The Slow Afterhyperpolarization: A Target of β_1 -Adrenergic Signaling in Hippocampus-Dependent Memory Retrieval

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In rodents, adrenergic signaling by norepinephrine (NE) in the hippocampus is required for the retrieval of intermediate-term memory. NE promotes retrieval via the stimulation of β_1 -adrenergic receptors, the production of cAMP, and the activation of both protein kinase A (PKA) and the exchange protein activated by cAMP. However, a final effector for this signaling pathway has not been identified. Among the many targets of adrenergic signaling in the hippocampus, the slow afterhyperpolarization (sAHP) is an appealing candidate because its reduction by β_1 signaling enhances excitatory neurotransmission. Here we report that reducing the sAHP is critical for the facilitation of retrieval by NE. Direct blockers of the sAHP, as well as blockers of the L-type voltage-dependent calcium influx that activates the sAHP, rescue retrieval in mutant mice lacking either NE or the β_1 receptor. Complementary to this, a facilitator of L-type calcium influx impairs retrieval in wild-type mice. In addition, we examined the role of NE in the learning-related reduction of the sAHP observed *ex vivo* in hippocampal slices. We find that this reduction in the sAHP depends on the induction of persistent PKA activity specifically in conditioned slices. Interestingly, this persistent PKA activity is induced by NE/ β_1 signaling during slice preparation rather than during learning. These observations suggest that the reduction in the sAHP may not be present autonomously *in vivo*, but is likely induced by neuromodulatory input, which is consistent with the idea that NE is required *in vivo* for reduction of the sAHP during memory retrieval.

Introduction

Although considerable inroads have been made into understanding the molecular mechanisms that contribute to memory consolidation in the hippocampus, much less is known about the molecular mechanisms that contribute to hippocampus-dependent memory retrieval. A simple hypothesis is that during retrieval, fast excitatory transmission reactivates neurons that underlie memory storage, without the need for neuromodulatory input that is often essential during memory consolidation. However, an initial screen of neuromodulatory requirements in retrieval indicated that β -adrenergic, D1-class dopaminergic and muscarinic signaling in the dorsal hippocampus (DH) may be required for inhibitory avoidance memory (Barros et al., 2001). Evidence also indicates that μ -opioid signaling may be required in the DH for spatial memory retrieval (Meilandt et al., 2004). However, a potential caveat is that retrieval may depend on the “state” of the organism. Indeed, treatment with either muscarinic or μ -opioid agents has provided evidence for state-dependent

effects on retrieval (Bruins Slot and Colpaert, 1999; Colpaert et al., 2001).

An example in which state-dependent effects have been ruled out is for the role of adrenergic signaling. Mice in which the gene for dopamine β -hydroxylase has been disrupted (*Dbh*^{−/−}) completely lack norepinephrine and epinephrine (NE/E) and exhibit deficits in spatial and contextual memory that are consistent with a role for NE/E in retrieval (Thomas and Palmiter, 1997; Murchison et al., 2004). In addition, β -adrenergic receptor antagonists impair memory when administered systemically or into the DH before testing or before both training and testing, but have no effect when administered only before training (Murchison et al., 2004; Ouyang and Thomas, 2005).

Given this distinct role for adrenergic signaling in retrieval, one goal has been to determine the downstream mechanisms involved. In particular, β_1 -adrenergic signaling, cAMP, protein kinase A (PKA), and the exchange protein activated by cAMP (Epac) are all required for the role of NE in retrieval (Murchison et al., 2004; Ouyang et al., 2008). However, the ultimate effector of this initial signaling remains unidentified. An appealing candidate is the slow afterhyperpolarization (sAHP) because NE, β_1 receptors, cAMP, and PKA all reduce the sAHP in the hippocampus (Madison and Nicoll, 1982, 1986; Pedarzani and Storm, 1993). The sAHP is responsible for the accommodation of firing that occurs during persistent excitatory input. As a result, reductions in the sAHP enhance the transmission of excitatory input as excitatory output. The development of selective, direct blockers of the sAHP provides a means in which to test its role in retrieval (Shah et al., 2001, 2006; Zunszain et al., 2002).

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Interestingly, reductions in the sAHP are transiently observed *ex vivo* for several days after conditioning (Moyer et al., 1996; Saar et al., 1998). This time course mirrors that for the role of NE in retrieval (Murchison et al., 2004). However, it is not clear why NE is required for retrieval if the sAHP is a relevant target but is already reduced *in vivo*. To clarify the role of NE, the sAHP was recorded *ex vivo* in slices from naive and conditioned *Dbh*^{+/-} and *Dbh*^{-/-} mice.

Materials and Methods

Animals. *Dbh*^{+/-}, *Dbh*^{-/-}, *Adrb1*^{-/-} (β_1 KO) and wild-type (WT) littermate mice were on a hybrid 129/Sv $\times$ C57BL/6 background (Thomas et al., 1995; Rohrer et al., 1996). Mice were generated by mating either heterozygotes or homozygotes, and genotype was determined by PCR. The prenatal loss of *Dbh*^{-/-} mice was rescued as previously described (Ouyang et al., 2004). *Dbh*^{+/-} mice were used in place of WT mice in some experiments because the former were littermate controls for *Dbh*^{-/-} mice, and because they have normal levels of NE/E and are phenotypically indistinguishable from *Dbh*^{+/+} mice (Thomas et al., 1998). No significant differences were found by sex or parental genotype, so data were combined. Animals were maintained on *ad libitum* food and water and a 12 h light/dark cycle, with lights on beginning at 7:00 A.M. Animals were housed in small, quiet rooms for at least 3 weeks before studies began. Mice were 3–5 months old when tested. Studies were performed during the light phase, with most experiments taking place between 9:00 A.M. and 5:00 P.M. Studies were in accordance with National Institutes of Health (NIH) guidelines and had the approval of the IACUC at the University of Pennsylvania.

Classical fear conditioning. Adjacent to the training room, animals were placed in pairs into opaque plastic holding buckets (12 cm diameter) with bedding and lids for 30–60 min before being manipulated further. For habituation, animals were given two 3 min preconditioning handling sessions >2 d in the training room, and saline was injected at the end of handling each day. For conditioning, animals were placed in the training apparatus (ENV-010MC with ENV-414S, Med Associates) for 2 min, after which an 84 dB, 4.5 kHz tone was activated for 30 s. Two seconds before the end of the tone, a 2 s footshock was delivered. Except where noted, the shock intensity was 1 mA. The animal was removed from the apparatus 30 s later and returned to its home cage. Pseudo-conditioned animals were handled identically except that footshock was delivered immediately after placing the animal in the conditioning apparatus rather than at the end of the tone. Context-only animals were exposed to the context for the same duration but no shock was delivered. The apparatus was cleaned with Versa-Clean (Thermo Fisher Scientific) between subjects. Contextual fear was tested for 5 min in the conditioning apparatus in the absence of the tone, unless a second test of context was performed the next day, in which case testing was for 3 min each day to minimize extinction. Cued fear was tested in a Plexiglas cylinder (21 cm diameter, 24 cm tall) with green wire grid floor and vertical green and white wall stripes 240° around, and was cleaned with lemon-scented Ajax between subjects. Two minutes after placing the animal in the apparatus, the training tone was turned on for 3 min. Percentage freezing was estimated by scoring the presence or absence of nonrespiratory movement every 5 s. Tests were conducted 1 d after training unless noted otherwise.

