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Cholinergic Neurotransmission Is Essential for Perirhinal Cortical Plasticity and Recognition Memory

E. Clea Warburton, Timothy Koder, Kwangwook Cho,[1] Peter V. Massey, Gail Duguid, Gareth R.I. Barker, John P. Aggleton,[2] Zafar I. Bashir, and Malcolm W. Brown*
MRC Centre for Synaptic Plasticity
Department of Anatomy
University of Bristol
Bristol BS8 1 TD
United Kingdom

Summary

We establish the importance of cholinergic neurotransmission to both recognition memory and plasticity within the perirhinal cortex of the temporal lobe. The muscarinic receptor antagonist scopolamine impaired the preferential exploration of novel over familiar objects, disrupted the normal reduced activation of perirhinal neurones to familiar compared to novel pictures, and blocked production of long-term depression (LTD) but not long-term potentiation (LTP) of synaptic transmission in perirhinal slices. The consistency of these effects across the behavioral, systems, and cellular levels of analysis provides strong evidence for the involvement of cholinergic mechanisms in synaptic plastic processes within perirhinal cortex that are necessary for recognition memory.

Introduction

Attempts to uncover the neural plastic mechanisms underlying learning and memory in the mammalian brain have largely focused on the hippocampus, amygdala, or cerebellum (Milner et al., 1998; Albright et al., 2000). However, the perirhinal cortex of the temporal lobe provides an important additional region where such mechanisms may be sought. First, this is because ablation of perirhinal cortex in rats and monkeys produces severe impairments in visual recognition memory tasks that can be solved by familiarity discrimination (Zola-Morgan et al., 1989; Gaffan and Murray, 1992; Otto and Eichenbaum, 1992; Gaffan and Murray, 1992; Meunier et al., 1993; Suzuki et al., 1993; Mumby and Pinel, 1994; Ennaceur et al., 1996; Meunier et al., 1996; Murray, 1996; Murray and Bussey, 1999; Buffalo et al., 2000; Brown and Aggleton, 2001). Second, there is now substantial evidence that the familiarity discrimination component of recognition memory depends on the reductions in the responses of perirhinal neurons that occur when a visual stimulus is reencountered (Brown and Xiang, 1998; Brown and Aggleton, 2001; Brown and Bashir, 2002). Furthermore, these neuronal response reductions on stimulus repetition are the only neuronal substrate so far identified within perirhinal cortex that could subserve general, long-term familiarity discrimination in the absence of specialized training, though this hypothesized relationship has so far been little tested (Brown and Xiang, 1998; Brown and Aggleton, 2001). Additionally, there is evidence that the response reductions are first generated in perirhinal and/or adjacent cortex (Xiang and Brown, 1998; Brown and Xiang, 1998) and that changes in synapses occur in perirhinal cortex following the viewing of novel stimuli (Thompson et al., 2002). This reduction in neuronal activation for familiar compared to novel stimuli is so large that it can be imaged in rats using immunohistochemistry for the protein products (Fos) of the immediate early gene c-*fos* (Zhu et al., 1995; Zhu et al., 1996; Wan et al., 1999). In addition, it has been hypothesized (Brown and Xiang, 1998; Bogacz and Brown, 2003; Cho et al., 2000; Bogacz et al., 2001; Brown and Bashir, 2002) that these perirhinal response reductions on stimulus repetition could arise from mechanisms that are also used in the production of long-term depression (LTD) in perirhinal brain slices. This hypothesis arises not only because of the observed direction of response change with stimulus repetition but also because computational modeling of familiarity discrimination establishes that synaptic weakening produces a major gain in storage capacity over synaptic strengthening (Bogacz and Brown, 2003).

In this report, we test the hypotheses that (1) perirhinal neuronal response reductions underlie recognition memory; (2) that these response reductions arise from a synaptic plastic mechanism used in LTD; and (3) that cholinergic neurotransmission is essential to both these processes as well as familiarity discrimination behavior. The tests were provided by looking for consistency across different levels of analysis in the role of the cholinergic system. Thus the action of scopolamine, a muscarinic cholinergic antagonist, was investigated upon familiarity discrimination behavior, upon differential neuronal activation to novel and familiar stimuli measured by immunohistochemistry, and upon LTD and long-term potentiation (LTP) in perirhinal slices.

