

Cell-Specific Extracellular Signal-Regulated Kinase Activation by Multiple G Protein-Coupled Receptor Families in Hippocampus

JENNIFER L. BERKELEY and ALLAN I. LEVEY

Department of Neurology and the Emory Center for Neurodegenerative Disease, Emory University, Atlanta, Georgia

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ABSTRACT

Several families of G protein-coupled receptors (GPCR) have been shown to activate extracellular signal-regulated kinase (ERK) in transfected cells and non-neuronal systems. However, little is known about GPCR activation of ERK in brain. Because ERK is an important component in the regulation of synaptic plasticity, in this study we examined ERK activation by three families of GPCR that respond to major neuromodulatory neurotransmitters in the hippocampus. We used an immunocytochemical approach to examine ERK activation by muscarinic acetylcholine (mAChR), metabotropic glutamate (mGluR), and β -adrenergic (β -AR) receptors in CA1 neurons of mouse hippocampal slices. Because these GPCR families comprise receptors coupling to each of the major heterotrimeric G proteins,

we examined whether ERK activation differs according to G-protein coupling. By using immunocytochemistry, we were able to examine not only whether each family of receptors activates ERK, but also the cellular populations and subcellular distributions of activated ERK. We demonstrated that M_1 mAChRs and group I mGluRs, both of which are G_q -coupled receptors, activate ERK in CA1 pyramidal neurons, although activation in response to mAChR is more robust. The $G_{i/o}$ -coupled group II mGluRs activate ERK in glia scattered throughout CA1, and G_s -coupled β -AR receptors activate ERK in scattered interneurons. Thus, we demonstrated that GPCR coupling to G_q , $G_{i/o}$, and G_s all activate ERK in the hippocampus, although each does so with unique properties and distributions.

Mitogen-activated protein kinases are a family of serine/threonine protein kinases that play a role in a large number of neural functions, including cell survival (Yan and Greene, 1998), differentiation (Cowley et al., 1994), nociception (Karrim et al., 2001), and learning and memory (Impey et al., 1999; Sweatt, 2001). Extracellular signal-regulated kinase (ERK 1/2), also known as p42/44 mitogen-activated protein kinase, has been shown to play an important role in learning at both cellular and behavioral levels (English and Sweatt, 1997; Blum et al., 1999), but little is known about the mechanisms leading to its activation. ERKs were originally discovered for their role in mitogenic signaling downstream of receptor tyrosine kinases. However, much recent research has shown ERK activation downstream of G protein-coupled receptors (GPCR). Many of the modulatory neurotransmitters that play crucial roles in learning and memory have also

been shown to activate ERK via their actions at GPCR. These include acetylcholine acting at muscarinic acetylcholine receptors (mAChRs) (Berkeley et al., 2001), glutamate acting at metabotropic glutamate receptors (mGluRs) (Peavy and Conn, 1998), and norepinephrine acting at β -adrenergic receptors (β -AR) (Williams et al., 1998; Winder et al., 1999; Watabe et al., 2000). Thus, each of these neurotransmitter systems provides a possible mechanism for the activation of ERK required for memory. Moreover, each neurotransmitter and respective family of receptors are localized in the hippocampus, a region that is critical for memory function.

The mAChR, mGluR, and β -AR families are composed of several members that have unique but overlapping distributions and activate different G proteins to signal through distinctive signaling pathways. For example, three mAChR subtypes (M_1 , M_3 , and M_5) couple to G_q , as do the group I mGluRs (mGluR1 and 5). Many of these receptor subtypes are expressed in pyramidal neurons in CA1 of the hippocampus (Testa et al., 1994b; Levey et al., 1995). Similarly, there are multiple G_i -coupled receptors, including M_2 and M_4

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ABBREVIATIONS: ERK, extracellular signal-regulated kinase; GPCR, G protein-coupled receptor; mAChR, muscarinic acetylcholine receptor; mGluR, metabotropic glutamate receptor; β -AR, β -adrenergic receptor; CCh, carbachol; DHPG, 3,5-dihydroxyphenylglycine; DCG-IV, (2S,2',3',R)-2-(2',3'-dicarboxycyclopropyl) glycine; MPEP, 2-methyl-6-(phenylethynyl)pyridine; AP5, DL-2-amino-5-phosphopentanoic acid; PRO, propranolol; ACSF, artificial cerebrospinal fluid; PB, phosphate buffer; TBS, Tris-buffered saline; NGS, normal goat serum; NMDA, N-methyl-D-aspartate.

mAChRs and the group II mGluRs mGluR2 and mGluR3. However, the distributions of these receptors differ. Whereas M_2 and M_4 are both neuronal in CA1 (found both pre- and postsynaptically on pyramidal and other types of neurons) (Rouse et al., 1998, 2000), mGluR3 is primarily glial, and mGluR2 is not expressed in this region (Testa et al., 1994b). Finally, the β -ARs, consisting of three subtypes (β_1 , β_2 , and β_3) couple to G_s and also are expressed in CA1. The distributions of the β -ARs overlap with those of the G_q -coupled receptors in that they are also expressed in CA1 neurons. However, the β -ARs are not found in the pyramidal cell layer itself; rather, β_1 is found in the stratum oriens and stratum radiatum, whereas β_2 is primarily in stratum lacunosum moleculare (Booze et al., 1993).

Much of the work examining ERK activation by these various GPCRs has been performed in cell culture, and therefore relatively little is known about ERK activation in brain. In cell-culture systems, many different GPCRs coupling to each of the major heteromeric G proteins, G_q , G_i , and G_s , have been shown to activate ERK by distinct mechanisms (Hawes et al., 1995; Williams et al., 1998). However, in cell culture, receptors are often overexpressed and can couple promiscuously to other G proteins than they might in vivo (Kenakin, 1988). In addition, the mechanisms of ERK activation differ by cell line, making results difficult to interpret. Thus, there is little known about which GPCRs activate ERK in endogenous systems, particularly in the brain. Specifically, there is little known about the cellular and subcellular distributions of activated ERK in response to stimulation of various GPCRs.