Drugs. UCL compounds were synthesized at University College London. Their chemical identities are as follows: UCL 1851: (2-chlorophenyl)-diphenylmethanol, UCL 1864: tris(4-chlorophenyl)methanol, UCL 2027: 2-(triphenylmethylamino)thiazole, UCL 2077: 3-(triphenylmethylaminomethyl)pyridine, UCL 2370: 5-amino-2-(triphenylmethylamino)pyridine.

TRAM 34, verapamil, Bay K8644 and betaxolol HCl were from Tocris Bioscience. (–)-Propranolol and (±)-isoproterenol were from Sigma. XE991 HCl and H89 HCl were from Abcam. Verapamil, betaxolol, and XE991 were dissolved in normal saline for DH infusion. The other compounds were dissolved in dimethyl formamide (DMF) and diluted into saline to give final concentrations that ranged from 50 to 100% DMF, depending on solubility. The highest percentage DMF used for each compound was also used as vehicle control. For central restoration of NE in *Dbh*^{-/-} mice, 1 g/kg L-DOPS (Dainippon Sumitomo Pharma) was

given together with 50 mg/kg benserazide (a peripheral aromatic L-amino acid decarboxylase inhibitor, Sigma) subcutaneously 5 h before training or euthanasia in a volume of 50 μ l/g (Thomas et al., 1998; Murchison et al., 2004). The 5 h lead results in near normal tissue levels of NE centrally. For slices, L-DOPS and H89 were dissolved in aCSF. The concentrations used for H89 was lower than that used previously to study the sAHP in CA1 (Oh et al., 2009).

CNS infusion. A double-guide cannula (C235 system, Plastics One) was implanted under pentobarbital anesthesia (72.5 mg/kg, i.p.) using a stereotax (SAS75/EM40M, Cartesian Research). The guide was placed 1.7 mm posterior to bregma and 1.5 mm bilateral for DH infusions. The guide projected 1.5 mm below the base and the dummy cannula extended 0.5 mm below the guide. One week after surgery, bilateral infusions were made into conscious mice while gently holding the nape of the neck. The dual-injection cannula extended 0.9 mm below the guide. Infusions were 0.4 μ l/min and the injection cannulae were left in place for 30 s before the mouse was returned to its home cage. Infusion volumes were 0.5 μ l/DH except for verapamil and XE991, which were 1 μ l/DH, and Bay K8644, which was 0.25 μ l/DH. Infusions were performed 30 min before testing *Dbh*^{-/-} and β_1 KO mice and 15 min before testing *Dbh*^{+/-} and WT mice. For *Dbh*^{-/-} mice, vehicle infusions 15 min before testing alter baseline freezing and so this time could not be used. Infusion location was assessed by infusing 1 μ l of 1% methylene blue after behavioral testing. Methylene blue was observed in the center of the DH in all cases, with spread reaching each hippocampal subfield but not outside the DH, except for some dye in the cannula track, as previously shown (Murchison et al., 2004). Thus, all animals were included in the current study unless blockage of the injection cannula was observed immediately after drug infusion.

Electrophysiology. Mice were killed by decapitation in the absence or presence (when noted) of anesthesia (intraperitoneal pentobarbital 15 min beforehand). The brain was rapidly removed and placed into ice-cold sucrose-aCSF (in mM: 220 sucrose, 3 KCl, 1.25 NaH₂PO₄, 1.2 MgSO₄, 26 NaHCO₃, 2 CaCl₂, and 10 dextrose, pH 7.3, bubbled with 95%/5% O₂/CO₂). Transverse slices (400 μ m) from the DH were cut (Vibroslicer, World Precision Instruments) and transferred to a holding chamber (BSC-PC, Harvard Apparatus) containing oxygenated aCSF (124 mM NaCl replacing sucrose). Slices were incubated at 35°C for 30 min and at room temperature for 1 h before the start of recording. Drugs were applied at the concentrations indicated starting at this time and their application continued during recording. For slices from mice treated with pentobarbital, the aCSF was changed once in the holding chamber at 30 min to remove any remaining pentobarbital. Individual slices were transferred to an interface-recording chamber (BSC-BU, Harvard Apparatus) at 30°C and continuously perfused (1–2 ml/min). Recording was performed using a CyberAmp preamplifier and an Axoclamp 2B amplifier (Molecular Devices) in bridge mode. Intracellular recordings were obtained from CA1 pyramidal neurons using 70–100 M Ω glass microelectrodes filled with 3 M K-acetate. Data were digitized at a sampling rate of 1–10 kHz, acquired and analyzed using pCLAMP 10 (Molecular Devices). Only putative CA1 pyramidal neurons with the following properties were used: minimal spontaneous activity, action potential amplitude $\geq$ 80 mV from threshold and duration $\geq$ 1.2 ms from threshold to recrossing of the membrane potential before the stimulus, input resistance $\geq$ 25 M Ω and a stable resting membrane potential negative to –60 mV. Cells were studied at a membrane potential of –65 mV ($\geq$ 0.2 nA constant current injection) to minimize variability caused by the influence of voltage-dependent changes on membrane conductance.

Neurons that were stable for at least 5 min after impalement were studied under current-clamp using the following protocol: (1) Current–voltage (*I*–*V*) relations were studied using 400 ms current injections (range –1.0 to +0.2 nA). Input resistance was determined by measuring the plateau voltage deflection (last 75 ms of the 400 ms pulse) using a –0.2 nA hyperpolarizing current injection. (2) The AHP was studied using a 100 ms depolarizing current injection sufficient to elicit reliably a burst of four action potentials. The peak of the fast AHP was measured as the most negative membrane potential between the first and second action potentials. The peak postburst AHP amplitude was calculated as the maximum negative voltage deflection (relative to prepulse baseline po-

tential) during the first 250 ms after current offset. A total of five AHP measurements (20 s intervals) were made from each cell. (3) Spike-frequency adaptation (accommodation) was studied using an 800 ms depolarizing current injection of the same stimulus intensity used to study the AHP. Three samples were taken per cell, at 20 s intervals, and the number of action potentials elicited was noted.

Statistics. Data were analyzed with Statistica 9.1 (StatSoft) using one-way or two-way ANOVA with $\alpha = 0.05$. For day 1–day 2 retrieval experiments, two-way ANOVA with repeated measures was used. The Bartlett chi-square test was used to analyze homogeneity of variances. *Post hoc* comparisons were made using Duncan's range test. Data are presented as mean $\pm$ SE. For all figures, * indicates $p < 0.05$, ^ indicates $p < 0.01$, and # indicates $p < 0.001$ for *post hoc* comparisons to the reference group or another group if indicated.

