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Removal of $G_{i\alpha1}$ Constraints on Adenylyl Cyclase in the Hippocampus Enhances LTP and Impairs Memory Formation

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Summary

Stimulation of adenylyl cyclase in the hippocampus is critical for memory formation. However, generation of cAMP signals within an optimal range for memory may require a balance between stimulatory and inhibitory mechanisms. The role of adenylyl cyclase inhibitory mechanisms for memory has not been addressed. One of the mechanisms for inhibition of adenylyl cyclase is through activation of G_i-coupled receptors, a mechanism that could serve as a constraint on memory formation. Here we report that ablation of $G_{i\alpha1}$ by gene disruption increases hippocampal adenylyl cyclase activity and enhances LTP in area CA1. Furthermore, gene ablation of $G_{i\alpha1}$ or antisense oligonucleotide-mediated depletion of $G_{i\alpha1}$ disrupted hippocampus-dependent memory. We conclude that $G_{i\alpha1}$ provides a critical mechanism for tonic inhibition of adenylyl cyclase activity in the hippocampus. We hypothesize that loss of $G_{i\alpha1}$ amplifies the responsiveness of CA1 postsynaptic neurons to stimuli that strengthen synaptic efficacy, thereby diminishing synapse-specific plasticity required for new memory formation.

Introduction

Long-term memory (LTM) depends on the convergence of diverse signal transduction pathways that mediate changes in gene expression and increased synaptic efficacy (for reviews see Kandel, 2001; Poser and Storm, 2001; Sweatt, 2001). Studies with invertebrates and vertebrates have identified the cAMP signal transduction system as a crucial signaling pathway for learning and memory. A role for cAMP in memory formation was first deduced from the characterization of *Drosophila* memory mutants, which identified defects in cAMP-phosphodiesterase in *dunce* (Byers et al., 1981), Ca^{2+}/calmodulin (CaM)-stimulated adenylyl cyclase in *rutabaga* (Livingston et al., 1984), and the cAMP-dependent protein kinase (PKA) in *DCO* (Skoulakis et al., 1993). Furthermore, expression of dominant-negative cAMP response element binding protein (*dCREB2*-b) in *Drosophila* is reported to disrupt LTM (Yin et al., 1994) whereas expression of an activator isoform of CREB (*dCREB2*-a) enhances LTM (Yin et al., 1995).

Related studies in mice have also implicated cAMP signaling for LTM formation in mammals. Animals that lack the Ca^{2+}/CaM-stimulated adenylyl cyclase (AC1) have impaired spatial memory when tested in the hidden platform version of the Morris water maze (Wu et al., 1995). Furthermore, the elimination of both Ca^{2+}/CaM-stimulated adenylyl cyclases (AC1 and AC8) ablates L-LTP and LTM for contextual and passive avoidance learning (Wong et al., 1999). Partial inhibition of PKA activity by the transgenic expression of a dominant-negative regulatory subunit for PKA [R (AB)] also impairs contextual memory and L-LTP (Abel et al., 1997). Furthermore, disruption of CREB activity causes defects in hippocampus-dependent memory (Bourtchuladze et al., 1994; Pittenger et al., 2002, Athos et al., 2002).

Although the data discussed above indicate that memory formation may depend upon synapse-specific increases in cAMP, other studies suggest that inhibition of adenylyl cyclase activity may be just as important for memory as stimulatory mechanisms. For example, the learning and memory defect of the *Drosophila* mutant *dunce* is due to a decrease in cAMP phosphodiesterase activity (Byers et al., 1981). In *Drosophila*, expression of a constitutively active form of $G_{s\alpha}$ (Connolly et al., 1996) that elevates adenylyl cyclase activity also causes memory defects. Collectively, these studies in *Drosophila* suggest the interesting possibility that memory formation depends upon a balance between mechanisms for increasing and decreasing cAMP.

One of the primary mechanisms for inhibition of adenylyl cyclase activity in animals is via G_i-coupled receptors. The objectives of this study were to determine which of the three $G_{i\alpha1}$ isozymes is important for attenuation of adenylyl cyclase activity in the hippocampus and to evaluate the role of this inhibitory mechanism for hippocampus-dependent memory. Our data identify $G_{i\alpha1}$ as a major contributor to inhibition of adenylyl cyclase in the hippocampus. Furthermore, we show that the specific loss of $G_{i\alpha1}$ causes defects in contextual, passive avoidance, and novel object recognition memory but enhances Schaffer collateral CA1 LTP. These data illustrate that hippocampus-dependent memory depends upon optimum cAMP signaling and that mechanisms for inhibition and stimulation of adenylyl cyclase are both important. We conclude that new memory formation requires tonic inhibition of adenylyl cyclase and that loss of this inhibitory constraint occludes synapse-specific events that mediate new memory formation in vivo.

Results

Pertussis Toxin Impairs Hippocampus-Dependent Memory and Lowers $G_{i\alpha1}$ in the Hippocampus

In preliminary studies, we assessed the general importance of G_i activity for passive avoidance memory forma-

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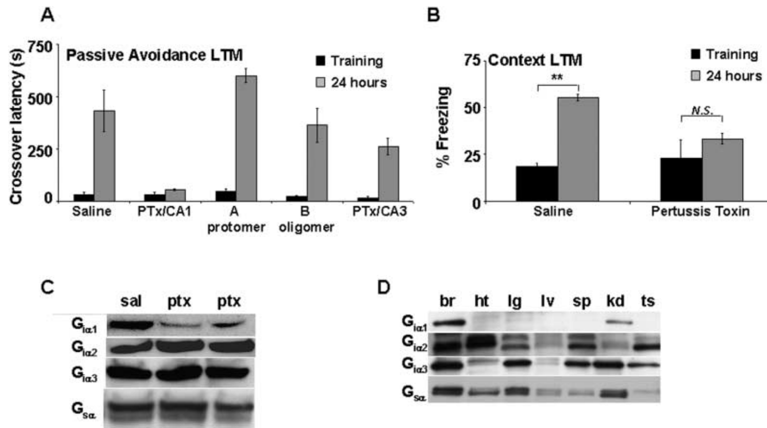

Figure 1. Intrahippocampal Injection of Pertussis Toxin Lowers $G_{i\alpha1}$ and Disrupts Memory for Associative-Fear Conditioning

(A) Mice that received either the A protomer or B oligomer of pertussis toxin into area CA1 showed memory for passive avoidance 24 hr after training ($n = 6$ each, $p < 0.01$). The bilateral administration of whole pertussis toxin to area CA1 ($n = 6$, $p = 1.0$) but not CA3 ($n = 4$, $p < 0.05$) blocked passive avoidance memory. A single administration of saline, whole pertussis toxin (50 ng/side), the A protomer (50 ng/side), or B oligomer (50 ng/side) into bilateral targets of the hippocampus preceded training for passive avoidance as described in Experimental Procedures. Data is expressed as mean $\pm$ SEM, and p values shown are testing versus training day behavior.

(B) The bilateral administration of whole pertussis toxin to area CA1 blocked contextual-conditioned memory measured 24 hr after training (mean $\pm$ SEM, $n = 7$, ** $p < 0.01$).

