interface recording chamber containing preheated

artificial cerebrospinal fluid (aCSF) of the following composition (in mM): 124 NaCl, 3 KCl, 1.25 KH₂PO₄, 1.5 MgSO₄, 2.5 CaCl₂, 26 NaHCO₃, and 10 glucose. Slices were continuously perfused with this solution at a rate of 1.75-2 ml/min while the surface of the slices were exposed to warm, humidified 95% O₂ / 5% CO₂. Recordings began following at least 1.5 hrs of incubation.

Field excitatory postsynaptic potentials (fEPSPs) were recorded from CA1b stratum radiatum using a single glass pipette (2-3 MΩ). Stimulation electrodes (twisted nichrome wires, 65 μm) were positioned at two sites (CA1a and CA1c) in the apical Schaffer collateral-commissural projections to provide convergent activation of CA1b pyramidal cells. Pulses were administered in an alternating fashion to the two electrodes at 0.05 Hz using a current that elicited a 50% maximal response. After establishing a 10-20 min stable baseline, LTP was induced with 10 “theta” bursts, each burst consisting of four pulses at 100 Hz with an inter-burst interval of 200 msec (i.e., theta burst stimulation or TBS). The stimulation intensity was not increased during TBS. In some slices, test compounds (see below) were introduced into the infusion line by switching from control aCSF to drug-containing aCSF prior to induction. Data were collected and digitized by NAC 2.0 Neurodata Acquisition System (Theta Burst Corp) and stored on a disk.

2.2 BDNF application

The perfusion system used for BDNF application during field recordings was employed as previously described (Kramár et al., 2004). In some experiments, BDNF was inactivated by boiling for 10 min before experimentation.

2.3 In situ phalloidin labeling

To assess filamentous actin (F-actin) within individual dendritic spines, we used a recently developed in situ labeling technique which entails applying fluorescent-tagged phalloidin, a mushroom toxin that penetrates the cell membrane (Bernstein et al., 1998; Geeraert et al., 2003) and binds to F-actin, to living tissue before fixation (Lin et al., 2005; Kramár et al., 2006; Rex et al., 2007). Epifluorescence wide field microscopy was then used to quantify dendritic spine levels of fluorescent-labeled F-actin in association with treatments and the various stages of LTP. Specifically, Alexa568-phalloidin (6 μM, Tocris Bioscience) was applied topically (2 μl) from a micropipette every 5 min for 20 min either at the end of the experiment in slices that received low-frequency stimulation or 60 min after the delivery of 10 theta bursts. Slices were then fixed in 4% paraformaldehyde (12-18 hrs), cryoprotected in 20% sucrose/0.1M phosphate buffer (PB) for 1 hr at 4°C and sectioned on a freezing microtome at a thickness of 20 μm (parallel to the broad surface of the slice). Sections were collected in PB, mounted onto Superfrost slides (Fisher Scientific) and coverslipped with Vectashield (Vector Laboratories).

2.4 Imaging and measures of dendritic spines

Alexa568-phalloidin labeling was visualized using a Zeiss Axioscop microscope equipped with an AxioCam CCD camera and AxioVision 4.0 software (for quantitative data collection). For identification of labeled spines, images were collected at 40× magnification from three serial sections beginning 60 μm below the surface of each hippocampal slice. The sampling zone, as previously described (Lin et al., 2005; Kramár et al., 2006; Rex et al., 2007), was within the area of physiological recording between the two stimulating electrodes positioned in the CA1 proximal stratum radiatum. For each field, 5 sequential images were collected at 2 μm focal steps totaling a thickness of 10 μm on the z-axis. Exposure times were adjusted for each experiment so that ~6-12 spines were visualized in images from control slices. Images used for comparisons were collected with the same exposure parameters.

