Kinase Suppressor of Ras1 Compartmentalizes Hippocampal Signal Transduction and Subserves Synaptic Plasticity and Memory Formation

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Summary

The ERK/MAP kinase cascade is important for long-term memory formation and synaptic plasticity, with a myriad of upstream signals converging upon ERK activation. Despite this convergence of signaling, neurons routinely activate appropriate biological responses to different stimuli. Scaffolding proteins represent a mechanism to achieve compartmentalization of signaling and the appropriate targeting of ERK-dependent processes. We report that kinase suppressor of Ras (KSR1) functions biochemically in the hippocampus to scaffold the components of the ERK cascade, specifically regulating the cascade when a membrane fraction of ERK is activated via a PKC-dependent pathway but not via a cAMP/PKA-dependent pathway. Specificity of KSR1-dependent signaling also extends to specific downstream targets of ERK. Behaviorally and physiologically, we found that the absence of KSR1 leads to deficits in associative learning and theta burst stimulation-induced LTP. Our report provides novel insight into the endogenous scaffolding role of KSR1 in controlling kinase activation within the nervous system.

Introduction

The ERK/MAP kinase pathway was first discovered for its role in regulating the cell cycle, but when members of the kinase cascade were found to be present in neurons, another role was ascribed to ERK/MAP kinase: regulating synaptic plasticity and memory formation. The ERK cascade is now known to be a convergence point in neuronal signaling and is considered to be a signal integrator in both synaptic plasticity and memory formation (for review, see Adams and Sweatt [2002]).

A large body of research has established the involvement of ERK in rodent behavior and memory, including associative fear conditioning (Atkins et al., 1998; Schafe et al., 2000), spatial learning (Blum et al., 1999; Selcher et al., 1999), and conditioned place preference (Gerdjikov et al., 2004; Miller and Marshall, 2005). ERK has also been implicated as critical for normal synaptic plasticity in various long-term potentiation (LTP) induction paradigms (English and Sweatt, 1997; Kelleher et al., 2004; Mazzucchelli et al., 2002; Selcher et al., 2003; Watabe et al., 2000). Moreover, biochemical experiments have identified different neuronal receptors that, when activated, can lead to activation of the ERK cascade (Krapivinsky et al., 2003; Morozov et al., 2003; Roberson et al., 1999; Watabe et al., 2000). Once activated, ERK phosphorylates many targets which are implicated in diverse cellular processes, including cellular excitability (Adams et al., 2000a; Yuan et al., 2002), transcription (Impey et al., 1998; Vossler et al., 1997), translation (Banko et al., 2004; Kelleher et al., 2004), and organization of the dendritic cytoskeleton (Goldin and Segal, 2003; Wu et al., 2001). In addition, although the majority of studies have emphasized mechanisms of postsynaptic ERK signaling, recent work has also established a role for presynaptic ERK signaling, such that presynaptic inhibition of ERK can inhibit LTP in the lateral amygdala (Apergis-Schoute et al., 2005) and ERK phosphorylation of synapsin can influence neurotransmitter release (Kushner et al., 2005). These recent findings further increase the number of roles that this ubiquitous kinase oversees.

Regulation of the ERK cascade is complex (for review, see Adams and Sweatt [2002] and Pearson et al. [2001]). The trikinase cascade consists of Raf-1 and B-raf, which activate MEK1/2, the obligate upstream activator of ERK 1/2. Upstream of the Raf-MEK-ERK pathway is an enormous variety of membrane receptors, channels, and protein kinases which invariably feed into ERK activation. In neurons, routes to ERK exist via both Ras/protein kinase C (PKC)-dependent activation of Raf-1 and cAMP/protein kinase A (PKA)-dependent activation of B-raf (Dhillon and Kolch, 2002; Vossler et al., 1997). However, it is becoming increasingly clear that the ERK activation necessary for synaptic plasticity and memory formation is not a linear signaling module but a complex network of tightly regulated events.

This complexity raises the important question of how specificity of signaling initiated at the cell surface is maintained. Despite our knowledge of the involvement of the ERK cascade in many memory-related processes, mechanisms by which signaling specificity is maintained remain largely unknown. Numerous processes trigger ERK activation, yet cells manage to link the diverse initiating signals with a proper biological response. A mechanism to segregate signaling from different ERK-coupled receptors would seem vital for proper signal integration. In other words, the cell should have the capacity to know how ERK came to be activated so that the correct downstream response can be initiated.

Scaffolding proteins potentially represent a means for achieving compartmentalization of signaling molecules, retaining upstream and downstream signaling integrity, and regulating activation of signaling modules. A scaffolding protein facilitates pathway activation by physically coupling members of a signaling pathway so that

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the local concentration of these proteins is increased or by targeting signaling components to specific subcellular locations. Mammalian MAPK scaffolds have been reported, although none have been investigated for a role within the central nervous system (for review, see Morrison and Davis [2003]).

Kinase suppressor of Ras 1 (KSR1) has been shown to act as a scaffolding protein for the ERK cascade in invertebrates and in cell culture (Cacace et al., 1999; Yu et al., 1998). It was originally described in *C. elegans* (Therrien et al., 1995) and *Drosophila* (Kornfeld et al., 1995) as a protein, that, when mutated, suppressed an oncogenic ras phenotype. Although KSR1 shares sequence homology with the Raf family of kinases, whether or not it possesses enzymatic capability remains to be definitively established (for review, see Morrison [2001] and Xing et al. [2004]).

Although KSR1 has limited expression elsewhere, highest expression of the protein is seen in the adult brain, particularly in the hippocampus (Muller et al., 2000). The structure of KSR1 reveals functional domains for interaction with various members of the ERK cascade and other regulatory molecules, further strengthening its claim as a scaffolding protein. For instance, a portion of the N terminus interacts inducibly with ERK through an FxFP motif (Muller et al., 2000; Xing et al., 1997). The C terminus interacts with MEK1 and 2, an interaction that appears to be stable and constitutive (Cacace et al., 1999; Muller et al., 2000).

The present study investigated the biochemical role of KSR1 in the hippocampus and its effects on synaptic plasticity and behavioral learning. Using KSR1 null mice (Nguyen et al., 2002), we found that KSR1 was important for long-term associative memory formation, as well as for specific forms of ERK-dependent synaptic plasticity. Interestingly, our results suggested agonist-specific deficits in a membrane pool of hippocampal ERK signaling in KSR1 null mice. Moreover, we observed that the downstream target of ERK, Kv4.2, was affected by the absence of KSR1, while another target, RSK, was not, arguing that the signaling specificity observed at the level of ERK was maintained to downstream effectors of ERK. Taken together, the data demonstrate that KSR1 functions to colocalize signal-transducing proteins in response to a specific initiating signal and to compartmentalize signaling within the hippocampal neuron. KSR1 thus represents a mechanism to keep a cellular record of specific routes of ERK activation and links ERK to specific learning and plasticity paradigms.

Results

KSR1 Is Expressed in the Mouse Brain, and Absence of KSR1 Preserves Brain Morphology

We first demonstrated KSR1 expression in the hippocampus, cerebellum, amygdala, and cortex of wild-type mice, but not null mice, using both Western blotting and immunohistochemical techniques (Figures 1A–1H). Among the areas evaluated, the hippocampal formation demonstrated the highest relative expression of KSR1 (Figure 1A). Within the hippocampal formation, the density of KSR1 was highest in region CA3 (Figures 1F–1H). KSR1 is expressed in both cell bodies and dendrites of neurons, but not glial cells, as demonstrated by dual-labeling studies with MAP-2A/2B, NeuN, and GFAP, respectively (Figures 1E–1H). Additionally, despite the absence of KSR1, Nissl-stained sections revealed that null mice did not demonstrate gross alterations in brain morphology compared to wild-type mice (Figure 1C).

Baseline Behaviors in KSR1 Null Mice

Baseline behaviors in KSR1 null and wild-type littermates were examined for control purposes and found to be similar (Table 1). KSR1 null and wild-type mice showed no significant differences in levels of activity in the open field or in center to distance ratios, which is viewed as an index of anxiety-like behavior. Performance on the accelerating rotarod was not impaired in KSR1 null animals, and baseline nociception was intact in KSR1 null mice as assessed by shock thresholds and the hot plate test. In addition, swim speeds in the hidden platform version and latencies on the visible platform version of the Morris water maze task were similar. Taken together, these data demonstrate that KSR1 null animals are not impaired in motor activity or sensation and do not display abnormal levels of anxiety-like behavior, all of which are important controls for further behavioral tests.

KSR1 Is Important for Hippocampus-Dependent Memory

As ERK has been demonstrated to be important for certain hippocampus-dependent forms of memory, we hypothesized that KSR1 may also be important for these functions. Associative fear conditioning trains a mouse to associate a mild footshock with both a paired context and an auditory cue. Learning can be assessed at the desired time points by monitoring the animal's freezing behavior, an indicator of learned fear.

Fear-conditioned learned behavior was assessed in KSR1 null animals and littermate controls. Animals received either a modest training, consisting of a single pair of cue and shock, or a stringent training, consisting of three pairs of cue and shock. When the modest-training protocol was used, KSR1 null animals, compared to wild-type controls, displayed significantly less freezing when reexposed to either the context or the cue 24 hr posttraining (Figure 2A, as mean percentage freezing; contextual test—KO = 17.2 ± 3.8 s, wt = 32.9 ± 4.2, $p = 0.0085$; cued test—KO = 23 ± 4.1 s, wt = 43.8 ± 3.1, $p = 0.0003$). However, this deficit was not apparent when the null animals were trained in a more robust training paradigm. When three pairs of cue shock were used, freezing behavior was not significantly different between KSR1 null and wild-type mice in either the contextual or cued tests (Figure 2B, as mean percentage freezing; contextual test—KO = 52.2 ± 5.4, wt = 39.8 ± 5.4, $p = 0.15$; cued test—KO = 37.2 ± 4.6, wt = 42.9 ± 6.5, $p = 0.48$). This reversal of the deficit indicates that KSR1 null mice are able to freeze as much as littermate counterparts with sufficient stimulus.