(C) The bilateral administration of whole pertussis toxin to area CA1 caused a significant decrease in $G_{i\alpha1}$ protein measured by Western analysis. The levels of G-coupling proteins were measured 1 week after pertussis toxin treatment. Extracts taken from a pool of three animals were loaded to each lane.

(D) Western analysis indicated that $G_{i\alpha1}$ protein is expressed predominantly in the brain. br, brain; ht, heart; lg, lung; lv, liver; sp, spleen; kd, kidney; ts, testes.

tion by bilateral, site-specific administration of pertussis toxin to area CA1 of the hippocampus. Pertussis toxin catalyzes the ADP-ribosylation of the three G_i isozymes and is a general inhibitor of G_i function (Bokoch et al., 1984). Wild-type mice that received bilateral injections of whole pertussis toxin into area CA1 of the hippocampus showed a severe deficit in memory for passive avoidance (Figure 1A). The individual components of the toxin, the enzymatically active A protomer, which by itself cannot penetrate the cell membrane, and the B oligomer, which facilitates the toxin's entry into the cell, had no effect on passive avoidance LTM (Figure 1A). Additionally, pertussis toxin injection into CA3 of the hippocampus failed to disrupt passive avoidance LTM, although it was lowered somewhat. This suggests that the defect in memory formation occurs via inhibition of pertussis toxin-sensitive G proteins and not through other cellular effects attributed to the toxin or its components. Furthermore, inhibition of LTM was relatively specific to area CA1 and did not occur when pertussis toxin was administered to area CA3 of the hippocampus. Delivery of pertussis toxin to area CA1 also inhibited LTM for contextual learning (Figure 1B). These data suggest that one or more pertussis toxin-sensitive G proteins are required for fear-associated memory.

Several G_α subunits are substrates for pertussis toxin, including the three isoforms of G_i and the ubiquitous G_o. Consequently, it is not clear from these pertussis toxin experiments which G protein or combination of G proteins is important for passive avoidance and contextual memory formation. However, we found that pertussis toxin administration to area CA1 reduced $G_{i\alpha1}$ protein without affecting the amount of $G_{i\alpha2}$ or $G_{i\alpha3}$ (Figure 1C). Although it is not clear why $G_{i\alpha1}$ protein levels were specifically reduced, ribosylated $G_{i\alpha1}$ protein may be more susceptible to proteases than the other G-coupling proteins. Western analysis indicated that $G_{i\alpha1}$ is expressed predominantly in the brain and to a lesser extent in the kidney (Figure 1D). In contrast, $G_{i\alpha2}$ and $G_{i\alpha3}$ show a much broader tissue distribution, although all three isozymes are found in the hippocampus. Since pertussis toxin treatment only reduced the levels of $G_{i\alpha1}$ protein, we considered the possibility that $G_{i\alpha1}$ may be particularly important for hippocampus-dependent memory formation.

Targeted Disruption of the $G_{i\alpha1}$ Gene Increases Adenylyl Cyclase Activity and Impairs LTM

To assess the role of $G_{i\alpha1}$ in hippocampus-dependent LTM, the mouse $G_{i\alpha1}$ gene was disrupted using a targeting construct containing a neomycin cassette spliced into exon 3 (Figure 2A). The ablation of $G_{i\alpha1}$ was confirmed by Southern (Figure 2B) and Western (Figure 2C) analysis. Adenylyl cyclase activity in hippocampal membranes from $G_{i\alpha1}{}^{-/-}$ and $G_{i\alpha1}{}^{+/-}$ mice was increased approximately 2-fold relative to wild-type mice (Figure 2D), indicating that $G_{i\alpha1}$-coupled receptors provide tonic inhibition of hippocampal adenylyl cyclase activity. The observation that heterozygotes showed comparable adenylyl cyclase activity as the homozygotes reflects the fact that $G_{i\alpha1}$ is in stoichiometric excess over adenylyl cyclase catalytic subunits. Furthermore, the hippocampus expresses a complex mixture of $G_{i\alpha1}$-sensitive and -insensitive adenylyl cyclases (Poser and Storm, 2001).

$G_{i\alpha1}{}^{-/-}$ mice exhibited normal open field activity ($p > 0.9$ for center and margin time behavior between genotypes, data not shown), and brain morphology appeared to be normal (data not shown).

Both $G_{i\alpha1}{}^{+/-}$ and $G_{i\alpha1}{}^{-/-}$ mice exhibited a partial defect in memory for passive avoidance training measured 24 hr after training (Figure 3A). After 8 days, $G_{i\alpha1}{}^{-/-}$ mice showed no memory for passive avoidance training. The fact that mutant animals lacking one copy of the $G_{i\alpha1}$ gene have deficits in passive avoidance memory is consistent with the significant reduction of $G_{i\alpha1}$ in heterozygous mice (Figure 2C). It is unclear why heterozygotes showed intermediate behavioral phenotypes while adenylyl cyclase activity was reduced to a comparable extent in heterozygotes and homozygotes. Adenylyl cyclase activities were measured in whole hippocampus

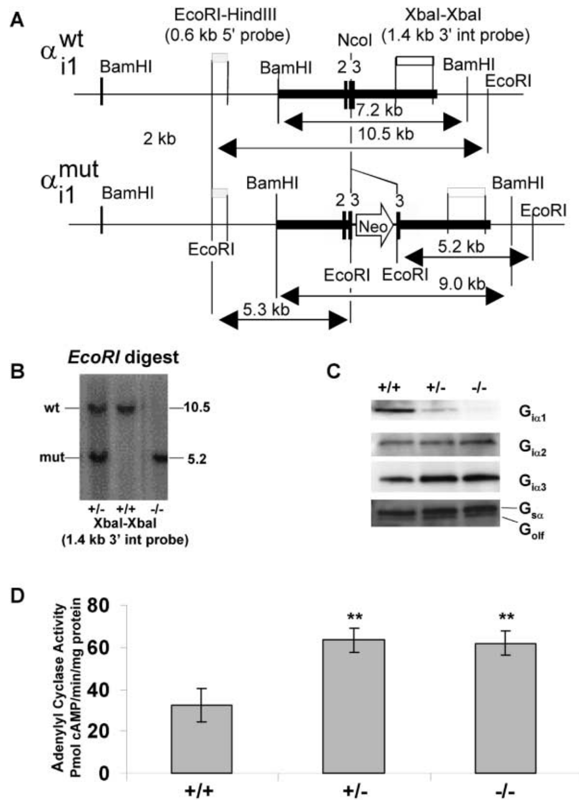

Figure 2. Disruption of the $G_{i\alpha1}$ Gene Increases Adenylyl Cyclase Activity in the Hippocampus

(A) Targeting vector for disruption of the $G_{i\alpha1}$ gene showing insertion of the neomycin cassette into exon 3. Restriction enzyme map shows fragment size.

(B) Representative Southern blots showing wild-type (+/+), heterozygous (+/−), and homozygous (−/−) genotypes.

(C) Western analysis for G protein α subunits in hippocampal membranes shows no $G_{i\alpha1}$ protein expression in knockout animals and low protein expression in heterozygote littermates.