Quantitative analysis of Alexa568-phalloidin labeled spines was conducted as previously described (Rex et al., 2007). Briefly, digital image Z-stacks were collapsed by extended focal imaging into a single image using AxioVision software. These images were then converted to grayscale and inverted, and intensity levels were scaled to values determined for each experiment (Adobe Photoshop 6.0) to visualize low-intensity labeling. Counting and analysis of labeled spine-like structures were conducted blind to sample identity on batches of slices that had been processed together using in-house developed software (Lin et al., 2005). A constant sized image ($37800 \mu\text{m}^2$) was converted to 8 bits/pixel gray scale and processed through a series of intensity thresholds to identify spine like-structures at varying levels of label intensities within a range of reliably counted dendritic spines. Pixel values for an image were normalized to a fixed, overall grey value background. This reduced contributions of differences in background intensity across the images. The normalized images were then binarized using each intensity threshold and finally cleaned by “erosion” followed by “dilation” filtering (Jain, 1989). Area and eccentricity were used to count objects as spines or to reject observations as artifacts. To verify accuracy of spine counts, images of objects identified as spines were overlaid semi-transparently with the original photomicrographs. In slices receiving low-frequency stimulation or TBS, the analysis used a single threshold set at 75% of maximum image intensity to detect only densely labeled spines. Spine counts from three serial sections were averaged to produce a mean value for each hippocampal slice. For each control and experimental group, slices were tested from a minimum of 4 animals.

2.5 Chronic estradiol and ampakine treatment

Long Evans rats (retired breeders) at middle-age (9-10 mo) were ovariectomized and 6 months later randomly divided into two groups: one group received estradiol replacement therapy and the other remained estradiol-depleted. For hormone replacement the estradiol analog 17- β estradiol benzoate (EB), maintained in a stock solution of 10 $\mu\text{g}/0.1\text{ml}$ in sesame oil, was injected subcutaneously once/day, every other day, for 2 weeks (Woolley and McEwen, 1993). Vehicle animals received similar injections of sesame oil. At 24 hrs after the last injection rats were decapitated, the right hippocampus was used for slice physiology experiments and the left was used for Western blot analysis of BDNF protein content (see below). To verify the ovariectomy and sufficiency of EB replacement, the uterus was severed from the cervix, cleared of fat on the uterine tubes, and blotted dry on tissue paper to remove luminal fluid before being weighed. Uteri from EB-injected animals weighed, on average ($\pm\text{SEM}$), twice as much as uteri collected from vehicle-injected rats and were highly vascularized and filled with fluid (uterine weight: $0.105 \pm 0.02\text{g}$, $0.364 \pm 0.3\text{g}$, and $0.126 \pm 0.2\text{g}$ for vehicle-, EB-, and CX929-treated rats, respectively; $n > 4/\text{group}$).

In vivo ampakine treatments were carried out as previously described (Rex et al., 2006). Middle-aged Long Evans rats (OVX for 6 mo) received two intraperitoneal (ip) injections per day, one between 8:00 and 9:00 a.m. and the second between 2:00 and 3:00 p.m., for a total of 8 days. For the first 4 days of acclimatization to the injection schedule, all animals received vehicle injections containing normal saline in 20% 2-hydroxypropyl- β -cyclodextrin (Sigma). For the remaining 4 days, animals were randomly divided into two groups, one continued to receive vehicle injections, while the other received the ampakine CX929 (5 mg/kg) in vehicle. Immediately after each injection, animals were placed in an enriched environment (an open field filled with novel objects) with other rats for 30 min of exploration and social interaction before returning to their home cages. At 18 hrs after the last ampakine injection, animals were sacrificed and brains removed. The two hemispheres were used for electrophysiology and BDNF protein measurement, respectively.

2.6 Western blot analysis

Hippocampi were homogenized in aCSF containing Phosphatase Inhibitor Cocktails 1 and 2 (Sigma) and Complete Protease Inhibitor Cocktail (Roche Diagnostics) (Rex et al., 2007), normalized for protein content (Bio-Rad Protein Assay), separated by 12% SDS-PAGE and processed for Western blot analysis using rabbit anti-BDNF (1:2000 dilution; Santa Cruz, sc-546) and the ECL Plus chemiluminescent detection system (Amersham). Blots were stripped (Millipore) and re-probed for β -actin (Sigma) as a control for loading. ImageJ (NIH) software was used to measure the intensity of immunoreactive bands at ~14kDa. The Q-test was used to identify outliers within groups (Dean and Dixon, 1951); only one animal met the criteria. Statistical significance for the effect of treatment was determined by one (outcome predicted) or two-tailed (no prediction) Student's t-test or Mann-Whitney U-Test for comparisons between vehicle-treated and drug-treated groups.