We recently reported that activation of the G_q -coupled M_1 mAChR leads to ERK activation in dendrites and cell bodies of CA1 pyramidal neurons in a hippocampal slice preparation (Berkeley et al., 2001). In the current study, we sought to compare the distribution of activated ERK in response to the stimulation of other GPCRs in hippocampal slices. We demonstrate that the activation of the G_q -coupled group I mGluRs and M_1 mAChR activates ERK in CA1 pyramidal neurons, whereas the G_i -coupled group II mGluRs activate ERK in glia. Finally, we demonstrate that the G_s -coupled β -AR receptors activate ERK in interneurons. Together, these results demonstrate that ERK activation occurs downstream of multiple GPCRs in the hippocampus, but that each activates in a distinctive spatial and temporal pattern that governs the downstream effects of this activation.

Materials and Methods

Materials. C57BL/6NCR1BR mice were purchased from Charles River Laboratories, Inc. (Wilmington, MA). All studies were conducted in accordance with the Guide for the Care and Use of Laboratory Animals and approved by the Emory University Institutional Animal Care and Use Committee. Antibodies to phospho-ERK 1/2 (rabbit polyclonal and mouse monoclonal) were purchased from Cell Signaling Technologies (Beverly, MA). 3,5-Dihydroxyphenylglycine (DHPG), LY341495, LY367385, (2*S*,2'*R*,3'*R*)-2-(2',3'-dicarboxycyclopropyl) glycine (DCG-IV), DL-2-amino-5-phosphonopentanoic acid (AP5), and 2-methyl-6-(phenylethynyl)pyridine (MPEP) were purchased from Tocris Cookson Inc. (Ballwin, MO). Isoproterenol, propranolol, and carbachol were purchased from Sigma (St. Louis, MO). Avidin-biotin complex and biotinylated goat anti-rabbit secondary antibody were purchased from Vector Laboratories, Inc. (Burlingame, CA).

Slice Preparation. Slices were prepared as described previously (Berkeley et al., 2001). Briefly, 5- to 12-week-old male C57BL6 mice were anesthetized and decapitated. Brains were rapidly removed into chilled and oxygenated chopping buffer (110 mM sucrose, 60 mM NaCl, 3 mM KCl, 1.25 mM NaH_2PO_4 , 0.5 mM CaCl_2 , 7 mM MgCl_2 , 0.6 mM ascorbic acid, 28 mM NaHCO_3 , and 5 mM D-glucose). Coronal sections (400 μm) were cut on a Vibratome (Technical Products International, St. Louis, MO). Hippocampi were gently dissected and transferred to wells containing 2 ml of an oxygenated 1:1 mix of chopping buffer and ACSF (125 mM NaCl, 2.5 mM KCl, 1.25 mM NaH_2PO_4 , 2 mM CaCl_2 , 1 mM MgCl_2 , 25 mM NaHCO_3 , and 10 mM D-glucose). Plates were maintained at room temperature in a closed container with 95% O_2 /5% CO_2 for 45 min. Slices were then transferred to wells containing 2 ml of ACSF and maintained at 25 to 27°C for an additional 90 min. Antagonists were added during this second equilibration period. Slices were then transferred to wells containing agonists \pm antagonists in ACSF maintained at 25 to 27°C for the indicated time.

Fixation and Immunohistochemistry. After treatments, slices were fixed for 1 h with ice-cold 3% paraformaldehyde in 0.1 M phosphate buffer (PB). Slices were washed for 30 min in PB. The slices were then embedded in gelatin blocks (12% 225-bloom calf-skin gelatin, 26% sucrose). The blocks, containing multiple hippocampi, were submerged in 1.5% paraformaldehyde and 15% sucrose for 1 h at 4°C, then placed in 30% sucrose overnight at 4°C. The next day, 50- μm slices were cut on a freezing microtome. Slices were then rinsed in PB and in TBS. Slices were treated with 3% H_2O_2 for 10 min at room temperature and then rinsed. Slices were blocked for 1 h in TBS containing 10 $\mu\text{g}/\text{ml}$ avidin, 0.1% Triton X-100, and 4% normal goat serum (NGS). After three rinses, slices were placed in TBS containing 50 $\mu\text{g}/\text{ml}$ of biotin, 2% NGS, and the primary antibody phospho-ERK 1/2 (1:1000) overnight at 4°C. The next day, slices were rinsed and placed in TBS containing 2% NGS and biotinylated secondary antibody (goat anti-rabbit 1:200) for 1 h at 4°C. Slices were rinsed and placed in avidin-biotin complex reagent for 1 h at 4°C. Slices were rinsed, developed using diaminobenzidine, and mounted onto glass slides.

Quantitation of Immunocytochemistry. Cell bodies and dendrites were counted using Stereologer Version 1.1a (Systems Planning and Analysis, Inc., Alexandria, VA). For each section quantified, 5 to 10 disector boxes (100 μm^2) were randomly placed in the pyramidal layer (cell bodies) or stratum radiatum (dendrites) of CA1. The number of neurons within each box or dendrites that intersected the bottom or right edges were counted. An average value was obtained for each section. Each n represents a separately treated hippocampal slice. Statistical analyses were done using SPSS 10.0.5 (SPSS Science, Chicago, IL). Groups were compared by one-way analyses of variance followed by least significant difference post hoc testing. Data are represented as means \pm S.E.M.