(D) Adenylyl cyclase activity is increased in the hippocampus of $G_{i\alpha1}{}^{+/-}$ and $G_{i\alpha1}{}^{-/-}$ mice. Pooled hippocampal tissue isolated from littermates were prepared and assayed for membrane adenylyl cyclase activity as described in Experimental Procedures. Error is expressed as ±SD, with n = 5 for each, **$p < 0.01$.

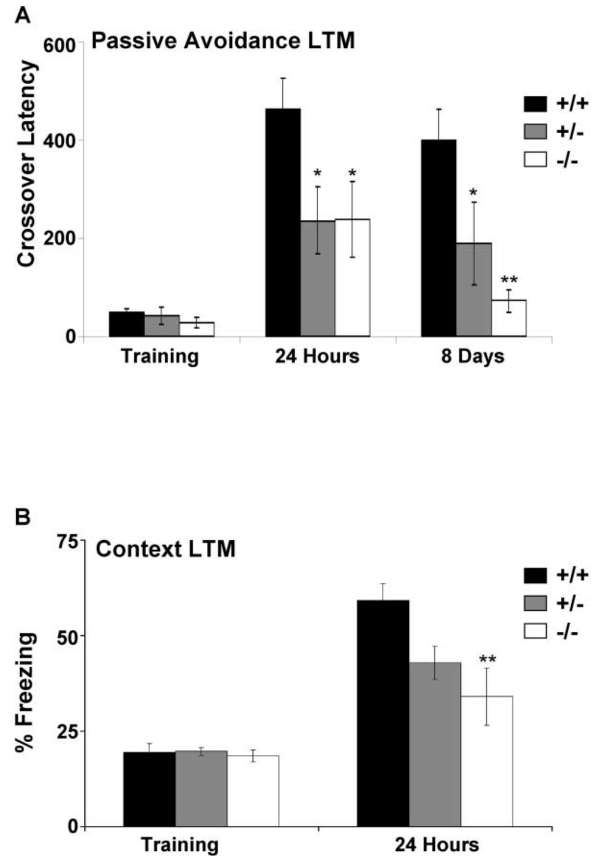

Figure 3. Disruption of the $G_{i\alpha1}$ Gene Impairs Hippocampus-Dependent Conditioned Fear Memory

(A) Memory for passive avoidance training measured after 24 hr and 8 days is reduced in $G_{i\alpha1}{}^{+/-}$ and $G_{i\alpha1}{}^{-/-}$ mice. Data are shown as mean ± SEM, *$p < 0.05$, **$p < 0.01$.

(B) Memory for contextual training measured 24 hr after training is reduced in $G_{i\alpha1}{}^{-/+}$ and $G_{i\alpha1}{}^{-/-}$ mice. Data are shown as mean ± SEM (n = 9 +/+, 13 +/−, 7 −/−), **$p < 0.01$ compared to wild-type controls.

Mice were trained for passive avoidance and contextual fear memory as described in Experimental Procedures.

extracts, and it seems likely that adenylyl cyclase activities within subregions of the hippocampus of heterozygous and homozygous mice may be different since the distribution of G_i-sensitive adenylyl cyclases varies within the hippocampus. Heterozygous and homozygous mice showed similar freezing behavior as wild-type littermates immediately after foot shock was presented, which suggests normal perception of the stimulus and intact short-term memory ($p < 0.01$ post- versus pre-shock for all genotypes $p > 0.96$ between genotypes—data not shown). $G_{i\alpha1}{}^{-/-}$ mice also had defects in memory for contextual training (Figure 3B), while $G_{i\alpha1}{}^{+/-}$ mice exhibited an intermediate but statistically nonsignificant phenotype.

Memory for object recognition, another form of hippocampus-dependent memory, was compromised in $G_{i\alpha1}{}^{+/-}$ and $G_{i\alpha1}{}^{-/-}$ mice (Figure 4A). In contrast, spatial memory, assessed by the Morris water maze, was normal for $G_{i\alpha1}{}^{+/-}$ and $G_{i\alpha1}{}^{-/-}$ mice (Figure 4C). Homozygote and heterozygote animals also exhibited normal memory for cued auditory training and cued memory extinction (Figure 5). The fact that cued memory is normal in $G_{i\alpha1}{}^{+/-}$ and $G_{i\alpha1}{}^{-/-}$ mice indicates that the mutant mice exhibit shock sensitivity comparable to wild-type mice. Collectively, these data indicate that loss of $G_{i\alpha1}$ perturbs several hippocampus-dependent forms of LTM, including contextual, passive avoidance, and novel object recognition. Normal spatial memory with $G_{i\alpha1}{}^{+/-}$ and $G_{i\alpha1}{}^{-/-}$ mice implies that unregulated cAMP signaling disrupts some but not all memory encoding pathways in the hippocampus.

The memory defects exhibited by the $G_{i\alpha1}{}^{-/-}$ mice may be a result of some unobserved peripheral defect; however, they show normal spatial and cued memory which depend upon visual acuity and shock sensitivity, respectively. To address this issue, we also depleted $G_{i\alpha1}$ using antisense oligodeoxynucleotides (asODN) bilaterally injected into area CA1 of the hippocampus in wild-type mice. Penetration of the oligonucleotide into hippocam-

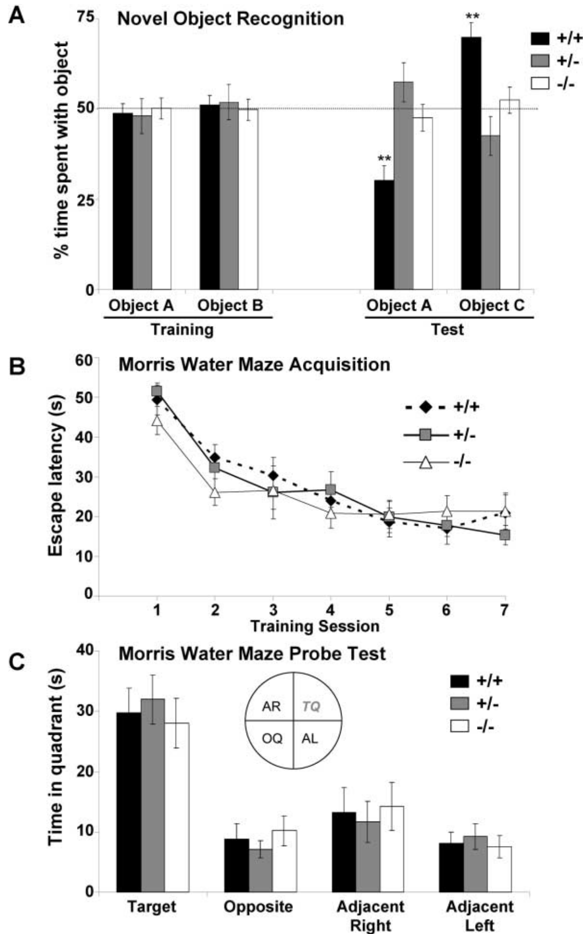

Figure 4. Impaired Object Recognition but Normal Spatial Memory in $G_{i\alpha1}$-Deficient Animals

(A) When presented with two novel objects (A and B) during training, all genotypes ($p > 0.99$) showed equal preference. When one of the objects was replaced with a different object (C), $G_{i\alpha1}{}^{+/+}$ ($n = 14$ $p < 0.01$) control animals showed a clear preference for the novel object. However, $G_{i\alpha1}{}^{+/-}$ ($n = 5$, $p = 0.527$) and $G_{i\alpha1}{}^{-/-}$ ($n = 10$, $p = 0.966$) mice exhibited no memory for object recognition. p values relative to training behavior.

