Acquisition of Contextual Discrimination Involves the Appearance of a RAS-GRF1/p38 Mitogen-activated Protein (MAP) Kinase-mediated Signaling Pathway That Promotes Long Term Potentiation (LTP)*

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Background: The ability to distinguish similar experiences is a critical aspect of memory.

Results: Contextual discrimination involves LTP promoted by calcium-permeable AMPA-type glutamate receptors, RAS-GRF1 and p38 MAP kinase.

Conclusion: A newly discovered, LTP-supporting, signaling pathway contributes to a key component of memory formation.

Significance: Defects in this signaling pathway may contribute to cognitive loss associated with neurological disorders.

RAS-GRF1 is a guanine nucleotide exchange factor with the ability to activate RAS and RAC GTPases in response to elevated calcium levels. We previously showed that beginning at 1 month of age, RAS-GRF1 mediates NMDA-type glutamate receptor (NMDAR)-induction of long term depression in the CA1 region of the hippocampus of mice. Here we show that beginning at 2 months of age, when mice first acquire the ability to discriminate between closely related contexts, RAS-GRF1 begins to contribute to the induction of long term potentiation (LTP) in the CA1 hippocampus by mediating the action of calcium-permeable, AMPA-type glutamate receptors (CP-AMPARs). Surprisingly, LTP induction by CP-AMPARs through RAS-GRF1 occurs via activation of p38 MAP kinase rather than ERK MAP kinase, which has more frequently been linked to LTP. Moreover, contextual discrimination is blocked by knockdown of Ras-Grf1 expression specifically in the CA1 hippocampus, infusion of a p38 MAP kinase inhibitor into the CA1 hippocampus, or the injection of an inhibitor of CP-AMPARs. These findings implicate the CA1 hippocampus in the developmentally dependent capacity to distinguish closely related contexts through the appearance of a novel LTP-supporting signaling pathway.

It is well known that the hippocampus plays a critical role in processing contextual information relating to episodic memory. Moreover, the contribution of the hippocampus to learning and memory develops late among brain regions, appearing first ~21 days after birth in mammals (1). Among this category of functions, the hippocampus is particularly important for discriminating similar contexts, which requires processing of not only spatial but also temporal and relational information (2). In fact, clearly distinct roles for the dentate gyrus and CA3 and CA1 regions of the hippocampus in this form of learning have been identified (3). However, the biochemical underpinnings of these functions remain poorly understood. Likely candidates include signaling components that regulate synaptic plasticity in each of these hippocampus segments.

One class of signaling molecules that have been implicated in synaptic plasticity consists of the RAS-GRF1 and RAS-GRF2 guanine nucleotide exchange factors (4). These proteins have the capacity to activate RAS and RAC GTPases through their CDC25 and DH domains, respectively. Both proteins can be activated by calcium-dependent binding of calmodulin to their IQ motifs. Beginning at ~1 month of age, RAS-GRF1 mediates NMDA receptor (NMDAR)2-induced LTD, whereas RAS-GRF2 mediates NMDAR-induced LTP in the CA3/CA1 synapse of the hippocampus (5). At this site, RAS-GRF2 mediates LTD induced specifically by NMDARs that contain GluN2A subunits (6). Consistent with their defective LTP, RAS-GRF2 knock-out mice display defective contextual fear conditioning memory. In contrast, RAS-GRF1 mice display more subtle fear memory defects in that contextual fear memory is weakly defective (7, 8), but contextual discrimination is completely lost (7). How RAS-GRF1 contributes to contextual discrimination is not known.

MAP kinases play integral roles in neuronal functions related to synaptic plasticity associated with learning and memory (9). ERK MAP kinase in synapses of mature animals can be activated by a variety of upstream receptors, including the Trk family of tyrosine kinase receptors (10) and both NMDA-type (11) and calcium-permeable AMPA-type glutamate receptors (11, 12). Its activation is a critical step in promoting many forms of LTP, including protein synthesis-independent early LTP

2 The abbreviations used are: NMDAR, NMDA receptor; CP-AMPAR, calcium-permeable 2-amino-3-(3-hydroxy-5-methyl-isoxazol-4-yl)propanoic acid receptor; RAS-GRF1, RAS guanine nucleotide-releasing factor 1; RAS-GRF2, RAS guanine nucleotide-releasing factor 2; LTP, long term potentiation; LTD, long term depression; ACSF, artificial cerebrospinal fluid; fEPSP, field excitatory postsynaptic potential; DG, dentate gyrus; TBS, theta-burst; HFS, high frequency stimulation; DMSO, dimethyl sulfoxide; AAV, adeno-associated virus; APV, DL-2-amino-5-phosphono pentanoic acid.