In order to assess whether KSR1 is specifically important for long-term memory, we investigated short-term memory by testing KSR1 null animals and littermates 1 hr after the modest training paradigm. One hour after training, KSR1 null and littermate mice displayed equivalent freezing behavior in both the cued and contextual

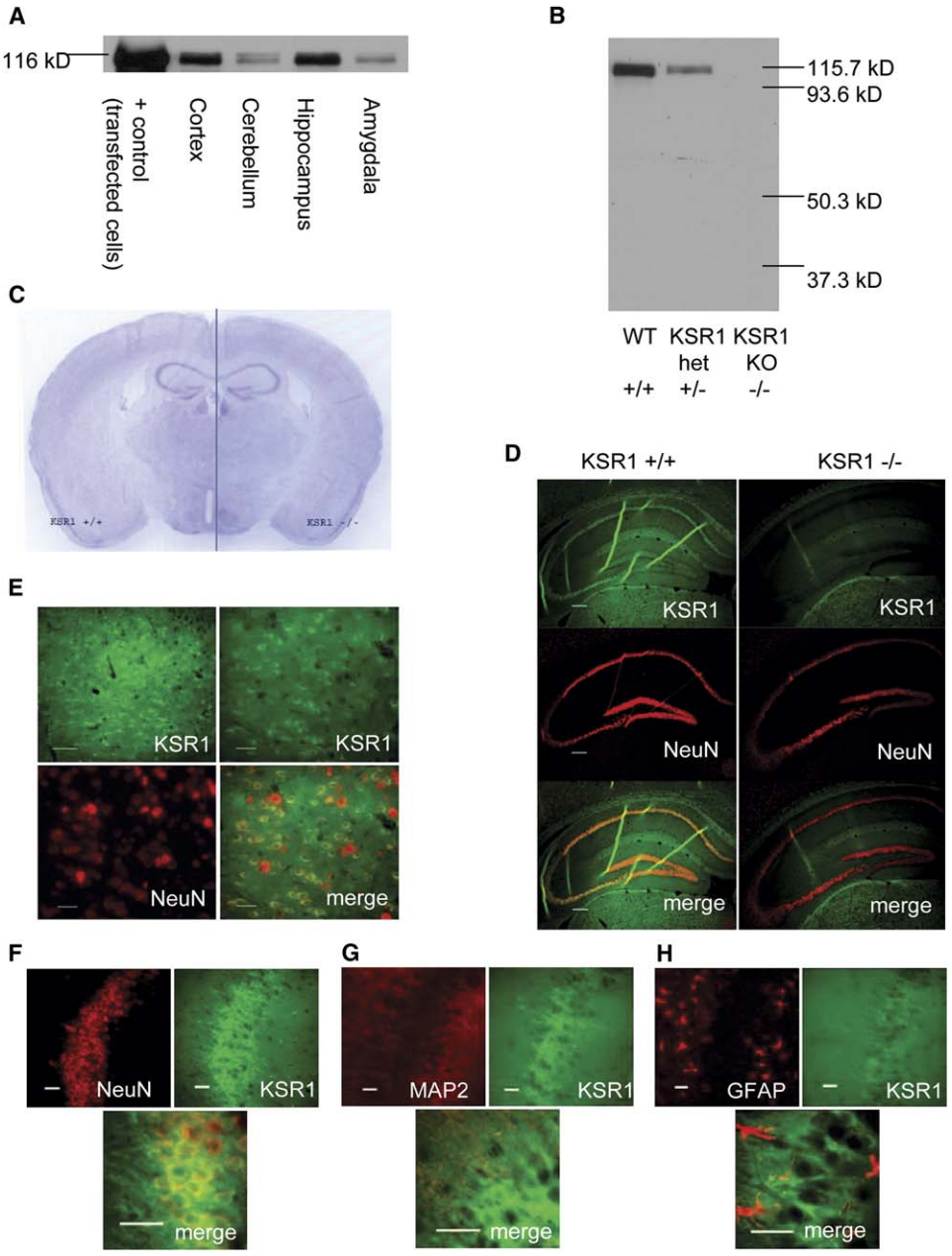

Figure 1. KSR1 Expression

(A) KSR1 was immunoprecipitated (Upstate antibody) from wt brain subregions, followed by probing for KSR1 (H70 Santa Cruz antibody). KSR1 is seen in cortex, cerebellum, hippocampus, and amygdala.

(B) KSR1$^{-/-}$ brain samples lack an immunoreactive band at the molecular weight of KSR1.

(C) Nissl stain of coronal hemisections from KSR1$^{+/+}$ (left) and KSR1$^{-/-}$ (right), indicating the absence of gross morphological abnormalities in the KSR1 null brain.

(D) Fluorescent immunohistochemistry comparing KSR1$^{+/+}$ and KSR1$^{-/-}$ hippocampus. KSR1 (green) specifically colocalizes with NeuN (red) in +/+ sections. Scale = 100 μm.

(E) KSR1 expression in the amygdala at the level of the basolateral nucleus: KSR1 (green) colocalizes with NeuN (red). Top left panel scale = 50 μm. Other panel scales = 20 μm.

(F–H) Higher magnification of area CA3 of hippocampus of KSR1$^{+/+}$ mice, showing NeuN (red, [F]), MAP2 (red, [G]), and GFAP (red, [H]) staining with KSR1 (green).

Scale = 20 μm.

tests (Figure 2C, as mean percentage freezing; contextual test—KO = 29.2 ± 5.1, wt = 27.4 ± 7.3, p = 0.84; cued test—KO = 28.6 ± 5.2, wt = 24.7 ± 4.2, p = 0.64). Thus, KSR1 null animals are able to process the sensory components of the training paradigm, form an association between one pair of cue and shock, and express this memory in the form of freezing behavior in the short term. However, by 24 hr, this memory is significantly impaired.

Table 1. Baseline Behaviors in KSR1 Null and KSR1 Wild-Type Littermates

Behavioral Test	Measurement	Testing For	KSR KO	Wild-Type Littermate	Statistical Significance	Statistical Test Used
Open field	Horizontal activity (beam breaks)	Activity levels	362.1 ± 8.49	334.9 ± 13.19	Not significant	t test
Open field	Vertical activity (beam breaks)	Activity levels	16.47 ± 0.77	16.44 ± 0.94	Not significant	t test
Open field	Center distance: total distance ratio	Anxiety	0.307 ± 0.013	0.318 ± 0.015	Not significant	t test
MWM	Swim speed (cm/sec)	Motor ability	25.9 ± 2.7	25.5 ± 2.1	Not significant	t test
MWM visible platform	Latency to visible platform—day 3 of training (s)	Visual acuity	2.95 ± 0.37	2.93 ± 0.38	Not significant	t test
Rotarod	Time spent on apparatus over eight trials, 2 days (s)	Motor ability; Motor learning	1^{st} trial—92 ± 16.6 8^{th} trial—287.7 ± 5	1^{st} trial—133 ± 20 8^{th} trial—283.4 ± 8	F7,448 = 57.8, p < 0.0001 (trial) Not significant (genotype) no interaction	Two-way ANOVA
Hot plate	Latency to lick or lift paw (s)	Sensation; Nociception	11.83 ± 0.61	11.79 ± 0.85	Not significant	t test
Shock thresholds	Shock threshold for flinching (f), jumping (j), or vocalization (v)—(mV)	Sensation; Nociception	f: 0.167 ± 0.008 j: 0.33 ± 0.04 v: 0.35 ± 0.03	f: 0.189 ± 0.16 j: 0.31 ± 0.03 v: 0.34 ± 0.02	f: Not significant j. Not significant v. Not significant	t test

This table depicts analysis parameters for baseline control behaviors in both KSR1 null and wild-type littermate mice. The table shows the test performed, what the test measured, mean values for the KSR1 null mice, mean values for wild-type mice, whether the statistical analysis yielded a significant result, and which statistical test was used for analysis. On all measures, KSR1 null mice displayed similar behavior patterns to their littermate counterparts.

Another test of hippocampus-dependent learning is the Morris water maze. A mouse learns to swim to a submerged platform using a pattern of visual cues placed on the wall of the training room. A probe trial with the platform removed is conducted after training to assess spatial learning. An animal that has learned a spatial search strategy is expected to spend the majority of the time in the quadrant where the platform had been located and to display many crossings at the previous locale of the escape platform.