(B) Target acquisition in the hidden platform version of the Morris water maze was similar in $G_{i\alpha1}{}^{-/-}$, $G_{i\alpha1}{}^{+/-}$, and $G_{i\alpha1}{}^{+/+}$ mice ($n = 8$, 9, and 7, respectively).

(C) Probe test reveals normal spatial memory in $G_{i\alpha1}{}^{-/-}$ and $G_{i\alpha1}{}^{+/-}$ animals compared to wild-type littermates ($p \geq 0.996$ between genotypes, target quadrant versus other quadrants $p \leq 0.05$ for all genotypes).

pal neurons when asODN was administered through cannulated mice was verified using a fluorescent-labeled asODN (Figure 6A). In cultured primary hippocampal neurons, we determined that treatment with asODN increased basal adenylyl cyclase activity approximately 2-fold compared to neurons treated with the scrambled oligonucleotide control (Figure 6B). When asODN was administered to area CA1 of mice, $G_{i\alpha1}$ protein was significantly reduced (Figure 6C), and memory for context was impaired compared to mice that received scrambled ODN as a control (Figure 6D). These data support the hypothesis that $G_{i\alpha1}$ plays a pivotal role in signaling mechanisms mediating hippocampus-dependent memory formation.

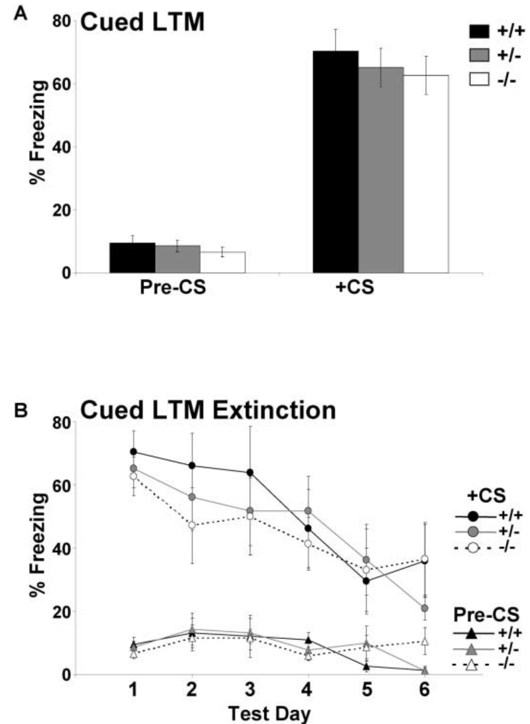

Figure 5. Disruption of the Gene for $G_{i\alpha1}$ Does Not Affect Memory for Auditory Cued Conditioning or Extinction of Cued Memory

(A) Wild-type as well as $G_{i\alpha1}{}^{-/+}$ and $G_{i\alpha1}{}^{-/-}$ mice showed memory for auditory cued conditioning measured 24 hr after training.

(B) Cued memory extinction was normal in $G_{i\alpha1}{}^{-/-}$ mice. After training for cued conditioning, mice were exposed to the conditioning tone once a day for 6 days without a paired shock and freezing behavior was recorded.

Mice were trained for auditory cued learning as described in Experimental Procedures.

Loss of $G_{i\alpha1}$ Results in Enhanced LTP in Area CA1

The loss of adenylyl cyclase inhibition in the hippocampus of $G_{i\alpha1}{}^{-/-}$ mice may hyperstimulate synapses and occlude new memory formation. To examine this possibility, CA1 LTP in hippocampal slices from wild-type and mutant mice were compared. In the rodent hippocampal slice, high-frequency stimulation (HFS) of the Schaeffer-collateral pathway elicits LTP in area CA1 pyramidal cells. Two forms of LTP are observed at this synapse: a decremental or early-phase (E-LTP) arises from covalent modification of synaptic proteins evoked by a single tetanic stimulus, while the long-lasting or late-phase form (L-LTP) depends on de novo protein synthesis and requires multiple high-frequency stimuli (Frey et al., 1993). L-LTP is dependent on cAMP signaling (Abel et al., 1997; Wong et al., 1999), whereas E-LTP is positively modulated by cAMP (Otmakhova et al., 2000). Disruption of the $G_{i\alpha1}$ gene had no effect on paired-pulse facilitation, a form of short-term plasticity and a measure of presynaptic function (Figure 7A). Additionally, input-output analysis showed no difference between genotypes in slope to fiber volley ratio (Figure 7B). In wild-type mice, a single 100 hz HFS induced E-LTP (Figure 7C) that decayed to baseline approximately 90 min after tetanic stimulation. In contrast, the same stimulus elicited persistent L-LTP in hippocampal slices from $G_{i\alpha1}{}^{+/-}$ or $Gi_{\alpha1}{}^{-/-}$ mice lasting for at least 3 hr (Figure 7C). As in the LTM

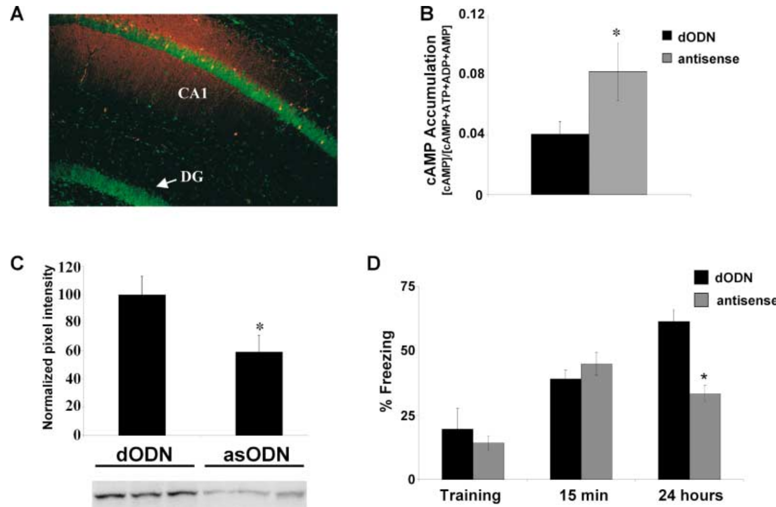

Figure 6. $G_{i\alpha1}$ Antisense Treatment In Vivo Disrupts Contextual Memory

(A) Confocal microscopy shows uptake of Cy_3-conjugated $G_{i\alpha1}$ antisense oligonucleotides (red) in area CA1 of the hippocampus (green-syto 13). Labeled oligonucleotide was injected into area CA1 as described in Experimental Procedures.

(B) $G_{i\alpha1}$ antisense treatment of cultured hippocampal neurons increased intracellular cAMP relative to neurons treated with the scrambled oligonucleotide (dODN) control (*$p < 0.05$). Intracellular cAMP was measured 24 hr after treatment of neurons with oligonucleotides as described in Experimental Procedures.