2.7 Data Analysis

All electrophysiological data presented in the text and figures are reported as means $\pm$ S.D. and means $\pm$ S.E.M., respectively. To assess theta train facilitation, the area of bursts 2-10 were expressed as a change in total area from the initial theta burst response. Burst responses 2-5 were then normalized to their appropriate controls and expressed as a percent change in facilitation from controls. The sample size for all experiments represents the number of animals used unless otherwise noted.

Student's t-tests, using $P < 0.05$ significance criteria, were used to compare groups for all electrophysiology and measures of spine labeling.

3. Results

3.1 BDNF rescues theta burst-induced actin polymerization

Multiple studies have shown that theta burst stimulation (TBS) triggers actin signaling and polymerization in spines within the dendritic lamina of hippocampal field CA1 containing the activated Schaffer-commissural synapses (Kramár et al., 2006; Rex et al., 2007; Chen et al., 2007). These effects are largely absent in hippocampal slices prepared from OVX rats (Kramár et al., 2009b), as confirmed here in experiments using fluorescence-tagged phalloidin to label spines containing high concentrations of filamentous (F-) actin. Sixty minutes after inducing LTP using TBS, fluorescent-tagged phalloidin was applied topically to the recording site for 20 min before the slice was collected for fixation. Relative to labeling in slices from OVX rats that received low frequency stimulation (OVX+LFS+veh) (Fig 1A), TBS caused a pronounced increase in densely phalloidin-labeled spines in slices from OVX rats that had been treated for two weeks with 17 β -estradiol benzoate (OVX+TBS+EB) (Fig 1B); the magnitude of the increase was comparable to that reported for slices from young adult male rats (Lin et al., 2005). It should be noted that while the effect was dramatic relative to labeling in slices that received only low frequency stimulation, the increase involved only a small percentage of the total spine population. This finding accords with evidence that the stimulation typically used in slice physiology experiments activates a small fraction of the synapses found in the proximal stratum radiatum (Lin et al., 2005; Chen et al., 2007; Rex et al., 2007). In marked contrast to the results for hormone-treated rats, TBS produced only a marginal increase in phalloidin-labeled spines in slices from OVX rats that received vehicle injections (OVX+TBS+veh) (Fig 1C).

We next tested if pretreatment of the slices with a low concentration (2 nM) of BDNF offsets the deficit in theta-induced assembly of actin filaments. A 60 min infusion of the neurotrophin enhanced the effects of TBS on F-actin labeling in slices from OVX rats to the levels seen in the OVX+TBS+EB group (Fig 1D). Heat-inactivated BDNF was ineffective

(Fig 1E). Blind quantitative analyses of phalloidin-labeled spines confirmed that estrogen pretreatments or acute infusion of active BDNF rescued theta-elicited filament assembly in OVX slices (Fig 1F).

3.2 Actin polymerization in OVX slices with enhanced levels of BDNF

Past studies have shown that four days of treatment with centrally active, positive modulators of AMPA-type glutamate receptors (ampakines) elevate hippocampal and cortical BDNF levels in rats and mice (Rex et al., 2006; Simmons et al., 2009). We used a short half-life (~15 min) compound of this type to determine if increased concentrations of endogenous BDNF protein rescue synaptic plasticity in the manner found with acute infusions of the neurotrophin.

The ampakine CX929 (5 mg/kg) or vehicle was injected ip twice daily for four days. The animals were placed in a large open field with other rats for 30 min following each injection, resulting in an extended period of intense exploration and social interaction. Slices were prepared 18 hrs after the last injection. Hippocampal concentrations of mature BDNF protein at this time point were approximately 25% higher in the ampakine-treated group than in the vehicle group (Fig 2A). There were no evident effects of ampakine pretreatment on baseline parameters for fEPSPs (see below) but the ampakine injections had a strong effect on TBS-induced actin polymerization (OVX+TBS+CX929) as compared to the effects of TBS in slices from OVX+vehicle-treated animals (OVX+TBS+veh) (Fig 2B). Automated analyses showed that the increase in phalloidin-positive spines at 60 min post-TBS in the CX929-treated slices was equivalent to that found in hormone replacement- or BDNF-treated slices (Fig 2C, compare to figure 1F).