KSR1 null and wild-type mice were trained in the Morris water maze using four trials per day for 10 days, with an intertrial interval of 20–30 min. As expected, the latencies to find the platform decreased with training in both

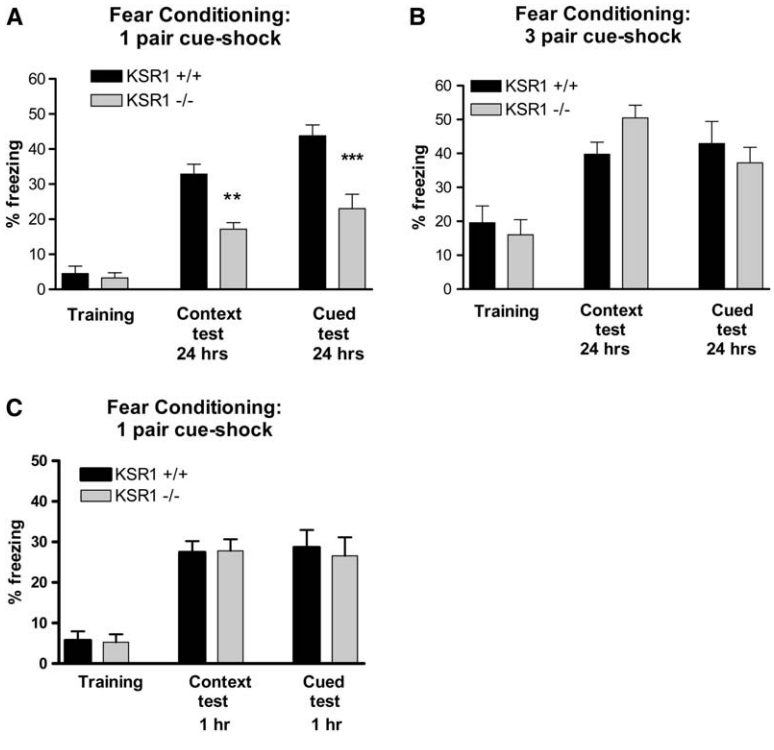

Figure 2. KSR1 Null Mice Show Deficits in Contextual and Cued Fear Conditioning

(A) Modest training (one exposure to cue and shock): $KSR1^{-/-}$ mice (gray bars, n = 19) froze significantly less than $KSR1^{+/+}$ (black bars, n = 18) in both contextual and cued tests at 24 hr posttraining.

(B) Robust, three-pairing protocol: $KSR1^{-/-}$ (gray bars, n = 24) and $KSR1^{+/+}$ mice (black bars, n = 12) displayed equivalent contextual and cued fear conditioning.

(C) $KSR1^{-/-}$ (gray bars, n = 15) and $KSR1^{+/+}$ mice (black bars, n = 7) showed equivalent freezing when tested one hour after the modest training.

Data are presented as mean ± SEM. **p < 0.01; ***p < 0.001.

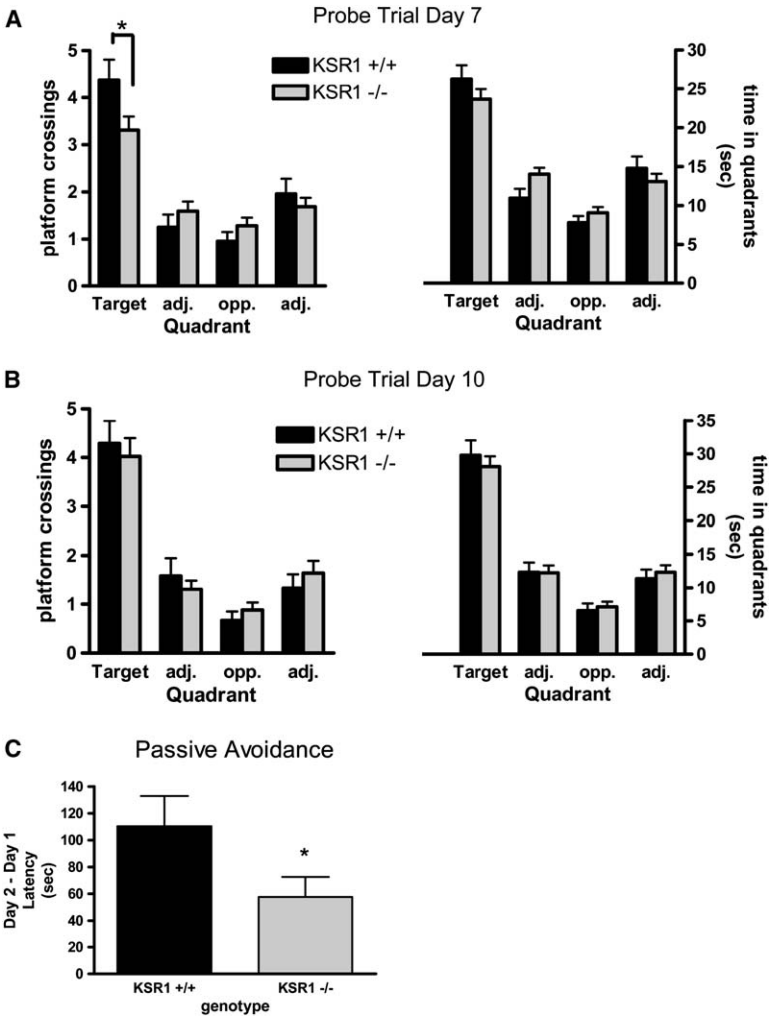

Figure 3. KSR1 Null Mice Show Deficits in Water Maze and Passive Avoidance

(A) Probe trial after day 7 of water maze training. KSR1$^{-/-}$ mice (n = 42, gray bars) show significantly fewer platform crossings than KSR1$^{+/+}$ (n = 24, black bars), but similar time spent in the target quadrant.

(B) Probe trial after day 10 of water maze training. KSR1$^{-/-}$ mice (n = 42, gray bars) show equivalent platform crossings and time spent in the target quadrant compared to KSR1$^{+/+}$ (n = 24, black bars).

(C) KSR1$^{-/-}$ mice (n = 30, gray bars) exhibit a passive avoidance deficit as evidenced by a less of a difference between first and second day latencies than KSR1$^{+/+}$ (n = 25, black bars).

Data are presented as mean ± SEM. *p < 0.05.

groups (data not shown). A probe trial performed after 7 days of training revealed that KSR1 null mice displayed fewer platform crossings than their littermate counterparts (Figure 3A, as mean crossings; KSR1 = 3.3, wt = 4.3, $p < 0.05$). However, this modest difference was not mirrored in another probe trial parameter. KSR1 null and wild-type littermates spent similar amounts of time in the target quadrant during the probe trial (Figure 3A, as mean time; KSR1 null = 23.6 s, wt = 26.3 s, $p > 0.05$). These data suggest that, while KSR1 null animals had learned the correct quadrant of the platform location, they were not as proficient as wild-type mice in pinpointing the exact location. Alternatively, a probe trial performed after 10 days of training revealed no discernable differences between the groups in either platform crossings or time spent in the target quadrants (Figure 3B).

To confirm this behavioral phenotype, we tested KSR1 null mice in another hippocampus-dependent task, passive avoidance. Because of their desire to avoid brightly lit spaces, mice will tend to quickly move from the well-lit to the darkened side of the testing chamber, whereupon a mild footshock is delivered. In subsequent trials, animals will display higher latencies to enter the darkened compartment, thus avoiding the associated footshock. KSR1 null and littermate animals displayed equivalent latencies to enter the darkened chamber on the first training day (before any shock had been delivered). Twenty-four hours after receiving a shock on the dark side, however, KSR1 null mice entered the darkened chamber earlier than their littermate counterparts (Figure 3C, as a mean difference score [day 2 latency − day 1 latency]; KO = 57.7 ± 14.9, wt = 110.5 ± 22.5, $p = 0.049$).

KSR1 Is Important for Certain Forms of ERK-Dependent Synaptic Plasticity

Having identified learning and memory impairments in KSR1 null mice and coupling this with the knowledge that ERK activation is vital for certain forms of synaptic plasticity, we next used electrophysiology to investigate whether KSR1 was required for ERK-dependent forms of plasticity in acute hippocampal slices. We specifically investigated synaptic connections at Schaffer collateral synapses. A general impairment in synaptic plasticity could provide a cellular mechanism to explain our behavioral data.

In evaluating the input-output curve for synaptic function, we found no differences in measures of baseline synaptic transmission. For a range of stimulation intensities, the slope of KSR1 null EPSP responses were not significantly different than EPSP responses from

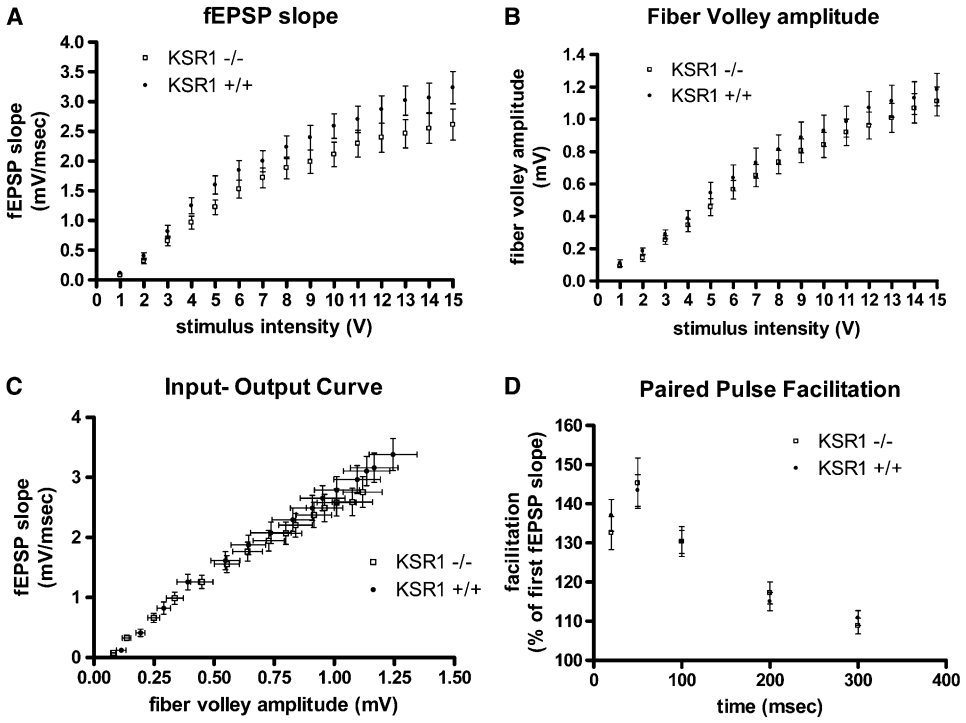

Figure 4. Baseline Synaptic Transmission in KSR1 Null Hippocampus

(A) fEPSP slopes are comparable in KSR1$^{-/-}$ (open squares, n = 48) and KSR1$^{+/+}$ (closed circles, n = 47) slices for a given range of stimulus intensities.