(C) Bilateral administration of $G_{i\alpha1}$ antisense oligonucleotide into area CA1 decreased $G_{i\alpha1}$ protein in the dorsal hippocampus. Antisense oligonucleotide (asODN) or scrambled oligonucleotide control (dODN) was bilaterally administered to area CA1. After 4 days, $G_{i\alpha1}$ protein levels were quantified by Western analysis as described in Experimental Procedures. The graph shows a decrease of about 50% in dorsal hippocampus $G_{i\alpha1}$ levels after asODN treatment ($n = 7$) compared to dODN-injected controls ($n = 8$) (*$p < 0.05$).

(D) Bilateral administration of $G_{i\alpha1}$ antisense oligonucleotide into Area CA1 decreased context-conditioned memory measured 24 hr after training. Antisense oligonucleotide (asODN, $n = 8$) or scrambled oligonucleotide control (dODN, $n = 7$) (*$p < 0.05$) were bilaterally administered to area CA1 4 days before training. Mean ± SEM for all treatment parameters.

assays, the heterozygotes exhibited an intermediate LTP phenotype. Furthermore, restimulation with a single 100 hz HFS at the end of the 3 hr recording period reestablished E-LTP in wild-type slices but failed to significantly elevate the potentiation in $G_{ia1}{}^{+/-}$ and $G_{ia1}{}^{-/-}$ slices (data not shown).

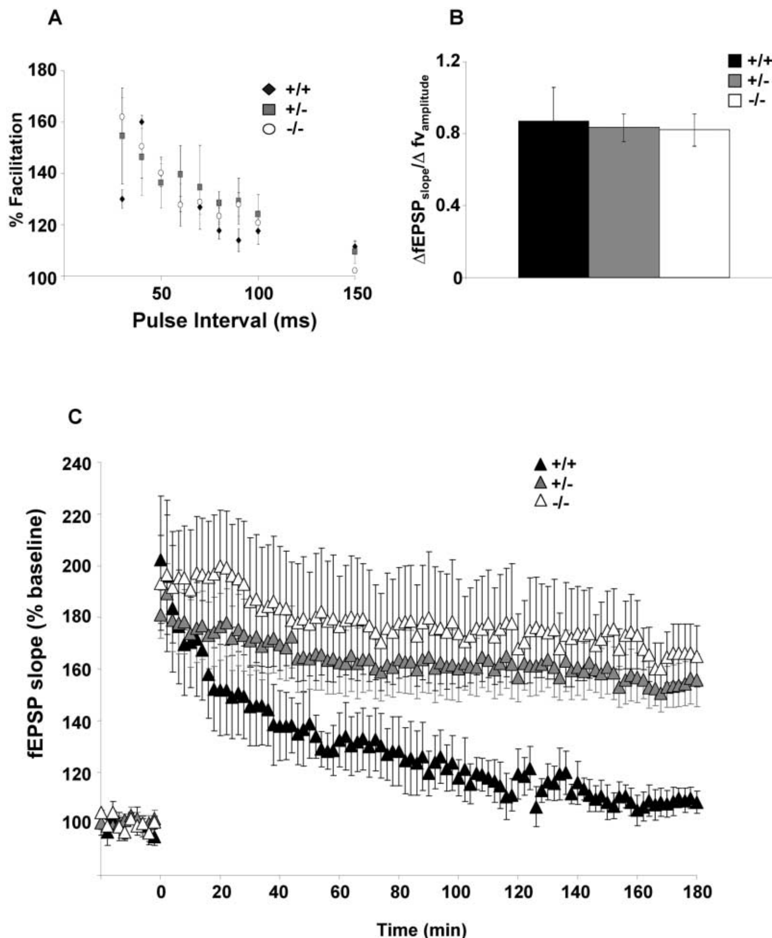

Figure 7. $G_{i\alpha1}{}^{+/-}$ and $G_{i\alpha1}{}^{-/-}$ Mice Exhibit Normal Basal Synaptic Transmission but Elevated LTP in Area CA1 of the Hippocampus

(A) CA1 paired-pulse facilitation is normal in $G_{i\alpha1}{}^{-/+}$ and $G_{i\alpha1}{}^{-/-}$ slices compared to wild-type littermate controls.

(B) Ratio of fEPSP slope to fiber volley amplitude is indistinguishable between $G_{i\alpha1}{}^{-/+}$, $G_{i\alpha1}{}^{-/-}$, and wild-type littermate control slices.

(C) A single 100 hz tetanic stimulus at $t = 0$ induced E-LTP in hippocampal slices from wild-type mice but elicited L-LTP in hippocampal slices from $G_{i\alpha1}{}^{-/+}$ and $G_{i\alpha1}{}^{-/-}$ mice. LTP at the Schaffer collateral CA1 synapse was measured as described in Experimental Procedures.

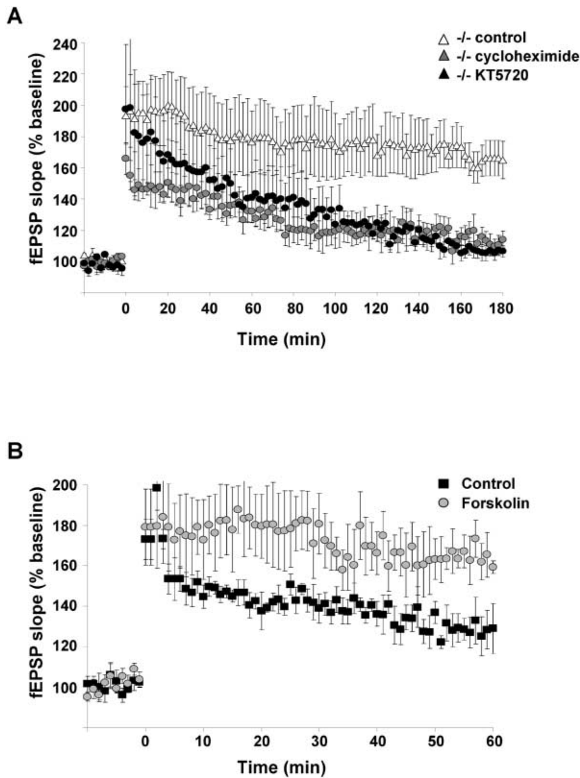

Figure 8. Enhanced LTP in $G_{i\alpha1}^{-/-}$ Slices Was Blocked by PKA and Cycloheximide

(A) A single 100 hz train failed to elicit L-LTP in area CA1 of $G_{i\alpha1}^{-/-}$ slices in the presence of 250 nM KT5720 (n = 4) and 60 μM cycloheximide (n = 4).

(B) Administration of 2 μM forskolin with a single 100 hz tetanic stimulus generated enhanced LTP in hippocampal slices from wild-type mice. Two μM forskolin stimulates adenylyl cyclase activity in the hippocampus approximately 2-fold but is not in itself sufficient to generate LTP.