Results

Blockers of the sAHP rescue memory retrieval in *Dbh*^{−/−} and β_1 KO mice

Blockers of the sAHP in the hippocampus were developed based on the original observation that the antimycotic drug clotrimazole blocks a related intermediate conductance Ca^{2+} -activated K^+ current in the periphery (Alvarez et al., 1992; Vandompe et al., 1998). Because clotrimazole can have effects on other K^+ and Ca^{2+} currents, derivatives of clotrimazole were tested for their efficacy in selectively blocking the sAHP over other K^+ and Ca^{2+} currents. Several compounds were identified that block the sAHP, with the most potent and selective compound initially being UCL 2027, which was eventually supplanted by UCL 2077 (Shah et al., 2001; Shah et al., 2006). As an example of their selectivity, concentrations of UCL 2027 and 2077 that block the sAHP have no effect on the L-type Ca^{2+} current that is necessary for activation of the sAHP.

For this study, experiments were initiated using UCL 2027, before the development of UCL 2077. With this compound, we set out to test the hypothesis that NE/E-deficient *Dbh*^{−/−} mice exhibit impaired hippocampus-dependent memory because they fail to reduce the sAHP during retrieval testing. This hypothesis predicts that treatment with UCL 2027 shortly before testing will rescue retrieval in these mice. The parameters for this and subsequent experiments are summarized in Figure 1. To test the hypothesis, UCL 2027 or vehicle was infused into the DH 30 min before testing contextual fear memory in *Dbh*^{−/−} mice 1 d after training. An enhancement in freezing was observed that reached normal levels at 5 $\mu\text{g}/\text{DH}$ (Fig. 2A).

To determine the nature of this enhancement in freezing, UCL 2027 (5 $\mu\text{g}/\text{DH}$) or vehicle was infused 30 min before testing DH-independent cued fear memory 1 d after training. Moderate shock intensity was used during conditioning to mimic the level of contextual freezing observed in vehicle-treated *Dbh*^{−/−} mice. No effect of UCL 2027 treatment was observed, indicating that it does not enhance fear or freezing per se (Fig. 2B). To distinguish between effects of UCL 2027 on retrieval from those on a later stage of memory consolidation, another cohort of mice was tested for retrieval after treatment 1 d after training, and then retested the next day in the absence of further treatment. The effects of UCL 2027 on *Dbh*^{+/−} mice that have normal tissue levels of NE/E were examined for comparison (Thomas et al., 1998). UCL 2027 rescued freezing in the *Dbh*^{−/−} mice on day 1, and freezing was significantly lower for these mice on day 2 in the absence of further treatment (Fig. 2C). UCL 2027 did not alter freezing relative to vehicle on either day in *Dbh*^{+/−} mice. The data demonstrate that the effects of UCL 2027 are transient, suggesting that it rescues retrieval rather than a later stage of consolidation.

Figure 1. Parameters for training and testing. There were three types of training regimens that differed by the application of footshock (triangle). The thick black boxes represent activation of the tone, and timelines are in minutes. There were also three types of testing regimens. Contextual testing was performed in the training apparatus, whereas cued testing was performed in a distinct, novel apparatus using the training tone. For electrophysiology, brain slices were prepared in the absence of behavioral testing.

To determine whether the rescue of retrieval by UCL 2027 is likely to be through reduction of the sAHP, several other derivatives of clotrimazole with varying potencies for blocking the sAHP were examined. As mentioned, UCL 2077 is more potent in blocking the sAHP than is UCL 2027, and UCL 2077 was also more potent in rescuing memory retrieval in the *Dbh*^{−/−} mice (Fig. 2D). UCL 1851 and 2370 also block the sAHP and rescued retrieval, but less potently than UCL 2027. In contrast, UCL 1864, which blocks the sAHP poorly, did not rescue memory retrieval. The previously determined 50% inhibitory concentration (IC_{50}) of these compounds for blocking the sAHP (Zunszain et al., 2002; Shah et al., 2006) correlates well with the estimated 50% effective dose (ED_{50}) for rescuing memory retrieval (Table 1). Further, another clotrimazole derivative, TRAM-34, which blocks IK1 Ca^{2+} -activated K^+ channels that do not contribute to the sAHP in the hippocampus (Wulff et al., 2000), also did not rescue retrieval when infused into the DH of *Dbh*^{−/−} mice (Fig. 2D).

As mentioned above, NE promotes retrieval by signaling through the β_1 -adrenergic receptor (Murchison et al., 2004). If the sAHP is indeed a critical target of β_1 signaling in retrieval, then UCL 2077 should also rescue retrieval in the specific absence of β_1 signaling (compared with the absence of all adrenergic signaling in *Dbh*^{−/−} mice). In support of this, UCL 2077 rescued the memory retrieval deficit present in β_1 KO mice (Fig. 2E). Demonstrating further spec-

Figure 2. Blockers of the slow afterhyperpolarization (sAHP) rescue memory retrieval in NE/E-deficient *Dbh*^{-/-} and β_1 KO mice. **A**, The sAHP blocker UCL 2077 was infused into the DH 30 min before testing DH-dependent contextual fear memory in *Dbh*^{-/-} mice 1 d after conditioning with 1 mA. The percentage time spent freezing was used as an indicator of fear memory. UCL 2077 at 5 μg/DH fully rescued contextual freezing behavior. $p = 0.0009$ for the main effect of dose ($n = 5$ /group). **B**, UCL 2077 was infused into the DH 30 min before testing DH-independent cued fear memory in *Dbh*^{-/-} mice 1 d after conditioning with 0.4 mA (to approximate the low level of contextual freezing observed in vehicle-treated *Dbh*^{-/-} mice). UCL 2077 at 5 μg/DH had no effect on cued freezing, indicating that it does not enhance fear or freezing per se ($p = 0.98$, $n = 5$ /group). **C**, Vehicle (Veh) or UCL 2077 (UCL) was infused into the DH 30 min before testing contextual fear memory 1 d after conditioning. Testing was for 3 min to minimize extinction. Testing (3 min) was performed again the next day in the absence of further treatment. Restoration of freezing by UCL 2077 was only apparent on day 1 during treatment, suggesting that it has an effect on retrieval rather than consolidation. $p = 0.14$ for the main effect of group; $p = 0.006$ for the main effect of day; and $p = 0.0014$ for the interaction of group and day ($n = 5$ /group). **D**, Other clotrimazole derivatives with varying potencies at blocking the sAHP (see Table 1) were infused into the DH 30 min before testing contextual fear memory 1 d after conditioning. TRAM-34 (TRAM) is a derivative that blocks the peripheral intermediate conductance Ca^{2+} -activated K^+ channel (IK1). $p < 0.0001$ for the main effect of treatment ($n = 5$ /group). **E**, UCL 2077 (2 μg/DH) was infused 30 min before testing contextual fear memory in β_1 KO mice 1 d after conditioning. UCL 2077 fully rescued contextual freezing ($p = 0.0002$, $n = 5$ /group). **F**, UCL 2077 (2 μg/DH) was infused 30 min before testing contextual fear memory in β_1 KO mice 1 d after pseudo-conditioning (Pseudo). UCL 2077 was without effect ($p = 0.71$, $n = 5$ /group). **G**, XE991 was infused 30 min before testing contextual fear memory in β_1 KO mice 1 d after conditioning. XE991 partially rescued contextual freezing at 1 μg/DH ($p = 0.0004$ for the main effect of dose, $n = 5, 5, 8, 5$ /group). $^*p < 0.05$, $^{\wedge}p < 0.01$, $^{\#}p < 0.001$ for post hoc comparisons, for all figures.

ificity for the effect of UCL 2077 on retrieval, UCL 2077 did not alter freezing in mice pseudoconditioned with the normal shock intensity of 1 mA (Fig. 2F).

