Regulation of Extracellular Signal-Regulated Kinase during Long-term Potentation in area CA1 of the Rat Hippocampus *in vivo*

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The extracellular signal-regulated kinase (ERK) cascade can transduce cell-surface signals to the nucleus in post-synaptic neurons during hippocampus-dependent learning and hippocampus-dependent synaptic plasticity, yet, whether the cascade can convey information about stimulus frequency or synaptic modification direction to the nucleus during plasticity events has not been addressed. The objective of the current study was to investigate whether ERK regulation differs as a function of stimulus frequency and in accordance with synaptic modification direction by comparing ERK regulation during LTP in area CA1 of the hippocampus *in vivo* to previous findings for ERK regulation during LTD in area CA1 *in vivo* (Thiels et al., 2002). The ultimate goal was to determine whether ERK functions as a general or as a specific plasticity kinase during synaptic plasticity events in the hippocampus. Using a combination of *in vivo* electrophysiology, pharmacology and Western blot analysis, I demonstrate that: (1) LTP induced by high-frequency stimulation applied to commissural fiber inputs to area CA1 pyramidal cells in the adult hippocampus *in vivo* is accompanied by a rapid yet transient increase in ERK2 activation; (2) blockade of NMDA receptors by MK-801 blocks both LTP induction and the associated increase in ERK2 activation; (3) HFS delivered in the presence of the ERK kinase inhibitor SL327 fails to produce a persistent potentiation; (5) phosphorylation of the transcriptional regulator cAMP response element-binding protein (CREB) is increased after HFS; and (6) inhibition of ERK activation by SL327 blocks this observed increase in pCREB. The similarity of the current findings with previous findings for ERK2 activation and regulation during LTD in area CA1 *in vivo*, suggests that the ERK cascade conveys a general as opposed to

a specific plasticity signal during these two forms of synaptic plasticity in area CA1 *in vivo*. Differences in the coupling of ERK2 activation to CREB phosphorylation between LTP and LTD (Thiels et al., 2002), suggest that other signaling cascades are most likely operative in determining the direction of synaptic modification during bidirectional synaptic plasticity in the hippocampus.

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1. GENERAL INTRODUCTION

1.1. Synaptic plasticity and memory: a brief history.

One prominent feature of learning is that a given experience, however momentary, can yield an enduring memory that long outlasts the experience itself. It is widely believed that the capacity to learn requires the presence of a neural mechanism by which information is encoded, stored and made available for future recall and use. In 1940, the psychologist Donald O. Hebb, borrowing insights from psychology and physiology, suggested that such information encoding is achieved by physiological changes localized to brain synapses. Hebb claimed that when two cells, A and B, which communicate under normal conditions, undergo a period of repeated and concurrent activation, the result is a strengthening of the connection between the two cells, exhibited by a subsequent change in the way cell A excites cell B (Hebb, 1949). The crux of this postulate, often referred to as "Hebb's rule", is that each learning event is accompanied by the brief *associated* activation of two neurons that comprise a synapse, which together, effectively store information in the form of a physiological change at the synapse.

Although Hebb's postulate was attractive, at the time of its introduction, no plausible candidates for a neural mechanism of learning that satisfied his described conditions had been located in the mammalian brain. In 1966, in the context of investigating the physiology of the dentate gyrus in the hippocampus of the adult anethetized rabbit, Terge Lømo observed an artificially induced physiological equivalent of a strengthening in synaptic efficacy (Lømo, 2003). After applying a brief yet repetitive stimulation to perforant path fibers that project from the entorhinal cortex to the dentate gyrus, he recorded, extracellularly, an enduring observable increase in the amplitude of the evoked field potential of post-synaptic dentate granule cells. This finding led to further investigations of the phenomenon in the early 1970's and the

publication of a famous paper that introduced the scientific community to "a long-lasting potentiation of synaptic transmission" (Bliss and Lømo, 1973)--the phenomenon later renamed "long-term potentiation" (LTP).

Long-term potentiation, as it is still defined today, is, roughly, an enduring increase in the strength of the communication between a pre- and post-synaptic neuron that can be induced artificially at specific synapses by particular patterns of afferent stimulation. In electrophysiological terms, if a pre-synaptic neuron is artificially stimulated it will elicit a recordable excitatory post-synaptic potential (EPSP) in the post-synaptic neuron to which it projects. If the pre-synaptic neuron is repeatedly stimulated during a relatively transient period, and in turn, repeatedly takes part in the firing of the post-synaptic neuron, an increase in the amplitude of the post-synaptic EPSP is subsequently observed. This is the physiological signature of LTP, and what was most striking for researchers in the mid to late 1970's, was the resemblance LTP bore to the mechanism that Hebb had postulated to underlie learning. First, the artificial stimulation used to induce LTP is relatively brief, similar, by extension, to the relative length of the duration an experience compared to the duration of a memory of that experience. Second, the effect is long-lasting. LTP was shown to last for up to an hour, post-tetanus, when it was first discovered, and recently, up to one year in the awake rat (Abraham, 2003). Third, LTP requires the rapid and coordinated activation of pre- and post-synaptic neurons that is achieved by specific stimulation parameters, just as Hebb had conceived of learning as requiring the concurrent activation of pre- and post-synaptic neurons. Fourth, LTP amounts to an observable physiological change in the way a presynaptic neuron excites the postsynaptic neuron to which it projects.

The discovery of a Hebbian-like mechanism at a synapse in the hippocampus led to immediate interest in the phenomenon for a variety of other reasons as well. First, by 1973, growing evidence suggested that the hippocampus was an important locus of memory storage. For example, Scoville and Milner had, in 1954, described the severe memory impairments exhibited by the patient H.M. after removal of his hippocampus. Second, the laminar organization of the hippocampus and the physiological properties of hippocampal synapses had been described. Per Anderson had explained in great detail how to record and measure evoked field potential responses in the hippocampus *in vivo* (1966), making these procedures easy for other neuroscientists to employ. Some went on to use Anderson's insights to employ the same techniques to study LTP at other hippocampal synapses, such as Schaffer collateral projections from area CA3 to area CA1 (e.g., Lynch, 1971). Third, a new technique that enabled field potential recordings to be performed in intact hippocampal slices provided a more direct method of recording from hippocampal synapses (Yamamoto and McIlwain 1966a, 1966b). In addition, the finding that hippocampal synapses were also capable of undergoing activity-dependent decreases in synaptic strength (Dunwiddie and Lynch 1978) led to the realization that synapses were potentially more physiologically mutable than Hebb's postulate captured, and, that changes in synaptic strength, depending upon the type of synaptic activation, could be bidirectional. Combined, these findings led to the hippocampus rapidly becoming a model system in which to undertake the study of activity-dependent changes in synaptic strength, which quickly were regarded to be the neural correlate of learning, and perhaps more controversially, memory.

Early research on LTP in the hippocampus yielded another intriguing finding. Hebb's rule required the concurrent activation of pre- and post-synaptic neurons at synapses in order for a given instance of learning to be encoded as a memory. It was discovered that this condition

could be met at specific hippocampal synapses during LTP and LTD by activation of the N-methyl-D-aspartate (NMDA) receptor (Collingridge, 1983 (LTP); Dudek, 1992 (LTD)). The pore of the channel of the NMDA receptor is, under basal conditions, blocked by Mg^{2+} ions that are only released when the post-synaptic neuron is sufficiently depolarized. In turn, NMDA receptor activation requires a combination of (1) glutamate binding and (2) post-synaptic depolarization, making it a likely candidate for the detection of the concurrent activation of pre-synaptic neurons (i.e., transmitter release) and post-synaptic depolarization (i.e., relief of Mg^{2+} block, Ca^{2+} influx).

Combined, the above findings provoked great interest in the plastic physiology of hippocampal synapses as a potential context in which to investigate the mechanisms that underlie learning and memory. Interest in both LTP and LTD has persisted over the past 30 years since the publication of Bliss and Lomo's original study. Of relevance to the current study are investigations into the molecular mechanisms that participate in the maintenance of activity-dependent changes in synaptic strength in the hippocampus. These enduring physiological changes are thought to require structural changes at activated synapses, and in turn one goal of the neurobiology of learning and memory is to outline, from the point of stimulation of afferent fibers to the point of observable physiological changes, the molecular events that enable enduring alterations in synaptic function (Dudai 1989, Sweatt, 1999a; Sweatt 1999b). The hope is that elucidation of these mechanisms will shed light on the molecular mechanisms operative in information encoding (learning) and information storage (memory) at brain synapses.

1.2. LTP and LTD: Induction, Expression and Maintenance

Both LTP and LTD are typically divided into three discrete phases: (1) induction (2) expression and (3) maintenance. First, certain events must occur in order for LTP or LTD to be induced at a given synapse. Second, LTP and LTD are essentially enduring changes in the physiological function of cells that comprise a synapse, and this is thought to require that specific kinds of changes (e.g., structural) be expressed at synapses that have undergone a plasticity inducing event (see below for references). Because these *expressed* changes are thought to maintain observed changes in physiological function, independent mechanisms must become operative to ensure that such functional changes occur and that they are maintained. In turn, LTP and LTD maintenance are thought to require new gene expression, which is thought to be set in motion by the activation of second messenger systems, including protein kinase and protein phosphatase signaling cascades, during the early stages of LTP and LTD.

The induction phases of both LTP and LTD are the first phases that occur shortly after tetanic stimulation. In the context of the current study, I am restricting my attention to NMDA-receptor dependent forms of LTP and LTD at the CA3-CA1 commissural fiber synapse of the rat or mouse hippocampus (Collingridge, 1983 (LTP); Dudek, 1992 (LTD)). The induction of NMDA receptor-dependent forms of LTP and LTD requires release of glutamate from pre-synaptic terminals and activation of post-synaptic alpha-amino-3-hydroxy-5-methyl-4-isoxazole propionate (AMPA) receptors via glutamate binding, which results in an influx of Na+ and the efflux of K+ ions and Cl- ions through the pores of the AMPA receptors (Muller et al., 1988). This results in depolarization of the post-synaptic membrane, which is sufficient to relieve the Mg^{2+} block from the NMDA-receptor, which, once activated, allows the influx of Ca^{2+} ions into the post-synaptic membrane (Collingridge et al., 1992). Glutamate released from pre-synaptic

terminals can also bind to metabotropic glutamate receptors (mGluRs), which are G-protein coupled receptors that, once activated, can trigger the activation of second messenger signaling cascades during the induction phase of LTP or LTD (Bashir et al., 1993a (LTP), 1993b (LTD)).

LTP and LTD are thought to be expressed at synapses in which they have been induced by specific changes in either or both pre- and post-synaptic neurons. Changes in pre-synaptic neurons are thought to include possible changes in (1) probability of transmitter release (Kullman et al., 1992; Isaac et al., 1996 (LTP); Debanne et al., 1996 (LTD)). Changes in post-synaptic neurons are thought to include: (1) functional changes in specific ion channels (e.g., potassium (K+) channels, Yuan et al., 2002), (2) changes in the number of AMPA receptors localized to the synapse (Shi et al., 1999), (3) changes in distribution of glutamate receptors (Carroll et al., 1999 (LTD)) and (4) growth or elimination of dedritic spines (Engert and Bonhoeffer 1999; Zhang and Benson, 2002). Any of these changes could potentially alter the excitability of a given synapse.