Results

Multiple G_q -Coupled Receptors Activate ERK in CA1 Pyramidal Neurons. Recently, we demonstrated that the M_1 mAChR subtype activates ERK in a hippocampal slice preparation (Berkeley et al., 2001). Because M_1 is a G_q -coupled GPCR highly expressed in the hippocampus, we examined whether another G_q -coupled receptor belonging to a different receptor family could also activate ERK in the hippocampal slice preparation. We also examined whether the distribution of ERK activated by another G_q -coupled receptor would be similar to that of M_1 mAChR-induced ERK activation. We selected the group I mGluRs because these receptors are also highly expressed in the hippocampus (Testa et al., 1994b), and like mAChR, they can modulate synaptic plasticity in the CA1 region (Auerbach and Segal, 1994; Mana-

han-Vaughan, 1997). In slices treated for 30 min with the group I mGluR agonist DHPG (100 μ M), ERK activation was found in CA1 pyramidal cell neurons, as demonstrated by increased immunoreactivity with a phospho-ERK-specific antibody (Fig. 1A). Because ERK is activated by dual phos-

phorylation, an increase in immunoreactivity with this antibody reflects an increase in activated ERK (Schramm and Limbird, 1999). We demonstrated previously that carbachol (CCh)-induced increases of phospho-ERK immunoreactivity are blocked by the mitogen-activated protein kinase kinase

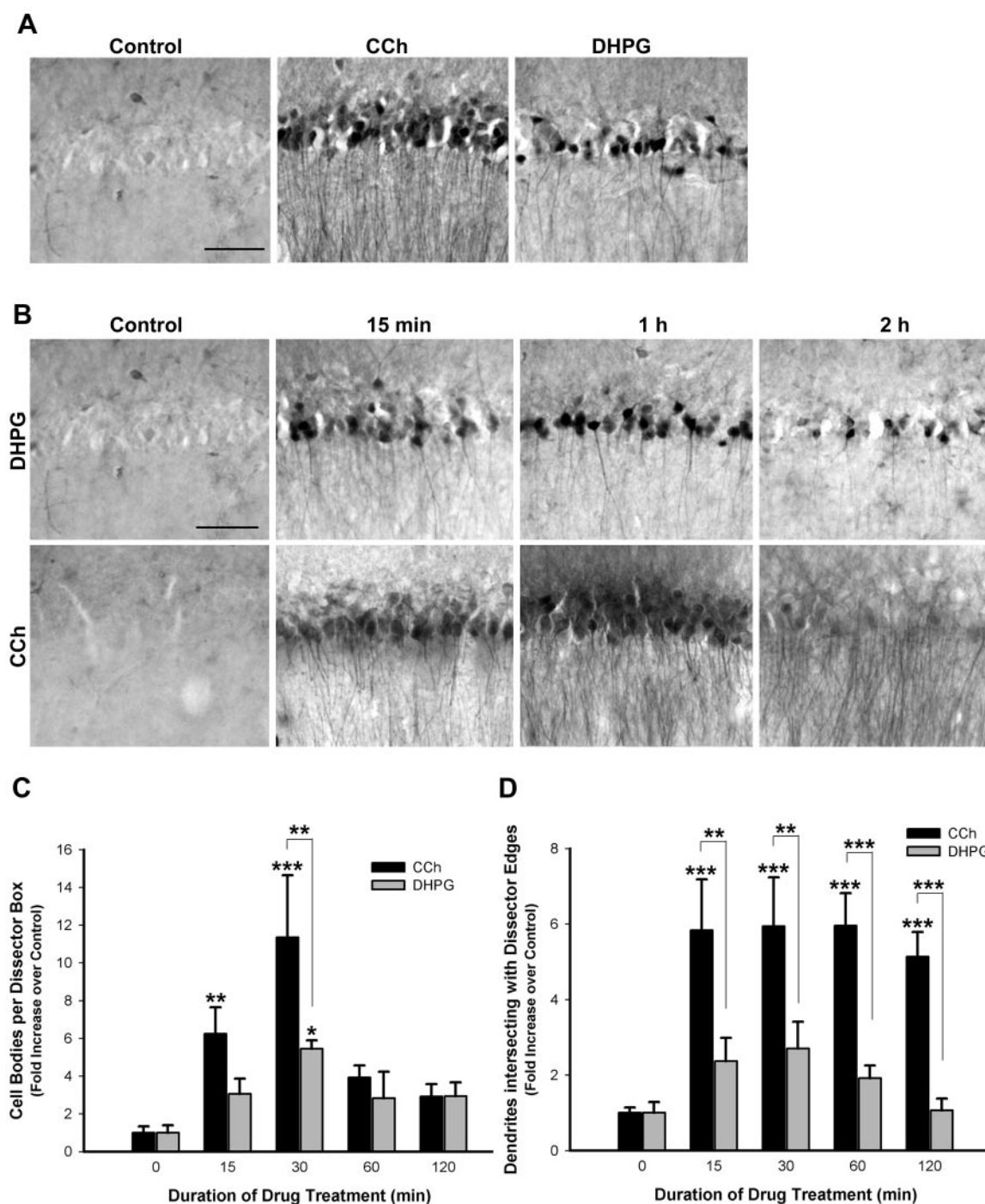


Fig. 1. G_q -coupled mAChRs and group I mGluRs activate ERK in CA1 pyramidal neurons. **A**, hippocampal slices were treated with 100 μ M CCh or 100 μ M DHPG for 30 min. Slices were immunostained with an antibody to dually phosphorylated ERK, which recognizes the active form of the protein. Both agonists increase phospho-ERK immunoreactivity in CA1 pyramidal neurons. **B**, slices were treated with either CCh (100 μ M) or DHPG (100 μ M) for the indicated time. Activation induced by both agonists seems to peak with 30 min to 1 h of treatment and decrease by 2 h. Scale bar, 50 μ m. **C** and **D**, quantitation of phospho-ERK immunoreactive pyramidal neurons (**C**) and dendrites (**D**) in CA1. **C**, both CCh and DHPG cause a significant increase in cell body immunoreactivity at 30 min; however, the difference between the treatment groups is also significant. **D**, CCh causes a sustained increase in the number of immunoreactive dendrites, whereas the increase in dendrites in DHPG-treated slices is not significant ($p = 0.089$). Bars represent mean \pm S.E.M. Asterisks directly over bars represent comparisons with control. Asterisks between bars represent comparisons between drug treatment groups. CCh, $n = 5$; DHPG, $n = 6$; *, $p \leq 0.05$; **, $p \leq 0.01$; ***, $p \leq 0.001$.

inhibitor SL327, demonstrating the antibody's specificity for the ERK 1/2 signaling pathway (Berkeley et al., 2001). Group I mGluR-induced ERK activation is considerably less pronounced than mAChR-mediated activation, as demonstrated in slices treated with the mAChR agonist CCh (100 μ M). In general, fewer pyramidal neurons are activated by DHPG, and the dendritic arbor is not as dramatically immunoreactive as with CCh treatment. Indeed, with 30 min of drug treatment, both CCh- and DHPG-treated slices show a statistically significant increase in the number of phospho-ERK immunoreactive pyramidal neurons. However, there is also a statistically significant difference in ERK activation between the two treatment groups (Fig. 1C). In addition, whereas CCh causes a statistically significant increase in dendritic staining, DHPG does not ($p = 0.089$; Fig. 1D).