(B) Fiber volley amplitudes are comparable in KSR1$^{-/-}$ (open squares, n = 48) and KSR1$^{+/+}$ (closed circles, n = 47) slices for a given range of stimulus intensities.

(C) Input/output curves are similar for KSR1$^{-/-}$ (open squares, n = 48) and KSR1$^{+/+}$ (closed circles, n = 47).

(D) KSR1$^{-/-}$ (open squares, n = 30) and KSR1$^{+/+}$ slices (closed circles, n = 15) have similar paired pulse facilitation.

Data are presented as mean ± SEM.

littermate slices (Figure 4A; $F_{1,1344}$ = 2.4 for genotype, p > 0.05). Likewise, measurements of the fiber volleys from KSR1 null and wild-type slices were similar (Figure 4B; $F_{1,1302}$ = 0.57 for genotype, p > 0.05), and there was no difference in an input/output curve (Figure 4C). Paired pulse facilitation was also normal in KSR1 null mice (Figure 4D; $F_{1,215}$ = 0.03 for genotype, p > 0.05). These data support the notion that baseline synaptic transmission is unaffected by the absence of KSR1.

A variety of investigators have determined that theta burst stimulation-induced LTP is ERK dependent in mice (Dudek and Fields, 2001; Selcher et al., 2003; Watanabe et al., 2002). Theta burst stimulation (TBS) consisting of three trains of ten high-frequency bursts delivered at 5 Hz (intertrain interval of 20 s) was delivered to hippocampal slices from both KSR1 null and KSR1 wild-type mice. KSR1 null animals showed diminished TBS-LTP compared to wild-type animals, especially during the first hour postinduction (Figure 5A; 30 min after induction, as mean percentage of baseline—KSR1 null = 135.7 ± 4.6, KSR1 wt = 163.4 ± 9.2, p = 0.01). Although slices from KSR1 null mice did develop LTP, they did not do so to the extent that wild-type slices did. In fact, the deficit in TBS-LTP in the KSR1 knockout animals mimicked the effect of U0126, a MEK inhibitor, on slices from wild-type mice. Application of U0126 caused no additional decrement in LTP induced in KSR1 null animals (Figure 5B, as percentage of baseline; KSR1 null ± U0126 = 135.4 ± 7.9, p = 0 .04; KSR1 wt ± U0126 = 129.2 ± 10, p = 0.02 compared to wild-type control).

Moreover, TBS-induced LTP was found to be dependent on PKC and mGluR5 activation. In wild-type animals, application of the PKC inhibitor GF109203x led to significantly reduced TBS-induced LTP (Figure 5C, as percentage of baseline; wt = 146.2 ± 7.1; wt + GF109203x = 122.2 ± 8.8, p < 0.05). Likewise, application of the specific mGluR5 antagonist MPEP on wild-type slices also resulted in diminished TBS-induced LTP (Figure 5D, as percentage of baseline; wt = 146.2 ± 7.1; wt + MPEP = 121.5 ± 8.5, p < 0.05).

As a control, an LTP-inducing protocol known to be non-ERK-dependent in mice was performed on slices from KSR1 wild-type and null slices. LTP induced by two high-frequency stimulations produced robust and equivalent levels of potentiation in both KSR1 null and wild-type slices (Figure 5E, as percentage of baseline; KSR1 null = 140.7 ± 8.7, KSR1 wt = 146.9 ± 7.5, p = 0.6). These data indicate that KSR1 null mice do not have a global impairment in synaptic function but do display a specific deficit in TBS-LTP.

Other forms of ERK-dependent LTP have been reported, including theta frequency stimulation in the presence of isoproterenol application (Winder et al., 1999). We were surprised to find that this form of LTP was equivalent in hippocampal slices from both KSR1 null and wild-type mice (Figure 5F, as percentage of baseline; KSR1 null = 129.1 ± 9.0, KSR1 wt = 122.5 ± 7.4,

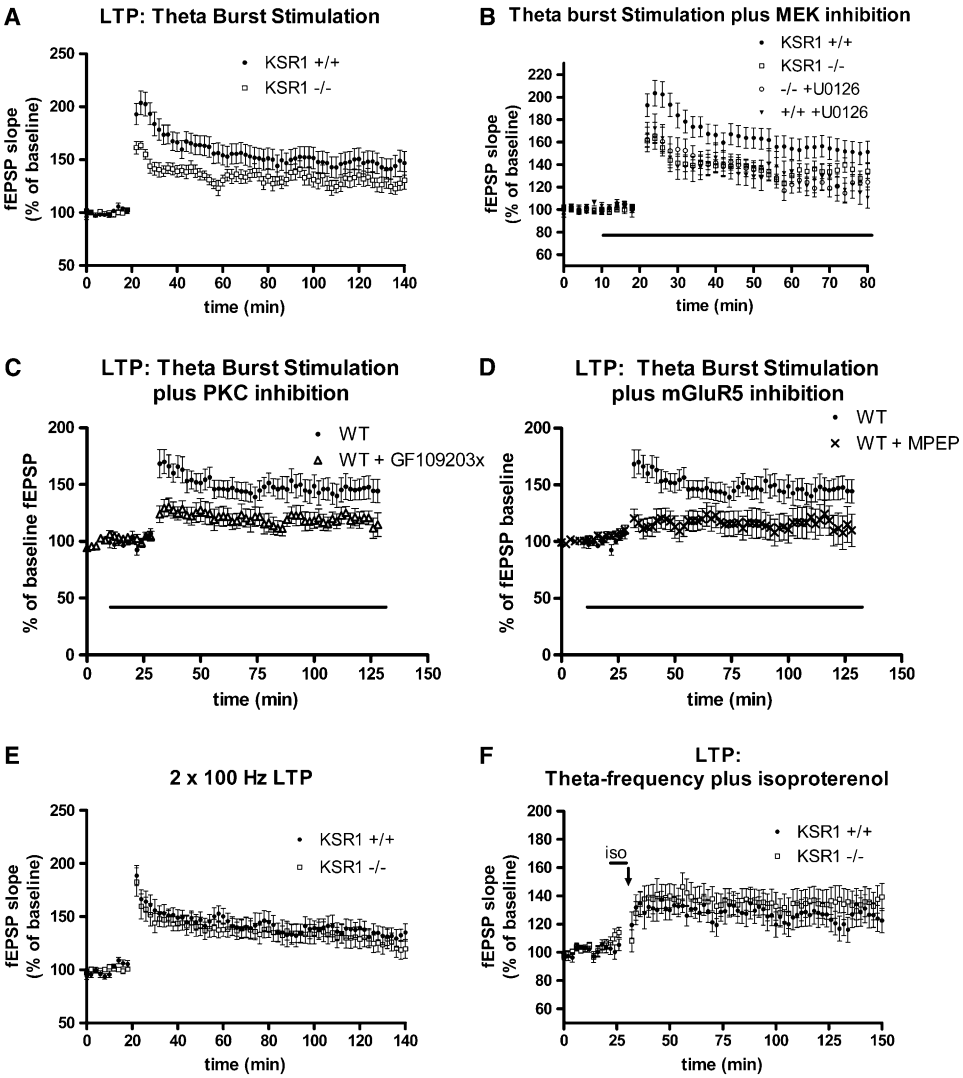

Figure 5. TBS LTP Is Diminished in KSR1 Null Hippocampal Slices

(A) KSR1$^{-/-}$ slices (open squares, n = 26) display diminished TBS-induced LTP compared to KSR1$^{+/+}$ slices (closed squares, n = 25).

(B) Application of the MEK inhibitor U0126 (20 μM) during induction of TBS LTP decreases LTP induced in KSR1$^{+/+}$ slices (closed triangles, n = 17), but not in KSR1$^{-/-}$ slices (open circles, n = 15).

(C) Application of the PKC inhibitor GF109203x (10 μm) reduces TBS-induced LTP (open triangles, n = 12) compared to TBS LTP in KSR1$^{+/+}$ slices (closed circles, n = 17).

(D) Application of the mGluR5 antagonist MPEP (10 μm) reduces TBS-induced LTP (exes, n = 11) compared to TBS LTP in KSR1$^{+/+}$ slices (closed circles, n = 17).

(E) High-frequency stimulation LTP is unimpaired in KSR1$^{-/-}$ mice (KSR1$^{-/-}$: open squares, n = 22; KSR1$^{+/+}$: closed squares, n = 20).

(F) LTP induced with theta frequency stimulation after application of 1 μM isoproterenol was unaffected in KSR1$^{-/-}$ mice (KSR1$^{-/-}$: open squares, n = 15; KSR1$^{+/+}$: closed squares, n = 15). Points on the graphs indicate mean percentage ± SEM of baseline fEPSP slope. Drug applications are indicated by solid lines.

Data are presented as mean ± SEM.

p = 0.58), indicating that KSR1 is important for some, but not all, forms of ERK-dependent LTP in the hippocampus.

In an effort to further analyze the observed deficits in TBS-induced LTP in the KSR1 null mice, we examined spiking within the cell body layer (Figures 6A–6D) during the theta burst stimulation delivery (Selcher et al., 2003). We found that the first train of theta burst demonstrated significantly less spiking in KSR1 null compared to wild-type slices (Figure 6B, $p < 0.01$) and that the second train of theta burst showed a trend toward significantly reduced spiking in KSR1 null compared to wild-type slices (Figure 6C, p = 0.0504). The decreased cell body spiking is likely to account for the observed reduction in TBS-LTP in KSR1 null mice.