3 L. A. Feig, unpublished observations.
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induced by theta-burst stimulation that is thought to contribute to short term memory (13). It is also critical for protein-dependent late LTP and long term memory (14). In contrast, p38 MAP kinase has been most often associated with LTD, although conflicting results have been obtained (5, 15–17). However, p38 MAP kinase activity has been associated with theta-burst-induced early LTP that is associated with enhanced learning after exposure to an enriched environment (18).

In this study, we demonstrate that p38 MAP kinase activation is also involved in an age-dependent LTP mechanism, mediated by calcium-permeable AMPA receptors and RAS-GRF1, that contributes to the acquisition at ~2 months of age of the ability of mice to distinguish closely related contexts.

EXPERIMENTAL PROCEDURES

Mice—ras-grf1−/− mice in a C57Bl6 background were described previously (5, 19). All procedures involving mice were in accordance with the animal welfare guidelines of Tufts University.

 Extracellular Field Experiments—Mutant and control mice (1–6 months old) were anesthetized with halothane and decapitated. The transverse acute hippocampal slices (350 μm) were cut in ice-cold oxygenated sucrose-enhanced artificial cerebrospinal fluid (ACSF) containing 206 mM sucrose, 2 mM KCl, 2 mM MgSO4, 1.25 mM NaH2PO4, 1 mM CaCl2, 1 mM MgCl2, 26 mM NaHCO3, 10 mM d-glucose, pH 7.4, saturated with 95% O2 and 5% CO2 (pH 7.4), in which they were allowed to recover for at least 90 min before recording. Recordings were performed in the same solution at room temperature in a chamber submerged in ACSF.

To record field EPSPs (fEPSPs) in the CA1, CA3, and DG regions of the hippocampus, respectively, standard procedures were used. 1) For CA1, Schaffer collaterals were stimulated with a unipolar stimulating electrode (World Precision Instruments) placed in the lateral CA1 subfield. A borosilicate glass recording electrode filled with ACSF was positioned in the stratum radiatum of CA1. 2) For CA3, the mossy fiber CA3 pyramidal neuron responses were induced by the stimulation of mossy fiber axons with a unipolar stimulating electrode. A borosilicate glass recording electrode filled with ACSF was placed at least 500 μm from the stimulating electrodes along the trajectory of the mossy fiber pathway. d-APV (50 μM) was added in ACSF to prevent contamination with the NMDA receptor-dependent pathway converging on CA3 neurons. 3) For DG, stimulating and recording electrodes were placed in the medial aspect of the dentate molecular layer. 10 μM bicuculline methiodide was added in ACSF to prevent GABA-arrested inhibition. Test stimuli were applied at low frequency (0.05 Hz) at a stimulus intensity that elicited an fEPSP amplitude that was 50% of maximum, and the test responses were recorded for 10 min before the experiment was begun to ensure stability of the response.

To induce LTP, we used three protocols in this study. 1) For high frequency stimulation (HFS)-induced LTP in the CA1 and CA3, two consecutive trains (1 s) of stimuli at 100 Hz separated by 20 s were applied to the CA1 and CA3 areas, respectively. 2) For theta-burst (TBS) stimulation in CA1, 15 bursts of four pulses at 100 Hz delivered at an interburst interval of 200 ms were applied to the CA1 area. 3) Four bursts of 50 pulses at 100 Hz, 30 s between bursts, were applied to the dentate gyrus.