Finally, it was recently reported that UCL 2077 can block KCNQ1 and KCNQ2-containing K^+ channels that might contribute to

the medium AHP but not to the sAHP in CA1 pyramidal neurons (Soh and Tzingounis, 2010). To determine whether rescue of retrieval by UCL 2077 might be due to blockade of KCNQ channels, the KCNQ channel blocker XE991 was infused into the DH before testing retrieval in β_1 KO mice (Zaczek et al., 1998). In contrast to UCL 2077, XE991 induced a partial rescue of retrieval with an inverted-U dose-response function (Fig. 2G).

Modulation of L-type voltage-dependent Ca^{2+} channels also affects retrieval

A major source of Ca^{2+} for the activation of the sAHP in the hippocampus is influx through L-type voltage-dependent Ca^{2+} channels (Moyer et al., 1992; Marrion and Tavalin, 1998; Lima and Marrion, 2007). Therefore, we asked whether blocking this influx of Ca^{2+} in the DH would also rescue memory retrieval in *Dbh*^{-/-} mice. Mice were infused with the L-type Ca^{2+} channel blocker verapamil 30 min before testing contextual fear memory 1 d after training. An enhancement in freezing that reached normal levels at 0.5 μg/DH was observed (Fig. 3A). Verapamil (0.5 μg/DH) was also infused 30 min before testing cued fear memory 1 d after training with a moderate shock intensity. No effect of treatment was observed, indicating that verapamil does not enhance fear or freezing per se (Fig. 3B).

To distinguish between effects of verapamil on memory consolidation and retrieval, another cohort of mice was tested for retrieval following treatment 1 d after training, and then retested the next day in the absence of further treatment. Verapamil rescued freezing in the *Dbh*^{-/-} mice on day 1; however, freezing was low for these mice on day 2 in the absence of further treatment, suggesting that verapamil rescues retrieval rather than consolidation (Fig. 3C). Finally, similar to UCL 2077, verapamil also rescues retrieval in β_1 KO mice (Fig. 3D). Demonstrating further specificity for the effect of verapamil on retrieval, verapamil did not alter freezing in mice pseudoconditioned with the normal shock intensity of 1 mA (Fig. 3E).

Although verapamil inhibits L-type Ca^{2+} channel activity, other compounds, such as Bay K8644, potentiate the activity of L-type Ca^{2+} channels, and secondarily the AHP (Marrion and Tavalin, 1998). Therefore, we asked whether potentiating L-type Ca^{2+} channel activity in the DH would impair retrieval in *Dbh*^{+/-} mice. Mice were infused with Bay K8644 (BayK) 15 min before testing contextual fear memory 1 d after training, and a significant impairment of freezing at 2

$\mu\text{g/DH}$ was observed (Fig. 4A). Mice were also infused with 2 $\mu\text{g/DH}$ BayK 15 min before testing cued fear memory 1 d after training. No impairment was observed, indicating that fear or freezing per se was not affected (Fig. 4B). To distinguish between effects of BayK on consolidation versus retrieval, BayK or vehicle was infused 15 min before testing contextual fear memory 1 d after training, and the mice were tested again the next day in the absence of treatment. BayK reduced freezing on day 1 but had no effect on day 2 freezing, indicating a transient effect on retrieval rather than a lasting effect on consolidation (Fig. 4C). Finally, we asked whether blocking the sAHP would rescue the impairment in retrieval induced by BayK, and found that retrieval was normal when BayK and UCL 2077 were coinfused (Fig. 4D).

If the sAHP is a target of NE in promoting memory retrieval, then it is predicted that the impairing effects of BayK on retrieval will be occluded in NE/E-deficient *Dbh*^{-/-} mice. Indeed, infusion of BayK before testing retrieval in these mice had no additional effect on retrieval (Fig. 4E). Further, NE, β_1 , cAMP, and PKA signaling all have time-limited roles in hippocampus-dependent memory retrieval (Murchison et al., 2004; Ouyang et al., 2008). For example, *Dbh*^{-/-} mice display deficits in contextual memory 2 h to 4 d but not at 1 h or ≥ 5 d after training, and mice and rats treated with a β antagonist or PKA inhibitor show compromised contextual memory over a similar time period. If the sAHP is a target of this initial signaling pathway, then enhancement of L-type Ca^{2+} channel activity might impair retrieval over a similar time course. To test this possibility, *Dbh*^{+/-} mice were infused with BayK 15 min before testing contextual memory 1–5 d after training. BayK impaired retrieval for the first 4 d after training, but had no effect 5 d after training (Fig. 4F).

NE is required for the reduction in the sAHP observed *ex vivo* after conditioning

Considerable evidence indicates that changes in intrinsic neuronal excitability occur after learning (Disterhoft and Oh, 2006; Saar and Barkai, 2009; Mozzachiodi and Byrne, 2010). In vertebrates this was first demonstrated with the observation that the sAHP is reduced in pyramidal neurons of hippocampal slices from eye blink-conditioned rabbits (Disterhoft et al., 1986). Interestingly, this reduction in the sAHP lasts for several days, after which the sAHP returns to preconditioned levels (Moyer et al., 1996; Saar et al., 1998). This time course parallels the time course over which NE is required for hippocampus-dependent memory retrieval. Thus, it is possible that NE has a role in the establishment or maintenance of the sAHP reduction observed *ex vivo*. To test this possibility, we asked whether sAHP reduction could be observed in hippocampal slices after single-trial contextual fear conditioning, and if so, whether the reduction depends on NE.

Mice were fear conditioned in an identical manner to that used for behavioral analysis. One day after conditioning, brain slices were prepared. To control for the experience of conditioning in the absence of learning, pseudo-conditioned mice that

Table 1. Comparison of *in vitro* and *in vivo* potencies for the UCL compounds

	UCL compounds				
	2077	2027	1851	2370	1864
IC ₅₀ (μM)	0.5	1.1	2.2	>3	>3
ED ₅₀ ($\mu\text{g/DH}$)	0.5	1.5	2	10	>10

IC₅₀ is for block of the sAHP in cultured rat hippocampal pyramidal neurons (Zunszain et al., 2002; Shah et al., 2006). ED₅₀ is for rescue of memory retrieval in *Dbh*^{-/-} mice (Fig. 1).