There are good grounds for choosing to test the effects of cholinergic antagonism. Acetylcholine has long been implicated in memory functions (Deutsch, 1971; Drachman and Leavitt, 1974), although there is no general agreement as to whether the memory impairments that follow muscarinic receptor blockade are due to a direct action on memory processes or an indirect effect, for example, as a result of impaired attention (Blokland, 1995; Hasselmo, 1995; Voytko, 1996; Everitt and Robbins, 1997; Steckler et al., 1998; Rasmusson, 2000). Additionally, cholinergic hypofunctionality has been widely suggested to be, at least in part, responsible for the memory impairments of early Alzheimer's disease; for review, see Bartus, 2000. Recent ablation experiments in monkeys have further emphasized the potentially important role of acetylcholine in memory (Easton et al., 2001). More specifically, the topical application of sco-

*Correspondence: m.w.brown@bris.ac.uk
[1] Present address: School of Medicine and Biomedical Science, University of Sheffield, Western Bank, Sheffield, S10 2TN, United Kingdom.
[2] Present address: School of Psychology, University of Cardiff, Cardiff, CF10 3YG, Wales, United Kingdom.

polamine to monkey perirhinal cortex produces an impairment in the performance of a visual recognition memory task (Tang et al., 1997). Importantly, a previous experiment has challenged the suggestion that neuronal response reductions are the basis of familiarity discrimination, as at short delays (<5 s) such reductions were unimpaired by systemic administration of the muscarinic antagonist, even though a monkey's performance of a delayed matching to sample task at such delays was impaired (Miller and Desimone, 1993). Here we report the effects of scopolamine on familiarity discrimination behavior in rats together for the first time (according to our knowledge) with its effects on neuronal activity changes at long delays and on perirhinal plasticity.

Results

Preferential Exploration of Novel Objects

Experiment 1: Systemic Administration of Scopolamine prior to Acquisition

Scopolamine impaired familiarity discrimination when present during sampling (acquisition). Thus, during the recognition memory test that occurred 15 min after the sampling period, the time spent exploring a novel object was significantly greater than that for a previously sampled (i.e., familiar) object for the control but not the scopolamine-treated rats [ANOVA of difference in exploration times—D1 index—between the two groups: $F(1,11) = 4.95$; $p < 0.05$]; see Figure 1A. The control rats reliably discriminated between novel and familiar objects [$t(11) = 5.31$; $p < 0.001$]. In contrast, scopolamine-treated rats failed to show significant discrimination [$t(11) = 1.56$; $p > 0.1$].

Experiment 2: Systemic Administration of Scopolamine after Acquisition

Scopolamine was without significant effect on familiarity discrimination when administered after acquisition. In this case, when sampling occurred without the drug, an analysis of the difference in exploration times during the recognition memory test revealed no significant difference in discrimination between the control and scopolamine-treated animals [D1 index; ANOVA, $F(1,11) < 1.0$]; see Figure 1A. Both the control and the drug-treated animals reliably discriminated novel from familiar objects [$t(11) = 5.13$ and 7.76, respectively; $p < 0.001$].

Exploration during the Sampling Period

There was no evidence of an effect of systemic scopolamine on general explorative behavior. To establish this, the total time required by the rats to complete 25 s of exploration of the objects presented for familiarization during the sampling period was recorded. ANOVA of these times revealed no significant differences [$F(1,11) < 1.0$] between the control (mean = 138.5 ± 11.1 s) and scopolamine-treated animals (mean = 147.4 ± 7.4 s) when scopolamine was administered before sampling commenced (experiment 1). Similarly, and as to be expected, the exploration times did not differ significantly when scopolamine was administered after sampling (experiment 2).