Virus vectors—Ras-Grf1 microRNA adenovirus-associated virus was generated as follows. An AAV8 shRNA vector pFB-U6-R2-CMVmCherry expressing microRNA against GRF1 (sequence, TGCTGTGACGAGGCGGCGTGATCAAATCTACA- GAAATAGTGACACCACAGATGTATTCTGTAGATAT- TGATCGACTGGTTACCTGCGGAA) or a similar control random sequence, along with mCherry or GFP under the control of the U6 and CMV promoters, respectively, were generated by Virovec and were produced at working concentrations of ~1.6 × 1013/ml. Adenovirus expressing Ras-Grf1 from the synapsin promoter was generated as described (20, 21). Briefly, the synapsin promoter and poly(A) regions were digested out of a plasmid provided by Dr. Colin Sumners (University of Flor-
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ida) and then ligated into pShuttle (20). Then Ras-Grf1 cDNA was inserted in the resulting plasmid. pShSynGRF1 was digested with Pinel and then used to co-transform Escherichia coli strain B5183 along with pAdEasy-I. Colonies were screened for the presence of Ras-Grf1 gene and the pAdEasy-I backbone by colony PCR. The positive clones were tested for the correct orientation by restriction enzyme digestion. The plasmid was then digested with PacI and transfected into 293 cells. Then the culture supernatant containing viral particles was harvested. Cleared supernatant was used for subsequent serial infection of 293 cells. Resultant viral samples were subjected to adenovirus purification using Adenopure kit (Puresyn Inc.). The virus was concentrated by filtration, yielding a concentration greater than 1 × 10^12 virus particles/ml.

Stereotaxic Surgery—2-Month-old ras-grf1 knock-out mice or WT mice were anesthetized with an intraperitoneal injection of ketamine (100 mg/kg)/xylazine (10 mg/kg). Once anesthetized, each mouse was placed in a stereotaxic frame (miNeuro-Lab, St. Louis, MO). A surgical incision was made along the midline of the head to expose the skull. Two holes were made in the skull overlying the dorsal hippocampus. Coordinates for CA1 injection or cannula implantation into the mice are: 2.0 mm posterior to bregma, ±1.9 mm lateral from the midline, 1.9 or 0.9 mm (cannula implantation) beneath the surface of the skull. Injections were performed with a 10-μl Hamilton syringe fitted with a custom made blunt-ended 30-gauge needle (Hamilton). Each injection consisted of 1 μl of adenovirus-expressing Ras-Grf1 or adeno-associated virus (AAV-8) expressing Ras-Grf1 shRNA with mCherry infused at a rate of 0.06 μl/min. An infusion pump controlling the plunger on the Hamilton syringe precisely regulated the rate of injection. The needle was then left in place for 8 min prior to withdrawal from the brain. Electrophysiological recordings or behavioral procedures began 7–11 days after stereotaxic injection of the viral vectors.

In some experiments, stainless steel double guide cannulas (21-gauge) were implanted bilaterally into the hemisphere. Mice were given at least 1 week to recover after cannula implantation. The 28-gauge injection cannula was 1 mm longer than the guide. For intrahippocampal infusion, BIRB0796 was prepared by dissolving in DMSO and was diluted in sterile saline to a concentration of 1.056 mg/ml in 10% DMSO. Vehicle-treated animals were infused with 90% saline and 10% DMSO. Vehicle-treated mice were shocked again in the paired context and exposed to the context A on day 1, day 2, and day 3, respectively. In another group of experiments, animals received an intrahippocampal infusion of vehicle, SB203580 (0.75 μg/side), or BIRB0796 (1.056 μg/side) each day of the experiments and 30 min later were trained in context A on day 1, day 2, and day 3, respectively. Animals received an intrahippocampal infusion of vehicle, SB203580, or BIRB0796 each day of the experiments and 30 min later were trained in context A on day 1, day 2, and day 3, respectively. Contextual fear conditioning was assessed by measuring the amount of freezing displayed by mice after they were placed back into the context where a single shock had been delivered 24 h previously. Mice were pretreated with shRNA against Ras-Grf1, BIRB0796, or IEM-1460 as described above. The results were tested with a one-way analysis of variance.