CCh and DHPG have similar affinities for their respective receptors, and in hippocampal slices, they exert similar effects at comparable concentrations. For example, both DHPG and CCh control Ca^{2+} release at concentrations between 3 and 5 μ M (Nakamura et al., 2000), and both potentiate NMDA currents at 10 μ M (Fitzjohn et al., 1996; Marino et al., 1998). We reported previously that 100 μ M CCh maximally activates ERK in CA1 (Berkeley et al., 2001); thus, to ensure maximal activation, we used this concentration for both CCh and DHPG in all studies comparing mAChR and mGluR activation of ERK. We observed similar patterns of activation, although less intense, at lower concentrations of both agonists (data not shown).

To determine whether the differences in ERK activation between the group I mGluRs and M_1 mAChR stimulation are caused by a difference in the kinetics of activation, we compared the time courses of DHPG- and CCh-induced ERK activation (Fig. 1B). At 15 min, the patterns of DHPG- and CCh-induced ERK activation seem similar in that both neurites and cell bodies of CA1 pyramidal neurons are prominently stained. However, more dendrites are stained in CCh-treated slices, and cell body staining is slightly more intense in DHPG-treated slices. Nonetheless, at this early time point, only the CCh-treated slices show a statistically significant increase in phospho-ERK immunoreactive cell bodies or dendrites. The distribution of staining in the somata with both CCh and DHPG treatments suggests that ERK has translocated to the nucleus. By 30 min of drug treatments, the time at which both DHPG- and CCh-treated slices had reached maximal ERK activation (Fig. 1A), the differences between DHPG- and CCh-treated slices are apparent. CCh-treated slices show strong dendritic and somatic staining, whereas the DHPG-induced staining is most intense in the somata and in fewer dendrites. These differences remain apparent with 60 min of drug treatment, although at this time point, only CCh-induced dendritic activation of ERK is statistically significant. Therefore, although there are differences in the patterns of phospho-ERK distribution between group I mGluR and M_1 mAChR-induced ERK activation, both activate ERK with similar kinetics. At 2 h, staining with both DHPG and CCh dramatically decreases. With DHPG, a few cell bodies remain prominently stained, whereas with CCh, there is little somatic staining, although dendritic immunoreactivity remains strong.

There are two molecularly identified subtypes in the group I mGluRs: mGluR1 and mGluR5. To determine the subtype responsible for DHPG-mediated ERK activation, we pre-

treated the slices with subtype-specific antagonists before a 1-h stimulation with DHPG (100 μ M). Interestingly, both the mGluR1 antagonist LY367385 (100 μ M) and the mGluR5 antagonist MPEP (5 μ M) inhibited DHPG-mediated ERK activation when slices were pretreated with these antagonists independently (Fig. 2). There was no additional inhibition of ERK activation when these antagonists were combined (data not shown). Because blocking either mGluR1 or mGluR5 alone prevents ERK activation, both receptors seem necessary for ERK activation by DHPG.

G_i -Coupled Group II mGluRs Activate ERK Glia. We have demonstrated that two different G_q -coupled receptors activate ERK in the same population of hippocampal cells, namely the CA1 pyramidal neurons, but each with distinct patterns of subcellular distribution. We next examined whether G_i -coupled GPCR could also activate ERK in hippocampal slices, and if so, in what cell population. We selected the group II mGluRs, which generally couple to G_i and are also highly expressed in the hippocampus. To determine