Experiment 3: Localized Infusion of Scopolamine in Perirhinal Cortex

Scopolamine also impaired familiarity discrimination when infused, via implanted cannulae, bilaterally into perirhinal cortex before, to be active during, sampling (acquisition); see Figure 1A. When saline was infused before sampling, the rats spent significantly more time exploring the novel than the familiar object [$t(5) = 3.52$; $p < 0.02$]; however, there was no significant such preference when scopolamine had been infused before sampling [$t(5) = 1.89$; $p > 0.1$], and the interaction between drug and preference was significant [repeated measures ANOVA, $F(1,5) = 12.9$, $p = 0.016$]. This impairment indicates that the action of scopolamine is produced even when administration is local to perirhinal cortex.

In sum, scopolamine impaired familiarity discrimination by an effect that was selectively on acquisition (or consolidation) rather than retrieval and impairment was produced even when drug administration was local to perirhinal cortex.

Preferential Neuronal Activation to Novel Pictures Measured by Fos Expression

Effects of Familiarity

Previous work (Zhu et al., 1996; Wan et al., 1999) has established reliable differences between the two hemispheres in counts of Fos stained nuclei in perirhinal cortex (PRH) and neighboring visual association cortex (area Te2 [TE]) but not in entorhinal cortex (ENT) or hippocampus (HPC), when one eye has been exposed to novel and the other previously presented stimuli in the paired viewing procedure (Figure 2A). As the behavioral results of experiment 1 indicated that the action of scopolamine was at acquisition, effects upon Fos expression were sought for scopolamine given before the familiarization trials but not during the final test comparison of novel and previously presented stimuli. Fos counts were made in PRH and TE, plus three control regions—ENT, dorsal CA1 field of the hippocampus (HPC), and primary auditory cortex (AUD)—where differences between novel and previously presented stimuli were not expected (Figure 2B). As in the previous work, the Fos counts were normalized across hemispheres and rats. These normalized counts from each of the five brain regions sampled were compared for the saline control and methyl scopolamine (a cholinergic antagonist which does not cross the blood-brain barrier) control groups using ANOVA with factors rat, stimulus repetition, and area; see Figure 2C. No significant differences in mean counts across, nor any significant interactions involving, these factors were revealed (all $Fs < 1.0$), and so the data from these two control groups were pooled for the subsequent analyses.

Differences in Fos counts between the combined control group and the scopolamine group were established in the following way. A repeated measures ANOVA with factors for area, stimulus repetition (novel or repeatedly presented), and drug (scopolamine or control) revealed significant interactions for stimulus repetition by drug [$F(1,276) = 19.89$; $p < 0.001$] and stimulus repetition by area [$F(4,276) = 2.72$; $p = 0.03$]; see Figure 2C. The analysis was therefore continued by subdividing the data into regions where an effect was (PRH and TE) or was not (AUD, HPC, and ENT) predicted. No evidence was found that scopolamine treatment had affected neuronal activation in the control regions. Thus the analysis for AUD, HPC, and ENT revealed only a significant