Behavior Assays—Mice were tested in contextual discrimination as described previously (7). In this task, the mice were trained to discriminate between two chambers, one in which they were shocked and another in which they were not. On day 1, the mice were pre-exposed to the two contexts for 10 min. On day 2, the mice were shocked in one context (paired); after 148 s, a 2-s shock (0.75 mA) was delivered, and the mice remained for another 30 s in the context. Additionally, the mice were exposed for 3 min to the other context (non-paired), during which time no shocks were delivered. On day 3, the mice were shocked again in the paired context and exposed to the non-paired context. On day 4, the amount of freezing was measured for 3 min in each of the two contexts. In some experiments, adeno-associated virus expressing GFP, control random shRNA, or shRNA against Ras-Grf1 was injected stereotaxically into the CA1 hippocampus 7–10 days before beginning the experiment. In other experiments, the CP-AMPA receptor selective blocker IEM-1460 (Tocris Bioscience, Ellisville, MO) was dissolved in filtered saline and administered via intraperitoneal injections. In another group of experiments, animals received an intraperitoneal injection of either saline or IEM-1460 each day of the experiments and 30 min later were trained in context A on day 1, day 2, and day 3, respectively. In another group of experiments, animals received an intrahippocampal infusion of vehicle, SB203580 (0.75 μg/side), or BIRB0796 (1.056 μg/side) each day of the experiments and 30 min later were trained in context A on day 1, day 2, and day 3, respectively. Animals received an intrahippocampal infusion of vehicle, SB203580, or BIRB0796 each day of the experiments and 30 min later were trained in context A on day 1, day 2, and day 3, respectively. Contextual fear conditioning was assessed by measuring the amount of freezing displayed by mice after they were placed back into the context where a single shock had been delivered 24 h previously. Mice were pretreated with shRNA against Ras-Grf1, BIRB0796, or IEM-1460 as described above. The results were tested with a one-way analysis of variance.

RESULTS

RAS-GRF1 Begins to Contribute to HFS-induced LTP at 2 Months of Age in the CA1, but Not CA3 or Dentate Gyrus, Regions of the Hippocampus—Using Ras-Grf1 knock-out mice, we previously showed that RAS-GRF1 is predominantly involved in mediating NMDAR activation of LTD, whereas RAS-GRF2 is predominantly involved in mediating NMDAR activation of LTP at the CA3/CA1 synapse beginning at approximately day 30 (early adolescence) in mice (5). However, because most behavior assays are performed with mice that are at least approaching late adolescence at 2 months of age, synaptic plasticity was restested in older Ras-Grf1 knock-out mice. To our surprise, 2-month-old Ras-Grf1 knock-out mice (as well as 6-month-old mice (data not shown)) displayed defective LTP induced by HFS (two trains of 100 Hz of tetanus) (Fig. 1A) (WT, 161.76 ± 4.73% (n = 11 slices from six mice) versus 117.41 ± 2.54% (n = 12 slices from eight grf1−/− mice; p < 0.01)). Interestingly, LTP generated by TBS (15 bursts of four pulses at 100 Hz delivered at an interburst interval of 200 ms) was normal in 2-month-old RAS-GRF1 knock-out mice (Fig. 1B) (WT, 159.43 ± 2.77% (n = 8 slices from five mice) versus 157.80 ± 1.69% (n = 9 slices from five grf1−/− mice; p > 0.05)) as we showed previously for 1-month-old mice (5). 2-Month-old Ras-Grf2 knock-out mice show the opposite results, normal HFS LTP but defective TBS LTP (data not shown).

Confirming that this defect was not due to the indirect effects of the loss of the RAS-GRF1 in neighboring brain regions or during development, defective HFS LTP in Ras-Grf1 knock-out
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FIGURE 1. Ras-Grf1 knock-out mice display defective HFS-induced but not TBS-induced LTP in CA1 hippocampus of 2-month-old mice. A and B, hippocampus slices from 2-month-old wild-type (filled squares) or Ras-Grf1 knock-out mice (open squares) were stimulated with HFS (A) or TBS (B). The recorded fEPSP slope using field recordings is expressed as a percentage of baseline ± S.E. For HFS: WT; n = 11 slices from six mice; grf1−/−; n = 12 slices from eight grf1−/− mice. For TBS: WT; n = 8 slices from five mice; grf1−/−; n = 9 slices from five mice. Insets, representative fEPSPs recorded. C, control GFP-expressing adenovirus (filled diamonds) or GRF1-expressing adenovirus (open diamonds) was injected into the CA1 hippocampus. 9–11 days later, HFS LTP was measured in isolated hippocampal slices. Data represent the mean ± S.E. (open diamonds). WT, 154.88 ± 1.22% (n = 9 slices from six mice) versus 147.48 ± 1.63% (n = 9 slices from five mice; p < 0.05). Overall, these findings show that Ras-Grf1 plays an age- and synapse-dependent role in promoting LTP induction in the hippocampus.