KSR1 Displays Preference for Specific Agonists that Activate ERK

Intrigued by the fact that one ERK-dependent form of synaptic plasticity (TBS) was impaired in KSR1 null mice while a different form (theta frequency stimulation with coapplication of isoproterenol) was intact, we investigated the molecular events leading to ERK activation as a possible explanation for this surprising result.

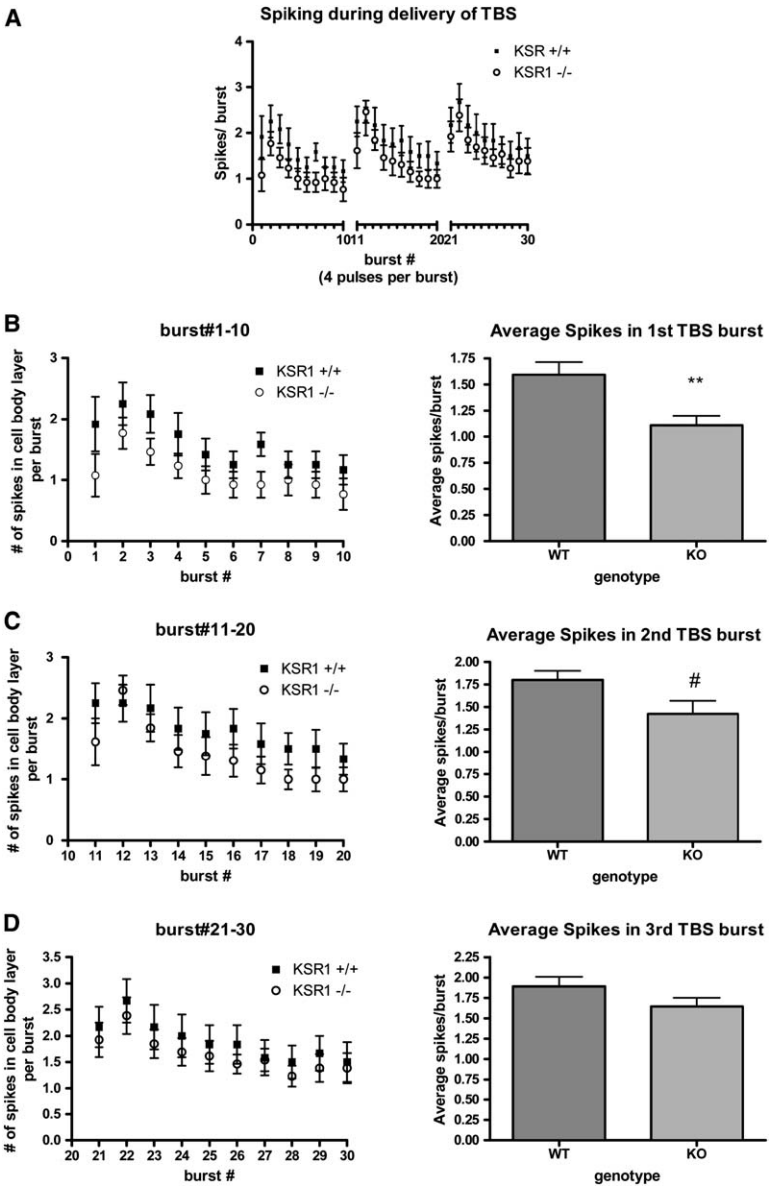

Figure 6. Cell Body Layer Spiking during Theta Burst Delivery Is Decreased in KSR1 Null Mice

(A) Spiking within the cell body layer was monitored during the delivery of theta burst stimulation in both +/+ (closed squares) and KSR1$^{-/-}$ (open circles) slices.

(B) Spikes during the first theta burst train.

(C) Spikes during the second theta burst train.

(D) Spikes during the third theta burst train.

**$p < 0.01$; # $p = 0.0504$.

Various pharmacological agents can trigger ERK activation through either a PKC/Ras-dependent pathway or a cAMP/PKA-dependent pathway. Forskolin activates adenylyl cyclase and thus PKA, while phorbol diacetate (PDA), a phorbol ester, leads to PKC activation. The effect of these drugs on ERK signaling in KSR1 null and wild-type hippocampus was evaluated by Western blotting. Initial results demonstrated impaired phosphorylation of ERK and MEK in slices from KSR1 null mice when treated with PDA but not forskolin (Figure 7A). This finding suggested the novel possibility that KSR1 might selectively couple the PKC-dependent pathway to ERK activation.

In order to further investigate this intriguing result and to examine whether this deficit in signaling was occurring in a specific subcellular compartment, membrane and cytosolic fractions were prepared from hippocampal slices following a 10 minute drug application. Treatment with PDA led to impaired ERK phosphorylation in KSR1 null mice specifically in the membrane fraction, while treatment with forskolin demonstrated levels of phosphorylation similar to that seen in wild-type (Figure 7B, as percentage of control; KSR1 null PDA = 326.9, KSR1 wt PDA = 797.8, $p < 0.01$; KSR1 null forskolin = 319.3, KSR1 wt forskolin = 353.7, $p > 0.05$). The cytosolic fraction did not show any difference in phosphorylation with either PDA or forskolin in KSR1 null mice (Figure 7C, as percentage of control; KSR1 null PDA = 214.5, KSR1 wt PDA = 305.8, $p > 0.05$; KSR1 null forskolin = 197.9, KSR1 wt forskolin = 222.2, $p > 0.05$). Likewise, treatment of slices with DHPG, a metabotropic glutamate receptor agonist that activates ERK through PKC, produced diminished ERK activation in the membrane, and not cytosolic, fraction of KSR1 null hippocampal tissue (Figures 7B and 7C, as percentage of control; membrane—KSR1 null = 166.6, KSR1 wt = 306.1, $p < 0.01$; cytosolic—KSR1 null = 99.4, KSR1 wt = 123.0, $p > 0.05$). Treatment with isoproterenol, a

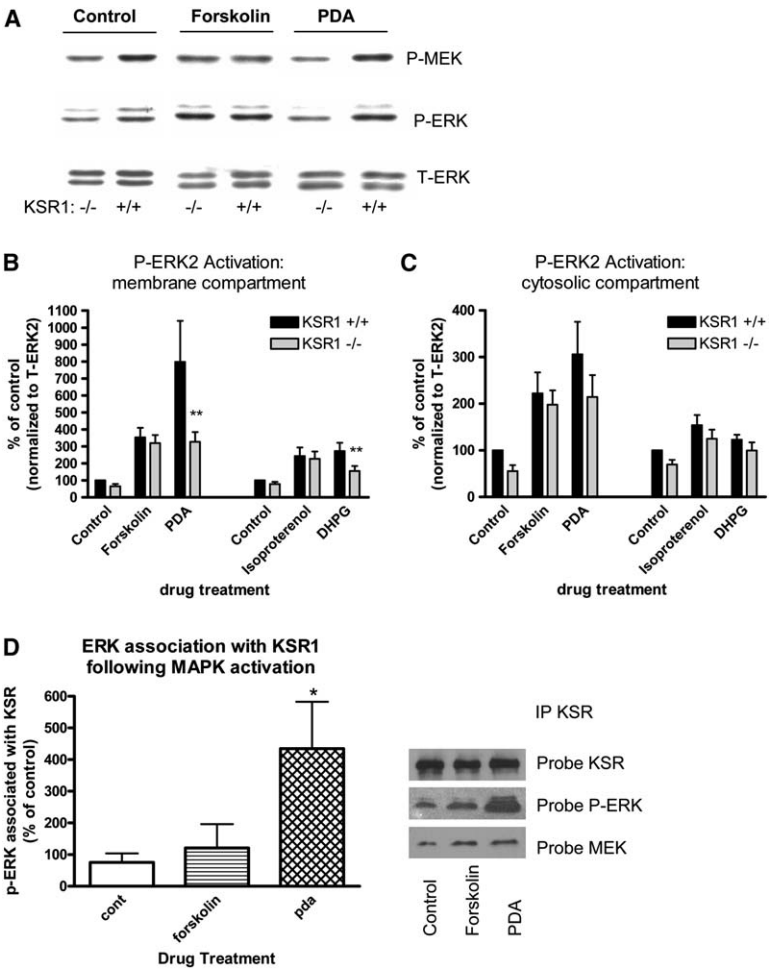

Figure 7. The Membrane Fraction of ERK Signaling Is Impaired when Stimulated via PKC in KSR1 Null Mice

(A) Representative Western blots from whole hippocampal homogenates suggest that MEK and ERK activations are impaired in KSR1$^{-/-}$ mice after PDA but not forskolin treatment.

(B and C) Hippocampal slices from KSR1$^{-/-}$ mice display impaired ERK2 phosphorylation in a membrane fraction following stimulation with activators of PKC (PDA or DHPG) but normal phosphorylation after treatment with activators of PKA (forskolin or isoproterenol). ERK2 phosphorylation in the cytosolic compartment of KSR1$^{-/-}$ and KSR1$^{+/+}$ slices is equivalent.

(D) ERK associates with KSR1 following stimulation with PDA. Representative western blots for KSR1, p-ERK, and MEK are shown following treatment of wild-type hippocampal slices with vehicle, forskolin, or PDA and subsequent immunoprecipitation of KSR1.

Graphs are averages of 8–12 experiments, presented as mean ± SEM. *p < 0.05; **p < 0.01.

β-adrenergic agonist that activates ERK through PKA, demonstrated equivalent levels of ERK phosphorylation in both null and wild-type slices (Figures 7B and 7C, as percentage of control; membrane—KSR1 null = 227.0, KSR1 wt = 243.3, p > 0.05; cytosolic—KSR1 null = 124.7, KSR1 wt = 153.9, p > 0.05).