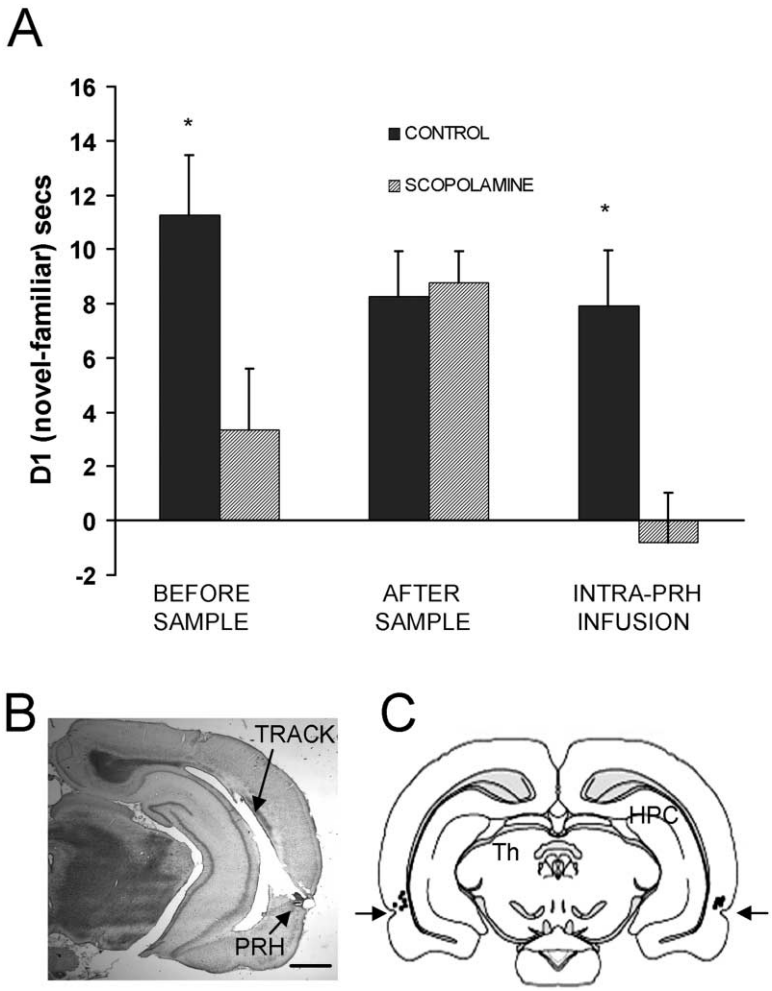

Figure 1. Muscarinic Antagonism Impairs Familiarity Discrimination

(A) Effect of scopolamine (0.05 mg/kg, i.p.) on spontaneous preferential exploration of novel compared to familiar objects when administered before (BEFORE SAMPLE: experiment 1) or after (AFTER SAMPLE: experiment 2) the sample (acquisition) phase. Scopolamine (10 μg/μl) also impaired preferential exploration when locally infused by a cannula implanted in each perirhinal cortex (INTRA-PRH: experiment 3). Note the significantly poorer performance (*$p < 0.05$) when scopolamine was administered systemically or directly into the perirhinal cortex before the sample phase but not when administered after.

(B) Photomicrograph of a coronal brain section showing the track (indicated by arrow) left by a perirhinal cannula. The perirhinal cortex is also indicated (PRH). Scale bar, 1 mm.

(C) Positions of the cannulae tips in all of the rats on a schematic illustration brain section. Arrows indicate location of the perihinal cortex. HPC, hippocampus; Th, thalamus.

main effect of stimulus repetition [$F(1,140) = 4.98$; $p = 0.027$]; there were no other significant main effects or interactions. In particular, as in previous work (Zhu et al., 1996; Wan et al., 1999), the hippocampal counts were low and showed no evidence of a difference for novel and familiar stimuli; neither was there evidence of a consistent difference between drug and control hippocampal counts.

In contrast to the control regions, scopolamine treatment was found to affect the differential neuronal activation produced by novel and repeated stimuli in PRH and TE. Thus the analysis for PRH and TE revealed a significant drug by stimulus repetition interaction [$F(1,102) = 19.88$; $p < 0.001$]. In controls, mean counts for PRH and TE combined were higher for the novel than for the familiar stimuli [$F(1,23) = 10.40$; $p < 0.005$], while oppositely, in the scopolamine-treated group, mean counts were higher, though not significantly so, for the repeatedly presented than for the novel stimuli [$F(1,11) = 2.94$; $p > 0.1$]; see Figure 2C. Thus, scopolamine treatment disrupted the normal differential expression of Fos for novel and previously seen stimuli in PRH and TE.

As a previous experiment (Tang et al., 1997) in the monkey indicated that scopolamine had an action in perirhinal cortex but not anterior visual association cortex, the data for PRH and TE were separately analyzed to test for such a dissociation in the rat. For the data for PRH alone, there was a significant drug by stimulus repetition interaction [$F(1,34) = 14.97$; $p < 0.001$]. In TE, the drug by stimulus interaction was not significant [$F(1,34) = 2.26$; $p = 0.142$]. However, as demonstrated above, there was not a significant interaction between the results for these two areas: for both, counts for novel stimuli were higher than for familiar stimuli in controls, with this difference being reversed in direction in the scopolamine-treated animals. Thus there was no evidence in the present experiment for a different action of scopolamine in PRH and TE.