Ras-Grf1 induces LTP in the CA1 of 2-Month-old Mice through Activation of p38 MAP Kinase—One of the known properties of Ras-Grf proteins is their ability to activate the ERK/ERK MAP kinase pathway, which can promote LTP. However, we showed previously that Ras-Grf1 is a weak inducer of NMDA-mediated LTP in the CA1 when compared with Ras-Grf2 in 1-month-old mice, at least in part, because it is less effective than Ras-Grf2 in activating ERK MAP kinase (5). Nevertheless, it was possible that Ras-Grf1 acquires the ability to promote LTP through ERK MAP kinase by 2 months of age. To test this possibility, hippocampal slices from 2-month-old wild-type mice were pre-exposed to U0126, an inhibitor of ERK activation, or buffer only for 30 min before LTP induction. Fig. 3A shows that as demonstrated previously (13, 22), HFS-induced LTP was not blocked by ERK inhibition (control, 161.86 ± 4.73% (n = 11); U0126, 154.34 ± 2.79% (n = 9); p > 0.05), although HFS-induced LTP was associated with an increase in ERK activity in CA1 neurons that was blocked by pretreatment with U0126 (data not shown). In contrast, TBS-
induced LTP in 2-month-old mice, which is mediated by RAS-GRF2/ERK signaling (Ref. 5), was suppressed by treatment with U0126 (Fig. 3B) (control, 159.43 ± 2.77% (n = 8); U0126, 133.46 ± 1.50%; p < 0.01).

RAS-GRF1 can also mediate p38 activation by NMDA receptors potentially through its RAC-activating DH domain or through interaction with JIP2, a p38 scaffold that binds to the N-terminal region of RAS-GRF1 (5, 23). Thus, we pretreated hippocampus slices from 2-month-old mice with the p38 inhibitor, SB203580 (10 μM), and found that HFS-induced LTP was suppressed (Fig. 3C) (control, 161.86 ± 4.73% (n = 11); SB203580, 126.95 ± 1.45%; (n = 8; p < 0.01)). Inhibition of p38 with SB20380 was quite specific because as expected it did not block TBS LTP in 2-month-old mice nor HFS LTP in 1-month-old animals. Neither of these forms of LTP is affected in \textit{Ras-Grf1} knock-out mice (data not shown). Nevertheless, because the SB203580 inhibitor is not totally specific for p38, we tested another p38 inhibitor, BIRB0796. It also suppressed HFS LTP (Fig. 3C) (BIRB0796, 136.87 ± 1.47%; n = 8) but not TBS LTP (Fig. 3D) in 2-month-old mice. Specific inhibition by both of these molecules is considered strong proof that p38 MAP kinase is involved (24).

To gain additional support for the idea that this form of LTP is mediated by p38 MAP kinase, HFS LTP was induced in hippocampal slices from 2-month-old mice and p38 activation was measured by immunofluorescence using antibodies specific for its active form. HFS activated p38 in principal cells of the CA1 hippocampus of control mice (Fig. 4A) but not in hippocampal

**FIGURE 2.** shRNA knockdown of \textit{Ras-Grf1} in the CA1 hippocampus of 2-month-old mice suppresses HFS-induced, but not TBS-induced, LTP. A, a mammalian expression vector expressing \textit{Ras-Grf1} or \textit{Ras-Grf2} was co-transfected with an empty control vector or a vector expressing shRNA against \textit{ras-Grf2} or \textit{Ras-Grf1} into HEK 293 cells. 3 days later, lysates of cells were probed with antibodies that recognize RAS-GRF1 and RAS-GRF2. B, either control scrambled shRNA-expressing or \textit{Ras-Grf1} shRNA-expressing adeno-associated viruses co-expressing the red-cherry fluorophore were stereotaxically injected into the CA1 hippocampus. 9–11 days later, brain slices were isolated. HFS (top graph) or TBS (lower graph)-induced LTP in the CA1 was then recorded (control AAV random shRNA (closed figures) n = 7; AAV \textit{Ras-Grf1} shRNA (open figures), n = 9). Data represent the mean ± S.E. Inset, a hippocampal slice from an infected mouse detecting red-cherry fluorescence. (scale bar, 250 μm).
slices from *Ras-Grf1* knock-out mice (Fig. 4B). Moreover, LTP induced by HFS treatment did not activate p38 in 1-month-old mice in which *Ras-Grf1* does not yet have the capacity to promote LTP (Fig. 4C). Finally, theta-burst LTP, which is not blocked by p38 inhibitors in 2-month-old mice, did not activate p38 (Fig. 4D) (for quantification of data, see Fig. 4E). Together these data strongly support the idea that HFS-induced LTP that begins at 2 month of age depends upon RAS-GRF1-mediated activation of p38 MAP kinase.