3.3 BDNF and ampakines rescue LTP in OVX rats

Harvesting slices at 7 min post-TBS, in the above phalloidin experiments, precluded tests of whether the restoration of TBS-induced actin polymerization is accompanied by a correction of the LTP impairments found in OVX slices. We therefore carried out additional studies using a conventional paradigm for analyzing the magnitude of post-TBS potentiation.

The amplitudes, waveforms, and input/output curves of fEPSPs recorded in field CA1 were not detectably different in slices prepared from OVX rats that did or did not receive estrogen treatments for two weeks; paired pulse facilitation, a measure of release kinetics, was also comparable in the two groups (Fig 3A, 3B). LTP, however, was clearly impaired in the OVX+vehicle treated animals (Fig 3C). Initial expression of potentiation, as measured at 1-2 min after TBS, was not measurably different between the groups (vehicle: $75 \pm 21\%$; hormone replacement: $89 \pm 15\%$; $p=0.30$). In contrast, LTP in the vehicle-injected rats decayed steadily towards baseline while that in the OVX+EB-treated group showed the typical stabilization at values well above pre-TBS amplitudes (Fig 3C). The differences in percent potentiation at 60 min post-TBS for slices from vehicle- and EB-treated rats were highly significant (vehicle: $21 \pm 6\%$; EB replacement: $50 \pm 12\%$; $p=0.001$).

Consistent with previous reports of studies using young adult male rats (Figurov et al., 1996; Tanaka et al., 1997; Frerking et al., 1998; Kramár et al., 2004), a 60 min infusion of 2 nM BDNF had no measurable effect on baseline synaptic responses in slices from OVX animals. It did, however, fully restore LTP: the percent potentiation vs. time curves were nearly identical, as was the degree of LTP, to those for slices from OVX+EB-treated animals at the end of the 60 min recording session ($51 \pm 14\%$ and $50 \pm 12\%$ for 2 nM BDNF vs. EB replacement, respectively) (Fig 4A). Slices collected from OVX rats infused for 60 min with heat-inactivated BDNF (Fig 4A) showed a similar gradual decay in LTP as observed in control OVX+vehicle slices (see Fig 3C).

Comparable results were obtained in slices from OVX rats given four days of ampakine injections. As in the experiment using acute BDNF treatments, a robust initial expression of LTP was followed by a gradual loss of potentiation over the next 60 min in the vehicle (no ampakine) group. However, LTP in slices from OVX rats injected with CX929 stabilized after 10-15 min with the responses remaining well above baseline for the remainder of the recording session (LTP at 60 min: $57 \pm 24\%$ for the ampakine group and $19 \pm 9\%$ for the vehicle group; $p < 0.001$) (Fig 4B). Importantly, the positive effects of ampakine pretreatment were highly selective: there were no evident differences in input/output curves or in paired pulse facilitation between slices from OVX+vehicle rats that did or did not receive the in vivo treatments (Fig 4C, 4D). Previous studies have shown that acute BDNF infusions similarly leave these baseline physiological measures unchanged (Figurov et al., 1996; Tanaka et al., 1997; Frerking et al., 1998; Kramár et al., 2004).

3.4 In vivo ampakine pretreatments reproduce effects of acute BDNF on theta burst responses

As described, the four days of ampakine injections selectively elicited two striking effects also obtained with acute treatments with low concentrations of BDNF: restoration of both TBS-driven actin polymerization and LTP consolidation. This, combined with the fact that the experiments were conducted 18 hrs after the last administration of a short half-life drug, suggests that the observed persistent elevations of BDNF were most likely responsible for the ampakine-induced changes in plasticity. We further tested this point by asking if in vivo ampakine pretreatment also reproduced an unusual influence of BDNF on theta burst responses.