Although there is no widespread agreement as to the locus of the expression of LTP and LTD, there is widespread agreement that enduring changes in synaptic strength require changes in gene expression. One way in which changes in gene expression can be achieved is via activation of protein kinase and protein phosphatase signaling cascades. Once activated, these signaling cascades can alter the phosphorylation states of specific transcriptional regulators, which can result in changes in gene expression. These signaling cascades may also target ion channels, cytoskeletal proteins and synaptic proteins, in addition to translational machinery.

1.3. The Extracellular Signal-Regulated Kinase Cascade

One signaling cascade that has been studied extensively in the context of synaptic plasticity and memory is the mitogen activated protein kinase (MAPK)/extracellular signal regulated kinase (ERK) cascade. Mitogen-activated protein kinases (MAPKs) comprise one superfamily of protein kinase signaling cascades that are expressed in both the developing and adult CNS (Thomas and Hunt, 1993). The extracellular-signal regulated kinases, ERK1 (p44 MAPK) and ERK2 (p42 MAPK), are members of a subset of the superfamily of MAPKs (Fiore et al. 1993). The ERK2 isoform, in particular, has been implicated in hippocampus-dependent synaptic plasticity (English and Sweatt, 1996, 1997; Davis et al., 2000; Rosenblum et al., 2002; Thiels et al., 2002) and hippocampus-dependent memory (Atkins et al., 1996; Selcher et al., 1999; Gooney et al., 2002; Boson et al., 2003). The ERK cascade is thought to contribute to synaptic plasticity and memory events in the hippocampus by targeting a diverse array of effectors that include: membrane associated proteins, cytosolic messengers and regulators, cytoskeletal proteins and nuclear transcription factors (for review see Blumer et al., 1994; Sweatt 2001).

The ERK signaling cascade consists of three kinases: Raf, MEK1/2 and ERK1/2 (Pearson et al., 2001). ERK1 and ERK2 are both activated through phosphorylation at threonine (T183/202) and tyrosine (Y185/204) sites by the dual specificity kinase MEK1/2 (Ahn et al., 1992a, 1992b; Crews et al., 1992). Although the events upstream of activation of the ERK cascade are not well understood, several activation pathways have been identified. First, ERK2 can be activated by the binding of growth factors, known as neurotrophins (e.g., BDNF), to specific post-synaptic receptors (e.g., TrkB), which, when activated, result in activation of the membrane associated GTPase ras, which triggers activation of the ERK cascade via

phosphorylation of Raf (Blanquet, 2000; Boulton, 1991). Second, studies suggest that Ca^{2+} entry through either NMDA receptors (Xia et al., 1996; Hardingham, 2001; Poser and Storm, 2001) or L-type VDCCs (Dudek and Fields 2001, 2002) also can result in activation of the ERK cascade. Release of calcium from intracellular stores may also contribute to activation of the ERK cascade. In addition, proteins that associate with NMDA receptors, such as SynGAP, have been shown to regulate activation of ERK2 via activation of RAS (Komiyama et al., 2002). In hippocampal neurons, activation of the ERK cascade is increased primarily through calcium influx in response to NMDA receptor stimulation, although the other sources of calcium and pathways coupled to receptors mentioned above may also contribute as well (Roberson et al., 1999).

As mentioned above, ERK2 activation can potentially impact on the activity of a variety of cytosolic and nuclear effectors. The focus of the current study is, however, restricted in so far as I am primarily interested in determining whether increases in ERK2 activation are accompanied by changes in the phosphorylation of the transcriptional regulator cyclic-AMP response element binding protein (CREB). Increased phosphorylation of CREB has been associated with LTP in the hippocampus (Deisseroth, Bito and Tsien, 1996; Bito et al., 1996). Decreased phosphorylation of CREB, on the other hand, has been associated with LTD.

ERK2 is thought to contribute to CRE-driven gene expression by activation of an intermediate cytosolic signaling protein, ribosomal S6 kinase (RSK), which enters the nucleus and phosphorylates directly the transcriptional regulator, cyclic-AMP response element (CRE) binding protein (CREB) at serine residue 133 (Ser-133) (Xing 1998; Frodin and Gammeltoft, 1999; Wang et al., 2003). Once phosphorylated, CREB recruits CREB binding protein (CBP) and can then drive cyclic AMP response element (CRE-) dependent gene expression. Examples

of immediate early genes that are expressed include c-fos and zif268 (Impey et al., 1996). Once expressed, these IEGs can regulate the expression of specific delayed-response genes, including growth factors that can potentially contribute to structural changes at the synapse (Murphy and Segal, 1997; Segal et al., 1998). Both *in vitro* (Impey et al., 1998; Kanterwicz et al., 2000; Ying 2002) and *in vivo* (Davis et al. 2002) electrophysiological studies have shown that increased ERK activation is accompanied by increased CREB phosphorylation and/or CRE-driven gene expression during LTP in the hippocampus. Another study demonstrated that increases in ERK are not always positively correlated with increases in CREB. More specifically, LTD in area CA1 of the hippocampus *in vivo* was shown to be accompanied by an increase in ERK2 activation, but a decrease below baseline levels in phosphorylated CREB (Thiels et al. 2002).

ERK2 can also contribute to SRE- driven gene transcription by translocating into the nucleus and directly phosphorylating the ternary complex factor Elk-1 at its serine residue 383 (Ser383) (Whitmarsh, 1995). Once phosphorylated, Elk-1 can regulate the expression of specific delayed-response genes, which are growth factors that can potentially contribute to structural changes at the synapse. Both *in vivo* LTP (Davis et al. 2002) and LTD (Thiels et al. 2002) studies undertaken at hippocampal synapses have shown that ERK2 activation is coupled to increased phosphorylation of Elk-1 during these plasticity events.

All of the aforementioned electrophysiological and biochemical studies were concerned with investigating the kinetics of ERK2 activation, and, in some cases, the kinetics of downstream effectors of the ERK cascade, during activity-induced synaptic plasticity events in the hippocampus. The primary focus of my master's thesis is similarly an investigation of the kinetics of the ERK2 signal during an activity-induced form of synaptic plasticity in the hippocampus. The methodological approach of identifying the role that specific enzymes play

in synaptic plasticity events by investigating the kinetics of these enzymes during such events is widespread in the field of synaptic plasticity primarily because a number of reliable biochemical techniques for studying enzyme kinetics are available. It should be noted, however, that the investigation of parameters other than enzyme kinetics is equally relevant to determining what role(s) a given enzyme plays in plasticity events. These other parameters of activity include, for example, the location of the activity of an enzyme within a cell, the movement of an enzyme from one cellular compartment to another, and the association and/or interaction of an enzyme with other molecules.

In this light, the aim of the current study is to make a small contribution to a vast body of research devoted to identifying the role(s) of the ERK cascade in synaptic plasticity events. Since ERK2 has the potential to be activated by a variety of pathways and to target a diverse array of downstream effectors during plasticity events, the role of ERK in synaptic plasticity is most likely quite complex, and will require the convergence of a variety of different approaches to grasp this complexity. One possible approach, which is the *modus operandi* of the current study, is to compare the kinetics of the ERK signal during two opposite forms of synaptic modification.

1.4. ERK2: A general or specific plasticity kinase?

Ca^{2+} entry through post-synaptic NMDA receptors during the induction phases of LTP and LTD is thought to function as a *specific* plasticity signal (for mini-review see Lisman, 2001). LTP and LTD are induced by either high-frequency or low-frequency stimulation, respectively. Differences in the frequency patterns of synaptic activation can impact directly on the amount of glutamate released from pre-synaptic neurons, and affect the subsequent probability of NMDA

receptor activation, which directly impacts on the total amount of Ca^{2+} that enters into post-synaptic neurons. During periods of high-frequency stimulation, a large amount of glutamate is thought to be released from pre-synaptic terminals and to result in prolonged activation of post-synaptic NMDA receptors and a large amount of Ca^{2+} influx. In contrast, during periods of low-frequency stimulation, a relatively small amount of glutamate is thought to be released from pre-synaptic terminals, which is thought to result in a shorter activation of post-synaptic NMDA receptors and comparatively less Ca^{2+} influx. In turn, a symmetry has been proposed to exist between stimulation frequency and the amount of postsynaptic Ca^{2+} influx. Moreover, this symmetry is thought to be preserved in the relation between the Ca^{2+} signal and the subsequent direction of synaptic modification. Less Ca^{2+} influx is thought to result in LTD; more Ca^{2+} influx is thought to result in LTP. Support for this symmetry arises in particular from studies conducted by Cummings and colleagues (1996), Yang and colleagues (1998) and Cho and colleagues (2001).

Ca^{2+} influx through NMDA receptors is the primary mechanism thought to trigger activation of the ERK cascade during plasticity events. Therefore, it is possible that phosphorylation of ERK2 might be sensitive to differences in Ca^{2+} influx, in so far as a large rise in intracellular Ca^{2+} could result in a significant increase in ERK2 activation, whereas a smaller rise in intracellular Ca^{2+} could result in a less significant increase in ERK2 activation. If this were the case, then ERK2 activation might be just as specific a signal during synaptic plasticity events as the Ca^{2+} signal is thought to be. However, if the profile of ERK2 activation were similar following both high-frequency and low-frequency stimulation, then this would suggest that ERK2 activation itself does not convey specific information about stimulation frequency nor the polarity of the subsequent synaptic change.

The events upstream of ERK2 activation are still under investigation, and whether the amount of Ca^{2+} influx during bidirectional plasticity events in the hippocampus impacts differentially on ERK2 activation is not known. However, I find it worthwhile to use the Ca^{2+} signal as a paradigmatic example of a common way in which plasticity researchers differentiate between general and specific plasticity kinases. Typically, increases in either the amount of intracellular enzymes and ions, or increases in their activity are thought to accompany LTP, and smaller increases or decreases with respect to these parameters are thought to accompany LTD. So, a symmetry has been proposed to exist between the amount or extent of enzymatic activity and the direction of synaptic modification.

The primary aim of the current study was to determine whether activation of ERK2 during bidirectional synaptic plasticity events in the hippocampus is affected by differences in stimulation frequency in the same way that rises in intracellular calcium in post-synaptic neurons are thought to be affected by differences in stimulation frequency. The rationale for undertaking such an investigation was that results from such an analysis might yield insight into whether ERK2 functions as a general or specific plasticity kinase during plasticity events in the hippocampus.

2. ERK: One Signaling Cascade, Two Forms of Synaptic Plasticity

2.1 Introduction

Activity-dependent changes in synaptic strength at hippocampal synapses can be bidirectional: whereas high-frequency stimulation applied to afferent fibers induces a persistent increase in synaptic strength (LTP), low-frequency stimulation induces instead an enduring decrease in synaptic strength (LTD). Such differences in the direction of synaptic modification

are thought to result in part from the impact of stimulation frequency on calcium entry into post-synaptic neurons (Cummings et al., 1996). Significant increases in intracellular calcium concentration have been associated with long-term increases in synaptic strength; slight increases in intracellular calcium concentration have been associated with long-term decreases in synaptic strength. In turn, the post-tetanus calcium signal is thought to be a specific plasticity signal in so far as it is predictive of the subsequent direction of synaptic modification (Cummings, et al., 1996). However, the calcium-dependent mechanisms, through which the signal is ultimately translated into directional changes at the synapse, are still under investigation. Of particular interest to this area of research are specific protein kinase signaling cascades that are directly activated by Ca^{2+} and poised to convey information from the cytosol to the nucleus.