Figure 3. Blockade of L-type voltage-dependent Ca^{2+} channels rescues memory retrieval in *Dbh*^{-/-} and β_1 KO mice. **A**, The L-type voltage-dependent Ca^{2+} channel blocker verapamil was infused into the DH 30 min before testing contextual fear memory in *Dbh*^{-/-} mice 1 d after conditioning. Verapamil at 0.5 $\mu\text{g/DH}$ rescued contextual freezing. $p = 0.0004$ for the main effect of dose ($n = 5/\text{group}$). **B**, Verapamil (Ver) was infused into the DH 30 min before testing cued fear memory in *Dbh*^{-/-} mice 1 d after conditioning with 0.4 mA. Verapamil at 0.5 $\mu\text{g/DH}$ had no effect on cued freezing, indicating that it does not enhance fear or freezing per se ($p = 0.93$, $n = 5/\text{group}$). **C**, Verapamil (0.5 $\mu\text{g/DH}$) was infused into the DH 30 min before testing contextual fear memory 1 d after conditioning. Testing was for 3 min to minimize extinction. Testing (3 min) was performed again the next day in the absence of further treatment. Restoration of freezing by verapamil was only apparent on day 1, suggesting that verapamil has an effect on retrieval rather than consolidation. $p = 0.056$ for the main effect of group; $p = 0.0037$ for the main effect of day; and $p = 0.026$ for the interaction of group and day ($n = 6/\text{group}$). **D**, Verapamil (0.5 $\mu\text{g/DH}$) was infused 30 min before testing contextual fear memory in β_1 KO mice 1 d after conditioning, rescuing contextual freezing ($p = 0.0025$, $n = 5/\text{group}$). **E**, Verapamil (0.5 $\mu\text{g/DH}$) was infused 30 min before testing contextual fear memory in β_1 KO mice 1 d after pseudo-conditioning (Pseudo). Verapamil was without effect ($p = 0.31$, $n = 5/\text{group}$).

received footshock immediately after being placed in the training apparatus were used. Electrophysiologic properties of CA1 pyramidal neurons were recorded intracellularly in current-clamp mode using glass microelectrodes. There were no significant differences in resting membrane potential, input resistance, or action potential threshold, height or width between neurons from naive, pseudo-conditioned, and conditioned mice (Table 2). In contrast, there were significant differences in several parameters related to the AHP. In particular, the peak of the AHP and the magnitude of the AHP at 1 s (a measure of the sAHP) were both significantly reduced in the conditioned group relative to the pseudo-conditioned and naive groups (Fig. 5A–D). In addition, the number of action potentials elicited during 0.8 s of depolarization was significantly increased relative to the two control groups (Fig. 5E). These findings are similar to those reported for CA1 pyramidal neurons using multi-trial conditioning paradigms (Moyer et al., 1996; Oh et al., 2003; McKay et al., 2009). In contrast, the fast AHP did not differ between naive and conditioned groups, as assessed by both the width of the action potential (Table 2) and the peak negative membrane potential of the fast AHP (naive -58.0 ± 0.5 mV, $n = 27$; conditioned -57.9 ± 0.6 mV, $n = 25$; $p = 0.8$).

The peak of the postburst AHP can reflect contributions from both the medium AHP and the sAHP. Because of this mixed contribution and the smaller effect of conditioning on this parameter, we focused on the magnitude of the sAHP and the number of action potentials generated after depolarization in subsequent experiments. Because studies indicate that reductions in the sAHP and in accommodation after conditioning can be transient (Moyer et al., 1996; Saar et al., 1998), we also examined these parameters at 2 d and at 5 d after single-trial context conditioning. The magnitude of the sAHP was reduced at 2 d but not

Figure 4. Enhancement of L-type voltage-dependent Ca^{2+} channels impairs memory retrieval in WT mice. **A**, The L-type voltage-dependent Ca^{2+} channel enhancer Bay K8644 (BayK) was infused into the DH 15 min before testing contextual fear memory in *Dbh*^{+/-} mice (which have normal levels of NE/E) 1 d after conditioning. BayK at 2 $\mu\text{g}/\text{DH}$ impaired contextual freezing. $p = 0.0006$ for the main effect of dose ($n = 5/\text{group}$). **B**, BayK was infused into the DH 15 min before testing cued fear memory 1 d after conditioning. BayK at 2 $\mu\text{g}/\text{DH}$ had no effect on cued freezing, indicating that it does not impair fear or freezing per se ($p = 0.74$, $n = 5/\text{group}$). **C**, Vehicle (Veh) or BayK (2 $\mu\text{g}/\text{DH}$) was infused into the DH 15 min before testing contextual fear memory 1 d after conditioning. Testing was for 3 min to minimize extinction. Testing (3 min) was performed again the next day in the absence of further treatment. Impairment of freezing by BayK was only apparent on day 1, suggesting that BayK has an effect on retrieval rather than consolidation. $p = 0.001$ for the main effect of group; $p = 0.002$ for the main effect of day; and $p = 0.0001$ for the interaction of group and day ($n = 5/\text{group}$). **D**, BayK (2 $\mu\text{g}/\text{DH}$) alone or with UCL 2077 was infused into the DH 15 min before testing contextual fear memory. UCL 2077 completely mitigated the retrieval deficit induced by BayK ($n = 5/\text{group}$). **E**, BayK was infused 30 min before testing contextual fear memory in *Dbh*^{-/-} mice 1 d after conditioning. No further impairment in contextual freezing was observed ($p = 0.84$, $n = 5/\text{group}$). **F**, BayK was infused into the DH 15 min before testing contextual fear memory. Impairment of retrieval was observed during the first 4 d after conditioning. Separate subjects were tested on each day. $p < 0.0001$ for the main effect of day, the main effect of treatment, and the interaction of day and treatment ($n = 5/\text{group}$).

Table 2. Electrophysiologic properties of CA1 pyramidal neurons

	V_{rest} (mV)	R_{input} (M Ω)	$V_{\text{threshold}}$ (mV)	Height _{AP} (mV)	Width _{AP} (ms)
Naive <i>Dbh</i> ^{+/-} (27)	-65.5 ± 1.6	35.5 ± 3.9	-55.1 ± 1.0	92.2 ± 2.5	2.14 ± 0.09
Pseudo <i>Dbh</i> ^{+/-} (22)	-65.3 ± 2.6	33.3 ± 4.3	-55.1 ± 1.0	90.8 ± 3.7	2.17 ± 0.06
Cond. <i>Dbh</i> ^{+/-} (25)	-66.7 ± 2.2	38.5 ± 5.7	-55.4 ± 1.1	89.7 ± 3.5	2.13 ± 0.12
Naive <i>Dbh</i> ^{-/-} (24)	-66.9 ± 2.2	37.2 ± 4.8	-55.2 ± 0.9	89.7 ± 3.1	2.12 ± 0.19
Cond. <i>Dbh</i> ^{-/-} (23)	-68.1 ± 2.7	35.0 ± 4.1	-55.0 ± 0.9	87.1 ± 2.7	2.15 ± 0.11
ANOVA p value	0.91	0.95	1.0	0.83	1.0

V_{rest} is the resting membrane potential, R_{input} is the input resistance at -65 mV, $V_{\text{threshold}}$ is the threshold for action potential (AP) generation, Height_{AP} is the height of the AP from threshold, and Width_{AP} is the width of the AP at threshold.

at 5 d after conditioning, whereas the number of action potentials showed a trend toward an increase at 2 d ($p = 0.06$ compared with naive and pseudo-conditioned) but not at 5 d after conditioning (Fig. 5C–E).