**CP-AMPARs Mediate HFS-induced LTP in the CA1 but Not CA3 or Dentate Gyrus**—Both the NMDA-type and the calcium-permeable, AMPA-type glutamate receptor (CP-AMPAR)-type of glutamate receptors are known to bind to RAS-GRF1 (12, 25), and both permeate calcium, an activator of RAS-GRF proteins. Thus, to determine which receptor type mediates HFS induction of LTP by RAS-GRF1 in the CA1 hippocampus of 2-month-old mice, hippocampal slices were pretreated with inhibitors of either NMDARs (APV) or CP-AMPARs (IEM-1460). Either inhibitor blocked HFS LTP (Fig. 5A) (control, 161.86 ± 4.73% (n = 11); IEM-1460, 118.15 ± 1.61% (n = 8); p < 0.01; control, 161.86 ± 4.73% (n = 11); APV, 104.12 ± 0.71% (n = 7); p < 0.01), but only the CP-AMPAR inhibitor blocked HFS-induced p38 activation (Fig. 5B) These findings imply that CP-AMPAR/Ras-Grf1/p38 signaling adds an additional component to LTP induction that complements that involving NMDARs.

Moreover, the CP-AMPAR inhibitor did not block LTP induction in the dentate gyrus or CA3 regions of the hippocampus from 2-month-old mice or the CA1 region from 1-month-old mice, situations in which RAS-GRF1 and p38 do not contribute to LTP (DG, control, 154.68 ± 1.22% (n = 9) versus IEM-1460, 155.51 ± 1.09% (n = 8); p > 0.05; CA3, control, 148.69 ± 1.51% (n = 8) versus IEM-1460, 153.81 ± 1.64% (n = 8); p > 0.05 and CA1 (1-month-old), control, 161.10 ± 3.32% (n = 8) versus 157.82 ± 2.49% (n = 9); p > 0.05). Also, the p38 inhibitor has already been shown not to block LTP in the dentate gyrus (26). Together, the results described above define a novel CP-AMPAR/RAS-GRF1/p38 MAP kinase signaling cascade that promotes LTP specifically in the CA1 only after mice reach ~2 months of age.

**Contextual Discrimination Is Mediated by RAS-GRF1 in the CA1 Hippocampus, p38 MAP Kinase, and CP-AMPARs**—We previously showed that 2-month-old *Ras-Grf1* knock-out mice fail to display contextual fear discrimination but have little if any defect in contextual fear conditioning (7). Thus, when 2-month-old *Ras-Grf1* knock-out mice were exposed to foot shock in a specific environment and then placed back in that environment 24 h later, they recognized the context and became immobile (froze), expecting a shock with a frequency that was similar to control mice treated similarly. However, *Ras-Grf1* knock-out mice failed to detect similar contexts. In particular, mice were trained to discriminate two closely related contexts, only one in which they received foot shocks. Freezing was then assessed in the non-shock environment 2 days later. Fig. 6A shows that wild-type mice recognized that the related environment was different from the one in which they received
a foot shock and froze less than when placed in the shock context. Consistent with our previous results, \textit{Ras-Grf1} knockout mice froze equally in the non-shock-related context as they did in the shock context (Fig. 6A) (WT (n = 7), froze 31.35 ± 5.90% of the testing time in the paired context versus 18.25 ± 6.06% in the non-paired context, F(1,12) = 5.39, p = 0.05; \textit{Ras-Grf1} KO (n = 8), froze 27.09 ± 5.93% of the testing time in the paired context versus 25 ± 6.03% in the non-paired context, F(1,14) = 0.06, p = 0.81).

Because these mice lacked RAS-GRF1 in all tissues, we tested its role in the CA1 hippocampus by assaying the consequence of knocking down RAS-GRF1 expression specifically in the CA1 as described in the legend for Fig. 2. In particular, adeno-associated viruses expressing shRNA against \textit{Ras-Grf1} or control viruses were injected bilaterally into the CA1 hippocampus of 2-month-old mice. 7–9 days later, mice were compared in contextual discrimination assays. Fig. 6B shows that control, but not \textit{Ras-Grf1} shRNA, virus-treated mice displayed contextual discrimination (AAV control (n = 8), froze 55.13 ± 5.56% of the testing time in the paired context versus 40.08 ± 4.37% in the non-paired context, F(1,14) = 4.53, p < 0.05; AAV \textit{Ras-Grf1} shRNA (n = 8), froze 57.54 ± 6.25% of the testing time in the paired context versus 56.56 ± 3.49% in the non-paired context, F(1,14) = 0.02, p = 0.89).