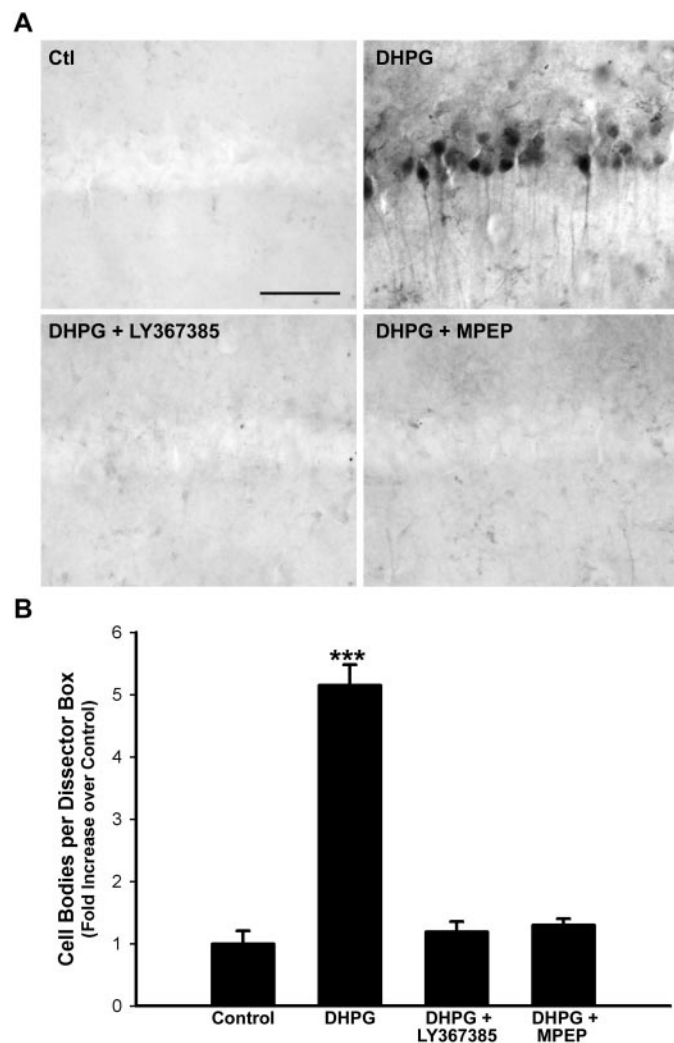


Fig. 2. mGluR1 and mGluR5 antagonists both inhibit DHPG-induced ERK activation. A, hippocampal slices were pretreated for 90 min with the mGluR1 antagonist LY367385 (100 μ M) or the mGluR5 antagonist MPEP (5 μ M) before 30-min stimulation with DHPG. Scale bar, 50 μ m. B, quantitation of phospho-ERK immunoreactive pyramidal neurons in CA1. Control, $n = 7$; DHPG, $n = 7$; DHPG + LY367385, $n = 4$; DHPG + MPEP, $n = 5$; ***, $p \leq 0.001$.

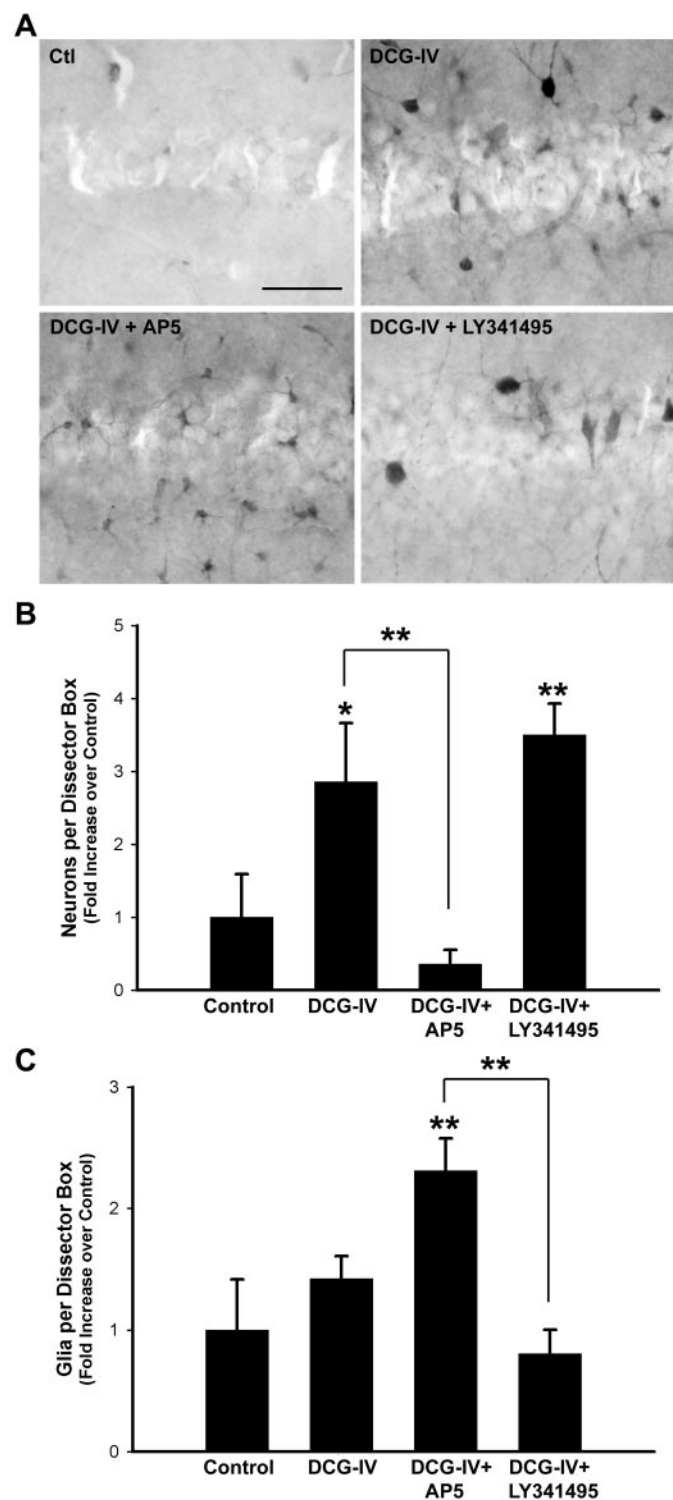


Fig. 3. $G_{i/o}$ -coupled group II mGluRs activate ERK in glia in CA1. **A**, hippocampal slices were treated with the group II mGluR agonist DCG-IV (10 μ M) for 30 min (top right). DCG-IV treatment causes increased phospho-ERK immunoreactivity in both interneurons and glia in CA1. Slices were pretreated for 15 min with the NMDA receptor antagonist AP5 (50 μ M) before treatment with DCG-IV to eliminate nonspecific activation of NMDA receptors (lower left). AP5 pretreatment eliminates phospho-ERK immunoreactivity in interneurons, but glial staining remains prominent. Slices were pretreated with the group II mGluR antagonist LY341495 for 90 min (5 μ M) before DCG-IV stimulation (lower right). LY341495 pretreatment reduces DCG-IV-induced glial immunoreactivity but has little effect on phospho-ERK immunoreactivity in inter-

whether these receptors activate ERK in hippocampus, slices were treated with the group II agonist DCG-IV. Treatment with DCG-IV (10 μ M) results in a dramatic increase in phospho-ERK immunoreactivity in interneurons and glia scattered throughout the hippocampus (Fig. 3). Neurons and glia were differentiated using morphological criteria. Cell bodies greater than 10 μ m in diameter and having a prominent axon or dendrite were considered to be neurons, whereas smaller cells with many short processes were counted as glia. Interneurons were distinguished from pyramidal neurons by their prominent processes that were oriented perpendicularly to or away from the stratum radiatum. In addition, the interneurons were not strictly localized to the pyramidal cell layer. Because DCG-IV can also activate NMDA receptors, slices were pretreated with the NMDA antagonist AP5 to determine whether the increased ERK activation was mediated by NMDA receptors. AP5 (50 μ M) pretreatment abolishes the ERK activation in the hippocampal interneurons, but it increases phospho-ERK-immunoreactive glia. This increase in glial staining is not present in slices treated with AP5 alone (data not shown), suggesting that it is caused by the activation of the group II mGluRs by DCG-IV. In addition, in slices pretreated with the group II mGluR antagonist LY341495 (1–10 μ M), ERK activation in glia is reduced to basal levels, whereas some interneuron staining remains.