One pathway through which calcium entry can impact on downstream changes at the synapse is the mitogen-activated protein kinase/extracellular signal-regulated kinase (ERK) cascade. The (ERK) cascade has been widely studied in the context of synaptic plasticity due to its capacity to transduce cell-surface signals, such as receptor or channel activation, to the nucleus where it can impact on the activity of specific regulators of gene transcription such as cyclic-AMP response element binding protein (CREB) and Elk-1 (Impey et al., 1998; Davis et al., 2000; Thiels et al., 2002). Calcium entry through either NMDA receptors or L-type voltage-dependent calcium channels during plasticity events is thought to result in the calcium-dependent phosphorylation of the membrane associated GTPase ras, which triggers activation of the ERK cascade (Ida et al., 2001). Previous studies have shown that ERK is activated during LTP induction in area CA1 of the hippocampus *in vitro*, and that it is necessary for maintenance of this form of LTP (English and Sweatt, 1996, 1997). In this context, ERK phosphorylation was shown to impact on phosphorylation of CREB (Impey et al., 1998). Similarly, it has been shown

that ERK is activated during and necessary for LTP in the dentate gyrus *in vivo*, where it impacts on phosphorylation of both CREB and Elk-1 (Davis et al., 2000). ERK is also required for LTD in area CA1 *in vivo*, where it impacts on phosphorylation of Elk-1 but is not coupled to CREB phosphorylation (Thiels et al., 2002). In each of these contexts ERK2 activation was shown to be NMDA-receptor dependent.

Although the type of information carried by the ERK2 signal is not known, its potential to be activated and influenced by intracellular calcium concentrations in addition to other factors, leaves open the possibility that ERK2 activation may differ as a function of stimulus frequency and may predict the polarity of synaptic modification during plasticity events. In a recent study, a paired-pulse stimulation (PPS) protocol was employed to induce LTD in area CA1 of the hippocampus *in vivo* (Thiels et al., 2002). In this study, ERK2 activation was shown to be increased at 5 and 15 min after LTD induction, and to return to baseline levels thereafter. This form of LTD and the accompanying increase in ERK2 activation were found to be NMDA receptor-dependent. In addition, increased ERK2 activation was not correlated with an increase in CREB phosphorylation during LTD. The findings from this LTD study offered a novel opportunity to investigate whether ERK2 activation was similar or different during LTP in area CA1 *in vivo*.

In the current study, I induced LTP in area CA1 of the hippocampus *in vivo*, employing a stimulation protocol that differed from the paired-pulse stimulation protocol that had been previously employed to induce LTD at this same synaptic location (Thiels et al. 2002) only in terms of stimulus frequency pattern. First, I investigated the post-tetanus profile of ERK2 activation 5, 15, and 35 min after LTP induction in area CA1 *in vivo*, in order to establish that ERK2 was activated during this form of synaptic plasticity, and to determine whether the profile

of ERK2 activation during LTP was similar to or different from the ERK2 activation profile during LTD. I found that ERK2 activation was increased significantly 5 and 15 min after LTP induction, but returned to near baseline levels within 35 min after LTP induction. This activation profile for ERK2 was very similar to that previously found to accompany LTD (Thiels et al. 2002).

In turn, I sought to determine if the route of increased ERK2 activation differed between LTP and LTD, in order to rule out that differences in the route of increased ERK2 activation were responsible for differences in the direction of synaptic modification. Since Ca^{2+} entry through NMDA receptors is thought to be the primary pathway by which ERK2 is activated in hippocampal neurons (Dudek and Fields, 2001), I induced LTP in the presence of the NMDA-receptor antagonist MK-801. I observed not only that LTP induction was blocked by this manipulation, but also that the increase in ERK2 activation observed to accompany LTP in area CA1 was also blocked. These findings closely resembled previous findings for the dependence of LTD induction and the LTD-associated increase in ERK2 activation on NMDA receptor activation (Thiels et al., 2002).

Next, in order to assess whether the observed increase in ERK2 phosphorylation was necessary for the form of LTP under study, I induced LTP in the presence of SL327, which has been shown to inhibit activation of ERK2 via inhibition of the kinase upstream from ERK2 activation, MEK. I found that SL327 blocked the maintenance of robust LTP in area CA1 *in vivo*, which suggested that ERK2 activation is necessary for LTP maintenance.

Having established that ERK2 activation is necessary for LTP in area CA1 *in vivo*, I then wanted to determine if the phosphorylation of one nuclear effector of the ERK cascade, CREB, was altered during LTP. ERK2 activation during LTD was shown to be accompanied by a

dephosphorylation of CREB (Thiels, et al., 2002), and in turn, I wanted to investigate the phosphorylation status of CREB during LTP to determine if it was similar or different. I found that CREB phosphorylation was increased 15 and 35 min after LTP induction in area CA1 *in vivo*.

Finally, in order to assess whether the observed increase in CREB phosphorylation was dependent upon prior ERK2 phosphorylation, I induced LTP in the presence of SL327 and investigated CREB phosphorylation 15 min after LTP induction in area CA1. I found that the increase in CREB phosphorylation observed to accompany LTP induction was essentially blocked in the presence of SL327, which suggests that the observed increase in CREB phosophorylation during LTP in area CA1 *in vivo* is ERK2 activation-dependent.

In conclusion, I sought to compare the results of the current study to previous findings for activation and regulation of ERK2 during LTD in area CA1 *in vivo* (Thiels et al. 2002). This comparison suggests that ERK2 functions as a general rather than a specific plasticity kinase during bidirectional synaptic plasticity events in area CA1 of the hippocampus *in vivo*, yet differences between LTP and LTD emerge with respect to the coupling of the ERK cascade to CREB. Differences in the coupling of the ERK signal to CREB during LTP and LTD suggest that other signaling cascades that become operative during bidirectional synaptic plasticity events in the hippocampus most likely determine the direction of synaptic modification.

2.2 Materials and Methods

Electrophysiology. Adult male rats (Sprague Dawley, 250-360 g; Hilltop, Scottsdale, PA) were anesthetized with chloral hydrate (400 mg/kg ip) and small holes were drilled into the skull

overlaying the cortex above the hippocampus. Bipolar metal stimulating electrodes (insulated except for 150-200 μm at the tip) were lowered into dorsal area CA3 of the left hippocampus and a glass recording pipette (.8-1.1 MΩ) was lowered into either *stratum pyramidale* or *stratum radiatum* of dorsal area CA1 of the right hippocampus. The animals were maintained under anesthesia with supplemental injections of chloral hydrate (60 mg/kg) via a tail vein cannula. Their body temperature was maintained at 37ºC with the aid of a heating pad and monitored continuously with a rectal thermometer. All procedures were in compliance with and approved by the Institutional Animal Care and Use Committee, University of Pittsburgh. Unless otherwise indicated, all electrophysiological procedures employed in this study were identical to those described previously for paired-pulse stimulation-induced long-term depression (PPS-LTD) (Thiels et al., 1994), with the exception of the stimulation paradigm used to induce high-frequency stimulation-induced long-term potentiation (HFS-LTP). Field responses evoked by test pulses (20-250 μA, 100 μsec duration) delivered to the dorsal commissural pathway were recorded in either *stratum pyramidale* or *stratum radiatum* of area CA1 of the right dorsal hippocampus. The stimulation intensity for test pulses was set to produce a response magnitude ~30% of the maximum magnitude as determined at the beginning of the experiment. Series of 10 test pulses (0.1 Hz) were delivered at 5 min intervals before and after LTP-inducing HFS. After stable responding to test pulses was established, a HFS that consisted of four trains of 100 pulses delivered at 100 Hz with an intertrain interval of 130 sec was delivered to the dorsal commissural pathway. Unless indicated otherwise, HFS was delivered using a stimulation intensity that produced an area CA1 population spike amplitude ~60% of the maximum amplitude, as determined at the beginning of the experiment. Responding to test pulses was monitored for up to 90 min after HFS. In some experiments, animals were injected intraperitoneally with either

the ERK kinase (MEK) inhibitor SL327 [50 mg/kg, dissolved in 100% dimethylsulfoxide (DMSO); DuPont, Wilmington, DE] or vehicle solution [100% DMSO 1 ml/kg; Sigma], or intravenously with the NMDA-receptor antagonist MK-801 [1 mg/kg, dissolved in 100% NaCl], 90 minutes before the onset of baseline recording. Recorded waveforms were amplified, filtered (0.1-10 kHz), digitized (10 kHz), and stored on computer disk for later analysis of the amplitude of the evoked area CA1 population spike or the initial slope (1.0 msec after onset) of the evoked CA1 population excitatory postsynaptic potential (EPSP).

For purposes of biochemical analyses, animals were killed either before or after HFS, and their right hippocampus was dissected out in the presence of ice cold artificial CSF (in mM: 124 NaCl, 5 KCl, 1.25 NaH_2PO_4, 26 $NaHCO_3$, 1.5 $MgCl_2$ and 2.5 $CaCl_2$). A block of area CA1 (~1 mm^3) was excised from the dorsal and the ventral portion of the hippocampus, and each was placed in individual, coded vials on dry ice, which then were stored at -80º C until biochemical analysis.

Biochemistry, tissue preparation, and Western blotting. The microdissected dorsal and ventral area CA1 tissue samples were placed in ice-cold buffer A (10 mM HEPES-OH, pH 7.9, 10 mM KCL, 1.5 mM $MgCl_2$, 1 mM DTT, 1 mM NaF, 1 mM sodium orthovanadate, 2 mM sodium pyrophosphate, 1 mM PMSF, 10 uM benzamidine, 1 µg/ml leupeptin, 1 µg/ml aprotinin, and 1 µg/ml pepstatin) and incubated on ice for 20 min. Cells were disrupted with a dounce homogenizer (~20-40 strokes) until nuclei were free of cytoskeletal attachments as detected microscopically with phase-contrast examination and then centrifuged at 14,000 rpm at 4º C for 2 min. The supernatant was decanted, saved, and used as soluble fraction, and the nuclear pellet was resuspended in 30 µl of ice-cold buffer B (10 mM HEPES, pH 7.0, 450 mM NaCl, 5 mM EDTA, 0.05% SDS, 1% Triton X-100, 2 mM DTT, 1 mM NaF, 1 mM sodium orthovanadate, 2

mM sodium pyrophosphate, 1 mM PMSF, 10 μM benzamidine, 1 μg/ml leupeptin, 1 μg/ml aprotinin, and 1 μg/ml pepstatin) and incubated for 45 min on ice with gentle rocking, followed by centrifugation at 14,000 rpm at 4º C for 10 min. The resulting supernatant was decanted, saved, and used as nuclear extract.