The conversion of E-LTP to L-LTP was further supported by inhibitor studies. Application of KT5720, an inhibitor of cAMP-dependent protein kinase, occluded L-LTP formation in $G_{i\alpha1}^{-/-}$ slices. The protein synthesis inhibitor cycloheximide also blocked the expression of long-lasting LTP (Figure 8A). This supports the hypothesis that elevated adenylyl cyclase activity stimulates downstream signaling components that promote L-LTP in the hippocampus. This interpretation is further supported by the observation that E-LTP in wild-type mice can be converted to L-LTP by application of 2.0 μM forskolin to hippocampal slices prior to stimulating with a single train of HFS (Figure 8B). Two μM forskolin was used in this experiment because it increases adenylyl cyclase activity in hippocampal neurons 2-fold, approximately the same increase caused by disruption of the $G_{i\alpha1}$ gene (Figure 2D). Although forskolin at higher concentrations can cause an increase in the field excitatory postsynaptic potentials, which mimics HFS-evoked LTP, the concentration used in this experiment was insufficient to generate long-lasting "chemo-LTP."

Discussion

Genetic approaches using flies and mice illustrate the importance of the cAMP signal transduction pathway in memory formation (Table 1). In *Drosophila*, insufficient cAMP generation (*amnesiac* and *rutabaga*), disruption of the effector molecule PKA (*DCO* and *PKA-RI*), as well as attenuation of the downstream transcription factor (*dCREB2*-b) all deleteriously affect memory formation. Interestingly, an enhancement in memory has also been reported when a constitutively active form of CREB (d-*CREB2*-a) is ectopically expressed, suggesting that positive effectors of this signaling pathway may improve synaptic plasticity. However, flies that lack the cAMP-phosphodiesterase (*dunce*) or unregulated AC activity (constitutively active $G_{s\alpha}$) show impairments in memory formation despite having mutations that elevate cAMP. Since the process of memory formation requires synapse-specific plasticity, unfettered cAMP increases may occlude this process despite possible gains in synaptic efficacy.

Although mice are clearly more complex than flies, there are striking similarities between memory phenotypes seen when components of the cAMP signal transduction systems are genetically modified in flies and mice (Table 1). PKA regulates important aspects of neuronal function, including ion channel activity, gene expression, neurotransmitter synthesis, and release, all of which can lead to enhanced synaptic efficacy (reviewed in Nairn et al., 1985). Consequently, cAMP and PKA play important roles in specific forms of synaptic plasticity and LTM. For example, cAMP-mediated transcription is implicated in the late form of LTP (L-LTP) in area CA1, mossy fiber, and the medial perforant pathways (Abel et al., 1997; Frey et al., 1991, 1993; Impey et al., 1996; Nguyen and Kandel, 1996). A role for cAMP in LTP and hippocampus-dependent memory has also been suggested by transgenic mouse studies. Ablation by gene targeting of C1β or R1β subunits of PKA leads to a defect in mossy fiber LTP (Huang et al., 1995). Reduction of PKA activity in transgenic mice expressing a dominant-negative R subunit, R (AB), causes defects in L-LTP, spatial memory, and long-term contextual fear conditioning (Abel et al., 1997). Mice lacking type I adenylyl cyclase are deficient in spatial memory (Wu et al., 1995) and mossy fiber LTP (Villacres et al., 1998). Furthermore, mice lacking both type 1 and 8 adenylyl cyclases lack L-LTP and LTM for passive avoidance and contextual training (Wong et al., 1999).

Although the studies cited above indicate that synapse-specific increases in cAMP contribute to enhanced synaptic efficacy and support hippocampus-dependent memory formation, the importance of adenylyl cyclase inhibitory mechanisms for memory formation had not been evaluated. Based on the published literature, one might expect that G_i serves as a constraint on learning and memory since it opposes cAMP formation in the hippocampus. On the other hand, tonic suppression of adenylyl cyclase activity in the hippocampus may be required to allow an appropriate cAMP signaling differential when specific synapses are activated. Furthermore, chronic increases in cAMP have the potential to suppress specific signaling pathways, including the PI3 kinase pathway (Poser et al., 2003), which is required for the expression of synaptic plasticity (Sanna et al., 2002). The objectives of this study were to determine if adenylyl cyclase activity in the hippocampus is tonically suppressed through G_i-mediated inhibition and to evaluate the importance of this inhibitory mechanism for

Table 1. cAMP-Mediated Signaling in Learning and Memory

Drosophila	Mouse	Gene Product/Targeted Cellular Effector	Memory Phenotype	Hippocampal Long-Term Potentiation
Reduced cAMP Signal Transduction				
rutabaga (1)	AC1 KO (9) AC 1/8 DKO (10)	Ca^{++}-stimulated adenylyl cyclase	STM (D) LTM (M)	mf-CA3 defect CA1 L-LTP intact in single KO, ablated in DKO
amnesiac (2)	N.D.	pituitary adenylyl cyclase-activating peptide (PACAP) homolog	LTM	N.D.
PKA-RI (3)	R (AB) (11)	PKA inhibitory subunit	LTM	L-LTP defect
DCO (4)	C1β KO (12)	cAMP-dependent protein kinase (PKA) catalytic subunit	LTM (D) no phenotype (M)	reduced CA1 L-LTP mf-CA3 defect
dCREB2 - b (5)	αδCREB KO (13)	CREB repressor transgene in flies, isoform knockout in mice	LTM	L-LTP defect
Enhanced cAMP Signal Transduction				
dunce (6)	PDE 1B KO (14)	phosphodiesterase (non-homologs)	STM (D) mild LTM (M)	N.D.
CA $G_{s\alpha}$ (7)	$G_{i\alpha1}$ KO	unregulated adenylyl cyclase activity	LTM	E-LTP converted to L-LTP
dCREB2 - a (8)	VP16-CREB (15)	activated CREB	enhanced LTM (D) N.D. (M)	enhanced LTP

Genetic mutations that have positive and negative effects on the cAMP signal transduction pathway are shown along with the associated memory and LTP phenotypes. The fly and mouse mutants shown that have homologous gene products demonstrate conserved cAMP signaling components that mediate memory formation. In general, parallel memory phenotypes are observed when mutant flies and mice show analogous perturbations in cAMP signaling. N.D., no data available; CA, constitutively active; KO, single gene knockout; DKO, double knockout; STM, short-term memory; LTM, long-term memory; D, *Drosophila*; M, mouse; mf-LTP, mossy fiber/CA3 LTP; E-LTP, decremental CA1 LTP; L-LTP, long-lasting LTP. References: (1) Livingston et al., 1984; (2) Quinn et al., 1979; (3) Drain et al., 1991; (4) Skoulakis et al., 1993; (5) Yin et al., 1995; (6) Byers et al., 1981; (7) Connolly et al. 1996; (8) Yin et al., 1994; (9) Wu et al., 1995; (10) Wong et al., 1999; (11) Abel et al., 1997; (12) Huang et al., 1995; (13) Bourtchuladze et al., 1994; (14) Reed et al., 2002; (15) Barco et al., 2002.

memory. Does the reduction or ablation of G_i activity in the hippocampus enhance or inhibit hippocampus-dependent memory formation?