To eliminate the ERK activation caused by NMDA receptor stimulation, all additional experiments were performed in the presence of AP5. The time course for group II-mediated ERK activation is comparable with G_q -coupled receptor-mediated ERK activation, although it seems that it took longer for the glial staining to reach its maximum intensity (Fig. 4). Glial staining was apparent with 30 min of DCG-IV treatment and remained prominent with 60 min of drug treatment. At 60 min, the glial staining seems more intense than at 30 min, although there is not a statistically significant increase in the number of immunoreactive glial cell bodies. Also, at 60 min, there was a general increase in neuropil staining. After 2 h of DCG-IV treatment, there were fewer immunoreactive glia, and the staining in those that remained was less intense.

G_s -Coupled Receptor Signaling Pathways Activate ERK in Interneurons and Pyramidal Neuron Dendrites. Although mGluRs and mAChRs couple to G_i or G_q , neither family contains G_s -coupled GPCRs. To determine the effect of G_s stimulation in hippocampal slices, we examined whether the activation of downstream components of G_s -coupled receptor-signaling pathways activates ERK. We treated the slices with the adenylyl cyclase activator forskolin, which has been shown previously to potentiate LTP via ERK activation (Martin et al., 1997). Slices treated with 50 μ M forskolin show a dramatic increase in ERK activation in interneurons throughout the hippocampus (Fig. 5). However, because forskolin is an extremely potent activator of adenylyl

neurons. Scale bar, 50 μ m. **B** and **C**, quantitation of phospho-ERK immunoreactive interneurons (**B**) and glia (**C**) in CA1. **B**, DCG-IV causes a significant increase in the number of immunoreactive interneurons in CA1. Pretreatment with AP5 significantly reduces the number of immunoreactive neurons activated by DCG-IV treatment. **C**, glial activation by DCG-IV is only significant in the presence of AP5 and is eliminated by pretreatment with LY341495 (1–10 μ M); $n = 5$; *, $p \leq 0.05$; **, $p \leq 0.01$; ***, $p \leq 0.001$.

cyclase, we sought to examine whether receptor-mediated G_s activation similarly activated ERK.

Because much of the research examining GPCR activation of ERK in cell culture has been performed using β -adrenergic receptors (Williams et al., 1998), we examined whether these receptors also activate ERK in hippocampus and whether the pattern of ERK activation is similar to that of other receptor families. Previous studies have demonstrated ERK activation in pyramidal cell dendrites in response to β -AR stimulation (Winder et al., 1999), but there was no indication of ERK activation in interneurons, as our forskolin data would suggest. Slices stimulated with the β -adrenergic agonist isoproterenol (10 μ M) demonstrate increased phospho-ERK immunoreactivity in interneurons scattered throughout the hippocampus, similar to the pattern seen with forskolin. In isoproterenol-treated slices, there seems to be some staining of CA1 pyramidal cell neurons and dendritic immunoreactivity in stratum radiatum, similar to the dendritic activation reported previously (Winder et al., 1999). However, our statistical analysis did not show a significant increase in the number of stained dendrites over control cells (data not shown). Pretreatment with the β -adrenergic antagonist propranolol (10 μ M) markedly reduced phospho-ERK immunoreactivity in the interneurons, but it had no effect on ERK activation by forskolin (Fig. 5).

Discussion

ERK is required for one of the primary functions of the hippocampus: learning and memory. However, it is unclear how ERK is activated to play its role during memory formation. mAChRs, mGluRs, and β -ARs all have been shown to modulate LTP, a cellular model of learning and memory (Katsuki et al., 1992), and by Western blot, each family of GPCR has been shown to activate ERK in the hippocampus (Roberson et al., 1999). One might wonder, then, why each of these families is necessary if they are all expressed in CA1, and regardless of the G protein through which they signal, they all activate ERK. Here, we show that each receptor family activates ERK in a unique spatial and temporal pattern. Remarkably, there are cellular and subcellular differ-

ences in ERK activation that may provide a mechanism for the unique way in which each neurotransmitter modulates signaling in the hippocampus.

Generally, ERK activation by each receptor family correlates with receptor distribution. Group I mGluRs in CA1 are expressed primarily in pyramidal neuron cell bodies and dendrites (Lujan et al., 1996). This is also the distribution of ERK activation induced by treating the slices with the group I agonist DHPG. Similarly, M_1 mAChRs are expressed and activate ERK in the same cells (Berkeley et al., 2001). Because both of these receptors couple to the same G protein, G_q , and therefore presumably activate the same downstream signaling pathway, it would seem that they are redundant.