To test the role of p38 MAP kinase in contextual discrimination, the p38 MAP kinase inhibitors, SB203580 or BIRB0796, or vehicle were infused through a cannula placed above the CA1 region 30 min before mice were habituated to the two related contexts. Mice were then used in contextual discrimination assays. Although vehicle-infused mice retained the ability to distinguish related contexts, BIRB0796- or SB203580-infused mice did not (Fig. 6, C and D) (vehicle (n = 11), froze 53.01 ± 2.86% of the testing time in the paired context versus 36.94 ± 4.81% in the non-paired context, F(1,20) = 8.24, p < 0.01; BIRB0796 (n = 9), froze 59.03 ± 5.79% of the testing time in the paired context versus 62.37 ± 5.77% in the non-paired context, F(1,16) = 0.17, p = 0.69); SB203580 (n = 9), froze 38.29 ± 9.89% in paired versus 33.75 ± 6.45% in “unpaired” context, F(1,16) = 0.52, p = 0.48). Importantly, like \textit{Ras-Grf1} shRNA knockdown mice, both sets of p38 inhibitor-infused mice displayed normal contextual fear conditioning (data not shown).

Finally, to test the role of CP-AMPARs in contextual discrimination, the CP-AMPAR inhibitor, IEM-1460, or saline was introduced via intraperitoneal injection 30 min before mice were habituated to the related contexts. Like mice with suppressed GRF1 expression and inhibited p38 MAP kinase in the CA1, these mice also did not recognize a difference in the non-
shock context and froze just as often when placed in the non-shock context as when they were placed in the shock environment (Fig. 6E) (saline (n = 10), froze 47.48 ± 4.10% of the testing time in the paired context versus 31.19 ± 5.36% in the non-paired context, F(1,20) = 5.83, p < 0.05; IEM-1460 (n = 6), froze 42.52 ± 12.98% of the testing time in the paired context versus 44.48 ± 10.05% in the non-paired context, F(1,10) = 0.01, p = 0.91) but did display normal contextual fear conditioning (data not shown).

Because we did not detect a contribution of CP-AMPARs, GRF1, or p38 MAP kinase in HFS-induced LTP in the CA1 hippocampus of 1-month-old mice, we tested them for the ability to distinguish closely related contexts. Fig. 6F shows that, in contrast to 2-month-old mice, 1-month-old mice do not yet have the capacity to distinguish related contexts.

Overall, these findings implicate the appearance of a CP-AMPAR/RAS-GRF1/p38 MAP kinase LTP pathway in the acquisition of the ability to distinguish closely related contexts during early adolescent development in mice.

DISCUSSION

This study uses electrophysiology, genetics, immunohistochemistry, and behavioral analysis to reveal that the postnatal emergence of the ability of mice to distinguish closely related contexts at ~2 months of age involves the appearance of a newly discovered signaling pathway involving CP-AMPARs, RAS-GRF1, and p38 MAP kinase that contributes to LTP in the CA3/CA1 synapse of the hippocampus. One of the most striking findings of this work is the detection of a role for p38 MAP kinase, rather than ERK MAP kinase, in promoting LTP because p38 has mainly been implicated in promoting LTD (5, 15–17) or inhibiting LTP (27, 28). For example, LTD induced through metabotropic glutamate receptors in the CA1 (15, 29) and dentate gyrus of the hippocampus functions through p38. Evidence suggests p38 also mediates NMDA receptor-induced LTD, although the degree of inhibition of this form of LTD by p38 inhibitors has ranged from complete (16), to partial (5) to none (17). Some of this discrepancy may arise from the fact that these studies used varied methods to detect LTD, different rodents, and/or animals of different ages.