Protein concentrations in the respective fractions were determined according to the method of Bradford (Bradford, 1976) using bovine serum albumin (BSA) as standard. Equivalent amounts of protein for each sample were resolved by 10% SDS-PAGE, blotted electrophoretically onto Immobilon membranes (Millipore, Bedford, MA), blocked for 30 min with B-TTBS [50 mM Tris-HCL, pH 7.5-8.0, 150 mM NaCl, 0.1% Tween 20, and 3% BSA (phosphoERK) or 5% BSA (phosphoCREB)], and then incubated in B-TTBS with an antibody that recognizes either Thr202/183- and Tyr204/185-phosphorylated ERK1/2 (1:5000; Promega, Madison, WI) or Ser133-phosphorylated CREB (1:500; Cell Signaling Technology, Beverly, MA) or NUMA (1:250; BD Biosciences Transduction Laboratories, Lexington, KY). After incubation with the primary antibody, the membrane was washed four times with TTBS buffer (50 mM Tris-HCL, pH 7.5-8.0, 150 mM NaCl, 0.1% Tween 20), the blots were exposed to a donkey anti-rabbit IgG peroxidase-linked antibody (Amersham Biosciences, Piscataway, NJ), the membrane was washed four times with TTBS and developed using an enhanced chemiluminescence reagent (DuPont NEN, Boston, MA), and the films were analyzed densitometrically using NIH Imaging software. The membranes were then stripped in a heated (50º C) bath containing 50 mL activated stripping buffer (7.5 g Tris, 100 mL SDS 20%, 990 mL dH_2O, and 315 μL mercaptoethanol), blocked for 30 min with B-TTBS [50 mM Tris-HCL, pH 7.5-8.0, 150 mM NaCl, 0.1% Tween 20, and 3% BSA (phosphoERK) or 5% BSA (phosphoCREB and NUMA)], and then reprobed in B-TTBS with an antibody raised against

either total ERK (1:5000; Promega, Madison, WI) or total CREB (1:1000; Cell Signaling Technology). After incubation with the primary antibody, the membrane was washed four times with TTBS buffer (50 mM Tris-HCL, pH 7.5-8.0, 150 mM NaCl, 0.1% Tween 20), the blots were exposed to a donkey anti-rabbit IgG peroxidase-linked antibody (Amersham Biosciences, Piscataway, NJ), the membrane was washed four times with TTBS and developed using an enhanced chemiluminescence reagent (DuPont NEN, Boston, MA), and the films were analyzed densitometrically using NIH Imaging software. Statistical analyses of data obtained from biochemical analysis were conducted using a Student's *t* test.

2.3. Results

2.3.1. ERK2 phosphorylation is increased after LTP-inducing HFS in area CA1 of the adult hippocampus *in vivo*

I induced LTP in area CA1 of adult male rats, using a protocol that consisted of four trains of high-frequency stimulation applied to dorsal commissural pathway fibers that project contralaterally from dorsal area CA3 to dorsal area CA1 but not ventral area CA1 (Laurberg, 1979; Ishizuka et al., 1990). This HFS protocol produced a rapid and persistent increase in both the amplitude of the CA1 population spike (Fig. 1a) and the initial slope of the CA1 population EPSP (Fig. 1b), as determined by test pulses delivered to the commissural fibers before and after HFS.

In order to determine whether this type of LTP is accompanied by a change in ERK2 activation over a specified time course, I removed tissue from dorsal area CA1 near the recording site (experimental sample) and from ventral area CA1 from the same animal (control sample). Tissue blocks were collected either immediately before HFS (baseline) or either 5, 15, or 35 min after termination of HFS. These samples were then homogenized and separated into soluble

fractions and nuclear extracts. The soluble fractions were resolved by gel electrophoresis and blotted electrophorically to Immobilon membranes. Membranes containing the soluble fraction of the homogenized tissue samples were probed with an antibody that selectively recognizes ERK1/2 when phosphorylated at both Thr-202/183 and Tyr-204/185 (pERK). These membranes were then stripped and reprobed with an antibody that selectively recognizes total ERK (tERK). In Figure 2, antibody binding to pERK2 in dorsal CA1 samples is expressed as percent of antibody binding to pERK2 in ventral CA1 and normalized for differences in protein level (tERK) between dorsal and ventral samples. Figure 2 shows that, under basal conditions, pERK immunoreactivity normalized to tERK immunoreactivity was comparable in dorsal and ventral area CA1 (baseline: Student's *t* test for dorsal/ventral ratios vs. 1.0, $p > 0.5$, $n = 6$). In contrast, within 5 min after LTP-inducing stimulation, normalized pERK2 immunoreactivity in dorsal area CA1 was increased two-fold above levels in ventral area CA1, i.e., above control levels (5 min group: dorsal/ventral ratios vs. 1.0, $p < 0.01$, $n = 6$). The increase in ERK2 phosphorylation persisted for at least 15 min (15 min group: dorsal/ventral ratios vs. 1.0, $p < 0.02$, $n = 9$), but dissipated thereafter, so that 35 min after LTP-inducing stimulation, normalized pERK2 immunoreactivity in dorsal area CA1 no longer differed from control levels (35 min group: dorsal/ventral ratios vs. 1.0, $p > 0.2$, $n = 10$).

It was shown previously that LTP induction in area CA1 *in vitro* and ERK2 activation that accompanies it require activation of NMDA-receptors (English and Sweatt, 1996). Since the increase that I observed in the ERK2 signal after HFS could potentially have resulted from repetitive stimulation rather than the prior induction of LTP in area CA1, I wanted to test whether the observed increase in ERK2 activation was a direct result of prior LTP-induction mediated by NMDA-receptors. I therefore delivered four trains of HFS to the commissural

fibers in the presence of the noncompetitive NMDA receptor antagonist MK-801 (1mg/kg, iv), which produces a use-dependent blockade of the NMDA receptor-coupled channel (Morgan and Teyler, 1999; Abraham and Mason, 1988). As expected based on previous reports examining the NMDA receptor dependence of LTP at this synapse (Collingridge et al., 1983; Thiels et al. 1992), Figure 3a shows that LTP induction was blocked when HFS was delivered in the presence of MK-801, as determined by measurement of the initial slope of the CA1 population EPSP. In conjunction with this observed lack of LTP, Figure 3b shows that delivery of HFS in the presence of MK-801 was not accompanied by an increase in pERK immunoreactivity normalized to tERK immunoreactivity 15 min after HFS (dorsal/ventral ratios vs. 1.0; $p > 0.5$, $n = 5$). These findings suggest that HFS alone is not sufficient to enhance ERK2 phosphorylation and, in turn, that induction of NMDA receptor-dependent LTP is necessary for the increase in ERK2 activation observed to follow HFS.

2.3.2. Inhibition of ERK2 activation blocks maintenance of HFS-LTP in area CA1 of the adult hippocampus *in vivo*

It is possible that the observed increase in the ERK2 signal, while dependent on prior induction of LTP, is not necessary for LTP. I therefore tested whether inhibition of ERK activation interferes with the establishment of LTP by delivering HFS in the presence of an inhibitor of MEK, the kinase directly responsible for ERK1/2 activation. I administered the MEK inhibitor SL327 intraperitoneally 60-90 min before HFS at a dose of 100 mg/kg. Intraperitoneal administration of SL327 at this dosage was previously found to prevent ERK1/2 activation in CA1 (Favata et al., 1998; Atkins et al., 1998; Thiels et al., 2002). Figure 6a shows that the dosage of SL327 used in the current study effectively blocked ERK2 phosphorylation. I found that HFS delivered in the presence of SL327 resulted in an immediate increase in the

amplitude of the evoked area CA1 population spike that was nearly identical to that observed in (DMSO)-injected controls (Figure 4). However, in comparison to control animals in which the increase in the evoked response was maintained for up to 90 min after termination of HFS, in animals injected with SL327, the initial increase in the evoked response decayed gradually over time and returned close to baseline levels within 90 min after termination of HFS. These findings dovetail with observations made in area CA1 *in vitro* and the dentate gyrus *in vivo* that high-frequency tetanic stimulation delivered in the presence of MEK inhibitors results in a gradually decaying LTP (English and Sweatt, 1997; Coogen et al., 1999; Davis et al., 2000). The current findings indicate that ERK activation plays a specific role in LTP-maintenance rather than in LTP-induction because the increase in the evoked response was not abolished immediately after HFS but declined gradually after termination of LTP-inducing stimulation. The finding that the early phase of LTP maintenance was relatively unaffected by the inhibitor suggests LTP immediately after its induction is maintained by other ERK independent mechanisms.

2.3.3. CREB phosphorylation is increased during LTP in the adult hippocampus *in vivo*

It has been demonstrated in hippocampal neurons that ERK activation during LTP can result in phosphorylation of the transcriptional regulator cyclic-AMP response element binding protein (CREB) (Impey et al., 1996, 1998a; Davis et al., 2000; Schulz et al., 1999). On the other hand, ERK activation during LTD was shown to not couple to CREB phosphorylation (Thiels et al., 2002). Interestingly, the profile of ERK2 activation after LTD in the aforementioned study resembled closely the profile of ERK2 activation after LTP induction in the present study. Therefore, to determine whether ERK activation and CREB phosphorylation are coupled during

LTP in area CA1 *in vivo*, I prepared nuclear extracts from tissue of dorsal (experimental sample) and ventral (control sample) area CA1 extracted either 5 min after baseline recording or either 15 or 35 min after the induction of HFS-LTP and probed the extracts with an antibody that recognizes SER-133 (S-133) phosphorylated CREB. I then reprobed these extracts with an antibody that recognizes total CREB (tCREB) and an antibody that recognizes NUMA. In cases in which the signal for tCREB could not be read and scored with confidence, I compared phosphorylated CREB to NUMA. Since this method is essentially equivalent to comparing the signal for pCREB to CREB, it provided a reliable means to detect changes in pCREB. Figure 5 shows that no difference in pCREB immunoreactivity normalized to tCREB or NUMA immunoreactivity was observed between dorsal and ventral area CA1 nuclear extracts before HFS (dorsal ventral ratios vs. 1: $p > 0.5$, $n = 5$), but pCREB immunoreactivity normalized to tCREB or NUMA immunoreactivity was increased significantly relative to controls, 15 min after HFS ($.05 < p < .1$, $n = 4$). This observed increase in pCREB persisted for up to 35 min after HFS ($.05 < p < .1$, $n = 4$). These findings are in stark contrast to findings from the aforementioned study that ERK2 activation is accompanied by a dephosphorylation of CREB (Thiels et al., 2002). In turn, interestingly, the current findings in combination with these previous findings suggest that the same ERK2 signal can be accompanied by either increases or decreases in phosphorylation of CREB in area CA1 of the hippocampus *in vivo*.