These data suggest that KSR1 is preferentially influencing ERK signaling depending on the mechanism of signal initiation. To further investigate this possibility, we took advantage of the previously observed transient and inducible association of KSR1 with ERK following activation of the MAPK cascade in several cell culture systems (Cacace et al., 1999; Muller et al., 2000). Forskolin or PDA was used to trigger activation of the ERK cascade in hippocampal slices from wild-type C57/Bl6 mice. We found that KSR1 coimmunoprecipitated a significantly higher level of phospho-ERK following treatment with PDA than control. Treatment with forskolin did not yield a significant increase in KSR1 coimmunoprecipitation with phospho-ERK (Figure 7D, as percentage of control; PDA = 434.8 ± 147.8, p = 0.04; forskolin = 120.8 ± 75.6, p = 0.59).

Signaling Specificity Is Maintained Downstream of ERK

Having established that KSR1 plays a preferential role in PKC-directed ERK signaling in the membrane compartment, we next investigated whether this specificity extended to downstream targets of ERK. We first examined a membrane ERK target, the potassium channel subunit Kv4.2. Using Western blotting, we determined that PDA treatment resulted in decreased levels of ERK-phosphorylated Kv4.2 in KSR1 null compared to wild-type hippocampal slices (Figure 8A, as mean percentage of control; KSR1 null = 110.9 ± 19.75, KSR1 wt = 196.0 ± 34.9, p < 0.05). In contrast, PDA-stimulated, ERK-dependent phosphorylation of RSK, another target of ERK primarily located in the cytosol and nucleus, was unaffected by the absence of KSR1 (Figure 8B, as mean percentage of control; KSR1 null = 377.9 ± 112.0, KSR1 wt mean = 403.7 ± 123.4, p > 0.05). Overall, we conclude that KSR1 represents a mechanism to bridge particular upstream signaling events with certain specific downstream effectors of ERK (Figure 9).

Discussion

In the present study, we show that KSR1, a scaffolding molecule for the ERK cascade, functions to compartmentalize hippocampal signal transduction pathways and to mediate learning and certain types of ERK-dependent synaptic plasticity in hippocampus. KSR1 null mice show deficits in contextual and cued associative fear conditioning, passive avoidance, and the Morris

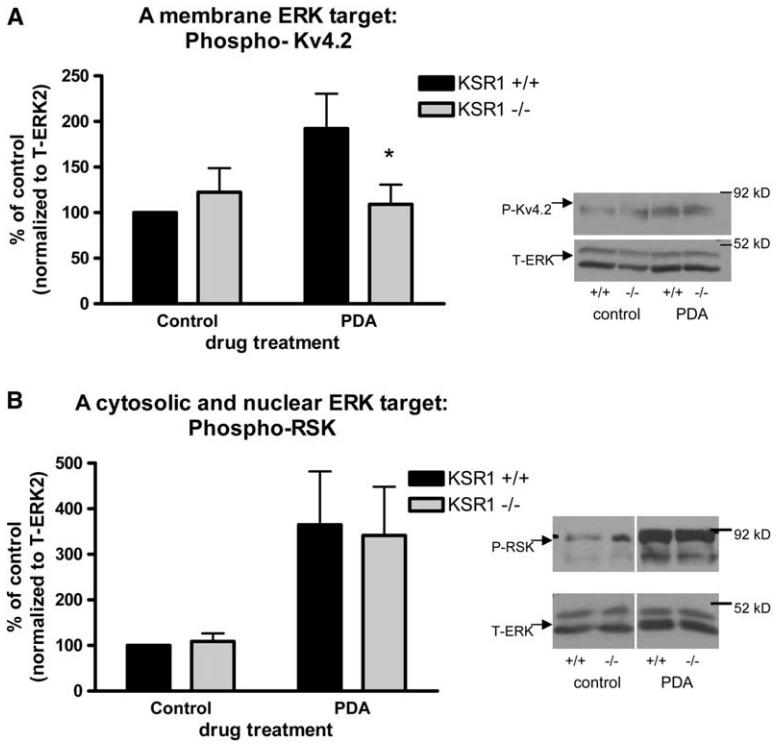

Figure 8. The Absence of KSR1 Affects Kv4.2 Phosphorylation

(A) Hippocampal slices from KSR1$^{-/-}$ mice display impaired ERK-phosphorylation of Kv4.2 when stimulated with PDA.

(B) Phosphorylation of RSK in KSR1$^{-/-}$ slices is normal when stimulated with PDA.

Representative Western blots are shown. Graphs are averages of 9–11 experiments, presented as mean ± SEM. *$p < 0.05$.

water maze. These mice also display decreased TBS-induced LTP, which represents a possible cellular mechanism for the observed behavioral phenotype. Most surprising and intriguing, however, is our finding that KSR1 specifically facilitates activation of a membrane pool of ERK that is preceded by activation of PKC but not by PKA, yielding a possible molecular mechanism for the reported cellular and behavioral deficits. This finding is supported by our observations that TBS-induced LTP shows a dependence on both PKC and mGluR5, which would explain why KSR1 null mice show a deficit in this type of plasticity. Moreover, this specificity of signaling is maintained in downstream targets of ERK, such that one target, the potassium channel subunit Kv4.2, is affected by the loss of KSR1 whereas another ERK target, RSK, is not. These findings provide new insight into the regulation of the ERK cascade in neurons, as KSR1 represents both a mode for compartmentalization of signaling (to plasma membrane and membrane targets) and a mode for maintaining signaling (PKC-initiated) specificity. Until now, no such compartmentalization or scaffolding

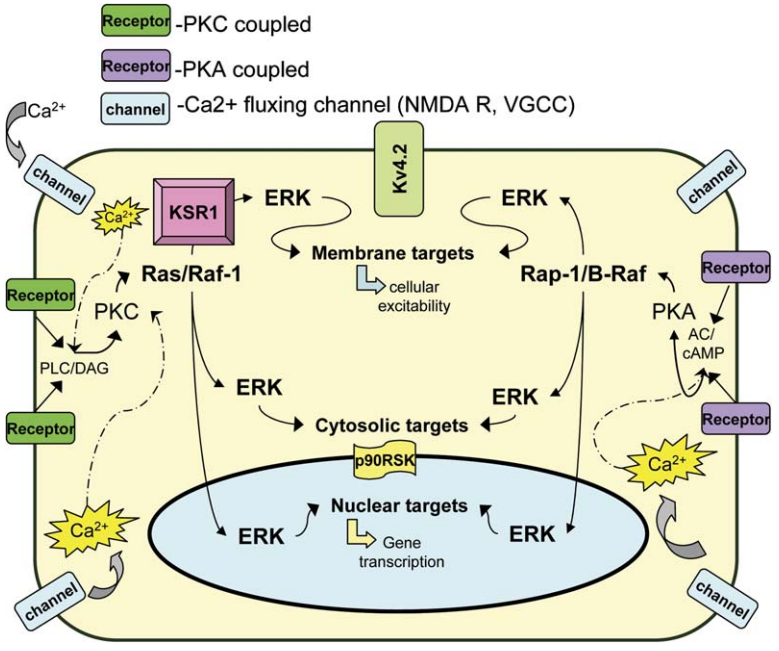

Figure 9. Model of KSR1 Function

Receptors on a neuron's surface couple to either PLC/DAG production, which activates PKC, or to adenylyl cyclase/cAMP generation, which leads to PKA activation. PKC feeds into the ERK cascade via activation of Ras and/or Raf-1. PKA triggers ERK activation through the intermediates Rap-1 and B-raf. Calcium entry from NMDA receptors, voltage-gated calcium channels, and internal stores also contribute to ERK activation through either PKC or PKA. PKC and PKA activate membrane, cytosolic, and nuclear pools of ERK, each of which have downstream targets to phosphorylate: Kv4.2 is a membrane target; RSK is a cytosolic and nuclear target. KSR1 is uniquely responsible for scaffolding components of a membrane pool of ERK that has been preceded by a PKC-initiated event.

mechanism for regulating ERK in the nervous system has been described.

KSR1 null mice displayed both contextual and cued long-term memory deficits with a one-pairing training paradigm. This memory deficit was not apparent 1 hr posttraining or when training involved a more stringent, three-pairing design. These data suggest that KSR1 plays a role in long-term memory formation when repeated reinforcement of the learning paradigm is lacking. Similarly, when trained in the water maze, KSR1 null mice showed fewer platform crossings than did littermates after 7 days of training but equivalent platform crossings after 10 days of training. These results are provocative because they emphasize the probable importance of multiple, converging inputs onto ERK activation in order to achieve a long-term, robust, ERK-dependent behavior. Additional training, such as three pairings of cue shock in the fear-conditioning paradigm instead of only one or extra training in the Morris water maze, might enhance the number of signaling systems neuronal networks recruit in order to form a memory. Less exposure to the conditions to be learned might not recruit all of these systems. Thus, the absence or diminishment of a particular signaling cascade, such as PKC-mediated ERK activation involving KSR1, might lead to a behavioral phenotype.

Alternatively, a composite minimum threshold of ERK activation from any source may be required in order to form a memory. It is possible that the absence of KSR1 sufficiently diminishes the total pool of ERK that is activated, thereby impairing the formation of the memories for which exposure to the training situation is limited. Therefore, the contribution of KSR1-dependent signaling may be less requisite when sufficient exposure to the training conditions exist.

Contextual-fear learning recruits both hippocampus and amygdala, while cued-fear learning relies on the amygdala (Phillips and LeDoux, 1992; Kim et al., 1993; Frankland et al., 1998). KSR1 null mice displayed deficits in both contextual and cued one-pairing fear conditioning. As such, the observed learning deficits argue that KSR1 is also important in the amygdala. Although KSR1 is expressed in amygdala, albeit to a lesser degree than in the hippocampus, ERK signaling and the importance of KSR1 in the amygdala have yet to be thoroughly investigated. We hypothesize that the contextual-learning deficit we observed is, at least, in part due to a lack of hippocampal KSR1. Not only is signal transduction altered in this region, but KSR1-deficient animals also demonstrate a passive avoidance learning deficit, which classically relies on the hippocampus (Izquierdo and Medina, 1997).