One example where p38 has been implicated in LTP induction mediated by NMDA receptors is via theta-burst stimulation of the CA1 after exposure of adolescent mice to an enriched environment (18). In that case, a cAMP/p38 MAP kinase signaling pathway emerges after enrichment that compliments the existing ERK MAP kinase pathway regulated by RAS-GRF2. Apparently, p38 MAP kinase can have multiple functions in cells, a concept consistent with the fact that kinases

FIGURE 5. HFS-induced LTP is blocked by inhibitors of either NMDARs or CP-AMPARs, but only inhibitors of CP-AMPARs block HFS-induced p38 activation. HFS LTP was induced in brain slices from WT mice in the presence of saline, NMDA receptor inhibitor, APV, (100 μM), or the CP-AMPA receptor inhibitor IEM-1460 (IEM, 30 μM). A and B, fEPSP slopes were then measured in some slices (A) (data represent the mean ± S.E. of ~6 slices), and others were stained with phospho-p38 MAP kinase antibodies (B). Data are representative of three fields (scale bar, 50 μm).
exist in distinct signaling complexes containing different scaffold proteins that can target them to specific subsets of their many substrates in cells (30). Of the ~100 p38 putative substrates identified (31), most are involved in gene transcription or translation regulation, which occurs at a time scale too slow to contribute to HFS LTP. However, some tyrosine kinase receptors, cytoplasmic kinases, and proteins involved in endocytosis also have been proposed to be substrates and thus could potentially influence synaptic plasticity. Which p38 substrates are involved here remains to be determined. This form of LTP appears to be a postsynaptic process because no change in paired pulse facilitation was observed, indicating a lack of presynaptic involvement (data not shown).

Another interesting aspect of this CP-AMPAR/RAS-GRF1/p38 MAP kinase pathway is that it contributes to HFS-induced, but not TBS-induced, early LTP. HFS usually involves one train of 100 stimuli at 100 Hz, whereas theta-burst stimulation is typically four 100-Hz pulses with 200-ms interburst intervals, a protocol designed to match endogenous theta waves (32). Although both of these types of tetanization produce similar magnitudes of early LTP when applied to the Schaffer collateral innervating synapses in the CA1 hippocampus, differences in their mechanisms have been noted. For example, although HFS is often described as a more intense form of stimulation, theta-burst actually leads to more calcium influx (33). In addition, theta-burst stimulation leads to significant inhibitory postsynaptic potentials soon after tetanus, whereas HFS stimulation produces a distinct NMDA-mediated suppression of these inhibitory responses (33). Which, if any, of these differences contribute to the specificity in evoking the CP-AMPAR/RAS-
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GRF1/p38 pathway at 2 months of age remains to be determined. Finally, although theta-burst LTP is considered a more “physiological” stimulus, our finding that the appearance of contextual discrimination at ~2 months of age correlates with the appearance of HFS LTP mediated by RAS-GRF1 and p38, rather than theta-burst LTP mediated by ERK MAP kinase that has been present since birth, suggests that HFS LTP contributes to physiological events in the hippocampus.

Furthermore, our finding that a new signaling cascade contributes to HFS-induced LTP only after 2 months of age during late adolescence highlights how the biochemical underpinnings of LTP change with age along with the changing role of synaptic attributes to HFS-induced LTP only after 2 months of age during physiological events in the hippocampus.

The present study also reveals that the contribution of CP-AMPARs, RAS-GRF1, and p38 to HFS LTP is limited to the CA1 region as inhibition of any of these proteins in the CA3 or dentate gyrus had no effect on this form of synaptic plasticity. Most studies have pinpointed specific roles for the dentate gyrus and CA3 regions in pattern separation, an important component of contextual discrimination (47). In particular, the DG and CA3 have been proposed to play contrasting roles in memory processing, with the dentate gyrus functioning as a pattern separator splitting the signal input from the entorhinal cortex into distinct parts and encoding stimuli as novel. The CA3 contributes to pattern completion that matches incoming stimuli from entorhinal cortex with previous familiar experiences. The CA1 generates the output of the hippocampus and therefore the end point of its processing properties. The CA1 has been proposed to be a mismatch detector (48) because it receives both direct input about current events from entorhinal cortex and stored information from the CA3. However, evidence suggests that this region also adds a temporal context to events (49), another key aspect of pattern separation involved in contextual discrimination studied here, where contexts are experienced temporally and retrieval of stored information during testing is required. Which of these aspects of contextual discrimination involve synaptic plasticity provided by the CP-AMPAR/RAS-GRF1/p38/LTP pathway is presently under investigation.

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