Because the role for NE in retrieval lasts for several days after conditioning, but is no longer required 5 d after conditioning, we asked whether NE has a role in establishing the reduction in the sAHP observed *ex vivo* after conditioning. *Dbh*^{-/-} mice were

handled and conditioned in an identical manner to that for the *Dbh*^{+/-} mice. For all parameters examined, there were no significant differences between slices from naive *Dbh*^{+/-} and *Dbh*^{-/-} mice (Table 2; Fig. 6A,B). Importantly, there were no significant differences between conditioned and naive *Dbh*^{-/-} mice, in contrast to what was observed for *Dbh*^{+/-} mice, suggesting that NE plays a role in the reduction in the sAHP observed *ex vivo*.

β_1 signaling during decapitation establishes the reduction in the sAHP observed *ex vivo* after conditioning

Based on the longstanding notion that NE is important for memory consolidation (McGaugh, 2000), it would be congruent to hypothesize that NE is required during or shortly after conditioning for the establishment of the reduced sAHP. To test this possibility, NE was restored in *Dbh*^{-/-} mice during acquisition and the initial consolidation period (i.e., for ~24 h) by injecting the NE precursor L-DOPS before conditioning (Thomas et al., 1998; Murchison et al., 2004). Brain slices were prepared 2 d after conditioning so that NE would no longer be present during recording. Interestingly, this treatment did not result in a reduction in the sAHP after conditioning. Therefore, we treated *Dbh*^{-/-} mice with L-DOPS 2 d after conditioning, shortly before slices were generated, using the same regimen that results in the rescue of memory retrieval in *Dbh*^{-/-} mice (Murchison et al., 2004). This treatment resulted in a significant reduction in the sAHP, as well as a tendency to increase in the number of action potentials during depolarization, equivalent to what was observed in slices from conditioned *Dbh*^{+/-} mice 2 d after conditioning (Figs. 5D,E, 6A,B).

Based on the results above, we considered the possibility that ongoing adrenergic signaling in the slice might be necessary to observe the sAHP reduction after conditioning. Two experiments were performed to test this possibility. First, slices from conditioned *Dbh*^{-/-} mice were incubated with L-DOPS for 2 h before recording. The highest concentration of L-DOPS that did not alter the sAHP in naive WT slices was used to restore NE in conditioned *Dbh*^{-/-} slices. Because aromatic L-amino acid decarboxylase, which converts L-DOPS to NE, resides in catecholaminergic terminals, this treatment is expected to restore vesicular NE content without directly affecting extracellular levels of NE. L-DOPS treatment did not lead to reduction of the sAHP, which might have been expected if there were ongoing release of NE-containing vesicles in the conditioned slices (Fig. 6C,D). Second, slices from conditioned *Dbh*^{+/-} mice were incubated with the β blocker propranolol before and during recording. The concentration of propranolol used (10 μM) was sufficient to completely block the effects of the β agonist isoproterenol (1 μM) on the sAHP in naive *Dbh*^{+/-} slices. This treat-

ment did not prevent observation of a reduced sAHP in the conditioned *Dbh*^{+/-} slices (Fig. 6C,D).

Finally, we considered the possibility that the reduction in the sAHP observed *ex vivo* may be due to release of NE during decapitation. To test this, our approach was to administer an adrenergic receptor antagonist shortly before decapitation to determine whether it would block the reduction in the sAHP. We considered that the β_1 receptor was likely to be the most relevant with respect to sAHP reduction. To confirm this, recordings in slices from naive and conditioned β_1 KO mice were made. Similar to the results for slices from conditioned *Dbh*^{-/-} mice, slices from conditioned β_1 KO mice did not exhibit a reduced sAHP (Fig. 7A,B). Based on this, we treated conditioned WT mice with the β_1 antagonist betaxolol before decapitation. We used a dose of betaxolol (1 mg/kg) that mimics the impairment of contextual memory retrieval observed in β_1 KO mice (Murchison et al., 2004). Betaxolol treatment before decapitation blocked the reduction of the sAHP that is observed after conditioning (Fig. 7A,B). To examine whether betaxolol might have nonspecific effects on the sAHP *ex vivo*, slices from pseudo-conditioned mice injected with vehicle or betaxolol before decapitation were compared. In this case, no effect of betaxolol was observed (Fig. 7A,B).

The *ex vivo* reduction of the sAHP depends on the level of anesthesia

The observation that betaxolol blocks the *ex vivo* reduction in the sAHP after conditioning is consistent with the idea that the combination of conditioning and a surge of NE release during decapitation results in the sAHP being reduced *ex vivo* (Greene et al., 1988). Up to this point, our results were obtained from mice decapitated without anesthesia. However, others have reported that the sAHP is reduced after conditioning when anesthesia is administered before decapitation (Moyer et al., 1996; Saar et al., 1998; Oh et al., 2003; McKay et al., 2009). Those observations are of interest because we predicted that sufficient anesthesia before killing would prevent the surge in NE release and the *ex vivo* reduction in the sAHP. To examine the effects of anesthesia on the *ex vivo* reduction in the sAHP, mice were decapitated 15 min after administering one of several doses of pentobarbital 1 d after conditioning. The lowest dose of pentobarbital is comparable to what others have used when examining reductions in the sAHP (Schreurs et al., 1998; Seroussi et al., 2002). This dose did not significantly alter the reduction in the sAHP or accommodation (Fig. 7C,D). In contrast, two higher doses of pentobarbital resulted in a partial and a complete block of the reduction in the sAHP and accommodation. Treatment of naive mice with the highest dose of pentobarbital did not significantly alter the sAHP or accommodation (Fig. 7C,D).

Another difference between our study and the conditioning paradigms used in other studies is the number of training trials. For contextual fear conditioning, three conditioning trials over one and a half days were used in conjunction with anesthesia

Figure 5. Single-trial fear conditioning results in enhanced intrinsic excitability of CA1 pyramidal neurons. **A, C**, The peak amplitude of the AHP is reduced 1–2 d (Cond D1, Cond D2) but not 5 d (Cond D5) after conditioning relative to naive, context-exposed (no shock = Context) and pseudo-conditioned (immediate shock = Pseudo) groups. $p = 0.001$ for the main effect of conditioning (for C–E, $n = 27, 22, 22, 25, 21, 23$ /group). **A, D**, The amplitude of the AHP at 1 s (defined as the sAHP) is reduced 1–2 d but not 5 d after conditioning relative to naive, context-exposed and pseudo-conditioned groups. $p = 0.001$ for the main effect of conditioning. **B, E**, The number of action potentials (AP) elicited during a 0.8 s depolarization step is reduced 1 d after conditioning relative to naive, context-exposed, and pseudo-conditioned groups. $p = 0.0033$ for the main effect of conditioning.

before decapitation (McKay et al., 2009). To examine whether the effect of anesthesia depends on the number of training trials, we also fear conditioned mice using three trials over one and a half days, and prepared slices the day after the third training trial. As expected, multiple spaced training trials resulted in a reduction in the sAHP and in accommodation (Fig. 7E,F). In contrast, the highest dose of pentobarbital completely prevented these reductions.

Persistent PKA activity is induced by β_1 signaling during decapitation of conditioned animals

The surge in NE release that accompanies decapitation should occur regardless of conditioning status (Greene et al., 1988). Yet reductions in the sAHP are observed in slices from conditioned mice relative to naive mice. One possibility is that these reductions occur in both groups, but are either greater or more persistent in the conditioned group. This implies that there is something unique to slices from conditioned mice that permits the greater or more persistent reduction in the sAHP. Indeed, signaling by extracellular signal-regulated kinase appears to maintain the reduced sAHP observed *ex vivo* in the piriform cortex after odor discrimination learning (Cohen-Matsliah et al., 2007).