2.3.4. Increase in CREB phosphorylation during HFS-induced LTP in area CA1 *in vivo* is ERK activation-dependent

Since it is possible that the increase in CREB phosphorylation that I observed to follow HFS was not directly coupled to ERK2 activation, as a second step towards determining whether ERK2 activation is coupled to CREB phosphorylation during LTP in area CA1 *in vivo*, I delivered four

trains of HFS to the commissural fibers in the presence of the MEK inhibitor SL327 (100 mg/kg, ip). Figure 6a shows that SL327 blocked effectively ERK2 activation (baseline, n = 12; SL327, n = 5) Figure 6b shows that the increase in pCREB immunoreactivity normalized to tCREB or NUMA immunoreactivity in dorsal compared to ventral area CA1 shown to accompany this form of LTP 15 min after termination of HFS was abolished in the presence of SL327 (Figure 6, $p > 0.5$, n = 5). CREB phosphorylation in these animals was instead comparable to that of baseline animals. These findings suggest that the increase in pCREB shown to follow HFS and accompany LTP in area CA1 *in vivo* is ERK activation-dependent.

3 DISCUSSION

Numerous studies have revealed that the ERK cascade plays a critical role in hippocampus-dependent learning and hippocampus-dependent synaptic plasticity (for review, see Thiels and Klann, 2001; Adams and Sweatt, 2002). During activity-dependent synaptic plasticity in the hippocampus, ERK2 activation is thought to be triggered by receptor activation and/or by receptor or channel mediated post-synaptic calcium entry (Xia et al., 1996; Hardingham, 2001; Poser and Storm, 2001). Once activated, ERK2 can target a diverse array of downstream effectors including nuclear transcription factors, membrane-associated proteins, cytosolic messengers, and cytoskeletal proteins. ERK2 activation has been shown to be necessary for LTP in area CA1 *in vitro* (English and Sweatt, 1996) and the dentate gyrus *in vivo* (Dudek et al., 2000) as well as LTD in area CA1 *in vivo* (Thiels et al., 2002). Despite the necessity of ERK2 activation for the aforementioned bidirectional modifications in synaptic function at hippocampal synapses, it is not known whether regulation of the ERK cascade differs between LTP and LTD.

Several studies have suggested that the amount of Ca^{2+} influx through the pore of the NMDA-receptor channel during activity-induced plasticity events varies as a function of stimulus frequency and that, in turn, the Ca^{2+} signal itself carries *specific* information about stimulus frequency and is predictive of the subsequent direction of synaptic modification. The potential sensitivity of activation of the ERK cascade to differences in the amount of Ca^{2+} influx raised a question regarding whether activation of the cascade might differ as a function of stimulus frequency, and whether the signal might carry specific information about the subsequent direction of synaptic modification. In order to address this issue in the current study, I investigated regulation of the ERK cascade during LTP in order to compare current findings to previously published results for LTD in area CA1 of the hippocampus *in vivo* (Thiels, et al., 2002). The fact that ERK2 activation had been shown previously to be necessary for LTD in area CA1 *in vivo,* and that the profile of ERK2 activation 5, 15 and 35 min after paired-pulse stimulation (PPS) had been investigated previously, provided a unique opportunity to investigate whether activation of ERK2 differed during LTD and LTP. Furthermore, the fact that the increase in ERK2 activation during LTD had been shown to be NMDA-receptor-dependent, and the transcription factor CREB was found to be dephosphorylated during LTD, offered further points of comparison for investigating events upstream and downstream of ERK2 activation between LTP and LTD.

First, I demonstrated that NMDA-receptor dependent LTP in area CA1 of the adult hippocampus *in vivo* is accompanied by a significant increase in ERK2 phosphorylation 5 min and 15 min after HFS, which returns to near baseline levels within 35 min after HFS. These findings are consistent with previous findings for ERK2 activation during LTP in area CA1 *in vitro* (English and Sweatt, 1996, 1997) and the dentate gyrus *in vivo* (Davis et al., 1996). This

suggests that ERK2 activation during LTP is similar in different hippocampal preparations and in different hippocampal areas. More interestingly, with respect to the specific aim of the current study to compare ERK2 activation during LTP and LTD in area CA1 *in vivo* (Thiels et al., 2002), the current findings for ERK2 activation during LTP are very similar to previous findings for ERK2 activation during LTD in area CA1 *in vivo* (Figure 7). These combined findings suggest that, at least with respect to these two forms of synaptic plasticity, the ERK signal does not carry specific information about stimulus frequency nor is it indicative of the direction of synaptic modification that will follow a given pattern of synaptic activation. I will elaborate on this interpretation of the current findings in more detail, at a later point in this section.

Next, I demonstrated that ERK activation is necessary for the maintenance of LTP in area CA1 *in vivo*. This finding is similar to *in vitro* and *in vivo* findings for HFS-induced LTP in both area CA1 (Rosenblum et al., 2002) and the dentate gyrus (Davis et al., 2002). Combined, these findings suggest that LTP, immediately after its induction, is maintained by ERK-independent mechanisms. ERK2 activation was also shown to be required for the persistence of LTD in area CA1 *in vivo* (Thiels et al., 2002), however, in the case of LTD, ERK2 activation appears to be necessary shortly after LTD induction, suggesting that although the maintenance of HFS-LTP and PPS-LTD requires ERK2 activation, the two forms of plasticity exhibit a differential dependence on ERK2 activation. This differential dependence may be due, in part, to differences in the targeting of downstream effectors of the ERK cascade during LTP compared to LTD. Since the ERK cascade can target a multitude of different effectors including cytosolic messengers and regulators, one suggestion is that during LTD in contrast to LTP, ERK may participate in some local mRNA synthesis event shortly after LTD induction. Future studies could be directed at determining if differences in early local mRNA synthesis exist between LTP

and LTD. Such studies could be combined with studies geared to determine the effect of inhibition of ERK activation on local mRNA synthesis during these two forms of plasticity. However, the aim of current studies is to investigate further the effects of inhibition of ERK activation on LTP by performing *in vivo* recordings in *stratum radiatum* in the presence of the MEK inhibitor SL327 before and after HFS. Such studies will enable us to determine more conclusively if the change in synaptic strength observed to follow HFS is ERK activation dependent.

Third, I demonstrated that phosphorylation of the transcriptional regulator CREB is increased during LTP and that this increase is ERK2 activation-dependent. This finding is in accordance with previous findings that CREB phosphorylation is increased during LTP in area CA1 *in vitro* (Impey et al., 1996) and the dentate gyrus *in vivo* (Davis et al., 2000), and that this increase is ERK2 activation-dependent. Interestingly, in contrast to the current findings for increased CREB phosphorylation during LTP, ERK2 activation was shown to be accompanied by a decrease in CREB phosphorylation during LTD in area CA1 *in vivo* (Thiels et al., 2002). One suggestion that has been offered for this difference in CREB phosphorylation is that certain protein phosphatases, protein phosphatases 1 and 2A (PP1 and PP2A) (Thiels et al., 2002) may become activated during LTD and disrupt the positive coupling of ERK to CREB through dephosphorylation of ribosomal S6 protein kinase (RSK), the intermediary kinase between ERK and CREB. Another possibility is that these phosphatases may directly dephosphorylate CREB during LTD. The activity of PP1 and PP2A was shown to be increased during PPS-LTD (Thiels et al., 2000). In turn, it would be interesting to investigate if differences in PP1 and PP2A activity exist between PPS-LTD and HFS-LTP, and to determine the effect of inhibiting these

phosphatases during PPS-LTD on the phosphorylation status of CREB. This is one focus of current research being conducted in the lab.

Differences in the phosphorylation status of CREB between LTP and LTD may also occur as a result of differences in the activation of other signaling cascades that act either in parallel or in concert with ERK to impact on the phosphorylation of nuclear effectors downstream. For example PKA, PKC and CAMKII can impact on ERK2 regulation and potentially affect its targeting of downstream effectors such as CREB (Adams and Sweatt 2002). In turn, it would be interesting to determine if inhibition of any of these kinases impacts on (1) the induction or maintenance of LTP or LTD, (2) the increase in ERK2 phosphorylation shown to accompany LTP and LTD and (3) the ERK-dependent phosphorylation of CREB during HFS-LTP.

In light of the above findings, the current study yields at least two testable working models of the potential role(s) of the ERK cascade in bi-directional modifications in synaptic strength at hippocampal synapses (figure 8). According to the first model (figure 8, top panel), the ERK2 signal, although requisite for the maintenance of both LTP and LTD in area CA1 of the hippocampus *in vivo*, is, unlike calcium, not sensitive to stimulus frequency, nor predictive of the direction of synaptic modification after a plasticity-inducing stimulus. It is, in turn, a *general* plasticity signal. As a general plasticity kinase, ERK2 could potentially target one or several downstream effectors during bi-directional modifications at hippocampal synapses. The activation of these *general effectors*, may be requisite for changes in synaptic function irrespective of the direction of synaptic modification. One candidate for such a general effector of ERK2 is the transcriptional regulator Elk-1. ERK2 can activate Elk-1 by means of direct phosphorylation. In support of the idea that the ERK2-Elk1 pathway may function as a general

plasticity pathway are findings showing that ERK2 activation during LTP in the dentate gyrus *in vivo* (Davis et al., 2000) is coupled to and necessary for increased phosphorylation of Elk-1 and that LTD in area CA1 *in vivo* is accompanied by increased phosphorylation of Elk-1 (Thiels et al., 2002). Further studies need to be undertaken to determine if LTP in area CA1 is accompanied by an increase in Elk-1 phosphorylation, and if this increase is ERK2 activation-dependent. Additionally, the first working model suggests that differences in the molecular pathways that become activated during LTP and LTD must exist, in order to account for the observed differences in synaptic modification direction demonstrated in the current study. In turn, either (1) one or more signaling cascades carry specific information relative to stimulus frequency and in turn are operative in determining the direction of synaptic modification during these two forms of plasticity or (2) some molecular pathways are activated during LTP that are not activated during LTD, and these differences in activated pathways result in differences in synaptic modification direction. In addition, in light of this model, one aim of future research would be to determine if the phosphorylation of other protein kinases such as PKA, PKC, or CAMKIV differs between LTP and LTD in order to assess whether the activation of any of these kinases is sensitive to stimulus frequency or predictive of the subsequent direction of synaptic modification. In turn, an analysis of the activity of these kinases during LTP and LTD could be performed in much the same way that the analysis of ERK activation was undertaken in the current study.

The second working model (figure 8, bottom panel) is meant to capture the idea that the ERK cascade may share certain targets in common with other signaling cascades. On this model, ERK2 could either (1) interact with or (2) work in parallel with other signaling cascades to affect the activity of specific downstream effectors. One possibility is that specific plasticity

signals are achieved at the level of transcriptional regulators that are capable of integrating converging inputs from different signaling mechanisms and transforming these combined inputs into specific consequences at the synapse. For example, differences in regulation of CREB during LTP and LTD could potentially yield a specific plasticity signal, in so far as increases in pCREB could result in increases in synaptic strength and decreases in pCREB could potentially yield decreases in synaptic strength. Another possibility is that ERK2 interacts with other signaling cascades to bring about specific kinds of synaptic changes. Although ERK2 activation may be a prerequisite for CREB phosphorylation during LTP, it is possible that activation of other molecular pathways, such as the cAMP-PKA, PKC, or Ca^{2+} may play a permissive role in enabling ERK2 to affect phosphorylation of CREB. Further studies would need to be undertaken to investigate these possibilities. For example, I am interested in determining if PKA is differentially recruited during LTP and LTD, and determining what effect, if any, inhibition of PKA has on ERK activation during these two forms of plasticity in area CA1 *in vivo*. In addition, another aim of future research might be to determine if the ERK-dependent phosphorylation of CREB shown to accompany LTP is mediated by activation of PKA (e.g., see Impey et. al., 1998).