Earlier work in rat slices showed that BDNF enhances the composite (four fEPSP) responses to the 2nd and subsequent stimulation bursts in a theta train, most likely by suppressing after-hyperpolarizing potentials (Kramár et al., 2004). We first confirmed that the effect is present in OVX slices. The total area of the four-EPSP burst responses was measured for bursts 1-10. The area of the burst response for OVX+vehicle and OVX+EB slices showed similar facilitation profiles, as reported earlier (Kramár et al., 2009b) in that bursts 2-10 are increased relative to the first, with the most reliable effects occurring on bursts 2-5 (Fig 5A). We then normalized burst responses 2-5 of the experimental groups (EB, BDNF infusion, and ampakine pretreatment) to their respective controls to compare results across treatment conditions. As expected from the results in figure 5A, slices from EB-injected OVX rats had the same relative degree of facilitation across bursts 2-5 as did slices from vehicle-treated OVX rats (dotted line at 100%) (Fig 5B, top left and right panels). It appears then that ovariectomy does not affect the diverse variables, including NMDA and GABA receptor activation, which shape responses to TBS (Larson et al., 1986; Arai and Lynch, 1992; Sah and Bekkers, 1996). In contrast, 45 min infusions of active BDNF, as predicted, increased burst facilitation in OVX slices above that found with heat-inactivated BDNF ($p = 0.037$, one-tailed t-test) (Fig 5B, middle panels). A four-day treatment with CX929 produced a similar effect (Fig 5B, bottom panels): facilitation of bursts 2-5 was about 40% greater in slices prepared 18 hrs after the last injection than was the case for slices from vehicle-injected OVX animals ($p = 0.016$).

Collectively, the data indicate that chronic increases in BDNF levels elicited by ampakines are associated with the same structural and physiological actions that follow BDNF infusions.

4. Discussion

Prompted by reports that reductions in circulating estrogen can result in significant impairments to human memory (Melleson, 1953; Kopera, 1973), a number of investigators

have examined learning in OVX rats (Singh et al., 1994; Daniel et al., 1997; Luine, 1997; Markowska and Savonenko, 2002; Rhodes and Frye, 2006; Wallace et al., 2006; Xu and Zhang, 2006) and monkeys (Lacreuse et al., 2002; Rapp et al., 2003; Hao et al., 2006). The results are reasonably consistent in showing that such animals have moderate to serious memory impairments across an array of behavioral protocols. A search for the origin of these deficits led to the discovery that hippocampal LTP fails to properly consolidate in OVX animals not given hormone replacement (Cordoba Montoya and Carrer, 1997; Gupta et al., 2001; Smith and McMahon, 2005, 2006; Kramár et al., 2009b), and thus to the broad hypothesis that estrogen in some way promotes plasticity. In accord with this, we recently found that brief infusions of estrogen activate sub-synaptic actin signaling pathways that play a central role in the production of stable LTP (Kramár et al., 2009b).

The present studies were concerned with the clinically important problem of plasticity losses in the middle-aged brain arising from chronic depression of estrogen levels. We therefore used rats ovariectomized at 9 months with experimental work beginning 6 months later. The synaptic effects of estrogen in the aged brain are less clear and likely more complex than those described in young adults. Age (Foster et al., 2003), along with dosage (Fernandez and Frick, 2004; Frick et al., 2002; Foster et al., 2003) and duration (Savonenko and Markowska, 2002; Luine and Rodriguez, 1994) of treatment, influences the behavioral effects of peripherally administered estrogen. There is also evidence that estrogen becomes less effective in reversing cognitive impairments after a long-term period of hormone depletion (Daniel et al., 2006; Gibbs, 2001; Savonenko and Markowska, 2003). Given these points, and evidence that daily injections of estrogen produce more consistently positive results on rodent learning than continuous administration (Savonenko and Markowska, 2002; Frick et al., 2002), we used every other day injections for two weeks to test if hormone replacement rescues structural and functional plasticity in the middle-aged, estrogen-deprived brain.

Our previous work showed that acute estrogen treatments restore actin filament assembly and LTP consolidation in slices prepared from middle-aged, long-term OVX rats, suggesting that the loss of plasticity is primarily due to the simple depletion of peripheral or locally synthesized estrogen pools (Kramár et al., 2009b). We therefore expected the two-week treatments would similarly restore LTP to levels found in young, adult slices. Confirmation of this prediction provided a middle-aged, ovariectomized control group (OVX + E2) against which to compare the effects of potential therapeutics that do not involve estrogen itself.