Our data indicate that adenylyl cyclase activity in the hippocampus of mice is normally restrained by $G_{i\alpha1}$-coupled inhibitory receptors, since basal adenylyl cyclase activity increased approximately 2-fold when the $G_{i\alpha1}$ gene was ablated. In contrast, there was very little effect on Ca^{2+}-stimulated adenylyl cyclase activity, presumably because AC8 is not inhibited through G_i-coupled receptors in vivo (Nielsen et al., 1996). Consequently, the cAMP differential caused by synapse-specific activation of adenylyl cyclases is actually lowered in $G_{i\alpha1}^{-/-}$ mice. The hippocampus expresses a number of G_i-coupled receptors that have the potential to tonically suppress adenylyl cyclase activity, including 5HT1A (Albert et al., 1990), mu opioid (Zastawny et al., 1994), metabotropic glutamate types II and III (Gereau and Conn, 1995), somatostatin (Breder et al., 1992), CB1 cannabinoid (Marsicano and Lutz, 1999), and melatonin receptors (Musshoff et al., 2002). Interestingly, disruption of the genes encoding several of these receptors perturbs synaptic plasticity and/or learning and memory (Dutar et al., 2002; Moneta et al., 2002; Sarnyai et al., 2000).

Reduction of $G_{i\alpha1}$ in area CA1 of the hippocampus of mice by three independent methods, including administration of pertussis toxin or antisense oligonucleotides to the hippocampus as well as gene ablation, caused significant defects in some forms of hippocampus-dependent memory. Interestingly, loss of $G_{i\alpha1}$ did not affect spatial memory, emphasizing that there are mechanistic differences between various forms of hippocampus-dependent memory. Loss of $G_{i\alpha1}$ enhanced synaptic plasticity in the hippocampus by lowering the threshold stimulus required for the generation of L-LTP. This increase in LTP response was mimicked by application of low levels of forskolin to hippocampal slices from wild-type animals, suggesting that normal increases in synaptic efficacy are dependent upon optimal cAMP signals. This suggests that the increase in adenylyl cyclase activity caused by ablation of $G_{i\alpha1}$ creates a state of hypersensitivity that perturbs normal synaptic plasticity and interferes with learning and memory. This idea was confirmed by demonstrating that application of high levels of forskolin to the hippocampus interferes with hippocampus-dependent memory (J.I.A. and D.R.S., unpublished data).

The enhancement of LTP seen with $G_{i\alpha1}^{-/-}$ mice provides a clue as to why these mice show memory defects. Encoding of hippocampus-dependent memory is thought to be a synapse-specific event with information storage due to activity-dependent enhancement in synaptic weights in the hippocampus (Martin and Morris, 2002). This hypothesis predicts that saturation of LTP should impair learning. $G_{i\alpha1}^{-/-}$ mice show a general enhancement of CA1 LTP, most likely because adenylyl cyclase activity is increased throughout the hippocampus. This increase in synaptic activity in the hippocampus caused by ablation of $G_{i\alpha1}$ may occlude synapse-specific events required for memory formation. This idea is supported by studies showing that saturation of LTP in vivo inhibits hippocampus-dependent memory (Moser et al., 1998). Presumably, neuronal activity involved in LTP and LTM formation share similar molecular mechanisms, and the ensuing enhancement of synaptic efficiency is needed for both processes. Alternatively, since we did not observe a defect in spatial memory, it is possible that the unrestrained activation or potentiation observed with $G_{i\alpha1}$ mutants only affects certain types of hippocampus memory formation.

In summary, hippocampus-dependent memory formation depends upon a critical balance between mechanisms for stimulating and inhibiting adenylyl cyclase activity. In this study, we identified one of the G_i isozymes, $G_{i\alpha1}$, as a crucial component of the signaling complex required for memory. We hypothesize that chronic activation of adenylyl cyclase activity in the hippocampus of $G_{i\alpha1}^{-/-}$ mice causes saturation, or partial saturation, of the intrinsic neuronal pathways required for new memory formation.

Experimental Procedures

Reagents

Pertussis toxin, B oligomer, and A protomer (List Biological Laboratories, San Jose, CA) were reconstituted in bacteriostatic 0.9% saline. Concentrated stock solutions of forskolin and KT5720 (Calbiochem, San Diego, CA) were prepared in DMSO while cycloheximide (Calbiochem) was dissolved in 100% ethanol. Drugs were diluted in ACSF to the final working concentration with DMSO and ethanol concentrations not exceeding 0.1%. All other reagents were dissolved in 1X PBS. Alpha {^{32}P}ATP and {^{3}H}cAMP were obtained from ICN, while all other salts were purchased from Sigma. Phosphothioate-modified control (dODN), antisense (asODN) oligodeoxynucleotides, and Cy_3-conjugated asODN (Integrated DNA Technology, Coralville, IA) were modeled after previously reported sequences (Galeotti et al., 2001). Lyophilized ODNs were reconstituted in bacteriostatic saline, aliquoted, and frozen until use.

Surgical Procedures

Six- to twelve-week-old (25–30 grams body weight) wild-type C57BL/6 (Taconic Farms) mice were used in pharmacological experiments. Mice were cannulated as described previously (Athos and Storm, 2001). Briefly, animals were anesthetized with an intraperitoneal injection (18–22 μl/g body weight) of a mixture of ketamine (7.0 mg/ml) and xylazine (0.44 mg/ml) dissolved in 0.9% bacteriostatic saline. Anesthetized mice were mounted on a stereotaxic frame (10 micron model, Cartesian Research, Sandy, OR), and cannulae (24 gauge) were implanted just dorsal to the CA1 region of the hippocampus (−1.5 mm AP, ±1.5 mm ML, −1.5 mm DV) (Slotnick and Leonard, 1975). The cannulae were affixed with dental acrylic and fitted with wire plugs (30 gauge) to maintain cannula patency. Mice were housed individually and allowed at least 1 week of postoperative recovery before being used in behavioral experiments.

In pertussis experiments, one-time site-specific infusions were performed using a frame-mounted stereotaxic injector (Cartesian Research, Sandy, OR). Administration of pertussis toxin and antisense deoxyoligonucleotide experiments did not exceed 0.5 μl/side delivered at a rate of 0.5 μl/min with an additional minute to ensure complete delivery of the reagents. Fifty ng of pertussis toxin, A protomer, and B oligomer and 5 μg/side antisense and scrambled control oligodeoxynucleotide were used.

Measurement of Memory

All animal experiments were done in accordance with IACUC guidelines and regulations. Experiments were preformed blind to genotype and treatment conditions. Age-matched (3–6 months), mixed-background mutant and littermate controls were used. C57Bl/6 wild-type mice were obtained from Taconic Farms for pharmacological experiments. Memory for passive avoidance learning was assessed using an automated light-dark shuttle box (Habitest System, Coulbourn Instruments, Allentown, PA) controlled by a Pentium-class computer running Winlinc (Coulbourn Instruments). The test animal was placed in the lighted compartment of a shuttle box, and after 10 s, a guillotine style door was raised. Once the mouse crossed