The findings from the comparative analysis undertaken in the current study and the two working models are in accordance with the idea that bidirectional modifications in synaptic function likely involve a variety of different signaling cascades. The molecular mechanisms that are operative in effecting bidirectional modifications at activated synapses during plasticity-inducing events most likely include molecular mechanisms that convey very general information in so far as they prime synapses for plastic changes irrespective of the subsequent direction of synaptic modification, and molecular mechanisms that convey very specific information about

the direction of changes in synaptic modification. Comparative analyses of activation of second messenger molecules during LTP and LTD offers one approach to determining what function these molecules play in synaptic plasticity events.

In light of the above conclusions, it is, however, necessary to add the following proviso: ERK2 activity between LTP and LTD could potentially differ with respect to parameters other than the kinetics of the enzyme, and one limitation of the current study is its strict focus on enzyme kinetics during activity-induced synaptic plasticity. For example, the observed increases in ERK2 phosphorylation shown to accompany LTP and LTD may be localized to different subcellular compartments during LTP compared to LTD, creating the potential for differences in the activity of targets downstream of the ERK cascade between LTP and LTD. Depending upon the location of rises in intracellular phosphorylated ERK2, the targets of ERK2 activation could potentially differ between LTP and LTD. The ERK cascade can target specific channels, such as the KV4.2 channel, which can affect cell excitability. The cascade can potentially impact on local protein synthesis and the activity of specific nuclear effectors. In turn, differences in how ERK2 impacts on any of these targets may be crucial in determining synaptic modification direction following LTP- and LTD- inducing events. In turn, although the emphasis on studying the role of enzyme kinetics in the context of synaptic plasticity has been effective historically in shedding some light on the role that specific molecules play in bidirectional modifications at the synapse, one limitation of such studies is their failure to provide a more inclusive picture of the role of specific enzymes in bidirectional modifications at the synapse. In turn, the task of specifying the role of specific molecules in activity-dependent changes in synaptic plasticity will most likely require the combined application of a variety of different yet complementary

experimental techniques that shed light on each of the aforementioned parameters of enzymatic activity.

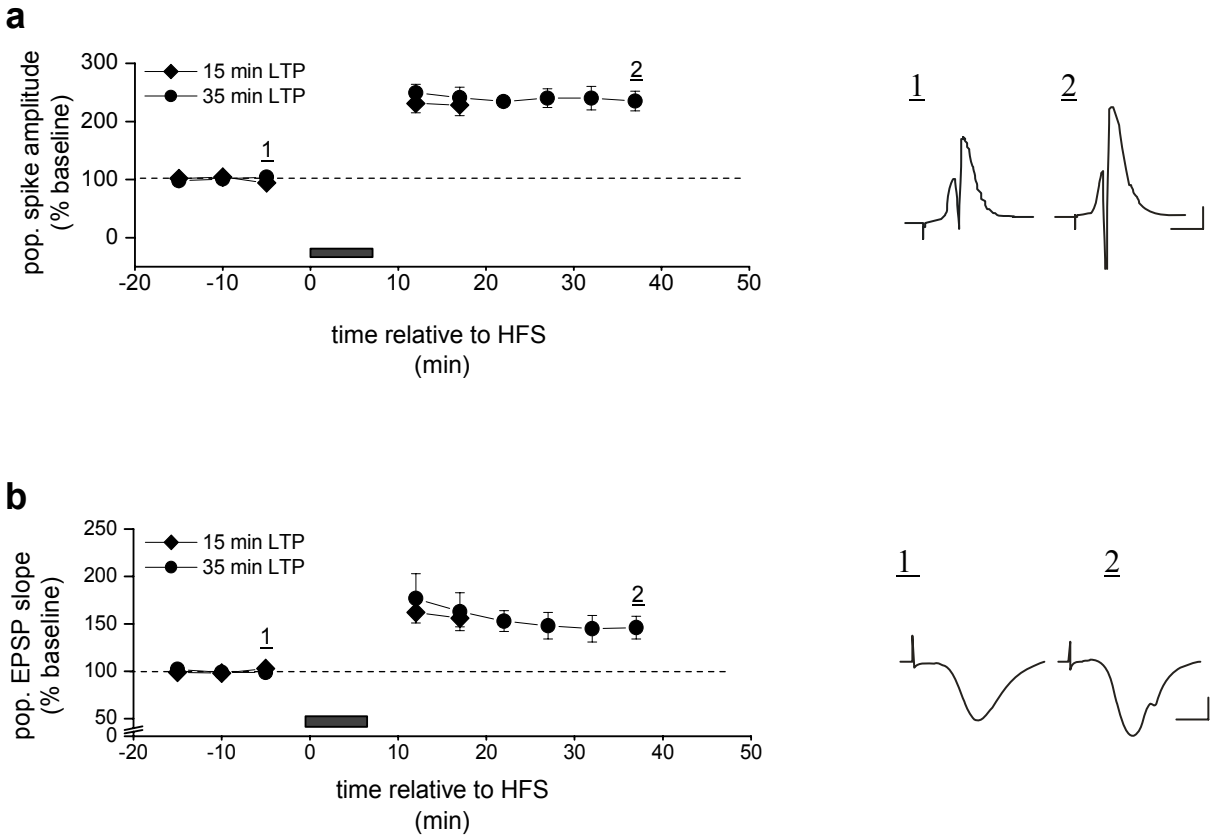

Figure 1. LTP in area CA1 *in vivo*. a, Group data (mean ± SEM) of the amplitude of the CA1 pyramidal cell population spike evoked by stimulation of commissural fibers before and after delivery of four trains of high-frequency stimulation (dark grey bar). The data are expressed as a percentage of the average population spike amplitude before high-frequency stimulation. Animals were killed either 15 min (circles; n = 5) or 35 min (diamonds; n = 5) after termination of high-frequency stimulation. Representative averages of 10 waveforms of population spikes recorded in the same animal before (1) and after (2) high-frequency stimulation are to the right. Calibration: 2 mv, 10 msec. b, Similar group data of the initial slope of the CA1 pyramidal cell population EPSP recorded in stratum radiatum before and after high-frequency stimulation (dark grey bar). Animals were killed either 15 min (circles, n = 4) or 35 min (diamonds; n = 5) after high-frequency stimulation. Representative averages of 10 waveforms of population EPSPs recorded in the same animal before (1) and after (2) high-frequency stimulation are to the right. Calibration: 2 mv, 10 msec.

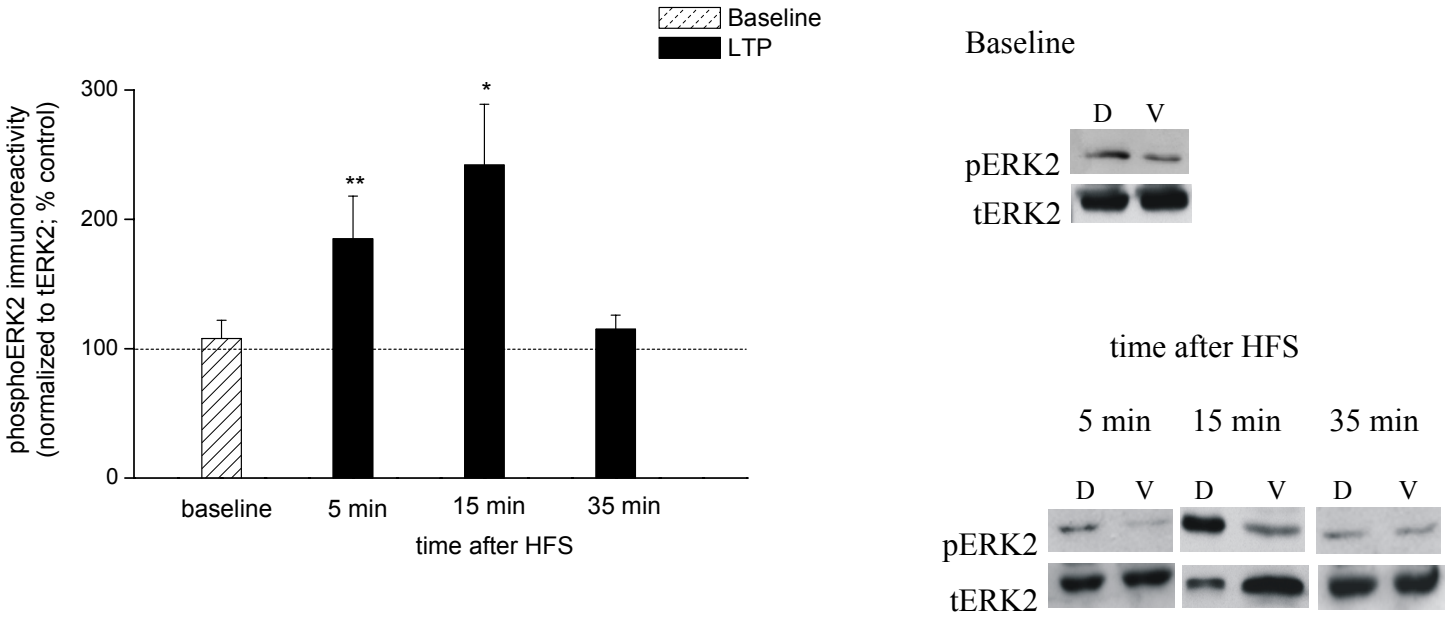

Figure 2. ERK2 activation is increased during LTP in area CA1 *in vivo*. Group data (mean ± SEM) of dual-phosphorylated ERK2 immunoreactivity, normalized to total ERK immunoreactivity, for dorsal area CA1 homogenates, expressed as a percentage of normalized pERK immunoreactivity for ventral area CA1, (baseline, striped bar; LTP, filled bars) derived from animals killed either 5 min after termination of baseline recording (baseline: n = 12) or 5 (n = 6), 15 (n = 9) or 35 min (n = 10) after termination of high-frequency stimulation. Total ERK levels were not affected by HFS. Representative Western blots of dual-phosphorylated ERK2 for ventral (V) and dorsal (D) area CA1 homogenates for the time points indicated on the x-axis are to the right. Asterisks indicate significant difference between control and LTP samples at the indicated time points (Student's t test for dorsal/ventral ratios vs. 1.0; *$p < 0.05$; **$p < 0.01$).