We observed a selective deficiency in PKC-dependent activation (through PDA stimulation or metabotropic glutamate receptor activation) of a membrane pool of ERK in KSR1 null mice. It is important to note that PKC activity itself does not appear to be impaired in these mice. PKC stimulation of ERK in the cytosolic fraction was equivalent in KSR1 null and wild-type hippocampal tissue, and levels of ERK-phosphorylated RSK were similar in both KSR1 null and wild-type hippocampal tissue. As such, this is the first report of a specific loss of PKC-dependent ERK activation in any model system. This finding raises the following question: are there specific PKC/ERK-dependent behaviors? The presence of a fairly robust associative fear memory deficit coupled with a subtle spatial learning impairment makes it tempting to hypothesize that PKC-coupling to ERK via KSR1 is involved in specific forms of memory. In other words, KSR1 might be selectively compartmentalizing processes not only at the molecular level but also at the behavioral level. Although it is surely an overgeneralization to say that KSR1 is important specifically for modest training of fear learning and that PKC-directed ERK activation contributes to this type of learning, it is reasonable to think that certain molecular events will contribute to different types or modes of learning more than others. Our current observations are consistent with this idea.

Likewise, the ERK-dependent LTP deficits we observed in KSR1 null mice were quite specific. TBS LTP (induced with three trains [intertrain interval = 20 s] of ten high-frequency bursts delivered at 5 Hz) was diminished in KSR1 null mice while LTP induced with theta frequency/isoproterenol (induced by a 10 min application of isoproterenol followed by 5 Hz stimulation for 3 min) was not. These data also support the notion that not all ERK activation is created equal—i.e., that certain modes of ERK activation can be channeled into different cellular outputs. Indeed, we found that TBS-induced LTP was partially dependent on PKC and mGluR5 activation in wild-type mice, in addition to its dependence on ERK. This finding suggests why KSR1 null mice displayed deficits in this form of synaptic plasticity. On the other hand, the isoproterenol required for the theta frequency/isoproterenol induction paradigm would be expected to recruit cAMP/PKA to activate ERK. Our biochemical data indicate that KSR1 is not involved in this type of ERK signaling, providing an explanation for the observed results.

In light of our biochemical results, we examined the role of metabotropic glutamate receptors and PKC in the generation of TBS-LTP. The mechanisms leading to this form of LTP have not been established, and we were interested in whether a PKC-dependence of TBS-LTP could explain the diminished form of this type of plasticity in KSR1 null mice. The observation that the specific mGluR5 antagonist MPEP substantially reduced TBS-LTP argues a critical role for mGluR5 in TBS-LTP induction. Metabotropic glutamate receptors have been linked primarily to LTD (Gallagher et al., 2004) and depotentiation of LTP (Delgado and O'Dell, 2005), so this apparent crucial involvement of mGluRs in the induction of LTP is somewhat surprising. However, a recent study found that while activating group I mGluRs with DHPG can cause depotentiation of LTP, it also led to a seemingly paradoxical increase in LTP-promoting GluR1 phosphorylation and to persistent activation of CaMKII (Delgado and O'Dell, 2005). In addition, other reports have found a role for group I mGluRs in priming LTP, such that preapplication of mGluR agonists can cause a weak tetanic stimulus to generate a persistent LTP (Raymond et al., 2000). Taken together, the data support an emerging role for metabotropic glutamate receptors in the generation of TBS-LTP.

The ERK/MAP kinase cascade has often been thought of as a final common pathway whereby many initiating signals feed indiscriminately into ERK activation. Our

data suggest a revision to this idea, as we found that PKC-dependent ERK signaling can be uniquely facilitated by the scaffolding protein KSR1. The ultimate test of whether these specific scaffolding mechanisms have functional relevance is whether or not specificity is maintained in the downstream phosphorylation targets of ERK. Kv4.2 displayed impaired phosphorylation in KSR1 null hippocampal slices following treatment with PDA, while phosphorylation of the ERK effector RSK was not diminished in KSR1 null slices after PDA application.

The phosphorylation state of Kv4.2 affects neuronal excitability, with phosphorylated subunits more likely to be inactivated, thus making the membrane more easily depolarized to threshold (Watanabe et al., 2002; Yuan et al., 2002). In this manner, phosphorylation of Kv4.2 may contribute to cell-body spiking observed during TBS-LTP (Selcher et al., 2003). In fact, we observed that KSR1 null mice displayed less spiking in the cell body layer during the delivery of the theta burst than did wild-type mice. These data explain, at least partially, the TBS-LTP deficit in KSR1 null animals. At higher membrane potentials, the majority of Kv4.2 channels will be rapidly inactivated, regardless of phosphorylation state. As such, for large depolarizations, LTP has a very high probability of being generated. In contrast, smaller depolarizations *are* sensitive to modulation by Kv4.2 and, by extrapolation, the presence or absence of KSR1. A higher probability of channel phosphorylation, and thus inactivation, means that LTP has a better chance of being induced. This observation has behavioral implications as well. A learning paradigm only exposing the mouse to one association pairing, as in the one cue-shock conditioning paradigm, would be expected to generate weaker cellular signals than would a paradigm utilizing multiple association pairings. Indeed, KSR1 null mice show deficits in this type of learning, and thus even the behavioral impairments may be linked to the decrease in Kv4.2 phosphorylation.

In contrast, we were unable to find a difference in phospho-RSK levels in KSR1 null mice when stimulated with PDA. RSK is a kinase known to phosphorylate CREB (Impey et al., 1998) and influence the gene expression required for long-term memory formation and for late-phase components of LTP (Adams et al., 2000b). KSR1 null mice were able to form long-term memories, although this required robust training. The mice generated persistent, albeit reduced compared to wild-type, TBS-LTP. These results, coupled with intact RSK phosphorylation, suggest that at least some protein synthesis-dependent processes are still intact in these mice. However, the observation of significant long-term learning impairments (fear conditioning, water maze, and passive avoidance) argues that there are other independent protein synthesis-dependent mechanisms which are not functioning optimally. Further experimentation will decipher how the absence of KSR1 affects protein-synthesis mechanisms to elicit long-term memory deficits.

As KSR1 appears to play a very specific role in ERK signaling, other mechanisms to compartmentalize signaling may exist to constrain different modes of ERK signaling. In fact, other scaffolding molecules have been postulated to serve the ERK cascade. MEK partner 1 (MP1) interacts with MEK1 and ERK1 to specifically facilitate the activation of ERK1 (Schaeffer et al., 1998). MAPK organizer (MORG)-1 scaffolds MAPK members in vitro only when certain agonists stimulate activation of the cascade (Vomastek et al., 2004). However, the effects of an ERK scaffolding protein have not been investigated in the nervous system until now, despite the large body of literature establishing a role for the ERK cascade in memory formation and CNS function. In addition, our study investigated an in vivo role of KSR1, rather than using cell culture and construct transfection, as was done in the majority of prior investigations.

In another vein of investigation, Morozov and colleagues (2003) used a dominant-negative Rap1 mutant mouse to examine the contribution of PKA-dependent ERK signaling. Rap1 is a Ras-related protein that serves to enhance cAMP/PKA-dependent ERK signaling (Morozov et al., 2003; Vossler et al., 1997). Like KSR1 null mice, Rap1 mutant mice showed defects in a membrane pool of ERK (although in the basal rather than stimulated state) and in Kv4.2 phosphorylation. However, the behavioral and physiological phenotypes of these mice were quite different from KSR1 null mice. Rap1 mutant mice showed deficits in water maze probe trial parameters, in context discrimination, but no impairments in a long-term cued- or contextual-fear memory. The mice also displayed deficits in theta-frequency- and forskolin-induced LTP (Morozov et al., 2003). These complementary studies indicate that even in the face of similar biochemical profiles, such as signaling defects in membrane-associated pools of ERK, additional mechanisms and levels of regulation exist to process ERK signaling and are reflected in the eventual behavioral phenotype.

It is also necessary to address the fact that at least one other mammalian KSR protein exists: KSR2. Although KSR2 displays a similar expression pattern as KSR1, there does not appear to be upregulation of KSR2 in KSR1 null mice (D.K. Morrison and M.K. Dougherty, personal communication). As such, KSR1 and 2 could have completely independent or overlapping, redundant roles. Ohmachi et al. (2002) reported the discovery of ksr2 in *C. elegans* and found instances of both distinct and redundant functions of the two proteins. Mammalian KSR1 and 2 could function similarly. KSR2 could serve as a scaffolding protein for PKC-initiated signaling as does KSR1, which could explain why PKC activation still yielded ERK phosphorylation in KSR1 null mice, albeit significantly less than in wild-type mice. Redundancy in function would also suggest why KSR1 null mice showed only modest long-term memory phenotypes and very defined synaptic plasticity deficits. It is also possible that KSR2 functions completely independently of KSR1, perhaps preferentially coupling PKA with ERK activation. Further experimentation is needed to clarify the role(s) of KSR proteins, and generation of a KSR1$^{-/-}$ KSR2$^{-/-}$ mouse would be ideal to truly decipher the importance of KSR in MAPK signaling.