Overall Counts

There was no evidence that scopolamine treatment during the paired viewing sessions prior to the final test session (when no drug was given) produced any effect on the neuronal activation evoked by visual stimuli when no account was taken of whether these stimuli had been shown previously. Thus, the mean Fos counts averaged for all stimuli, whether novel or previously presented, did not differ significantly (ANOVA) among the scopolamine, the methyl scopolamine control, and the saline control groups, either overall or for any individual area; see Table 1.

In sum, scopolamine selectively disrupted the normally greater neuronal activation produced by novel

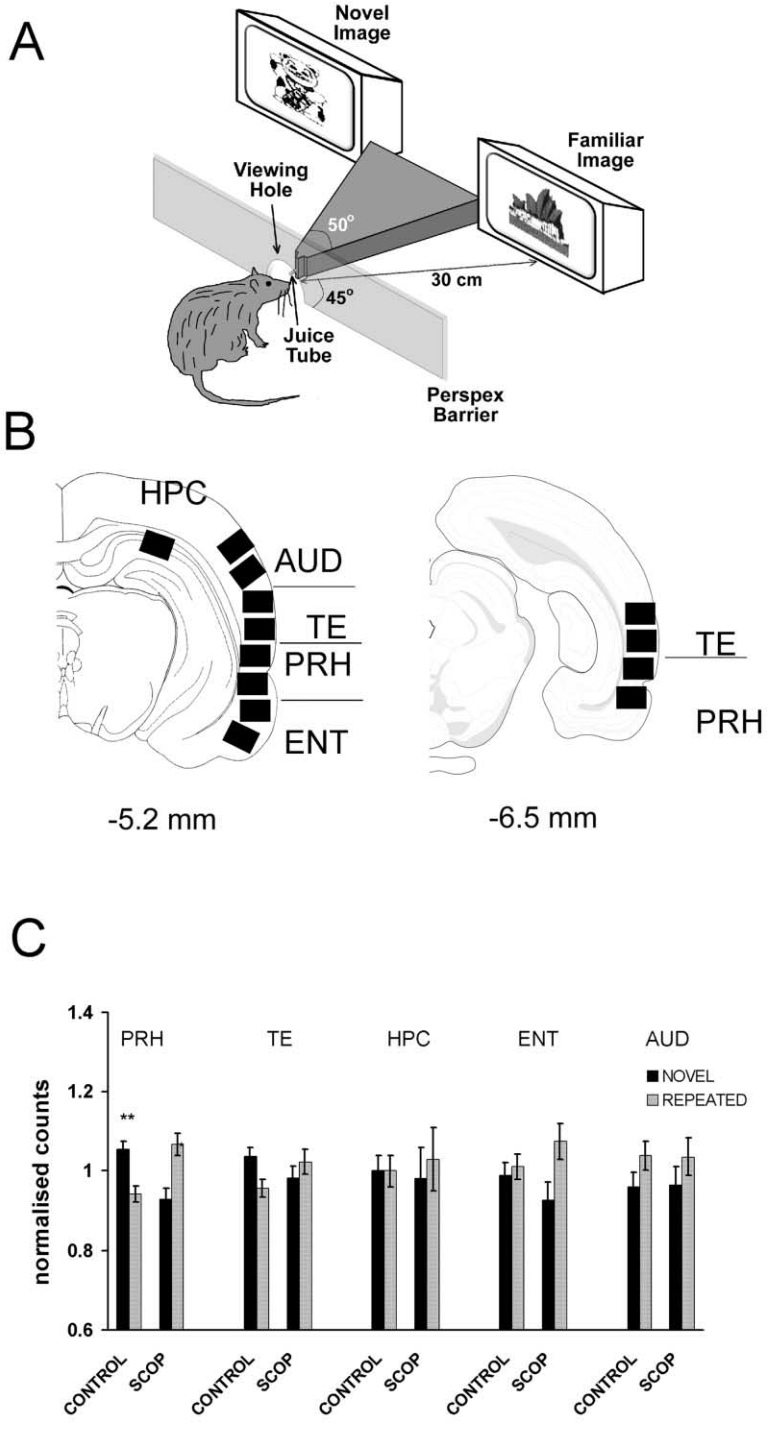

Figure 2. Muscarinic Antagonism Disrupts Fos Expression

(A) Paired viewing apparatus. The animal gains juice reward for keeping its head in the observing hole. Pictures, novel on one side and familiar on the other, are shown simultaneously on two monitors, each visible to only one eye. Figure modified from Brown and Aggleton (2001).