If the plasticity deficits caused by ovariectomy are due to the removal of a modulatory influence over spine actin networks, then other factors with comparable influence should substitute for the loss of estrogen. The present studies are in full agreement with this argument. Estrogen and BDNF both engage the cofilin signaling pathway that is critical to activity-induced actin polymerization and the production of stable LTP (Rex et al., 2007 and 2009; Kramár et al., 2009b). We tested if acute infusions of BDNF normalize actin filament assembly and LTP consolidation in long-term OVX rats. As described here, positive results were obtained.

The above conclusion that ovariectomy disrupts plasticity by removing one of a group of factors that act on the synaptic cytoskeleton helps explain why estrogen, and the estrus cycle, modifies spine morphology and numbers (Woolley et al., 1990; Warren et al., 1995). A substantial body of work shows that in cultured neurons spine morphology is strongly affected by actin signaling (Fischer et al., 2000; Zhang and Benson, 2002; Okamoto et al., 2004) and Rho GTPases (Gehler et al., 2004). LTP work indicates that these developmental signaling pathways are present, although suppressed, in adult brain and can be transiently

reactivated by appropriate afferent input (Rex et al., 2009). It follows from this that long-term removal of estrogen's influence over the cascades will gradually result in a shift in the morphology of adult spines.

The present demonstration that BDNF effectively reverses the effects of ovariectomy on plasticity raises issues of considerable therapeutic significance. Surgical ovariectomy is often followed by clinically significant complaints involving certain types of learning (Phillips and Sherwin, 1992; Henderson and Sherwin, 2007; Rocca et al., 2007). And, while the issue is still controversial, studies have described memory problems that emerge in the months following the onset of menopause (Devi et al., 2005; Wegesin and Stern, 2007). Therapeutic intervention in both cases would necessarily have to be minimally disruptive because the pertinent problems are both medically restricted and chronic in nature. These considerations rule out the use of direct application of BDNF as a substitute for the loss of estrogen but leave open the possibility of pharmacologically elevating expression of the neurotrophin.

Clinical issues of the type just noted led us to employ a short regimen of in vivo ampakine treatments to determine if up-regulation of BDNF normalizes plasticity in OVX rats. Past studies with cultured slices showed that periodic treatments with ampakines increase the production of BDNF mRNA and protein (Lauterborn et al., 2003). Despite the brevity of drug applications, the increases in neurotrophin protein concentrations were maintained for days, as expected given the relative stability of the protein (Lauterborn et al. 2003; Nawa et al., 1995). Consonant with the in vitro findings, subsequent work showed that daily injections of a short half-life ampakine cause significant and sustained increases in BDNF levels in the forebrains of rats (Rex et al., 2006) and mice (Simmons et al., 2009). We have here confirmed that this also holds for middle-aged OVX rats. Ampakines are not reported to produce significant side effects in rodent (Hampson et al., 1998; Rex et al., 2006), monkey (Porrino et al., 2005), and human studies (Goff et al., 2001; Wezenberg et al., 2007), and are currently in clinical trials for various brain-based disorders. Our findings thus point to a minimally invasive technology for increasing BDNF levels in conditions in which estrogen levels are severely depressed.

Increases in BDNF were accompanied by a full restoration of theta-induced actin polymerization and LTP consolidation in slices from OVX rats without evident changes in baseline physiological measures. It remains possible that the drug treatments produced these effects by initiating long lasting changes other than, or in addition to, the chronic elevation of BDNF. Two features of the studies are critical to the evaluation of mechanisms: the drug treatments were extremely brief and testing was carried out 18 hours after the last injection. Combined, these points require a process that has a low threshold and yet results in persistent modifications of the type observed here. A lasting change in synapses is one possibility but we found no evidence for this: there were no detectable modifications of baseline responses, input/output curves, paired-pulse facilitation, or resting levels of actin polymerization. An alternative explanation for the results, and the one advanced here, involves up-regulation of a gene whose product modifies the effects of TBS on actin signaling and LTP consolidation. Several candidates for this role could be considered but most of these (e.g., adenosine and/or its A1 receptor [Rex et al., 2005]) do not satisfy the requirement that the effective agent have little effect on baseline transmission. Estrogen synthesis in hippocampal terminals (Kretz et al., 2004) is an intriguing, although difficult to test, possibility; the steroid causes only modest increases in EPSPs and, as discussed, promotes actin polymerization and LTP consolidation. However, estrogen has no detectable influence on the composite responses to individual theta bursts and, as shown here, the ampakine treatment produced a characteristic change in burst responses during a theta train. However, the ampakine effect did closely resemble that produced by brief infusions of

BDNF. In all, then, it seems likely that the normalizing effect of ampakine treatment on synaptic plasticity in middle-aged OVX rats involves the up-regulation of either BDNF (as observed) or an unknown factor whose action profile closely resembles that of BDNF.