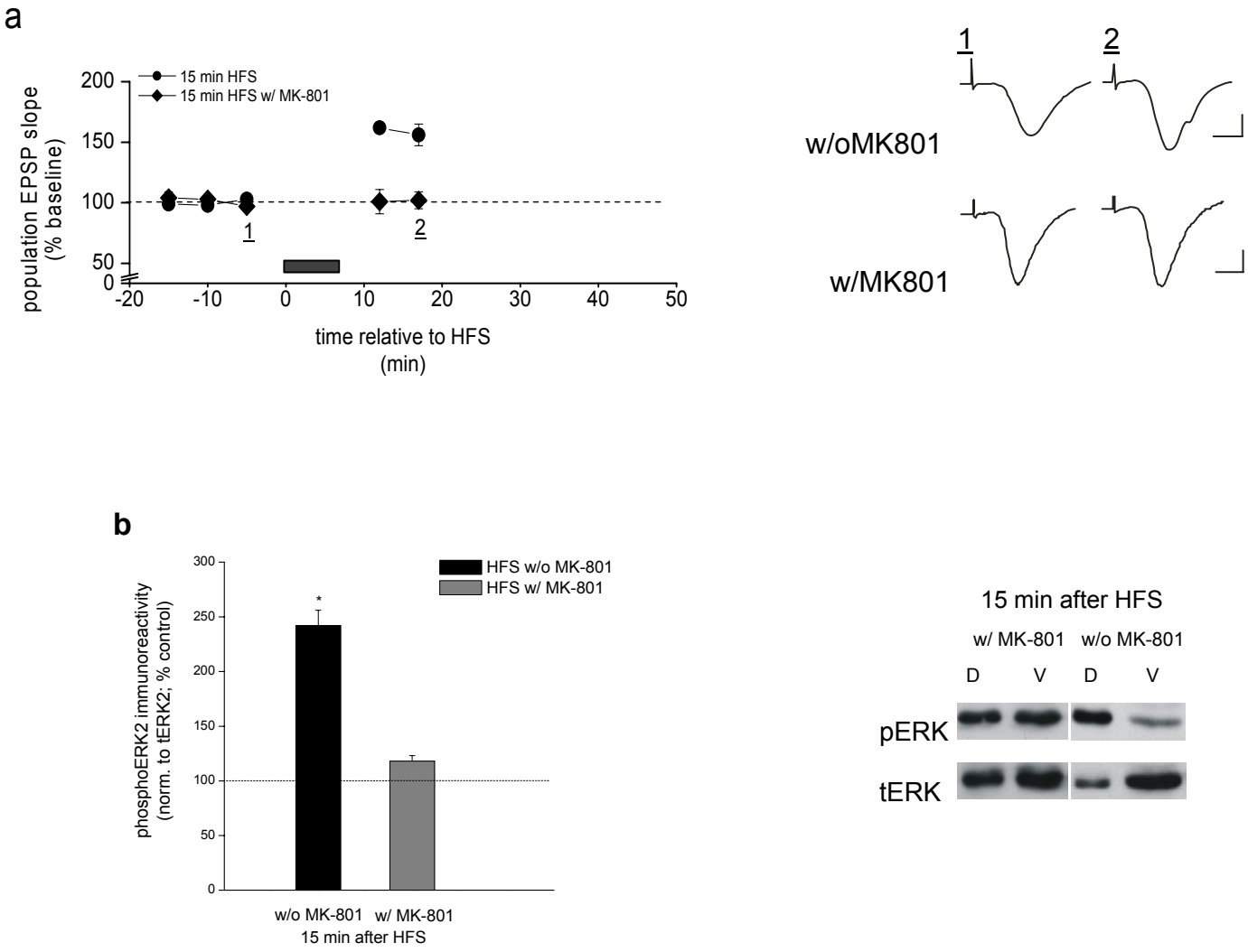

Figure 3. Blockade of NMDA receptors prevents both LTP induction and the associated increase in ERK2 activation. *a*, Group data (mean ± SEM) of the initial slope of the CA1 pyramidal cell population EPSP recorded in stratum radiatum before and 15 min after either HFS delivered in either the presence (diamonds, n = 5) or absence (circles, n = 4) of the NMDA-receptor antagonist MK-801. Representative averages of 10 waveforms of population EPSPs recorded 5 min prior to and 15 min after HFS delivered in the absence (top) or presence (bottom) of MK-801. Calibration: 2 mV, 10 ms. Group data (mean ± SEM) of dual-phosphorylated ERK2, normalized to total ERK, for dorsal area CA1 homogenates expressed as a percentage of normalized pERK immunoreactivity for ventral area CA1 homogenates (baseline, striped bar; LTP, black bar) derived from animals killed 15 min after termination of HFS delivered in either the presence (grey bar, n = 5) or absence of MK-801 (black bar, n =4). Representative Western blots of dual-phosphorylated ERK2 and total ERK2 for dorsal (D) and ventral (V) homogenates for the time points indicated on the x-axis are to the right. Asteriks indicate significant difference between control and LTP samples at the indicated time points (Student's t test for dorsal/ventral ratios vs. 1.0; *$p < 0.05$).

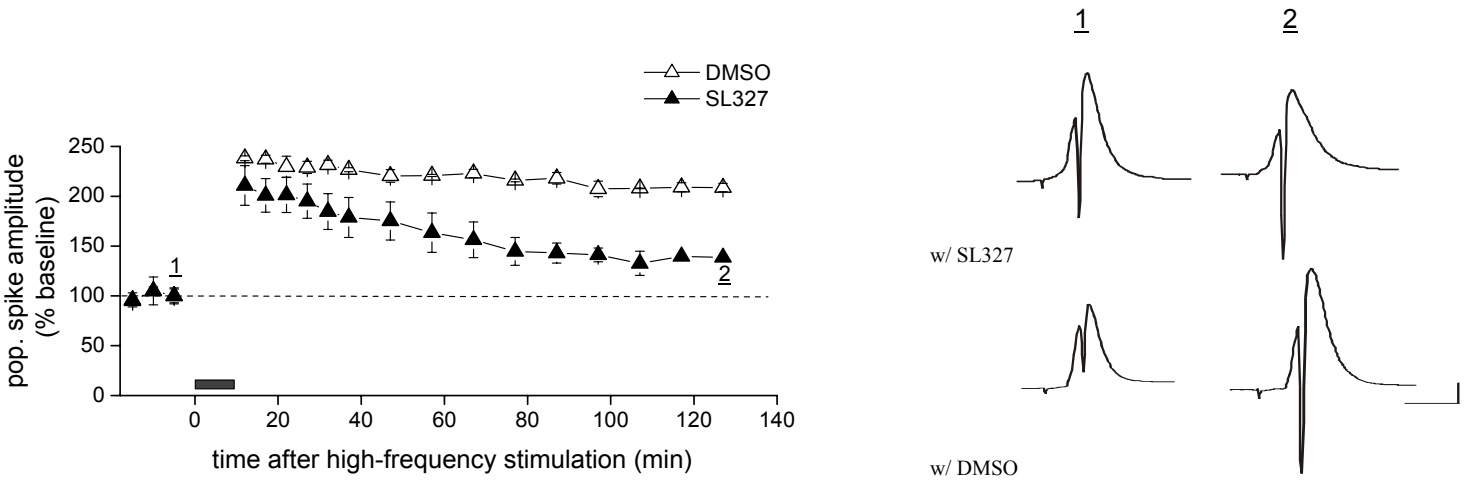

Figure 4. Inhibition of the ERK kinase MEK interferes with LTP maintenance in area CA1 of the adult hippocampus *in vivo*. Group data (mean ± SEM) of the amplitude of the CA1 pyramidal cell population spike evoked by stimulation of commissural fibers before and after delivery of four trains of high-frequency stimulation (dark grey bar) after animals were treated with either the MEK inhibitor SL327 (50 mg/kg, i.p.; filled diamonds; n = 3) or vehicle solution (100% DMSO, 1 ml/kg, i.p.; open diamonds; n = 3) 60-90 min before high-frequency stimulation. Average of 10 waveforms of population spikes recorded in the same SL327-treated (top) or DMS0-treated (bottom) animal before (1) and after (2) high-frequency stimulation at the times indicated. Calibration: 1mV, 15mS.

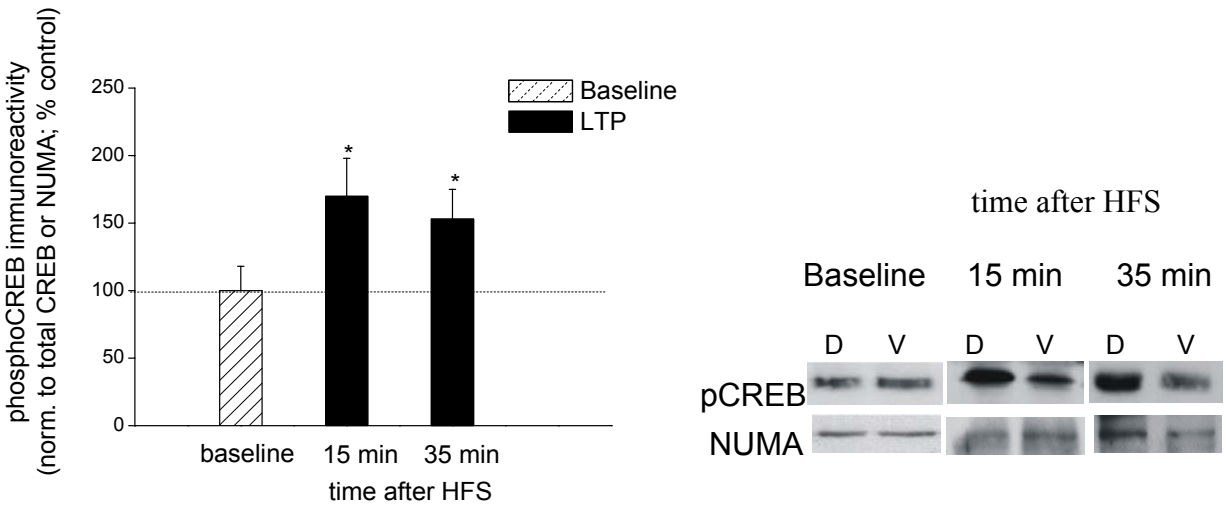

Figure 5. LTP in area CA1 of the adult hippocampus *in vivo* is associated with an increase in nuclear CREB phosphorylation. Group data (mean ± SEM) of SER133-phophorylated CREB immunoreactivity normalized to total CREB or NUMA immunoreactivity, for dorsal area CA1 homogenates, expressed as a percentage of normalized pCREB immunoreactivity for ventral area CA1, derived from animals killed either 5 min after termination of baseline recording (baseline, n = 5) or 15 (n = 7) or 35 (n = 5) min after termination of HFS. Representative Western blots of phosphorylated CREB and NUMA for dorsal (D) and ventral (V) area CA1 homogenates for the time points indicated on the x-axis are to the right. Asterisk indicates significant difference between control and LTP samples (Student's t test for averages; *$p < 0.05$).