However, within CA1, group I mGluRs and M_1 mAChR exert distinct functions. Group I mGluRs, particularly mGluR5, induce long-term depression, the functional converse of LTP, in many stimulation paradigms (Camodeca et al., 1999; Huber et al., 2001). On the other hand, M_1 mAChR generally potentiates or induces LTP (Auerbach and Segal, 1994). Consistent with their divergent functions, group I mGluRs and M_1 mAChR activate ERK in distinct patterns within the pyramidal neurons of CA1. M_1 -mediated ERK activation is much more prominent in the CA1 region of the hippocampus. The group I mGluR agonist DHPG seems to activate fewer pyramidal neurons than does the mAChR agonist carbachol, and the overall neuropil staining is considerably less. In addition, with CCh treatment, dendritic activation is much more prominent, whereas with DHPG, cell-body activation is the more striking localization of activated ERK. In fact, in some experiments (data not shown), we saw little ERK activation in cell bodies in response to CCh, but dendritic staining remained. In those same experiments, we saw the exact opposite with DHPG: little dendritic staining, but intense somatic phospho-ERK immunoreactivity. One possible explanation for the differences in DHPG- and CCh-induced ERK activation is differential uptake or metabolism of these drugs. Although our data cannot disprove this possibility, the fact that both drugs exert similar physiological effects at comparable concentrations (Fitzjohn et al., 1996; Marino et al., 1998; Nakamura et al., 2000) and that we observe differential localization of activation

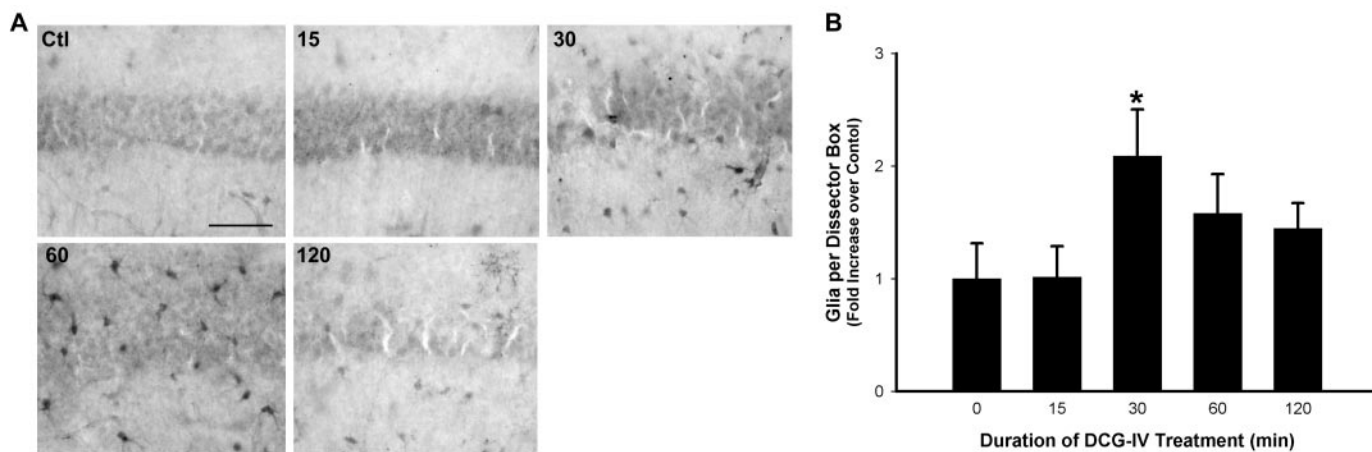


Fig. 4. Time course of DCG-IV-induced ERK activation in glia. **A**, hippocampal slices were treated with DCG-IV (10 μ M) for the indicated time in the presence of AP5. There is little phospho-ERK immunoreactivity with 15 min of DCG-IV treatment. Phospho-ERK immunoreactivity is most intense in glia with 60 min of agonist treatment. Immunoreactivity is reduced after 2 h of DCG-IV treatment. Scale bar, 50 μ m. **B**, quantitation of phospho-ERK immunoreactive glia in CA1. Only at 30 min is the number of immunoreactive glia significantly different from control glia, despite the apparent increase in the intensity of glial staining at 60 min; $n = 4$; *, $p < 0.05$.

with similar kinetics argues against it. In addition, it is possible that experiments that show a paucity of staining are caused by insufficient signal above the background level, because the signal-to-noise ratio can vary from experiment to experiment.

Although the distributions of activated ERK differ, the time courses of activation are remarkably similar, suggesting a similar mechanism of activation. Both CCh and DHPG induce ERK staining relatively rapidly, with both cell bodies and dendrites visible by 15 min, and both seem to achieve maximal activation by 30 min to 1 h of agonist treatment, which decreases by 2 h. However, DHPG-induced ERK acti-

vation in dendrites seemed to be more transient than that induced by CCh. By 60 min, there was a clear decline in the dendritic staining with DHPG, although cell-body staining maintained or even gained in intensity. On the other hand, CCh-induced dendritic staining remained elevated for the duration of the experiments. From these observations, mGluRs and mAChRs are most likely subserving different functions. The more prominent dendritic ERK activation induced by mAChRs suggests a role for mAChR-induced ERK activation in the dendrites themselves, such as altering dendritic morphology, causing local changes in neuronal excitability or modulating the responsiveness to other signals. On the other hand, the group I mGluR activation is primarily found in the cell bodies. Here, ERK activation could more rapidly effect changes throughout the entire cell. Such changes include alterations in cell excitability or inducing immediate early gene expression.

Another interesting aspect of ERK activation by the group I mGluRs is the apparent necessity for costimulation of both mGluR1 and mGluR5. There are other examples in which blocking either mGluR1 or mGluR5 reduces function, and there is no additional reduction when antagonists for both subtypes are combined (Karim et al., 2001). Many have hypothesized that these receptors may exist as heterodimers, but thus far, no one has been able to demonstrate an interaction between these receptor subtypes (J. Conn, personal communication). Regardless, there could be a functional dimerization in which both receptors are needed to activate a certain pathway, although there is no physical association. In this case, ERK activation could be a point of convergence between the signaling cascades initiated by each of these receptor subtypes.