If, as argued above, BDNF contributes importantly to the rescue of actin dynamics and LTP, then the question arises as to whether weeks-long up-regulation with brief ampakine treatments would elicit the positive trophic effects associated with the neurotrophin. This is particularly important with regard to the animal model under discussion here, which includes two conditions (aging, chronically low levels of estrogen) associated with impairments that go well beyond the rapid phase of LTP consolidation used as the endpoint measure in the present paper. It would, for example, be of great interest to test if prolonged treatments with the ampakine mitigate the calcium disturbances that accompany, and very probably contribute to, brain aging (Foster et al., 2001; Norris et al., 2010; Thibault et al., 2001) or the many alterations in neuronal gene expression found with abnormally low levels of estrogen (Adams et al., 2001; Brewer et al., 2009; Zhao et al., 2010).

In summary, a BDNF-based strategy for ameliorating plasticity losses associated with prolonged periods of low estrogen in middle-aged subjects appears feasible. There are thus reasons to think that the treatment regimens described here could translate into a viable strategy for addressing a major, unmet clinical problem for women.

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Fig. 1. Theta-induced actin polymerization in hippocampal slices prepared from ovariectomized (OVX) rats

Images were collected from the proximal stratum radiatum of field CA1b following stimulation of two populations of Schaffer/commissural projections that innervate that zone. **A-E)** Representative survey micrographs (scale bar: 5 μm) of phalloidin labeling from five groups of slices. **A)** Low frequency stimulation (LFS: 3 pulses/min) delivered to a slice from a vehicle-treated OVX rat (OVX+LFS+veh) did not increase phalloidin labeling. **B)** Theta burst stimulation (TBS) applied to the two inputs in a slice from a rat given two weeks of estrogen treatments (OVX+TBS+EB) caused a marked increase in the number of phalloidin-labeled puncta. **C)** TBS had little if any effect in a slice from an OVX+vehicle (OVX+TBS+veh) treated rat. **D)** A 60 min pretreatment with BDNF in a slice from an OVX+veh treated rat rescued the effect of TBS on phalloidin labeling (OVX+TBS+BDNF). **E)** Heat-

inactivated BDNF (iBDNF) had no effect on theta-induced phalloidin labeling (OVX+TBS +iBDNF). **F**) A computerized system was used to quantify the number of phalloidin-positive puncta in the CA1 stratum radiatum sample field. Planned comparisons between groups (LFS=15, TBS=19, EB=8 slices/group) showed that TBS produced a robust increase in densely labeled spines in slices from OVX rats injected with EB (EB, $*p<0.001$) but not in slices from OVX rats receiving vehicle ($p=0.10$). Acute infusion of BDNF, but not heat-inactivated BDNF, restored TBS-induced increases in phalloidin labeling in slices from OVX +vehicle treated animals ($**p<0.001$ and $p=0.48$, respectively).

Fig. 2. A four-day pretreatment with a short half-life ampakine (CX929) increased BDNF concentrations and rescued TBS-driven increases in phalloidin labeling

The ampakine CX929, or vehicle, was injected twice daily for four days and the animals were sacrificed 18 hr after the last injection. One hippocampus was used for Western blots and the other for slice physiology. **A)** Representative Western blot (top) shows that levels of mature BDNF (mBDNF, ~14-15kDa) were higher in samples from ampakine-treated animals (CX929, n=6) than those from yoked vehicle controls (veh, n=4). Densitometric analyses confirmed this point (bottom; $p=0.02$). **B)** Theta bursts had little effect on phalloidin labeling in slices from OVX+vehicle treated rats (TBS+OVX+veh) but caused an evident increase in slices from ampakine-treated rats (TBS+OVX+CX929). Scale bar: 5 μ m. **C)** Blind analyses of phalloidin labeling in the different treatment groups (LFS=15; TBS=19; CX929=10 slices/group) indicated that TBS had a four-fold greater effect on phalloidin labeling in slices from CX929-treated, as compared to vehicle-treated, OVX rats ($p<0.001$). The effect in slices from CX929-treated rats was numerically equivalent to that obtained with 60 min infusions of BDNF (see Fig 1B).