over into the darkened half of the chamber, the door was closed and a mild footshock was immediately administered (0.7 mV, 2 s). After training, the animals were promptly returned to their home cages. Memory for passive avoidance was assessed at various times post-training by placing the animal back in the lighted side of the chamber and measuring the time required to cross over into the darkened side. Contextual fear-conditioned memory was evaluated as previously described (Athos et al., 2002). Briefly, each animal was placed in a training chamber with a software-controlled shock floor and a 16 $\times$ 16 photobeam sensor grid (Truscan Mouse Chamber, Coulbourn Instruments, Allentown, PA). Baseline data were obtained while the test subject roamed freely for 2 min, after which the unconditioned stimulus (US) in the form of a mild footshock (0.7 mV, 2 s) was presented. An additional minute was allowed to elapse before returning the animal to its home cage. Memory for context was assessed by returning the animal to the conditioning chamber at 24 hr. Movement episodes, defined as consecutive beam breaks in the XY plane, were recorded in 100 ms intervals and summed into 5 s data bins. Taking the maximum number of observed movement episodes (limit of detection) per 5 s interval and correcting for background movement episode (noise), freezing behavior was obtained using the following equation ([maximum movement episodes $-$ observed movement episodes]/[maximum movement episodes] $\times$ 100). Data obtained from this equation is statistically indistinguishable from the sampling method we had used previously (Impey et al., 1998b). Training for cued-conditioned LTM was performed as previously described (Impey et al., 1998b), with some modifications. The conditioning stimulus (CS) in the form of a continuous 30 s tone (2600 hz, 110 dB) was presented before the unconditioned stimulus (US). Animals that received the CS during training were placed in a rat cage with peppermint oil-laced bedding. Movement behavior was observed for 2 min periods both before and during presentation of the CS. Spatial learning and memory using the Morris water maze was measured as previously reported (Wu et al., 1995).

Novel Object Recognition

Mice were individually housed in the test chambers (typical rat cage) for at least 2 hr of habituation before each experiment. Animals were first exposed to two objects, A and B, positioned at opposing ends of the rat cage. Since the rat cage is considerably larger than a mouse cage, an open field thigmotaxis behavior is predominantly observed. Placement of novel objects in this environment elicits a change in the wall-hugging behavior as the animals stop ambulating to investigate the new items. For a 5 min period, the number of approaches, which reflect time spent in close proximity to the introduced objects, were tallied and used to calculate the animals' preference for each object. This was reported as a percentage of the total number of approaches made for both objects. Testing occurred 1 hr after the initial exposure when a new object is presented along with one of the original or "familiar" objects. The animals' preference for the novel object (C) versus the "familiar" object (A) is used as an index of object recognition memory. Painted wooden playing blocks of varying shapes (triangle, square, and circle) and colors (red, yellow, green), measuring approximately 5 cm across were used as objects in these experiments. They were wiped down with 5% acetic acid followed by 70% ethanol prior to use to remove odorant cues.

Assay for Adenylyl Cyclase and cAMP

Adenylyl cyclase activity was measured as previously described (Wong et al., 1999). Briefly, the membrane fraction from hippocampi taken from a pool of four to five animals was resuspended using a glass dounce homogenizer in chilled buffer (Tris 50 mM, 2 mM $MgCl_2$, 1 mM EDTA, 0.5 mM dithiothreiotol, protease inhibitor cocktail (Roche Biochemicals). The assay cocktail (ATP, ^{3}HcAMP, ^{32}P-α-ATP, theophylline, CaM, creatine phosphate, creatine phosphokinase) was added to the membrane homogenate to start the reaction, and the mixture was incubated for 25 min at 34°C. Adding 1.5% sodium dodecyl sulfate and heating the mixture at 100°C for 2 min terminated the reaction. Upon cooling, each reaction mixture was poured into dowex columns and the flowthrough discarded. The nucleotides were eluted using water onto alumina-containing columns. The cAMP product eluted with imidazole into vials containing scintillation fluid (ICN). ^{3}H and ^{32}P counts were measured, and the ratios of ^{32}P-cAMP product to ^{3}H-cAMP internal control were used to calculate the reaction products. The total activity was normalized to protein concentration, which was determined using a commercially available BCA Assay (Pierce, Rockford, IL).

Primary hippocampal cultures were prepared as previously described (Impey et al., 1998a). After 7 days, the culture media was supplemented with ^{3}H-adenine (1 μCi/ml) and antisense or control ODN. Twenty-four hr after treatment, the media was aspirated and the cells were lysed with 5% trichloroacetic acid containing 1 μM cAMP. The cAMP activity was measured using the separation method described above with one modification. The first eluent was collected and the ^{3}H activity subsequently analyzed and treated as the total ^{3}H-labeled nucleotide fraction (ATP + ADP + AMP). This was used to normalize the activity measurements.

Western Analysis

Membrane fractions were dounce homogenized in Buffer H (50 mM glycerophosphate, 1.5 mM EGTA, 0.1 mM Na_3VO_4, 1 mM dithiothreiotol, Roche complete protease inhibitor cocktail). For equal loading, protein concentrations were measured using a commercial BCA assay kit. An appropriate volume of loading buffer was added, and the samples were denatured at 95°C for 10 min. Twenty μl of each sample was loaded onto a gradient (4%–20%) SDS-PAGE gel (Bio-rad) and electrophoresed using standard procedures. Once transblotted, the membrane (Immobilon P, Millipore) was blocked with blotto (10% milk in PBS). Antibodies against $G_{i\alpha1}$, $G_{i\alpha2}$, $G_{i\alpha3}$, and $G_{s\alpha}$ (Calbiochem) were diluted 1:1000 in PBST-BSA (PBS, 0.1% triton X-100, 5% g/v BSA). Signal was amplified using an alkaline phosphatase-conjugated secondary antibody (Cappel) and was visualized on film (KODAK) using CDP-star (ICN) as the ECL substrate.

Electrophysiology

Extracellular slice electrophysiology experiments were done using a submerged chamber as previously described (Impey et al., 1996; Wong et al., 1999). Briefly, 3-month-old mice were sacrificed by cervical dislocation and the brain rapidly excised and chilled in oxygenated ACSF (NaCl, 120 mM; KCl, 3.5 mM; $MgCl_2$, 1.3 mM; $CaCl_2$, 2.5 mM; NaH_2PO_4, 1.25 mM; $NaHCO_3$, 25.6 mM; Glucose, 10 mM). Transverse slices (400 μm) were obtained using a vibratome (Electron Microscopy Sciences), and slices containing the hippocampus were gently positioned on a nylon mesh within the chamber. Oxygenated (95% O_2/5% CO_2 mix) ACSF at a flow rate of approximately 1–2 ml/min continuously bathed the slices throughout the experiments. All fEPSP measurements were taken after the slices had equilibrated at 34°C for 1–2 hr. Test stimuli were delivered using an S88 Square Pulse Grass Stimulator (Astro-Med, West Warwick, RI) with a photoelectric stimulus isolation unit (Astro-Med) attached to a concentric 100 μM bipolar tungsten electrode (Rhodes Medical Instruments, Inc., Woodland Hills, CA) placed in the Schaeffer-Collateral inputs in the stratum radiatum. Field EPSPs for LTP measurements were sampled at 0.017 hz using a glass electrode filled with 3 M NaCl, and the intensity of the stimulus was adjusted to yield 50% maximal response. Potentials were amplified using an Axopatch 200B (Axon Instruments, Union City, CA), digitally converted by a Digidata 1200 (Axon), and compiled for subsequent analysis using Axoscope (Axon Instruments). Drugs were dissolved as previously described and perfused prior to and after tetanus was applied.

Statistical Analyses

One-way ANOVA with Tukey post-hoc tests were done to assess significant differences. Statistical p values are reported in figure legends.

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