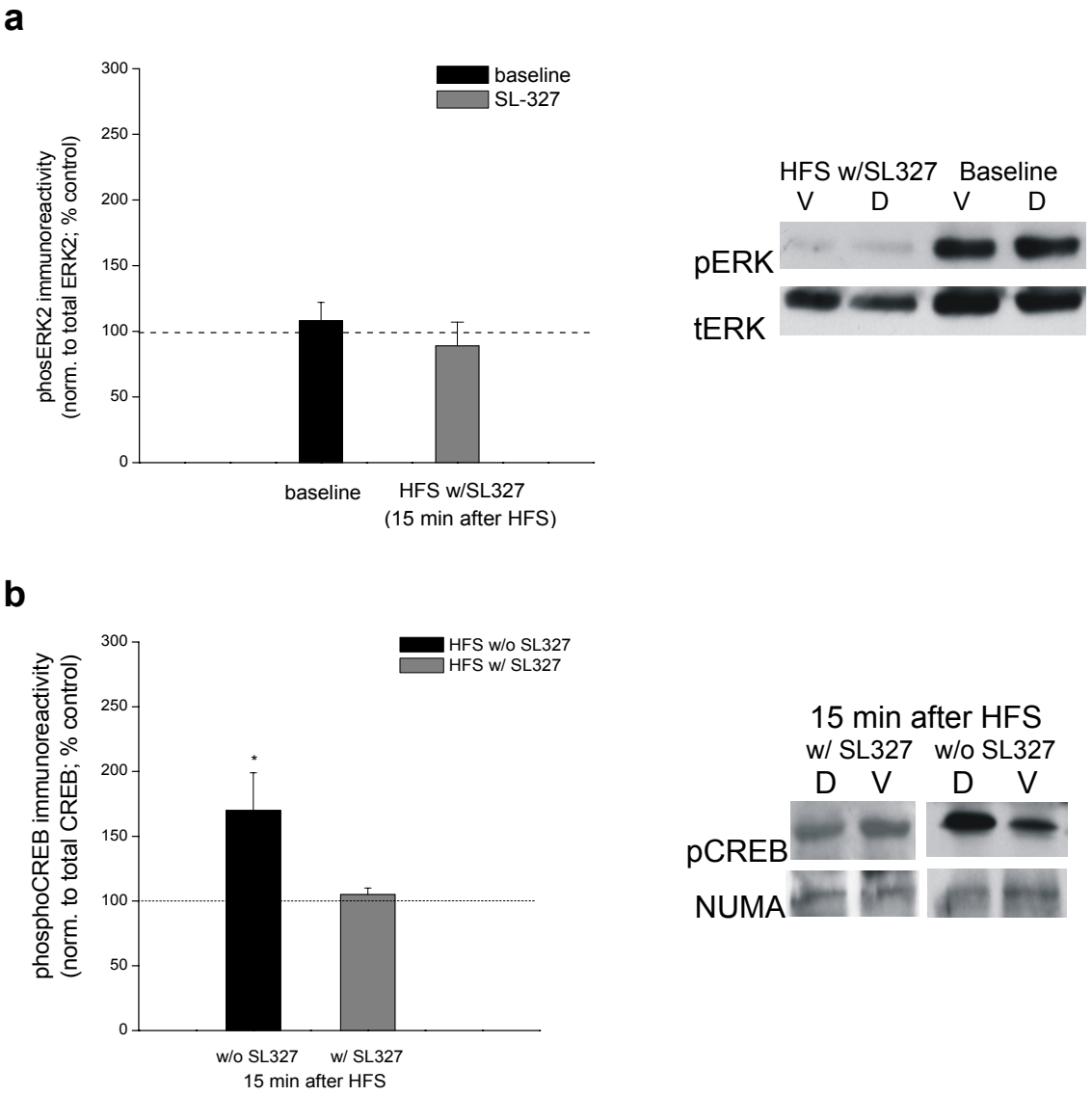

Figure 6. Increase in CREB phosphorylation during LTP in area CA1 *in vivo* is ERK activation-dependent. a, Group data (mean ± SEM) of dual-phosphorylated ERK2 immunoreactivity, normalized to total ERK immunoreactivity, for dorsal area CA1 homogenates, expressed as a percentage of normalized pERK immunoreactivity for ventral area CA1, (baseline, black bar; LTP, filled bars) derived from animals killed either 5 min after termination of baseline recording (baseline: n = 12) or 15 min after HFS delivered in the presence of SL327 (n = 5). Representative Western blots of dual-phosphorylated ERK2 for ventral (V) and dorsal (D) area CA1 homogenates for the time points indicated on the x-axis are to the right. (Tissue sections from an animal that received baseline stimulation and an animal that received HFS delivered in the presence of SL327 were processed simultaneously.) b, Group data (mean ± SEM) of SER133-phophorylated CREB immunoreactivity normalized to either total CREB or NUMA immunoreactivity, for dorsal area CA1 homogenates, expressed as a percentage of normalized pCREB immunoreactivity for ventral area CA1, derived from animals killed 15 min after HFS delivered in either the presence (grey bar, n = 5) or absence (black bar, n = 9) of the MEK inhibitor SL327. Representative Western blots of pCREB and NUMA for ventral (V) and dorsal (D) area CA1 homogenates for the time points indicated on the x-axis are to the right. Asterisk indicates significant difference between control and LTP samples. (Student's *t* test for dorsal/ventral ratios vs. 1.0; *$p < 0.05$; **$p < 0.01$).

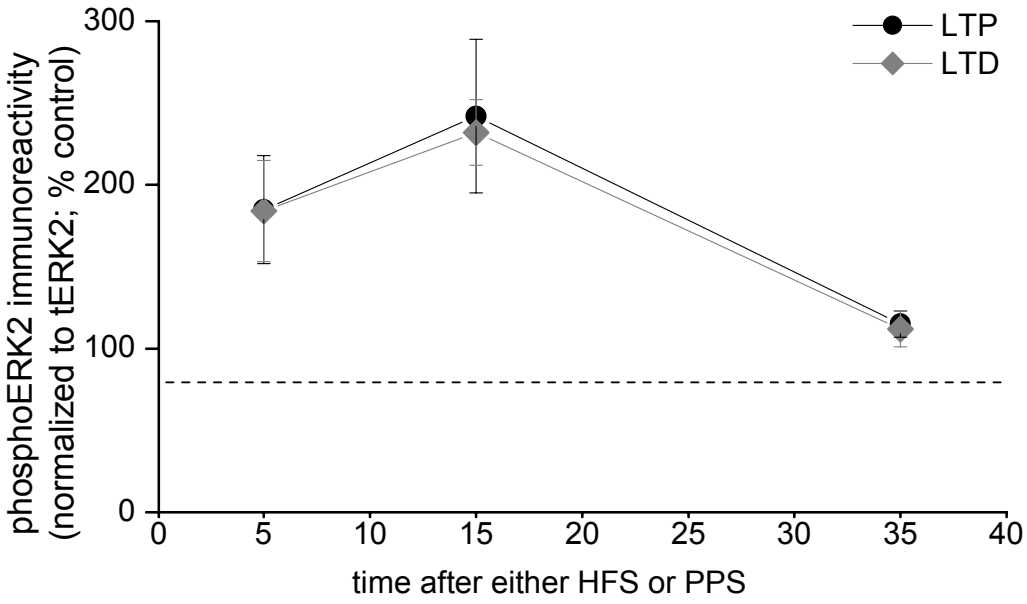

Figure 7. Profile of ERK2 activation is similar during LTP and LTD in area CA1 of the hippocampus *in vivo*. Group data (mean ± SEM) of dual-phosphorylated ERK2 immunoreactivity, normalized to total ERK immunoreactivity, for dorsal area CA1 homogenates, expressed as a percentage of normalized pERK immunoreactivity for ventral area CA1, (LTP, black diamonds; LTD; grey diamonds) derived from animals killed 5 (LTP, n = 6; LTD, n = 8), 15 (LTP, n = 9; LTD, n =12) or 35 (LTP, n = 10; LTD, n = 10) min after termination of high-frequency stimulation. LTD data contained in this graph were obtained from a previous study (Thiels et al., 2002) and are presented with LTP data from the current study in this figure in order to show the similarity of the ERK2 activation profile during LTP and LTD in area CA1 *in vivo*.

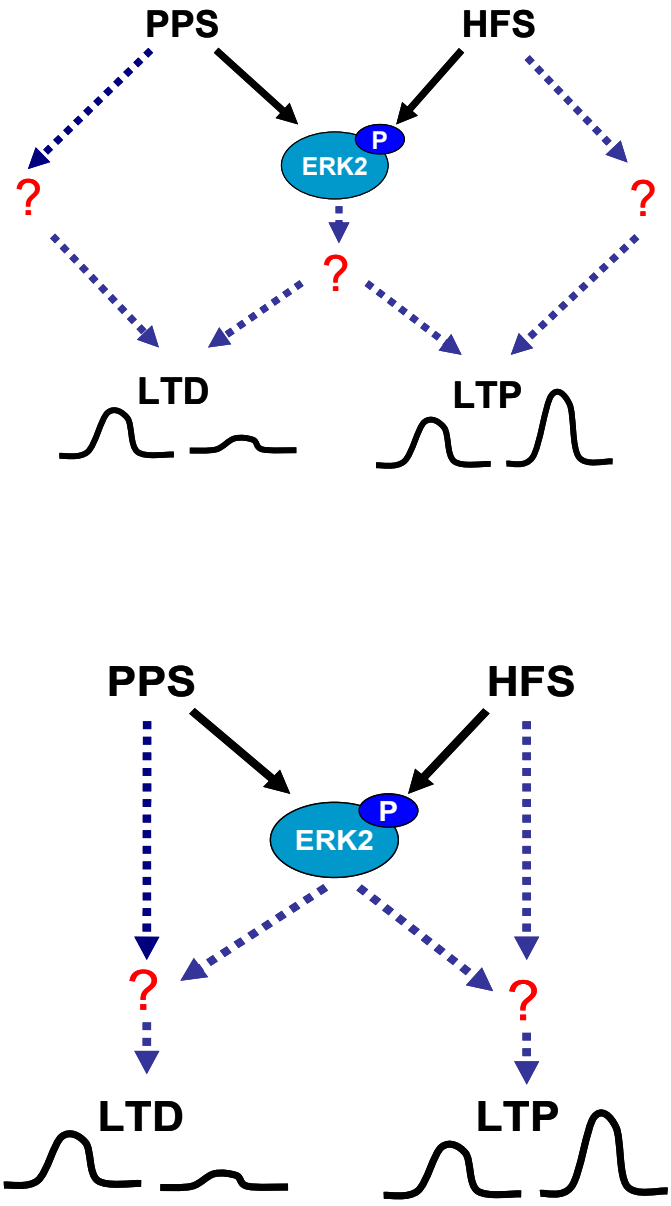

Figure 8. Two Working Models. Top panel, *ERK2 functions as a general plasticity kinase*. Both high-frequency stimulation (HFS) and paired-pulse stimulation (PPS) result in a similar ERK2 signal. This suggests that ERK2 functions as a general plasticity kinase, targeting one or multiple downstream effectors that are involved in and necessary for these two forms of plasticity area CA1 of the hippocampus *in vivo* irrespective of stimulus frequency and the ultimate direction of synaptic modification. Other molecular mechanisms must function as *specific* plasticity signals during these two forms of synaptic plasticity. Bottom panel, *ERK2 may share specific targets in common with other signaling cascades*. Both HFS and PPS result in a similar ERK2 signal. However, ERK2 may interact with or act in parallel with other signaling cascades and function as both a general and as a specific plasticity kinase. The fact that ERK2 can target a diverse array of downstream effectors supports the idea that it could function as both a specific and as a general plasticity kinase. In turn, these two working models are not mutually exclusive.

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