Like the group I mGluRs, the group II mGluRs activate ERK in the cells in which they are expressed. In this case, activation is primarily in glia. Few previous studies have examined ERK activation by endogenously expressed G_i -coupled receptors (Cook et al., 2000; Vanhose et al., 2002). Although there have been previous studies of mGluR-mediated ERK activation in glia, those studies showed that ERK activation was mediated by mGluR5. Those experiments differed in that they were performed in primary astrocyte cultures (Peavy and Conn, 1998), and it has been shown that astrocytes up-regulate mGluR5 expression when reactive or cultured (Cai et al., 2000; Ulas et al., 2000). In vivo, mGluR3 is the principal subtype expressed in glia (Testa et al., 1994a). The function of ERK activation in glia remains unclear. Previously, group II mGluR activation was shown to potentiate cellular responses to G_s -coupled receptors such as adenosine or β -AR receptors (Winder and Conn, 1995).

Perhaps the most surprising result of this study is that of forskolin treatment. Because forskolin activates not a specific receptor but the ubiquitously expressed enzyme adenylyl cyclase, one would expect it to activate ERK in every cell. However, the ERK activation by forskolin is remarkably specific for interneurons. Amazingly, these are the same cells in which ERK is activated in response to β -AR stimulation. These results indicate that there is something specific in interneurons that allows them to respond selectively to increases in cAMP and presumably protein kinase A activation.

Like mAChR stimulation, β -AR activation can potentiate LTP (Thomas et al., 1996), and β -ARs also have dramatic effects on the generation of postsynaptic complex spikes

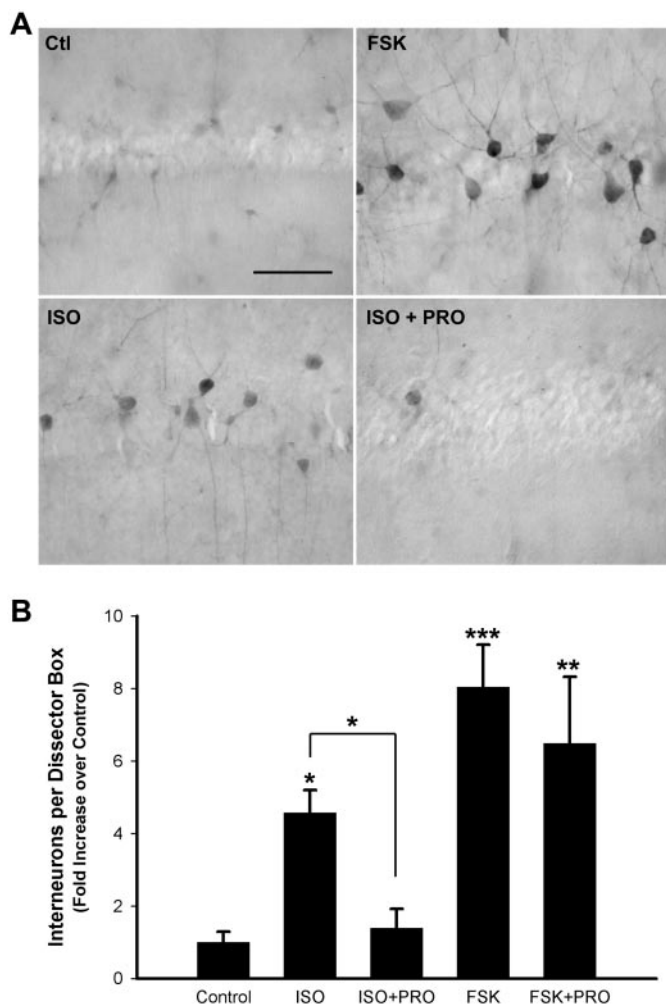


Fig. 5. G_s -coupled β -ARs activate ERK in interneurons in CA1. **A**, hippocampal slices were treated with the adenylyl cyclase activator forskolin (FSK, 50 μ M) for 30 min (top right). Forskolin treatment induces robust ERK activation in interneurons scattered throughout CA1. Slices were treated with the β -AR agonist isoproterenol (ISO, 10 μ M) for 30 min, resulting in increased phospho-ERK immunoreactivity in CA1 interneurons (bottom left). Pretreatment of the slices with the β -AR antagonist propranolol (PRO, 10 μ M) before isoproterenol stimulation reduces phospho-ERK immunoreactivity in interneurons (bottom right). Scale bar, 50 μ m. **B**, quantitation of phospho-ERK immunoreactive interneurons in CA1. Both ISO and FSK cause a significant increase in the number of immunoreactive interneurons. PRO pretreatment abolishes ISO-induced ERK activation, but it has no effect on FSK-induced activation. Control, ISO, ISO + PRO, and FSK: $n = 5$; FSK + PRO, $n = 3$; *, $p \leq 0.05$; **, $p \leq 0.01$; ***, $p \leq 0.001$.

(Winder et al., 1999). Furthermore, both of these effects of β -AR stimulation are mediated by ERK, although the cells in which ERK is activated have not been identified (Winder et al., 1999). ERK activation in interneurons provides a potential mechanism for these effects on postsynaptic responsiveness, because interneurons can regulate bursting (Dvorak-Carbone and Schuman, 1999). Additionally, adrenergic stimulation has previously been shown to increase the frequency of inhibitory postsynaptic currents by stimulation of both α - and β -ARs on CA1 interneurons (Bergles et al., 1996).

Taken together, our results demonstrate that signaling through ERK is a potential mechanism by which several GPCRs exert their modulatory effects on hippocampal function. mAChRs, mGluRs, and β -ARs each play important and distinct roles in learning and memory in the hippocampus, although in some experimental paradigms, these roles can seem redundant. We demonstrate that each of these receptor families activate ERK in a unique spatial and temporal pattern in CA1. Since the initial descriptions of ERK, it has been suggested that the different downstream effects of ERK activation are dependent on the duration of the ERK signal. Interesting future studies could examine how the duration and pattern of ERK activation contributes to the specificity of signaling by each of the different GPCRs that activate ERK.

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Address correspondence to: Allan I. Levey, M.D., Ph.D., Emory Center for Neurodegenerative Disease, Whitehead Biomedical Research Building, Room 505, Emory University, 615 Michael Street, Atlanta, GA 30322. E-mail: alevey@emory.edu