For our study, we considered whether persistent PKA activity might be responsible for the reduced sAHP *ex vivo* because sAHP reduction by the β agonist isoproterenol is occluded in CA1 from trace eyeblink-conditioned rats, and the effect of isoproterenol in CA1 from naive animals is occluded by the PKA inhibitor H89 (Oh et al., 2009). Thus, we asked whether slices from fear-conditioned mice would exhibit reductions in the sAHP and in accommodation after inhibition of PKA. Incubation of these slices with H89 resulted in a significant increase in the sAHP and in accommodation, such that they were not significantly different from those observed in slices from naive mice (Fig. 8). In con-

Figure 6. NE is required for conditioning-induced reduction of the sAHP. **A**, Relative to the sAHP from naive *Dbh*^{+/+} mice, the sAHP from naive or conditioned (Cond D1) *Dbh*^{-/-} mice is not significantly altered. To examine when NE might act to reduce the sAHP, NE was restored *in vivo* in *Dbh*^{-/-} mice by administering the precursor L-DOPS (L) either before conditioning (L-Cond D2) or before slice preparation (Cond D2-L). Slices were prepared 2 d after conditioning because some NE remains at 1 d but not 2 d after treatment with L-DOPS (Thomas et al., 1998). Restoration of NE during acquisition and initial consolidation did not result in sAHP reduction. In contrast, restoration of NE before slice generation reduced the sAHP. $p = 0.027$ for the main effect of conditioning (for *A* and *B*, $n = 27, 24, 23, 22, 24$ /group). **B**, Relative to the number of APs from naive *Dbh*^{+/+} mice, the number of APs from naive or conditioned (Cond D1) *Dbh*^{-/-} mice is not significantly altered. Restoration of NE during acquisition and initial consolidation did not increase the number of APs. In contrast, restoration of NE before slice generation tended to increase the number of APs, similar to what was observed in slices from WT mice 2 d after conditioning (Fig. 5C). $p = 0.28$ for the main effect of conditioning. **C, D**, To test whether constitutive adrenergic signaling in the slice is responsible for the conditioning-induced reduction in the sAHP, slices were treated in one of several ways. First, slices from *Dbh*^{-/-} mice conditioned 1 d earlier were incubated with vehicle or L-DOPS (20 μ g/ml). No significant difference in the sAHP or the number of APs was noted ($p > 0.3$, $n = 23, 20$ /group). Second, slices from WT mice conditioned 1 d earlier were incubated with either vehicle, the β blocker (–)–propranolol (Prop, 10 μ M), or the β_1 blocker betaxolol (10 μ M). There were no significant differences in the sAHP ($p = 0.95$ for the main effect of treatment) or the number of APs ($p = 0.53$ for the main effect of treatment, $n = 25, 21, 10$ /group).

trast, incubation of slices from naive mice with H89 had no effect on the sAHP or accommodation. Importantly, H89 also had no effect on the sAHP or accommodation in slices from conditioned mice treated with betaxolol shortly before decapitation. Together, these observations suggest that persistent PKA signaling is engaged selectively in conditioned animals as a result of β_1 receptor activation during euthanasia.

Discussion

Results from the current study provide substantial support for the idea that reduction of the sAHP is a critical event that mediates the role of NE and β_1 signaling in promoting hippocampus-dependent memory retrieval. Several of the UCL compounds studied here block the sAHP in CA1 pyramidal neurons *ex vivo* (Shah et al., 2001; Zunszain et al., 2002; Shah et al., 2006), and these same compounds rescue retrieval in NE/E-deficient *Dbh*^{-/-} mice. Further, the orders of potency for blocking the

sAHP and for rescuing retrieval correlate well. Of note, the KCNQ K⁺ channel blocker XE991 partially mimics the rescue of retrieval by UCL 2077. This is not surprising because KCNQ channels contribute to the medium AHP in CA1 pyramidal neurons (Gu et al., 2005; Gu et al., 2008). However, in contrast to the reduction of the sAHP by NE, KCNQ channel activity is enhanced by NE/ β_1 /cAMP/PKA signaling (Schroeder et al., 1998; Marx et al., 2002), suggesting that KCNQ channels may not be the relevant effector of NE in retrieval.

Because evidence indicates that influx of Ca²⁺ through L-type voltage-dependent Ca²⁺ channels is required for the activation of the sAHP, additional experiments examined the effects of modulating this Ca²⁺ influx on memory retrieval. The results are consistent with the sAHP being a target of NE in promoting retrieval. L-type Ca²⁺ channel blockers reduce the sAHP *in vitro* (Moyer et al., 1992; Marrion and Tavalin, 1998; Lima and Marrion, 2007), and the L-type blocker examined behaviorally (verapamil) rescues retrieval in the *Dbh*^{-/-} mice. Complementing these observations, the L-type Ca²⁺ channel enhancer Bay K8644 impairs retrieval in WT/*Dbh*^{+/+} mice. Further, concomitant treatment with UCL 2077 prevents the impairment induced by BayK, suggesting that regulation of the sAHP is the mechanism through which modulation of L-type Ca²⁺ channel activity influences retrieval.

The ability of the UCL compounds to block the sAHP was previously determined by recording the sAHP in CA1 pyramidal neurons (Shah et al., 2001; Zunszain et al., 2002; Shah et al., 2006). This is valuable because the molecular constituents of the channels underlying the sAHP in CA1 pyramidal neurons are unknown. The latter is exemplified by the recent identification of specific KCNQ channel subunits contributing to the sAHP in dentate granule cells and

in CA3 but not CA1 pyramidal neurons (Tzingounis and Nicoll, 2008; Tzingounis et al., 2010). Further, the intermediate conductance Ca²⁺-activated K⁺ channel (IK1) that is blocked by TRAM-34 is not expressed in brain (Ishii et al., 1997; Joiner et al., 1997). Importantly, the ability of NE to promote retrieval within the hippocampus appears to be through effects on CA1 pyramidal neurons (Zhang et al., 2005; Murchison et al., 2011).

Several behavioral roles for the sAHP in the hippocampus have been proposed. For example, the sAHP is proposed to limit excitability and possibly prevent seizure-like activity (Empson and Jefferys, 2001; Melyan et al., 2002). In addition, changes in intrinsic neuronal excitability that follow learning have been appreciated and studied for several decades (Disterhoft and Oh, 2006; Saar and Barkai, 2009; Mozzachiodi and Byrne, 2010). Based on the observation that the sAHP is reduced *ex vivo* for a limited period after conditioning, it is proposed that this reduc-

tion is also present *in vivo* during this period and that it acts to facilitate learning. However, each of these proposals is based on correlative data without direct manipulation of the sAHP, and thus more definitive evidence is currently lacking.