Fig. 3. Ovariectomy results in a pronounced but selective deficit in LTP consolidation

Slices were prepared from OVX rats that had received two weeks of injections with either 17 β -estradiol benzoate (OVX+EB, n=7) or vehicle (OVX+veh, n=7). Field EPSPs were elicited by stimulation of the Schaffer-commissural projections and recorded in the same CA1b lamina used in the phalloidin experiments. **A)** Input (stimulation current) / output (fEPSP amplitude) relationships were not detectably different for the OVX+veh vs. OVX+EB slices. **B)** Transmitter release kinetics, as assessed with paired pulse facilitation (ppf), were also comparable for the two groups of slices. **C)** Initial expression of LTP was comparable in the OVX+veh and OVX+EB slices but potentiation was notably reduced in the former group. Inset, traces collected during baseline (solid line) and 60 min post-TBS (dotted line). Calibration: 1 mV, 5 ms.

Fig. 4. Acute BDNF or in vivo ampakine treatments restore LTP consolidation in OVX rats
A) A 60 min infusion of 2 nM BDNF returned LTP (OVX+BDNF) to levels found in slices prepared from OVX rats that received estrogen replacement (OVX+EB, n=7). Heat-inactivated BDNF (OVX+iBDNF) had no detectable influence on LTP in slices (n=6) prepared from OVX+veh injected rats. Inset, traces collected during baseline (solid) and 60 min post-TBS. Calibration: 1 mV, 5 ms. **B)** LTP consolidation appeared normal in slices prepared from OVX rats that had received four days of twice daily ampakine injections (OVX+CX929, n=11; vehicle, n=7). Initial potentiation was comparable in the ampakine and vehicle groups. Inset, traces collected during baseline (solid) and 60 min post-TBS. Calibration: 1 mV, 5 ms. **C)** In vivo CX929 treatments did not affect input/output curves measured prior to the delivery of TBS. **D)** Similarly, paired-pulse facilitation (ppf) curves were equivalent in slices from OVX rats that had received either CX929 or vehicle treatments.

Fig. 5. In vivo ampakine treatments reproduce the effects of acute BDNF on theta burst responses in slices prepared from OVX rats

The extent to which the composite responses (four fEPSPs) to single theta bursts increase over the course of a theta train was measured. **A)** Grouped data summarize size of burst responses 1-10, expressed as the total area of the response, from OVX rats injected with vehicle (veh, $n=7$) or 17 β -estradiol benzoate (EB, $n=7$). Note that the increases are similar for the vehicle- and EB-treated groups. The inset compares responses elicited by the first (solid arrow) and fourth stimulation (dotted arrow) bursts in slices from OVX rats that did (EB) or did not (veh) receive hormone replacement. Note that the delayed response is substantially larger in both cases. Calibration: 1 mV, 10 ms. **B)** Comparisons of burst response facilitation in slices from OVX rats relative to their controls are summarized for three experiments: *top*) EB vs. vehicle pretreatment in vivo (see panel A); *middle*) OVX slices pretreated with active (aBDNF, $n=6$) vs. inactive BDNF (iBDNF, $n=6$); *bottom*) OVX rats that were given four days of vehicle (veh, $n=7$) or ampakine (CX929, $n=11$) injections.

In each case (left panels), the value for the within-train facilitation of bursts 2-5 in the experimental group (EB; OVX with active BDNF; OVX with in vivo ampakine) is expressed as a percent increase in facilitation relative to the indicated control group. The differences between the BDNF and ampakine curves did not approach statistical significance ($p=0.2$). The mean $\pm$ SEM increase in burst facilitation for bursts 2-5 between experimental and control groups is shown on the right. Note that both acute aBDNF and in vivo ampakine treatment enhanced the facilitation over levels in their corresponding inactive BDNF and in vivo vehicle controls. Insets comparing burst responses 1 and 4 in slices from OVX rats infused with BDNF or slices from CX929-treated animals. Calibration: 1 mV, 10 ms.