In summary, KSR1 represents a novel protein that appears to maintain signaling specificity both upstream and downstream of the ERK pathway. KSR1 coordinates PKC-directed ERK signaling in a membrane pool within the hippocampus. Because of the subtle fear associative learning deficits and TBS-LTP deficits in KSR1

null mice, we postulate that PKC-directed ERK signaling is specifically involved in these behaviors and cellular processes. Indeed, we were also able to show that TBS-induced LTP was reduced in wild-type slices when PKC was inhibited. Further investigation will continue to improve our understanding of how scaffolding proteins, such as KSR1, provide structural and mechanistic support for members of the ERK cascade in the mediation of memory formation and expression.

Experimental Procedures

Animals

KSR1$^{-/-}$ and *KSR1*$^{+/+}$ mice were provided by Dr. Deborah Morrison (NCI, Frederick, Maryland) and bred at Baylor College of Medicine. Animals were housed with same sex siblings with ad libitum access to food and water and maintained on a 12 hr light/dark cycle. All experiments were performed in accordance with Baylor College of Medicine Institutional Animal Care and Use Committee and with national regulations and policies. All behavior experiments were performed with the experimenter blind to genotype, using wild-type littermate controls from heterozygote breedings.

Baseline Behaviors

The following battery of behavioral tests were performed as previously described (Selcher et al., 2001). In the open field test, a mouse was placed in the center of a clear Plexiglas box (43 × 42 × 30 cm) under standard lighting conditions. Locomotor activity was tracked via automated computer system (VersaMax, Accuscan). Horizontal and vertical photoreceptor beam breaks were collected over the 15 min testing period.

Motor coordination, balance, and motor learning were assessed using the accelerating rotarod task (UGO Basile). Time was measured from the placement of the mouse on the rod until the mouse was unable to maintain balance. Over the 5 minute trial, the rod accelerated from 4 to 40 rpm. Mice underwent four trials per day for 2 consecutive days.

Nociceptive behavior and pain threshold was tested using the hot plate test. The animals were placed on a 52ºC surface. The time it took for the animal to lift or lick its paw was recorded. Shock threshold testing involved placing the animal in a chamber and administering successive shocks every 20 s from 0–1.0 mA. Thresholds to flinching, jumping, and vocalization were recorded.

Fear Conditioning

Fear conditioning was as previously described (Selcher et al., 2001) with several modifications. Animals were trained in one of two training paradigms. Intense training consisted of three pairings of a 30 s, white noise (90 dB) tone with a mild, 1 s footshock over the course of 8 min. Each pairing was separated by 90 s. Modest training consisted of a single pair of tone and footshock over the course of 4 min. Freezing behavior was measured during each training trial both automatically (FreezeFrame, Actimetrics) and manually. Twenty-five hours posttraining, context learning was assessed by monitoring freezing behavior during a 5 min reexposure to the fear-conditioning chamber. Cued-fear learning was assessed by placing the animal in a novel test cage with altered dimensions, colors, and smells, which the mouse explored for 3 min. The auditory cue was then re-presented for 3 min, and freezing behavior was measured.

Passive Avoidance

Training consisted of placing the mouse into a well-lit chamber with an opening leading to a darkened side. The time elapsed until the mouse entered the dark side of the chamber was recorded as the baseline latency. After entering the darkened side, a mild footshock was delivered to the mouse via a metal floor grid. Twenty-four hours later, the mouse was tested by placing him back into the light side of the chamber. The latency to enter the darkened side was recorded with a ceiling time of 5 min.

Morris Water Maze

The Morris water maze experiment was performed similarly to previous descriptions (Selcher et al., 1999). Briefly, animals were trained to find a hidden platform in an opaque pool using visual cues on the walls of the training room. Each animal underwent four trials (max = 60 s) a day for a total of 10 days with an intertrial interval of 20 min. If the mouse had not found the platform at the trial's end, it was directed to and placed on the platform. After certain days of training, the submerged platform was removed for probe trials, and swim patterns were tracked for 60 s (Noldus EthoVision).

Electrophysiology

For electrophysiological experiments, 400 μm hippocampal slices were prepared as previously described (Selcher et al., 2003). Glass recording electrodes (resistance, approximately 1 MΩ) were placed in stratum radiatum or cell body layer of area CA1, and a bipolar stimulating electrode was positioned along the Schaffer collateral afferents from CA3. Test stimuli were applied and EPSP slopes were measured to determine a stimulus intensity eliciting a slope 40%–50% of the maximum, thereby normalizing for any differences in maximal attainable EPSP slope. A minimum of 20 min of baseline stimulation (0.05 Hz) was recorded before the LTP induction. LTP was induced by one of several paradigms. Theta burst stimulation (TBS) LTP consisted of three trains of ten high-frequency bursts delivered at 5 Hz (intertrain interval = 20 s) as described (Selcher et al., 2003). In some experiments, 20 μM U0126 (Sigma, St. Louis, Missouri), 10 μm GF109203x (Tocris, Ellisville, Missouri), or 10 μm MPEP (Tocris) was perfused onto the slice for 10 min prior to TBS and maintained for the duration of recording. LTP was also induced using as described by Winder et al. (1999). Stimuli were delivered at a 5 Hz for 3 min immediately following a 10 min bath application of 1 μM isoproterenol (Sigma). High-frequency LTP was induced using two 1 s 100 Hz stimulations was performed as described (Selcher et al., 2001). All physiology was conducted at 30º.

Biochemistry

For biochemical experiments, 400 μm hippocampal slices were prepared from KSR1 null and wild-type littermate mice. Slices were incubated at room temperature in an oxygenated mix of cutting solution (110 mM sucrose, 60 mM NaCl, 3 mM KCl, 1.25 NaH_2PO_4, 28 mM $NaHCO_3$, 0.5 mm $CaCl_2$, 7 mM $MgCl_2$, 5 mM glucose, 0.6 mM ascorbate) and artificial cerebral spinal fluid (ACSF—125 mM NaCl, 2.5 mM KCl, 1.25 mM NaH_2PO_4, 25 mM $NaHCO_3$, 2 mM $CaCl_2$, 1 mM MgCl, 25 mM glucose) for 1 hr. Slices were transferred to oxygenated, pure ACSF for 2 hr at 32ºC. One of the following drugs or the appropriate vehicle was applied for 10 min: 50 μM forskolin, 3 μM phorbol diacetate (PDA), 10 μM isoproterenol (all from Sigma), or 50 μM DHPG (Tocris). One hundred micromolar Ro20-1724 (Sigma) was included in forskolin conditions to prevent cAMP breakdown.

For experiments requiring membrane and cytosolic fractions, fractionations were isolated using the methods of Morozov et al. (2003) or by using a compartmental protein extraction kit (Chemicon, Temecula, California). For immunoprecipitation experiments, tissue was homogenized in TBS Lysis buffer (0.137M NaCl, 20 mM Tris [pH 8.0], 10% glycerol, 0.5% Triton X-100, protease inhibitors, phosphatase inhibitors), then spun at 16,000 × g. The supernatant was collected and spun at 42,000 × g for 1 hr. The protein concentration of this new supernatant was determined, and all samples were equalized. KSR was immunoprecipitated using monoclonal KSR antibody (Upstate Biotechnology, Lake Placid, New York) and protein G beads (Amersham Biosciences, Piscataway, New Jersey) for 3 hr at 4ºC. Beads were collected, washed, and boiled in sample buffer.

Samples were run on SDS-PAGE gels and transferred to PVDF Immobilon membranes. Membranes were blocked in 3% BSA or 5% milk with 1 μg/ml microcystin. Blots were incubated in primary antibody for 1 hr at room temperature or overnight at 4ºC. The following antibodies were used: phospho-p42/p44 MAP kinase, phospho-MEK, total MEK, phospho-p90RSK (all Cell Signaling, Beverly, Massachusetts), KSR1 and total MAPK (both Upstate Biotechnology), KSR1 H70 (Santa Cruz, Santa Cruz, California), and phospho-Kv4.2 antisera (Adams et al., 2000a). Blots were washed and incubated in the appropriate HRP-conjugated secondary antibody (Cell Signaling) and developed with either ECL (Amersham

Biosciences) or Super Signal (Pierce, Rockford, Illinois). Immunoreactive bands were quantified with Image J software (NIH).

Immunohistochemistry

Animals were anesthetized with 0.2% avertin and sacrificed by transcardial perfusion with ice-cold PBS followed by 4% paraformaldehyde (PFA) in PBS. Whole brain was removed, processed for paraffin embedding, and sectioned at 10 μm. Following heated citrate buffer antigen retrieval, sections were blocked in 5.0% normal goat serum in PBS then incubated overnight at 4ºC in one of the following monoclonal antibodies diluted in PBS: NeuN or MAP2A/B (Chemicon), GFAP (Invitrogen, Carslbad, California). Following several washes in PBS, sections were incubated in the following, respectively: biotinylated mouse anti-IgG_1 (Jackson Immunoresearch, West Grove, Pennsylvania), ABC reagent (Vector Labs, Burlingame, California), and streptavidin-Cy3 (Jackson Immunoresearch). After washing, sections were incubated in rabbit anti-KSR1 (Santa Cruz), diluted in PBS, at 4ºC for 48 hr, which was recognized by AlexaFluor 488-conjugated goat anti-rabbit IgG (Invitrogen). Slides were coverslipped with Vectashield mounting media with DAPI (Vector Labs). Digital images were obtained with a Zeiss microscope (Axioskop 2). Nissl staining was performed on sections using a solution of 0.5% cresyl violet, 60 mM sodium acetate, 0.34 M acetic acid.

Acknowledgments

This study was supported by NIH grants F31MH071014 (to S.C.S.), NS37444 (to J.D.S.), MH57014 (to J.D.S.), and NS13546 (to J.D.S.). S.C.S. is supported by the Robert and Janice McNair Scholars Foundation and the BRASS Foundation.

Received: November 9, 2005
Revised: December 27, 2005
Accepted: April 18, 2006
Published: May 31, 2006

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