Our data do not exclude the above roles; instead they demonstrate a novel role for reduction of the sAHP in facilitating memory retrieval. They also raise the interesting question of whether or under what circumstances the sAHP may be reduced *in vivo*. The sAHP and accommodation are transiently reduced in the hippocampus *ex vivo* after conditioning, and the time course of these reductions parallels the time course over which NE is required for hippocampus-dependent memory retrieval (Moyer et al., 1996; Murchison et al., 2004). We hypothesized that NE might play a role in establishing these reductions during conditioning. Instead, we found that NE is required during decapitation for the *ex vivo* expression of enhanced intrinsic excitability. This was established both by restoring NE in *Dbh*^{-/-} mice and by blocking NE at β_1 receptors in WT mice. Although less specific in its effects, sufficient anesthesia before decapitation also prevents *ex vivo* reductions in the sAHP and in accommodation.

The above results suggested that the activation of β_1 receptors in naive and conditioned mice during euthanasia has differential effects that can be observed in slices. We hypothesized that persistent signaling by a kinase might be responsible for the differences in the sAHP. For example, persistent signaling by an isoform of protein kinase C is critical for memory maintenance after various learning paradigms (Sacktor, 2011), and persistent signaling by extracellular signal-regulated kinase is required for the reduction of the sAHP in the neocortex after olfactory conditioning (Cohen-Matsliah et al., 2007). Here we tested for persistent signaling by PKA because of the prominent role PKA plays in the regulation of the sAHP by NE and β_1 receptors in CA1 (Oh et al., 2009).

We found that inhibition of PKA increased the sAHP in conditioned but not naive slices. Importantly, this effect of PKA inhibition was absent when conditioned mice were treated with a β_1 antagonist shortly before euthanasia. This lack of an effect is unlikely to be due to a ceiling because the sAHP and accommodation in CA1 are enhanced with BayK (Rascol et al., 1991). These findings suggest that there is something unique about conditioning that exists for several days afterward and permits persistent PKA signaling to be induced by the release of NE during euthanasia. Of note, persistent PKA signaling due to degradation of the RI regulatory subunit has been observed in *Aplysia* during long-term facilitation (Chain et al., 1999; Kurosu et al., 2007).

Our observations raise the possibility that intrinsic excitability is not increased *in vivo* after conditioning, at least in the absence of additional neuromodulatory input. We are not aware of any studies demonstrating enhanced intrinsic excitability *in vivo* after conditioning, but approaches for doing so are challenging to per-

Figure 7. The *ex vivo* reduction in the sAHP after conditioning is the result of β_1 signaling during euthanasia. **A, B**, To determine whether β_1 signaling is relevant, slices from naive and conditioned (Cond D1) β_1 KO mice were compared. Similar to the *Dbh*^{-/-} mice, conditioned β_1 KO mice did not exhibit significant changes in the sAHP or number of action potentials relative to naive β_1 KO mice ($p > 0.3$, $n = 21$ /group). To determine whether β_1 signaling acts during euthanasia, conditioned WT mice were treated with vehicle or the β_1 antagonist betaxolol (1 mg/kg) 30 min before decapitation. Betaxolol blocked both the reduction in the sAHP and the increase in the number of APs ($n = 22$ /group). To determine whether β_1 blockade has nonspecific effects, pseudo-conditioned WT mice were treated with vehicle or the β_1 antagonist betaxolol (1 mg/kg) 30 min before decapitation. In this case betaxolol was without effect ($p > 0.4$, $n = 22$ /group). **C, D**, To examine the influence of anesthesia, mice conditioned with a single training trial were treated with various doses of pentobarbital 15 min before decapitation. The intermediate dose of pentobarbital diminished and the high dose of pentobarbital eliminated the reduction in the sAHP and the increase in the number of APs relative to slices from vehicle-treated conditioned mice. $p = 0.0084$ for the main effect of dose on the sAHP; $p = 0.0001$ for the main effect of dose on the number of APs ($n = 25, 23, 25, 21, 16$ /group). **E, F**, To examine the influence of training trial number of the effects of anesthesia, mice conditioned with three training trials were treated with one of two doses of pentobarbital 15 min before decapitation. The high dose of pentobarbital eliminated the reduction in the sAHP and the increase in the number of APs relative to slices from vehicle-treated conditioned mice. $p = 0.012$ for the main effect of dose on the sAHP; $p = 0.0013$ for the main effect of dose on the number of APs ($n = 23, 24, 15$ /group).

Figure 8. Persistent signaling by PKA is required for the *ex vivo* reduction in the sAHP after conditioning. **A, B**, To determine whether PKA signaling is involved, slices from naive and conditioned (Cond D1) mice were incubated with the PKA inhibitor H89 (2 μM). H89 selectively increased sAHP amplitude and decreased the number of action potentials in slices from conditioned mice. In contrast, no effect of H89 was observed when conditioned mice were treated with the β_1 antagonist betaxolol (1 mg/kg) 30 min before decapitation ($p > 0.5$, $n = 23, 15, 22, 15, 21, 15$ /group).

form and interpret. Interestingly, conditioning that induces enhanced intrinsic excitability in the hippocampus *ex vivo* can facilitate the acquisition of other hippocampus-dependent tasks in a time-limited manner (Zelcer et al., 2006). Whether this is due to enhanced intrinsic excitability *in vivo* is not clear. If it is, we hypothesize that the change in intrinsic excitability requires neuromodulatory input that is activated during acquisition of the second task. Further, pharmacologically enhancing the medium AHP in the DH can impair learning (McKay et al., 2012), and this is concordant with our view that changes in intrinsic excitability, whether driven by pharmacologic or neuromodulatory input, are likely to affect learning.

The possibility that the sAHP in the hippocampus may not be transiently reduced *in vivo* after conditioning is parsimonious with a role for NE in retrieval. If the sAHP was already reduced *in vivo* after conditioning, then it is not clear why NE would be required for retrieval if its effect is to reduce the sAHP. One could postulate that the reduction in the sAHP after conditioning occurs in cells other than those modulated by NE during retrieval. However, this seems unlikely because the *ex vivo* reduction after conditioning depends on NE and β_1 signaling for its expression. Instead, we hypothesize that the sAHP in the hippocampus is not reduced *in vivo* after conditioning unless additional neuromodulatory input is provided. We further hypothesize that during contextual fear memory retrieval; this neuromodulatory input is provided by the release of NE, which activates β_1 signaling to cause reduction of the sAHP.

The requirement for NE in retrieval is transient, beginning between 1 and 2 h after conditioning and lasting for ~ 4 d. The impairment of memory retrieval mediated by BayK also occurs during the first 4 d after conditioning. Thus, we hypothesize that intermediate-term memory retrieval depends on the enhancement of CA1 pyramidal neuron intrinsic excitability by NE. By 5 d after conditioning, a specific phase of memory consolidation within the hippocampus is likely complete and retrieval no longer requires nor is affected by modulation of the sAHP. The nature of this consolidation is unknown, but one could speculate that it is synaptically mediated and memory specific. Future studies will be required to address this idea.

Finally, our data also indicate that the method of euthanasia can have a significant impact upon *ex vivo* findings, and that caution is warranted in extrapolating *ex vivo* data to states *in vivo*. Although the need for caution is obvious when considering network properties in a slice relative to the intact brain, our data extend this need to parameters that are intrinsic to cells.

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