(B) The sampled areas are shown on sections at the indicated distances (in mm) behind bregma (Paxinos and Watson, 1986; Shi and Cassell, 1997). AUD, auditory cortex; ENT, entorhinal cortex; HPC, hippocampus; PRH, perirhinal cortex; TE, visual association cortex Te2.

(C) Effect of scopolamine on normalized Fos counts in the brain regions sampled. Note that novel stimuli no longer evoke significantly higher counts in perirhinal cortex (PRH) and Te2 (TE) in the scopolamine-treated animals. When the statistical analyses were conducted using data from only the methyl scopolamine controls and the drug group, the findings were the same as those when all control animals were included. Thus for PRH, there was a significant drug by stimulus repetition interaction [$F(1,22) = 11.45$; $p = 0.003$], and in TE, the drug by stimulus repetition interaction was not significant [$F(1,22) = 1.55$; $p = 0.23$]. When absolute rather than normalized counts were used in the analysis for PRH, there was still a significant drug by stimulus repetition interaction [$F(1,22) = 5.16$; $p = 0.03$]. $^{**}p < 0.01$.

than by previously presented stimuli in both perirhinal and neighboring visual association cortex while having no effect in control regions.

Perirhinal LTD and LTP

Previous work (Cho et al., 2000) has established that low-frequency stimulation (LFS; 1 Hz, 200 stimuli) of perirhinal slices can result in the induction of LTD, with the postsynaptic neuron either voltage clamped at −70mV or voltage clamped at −40mV. In the present study, control experiments carried out at −70mV verified that, in the absence of scopolamine, LTD was induced by LFS on each occasion [44% ± 5%, $n = 5$; $t(4) = 8.1$, $p < 0.005$]; see Figure 3A.

To test the effects of muscarinic antagonism, scopolamine (20 μM) was bath applied after obtaining a stable baseline of evoked excitatory postsynaptic currents (EPSCS). Scopolamine did not have any noticeable effect on synaptic transmission (Figures 3B and 3C). In the presence of scopolamine, however, LTD was not

Table 1. Fos Counts

	Control		Scopolamine	
Areas	Novel	Familiar	Novel	Repeated
PRH	42.5 ± 3.8	38.4 ± 3.2	36.1 ± 8.1	39.8 ± 7.3
TE	68.4 ± 7.0	61.4 ± 5.4	53.8 ± 14.9	54.4 ± 12.6
ENT	35.3 ± 6.4	36.4 ± 6.4	40.0 ± 11.6	43.9 ± 10.4
HPC	8.3 ± 0.7	8.2 ± 0.8	6.2 ± 1.2	6.4 ± 1.1
AUD	93.0 ± 14.4	93.2 ± 12.8	63.4 ± 22.4	76.8 ± 33.4

The mean counts (± SEM) of Fos stained nuclei in the sampled areas for the novel and repeated stimuli. The counting frame size was 0.63 mm^2.

induced following either of the above LFS protocols (−40mV: 5% ± 5%, $n = 5$, $p > 0.05$; −70mV: 3% ± 6%, $n = 5$, $p > 0.05$). This difference between the control and scopolamine-treated slices was highly significant [$F(1,10) = 25.71$, $p < 0.001$].

To test the specificity of action of cholinergic antagonism on perirhinal plasticity, the effects of scopolamine were sought on LTP induced by high-frequency stimulation (HFS; four 1 s trains, 100 Hz). After a stable baseline had been established for each of two inputs that were alternately stimulated, LTP was induced [29% ± 7%, $n = 5$; $t(4) = 4.14$, $p < 0.05$] by HFS applied to one input while baseline stimulation continued for the second input; see Figure 4. After 1 hr, scopolamine (20 μM) was bath applied and HFS applied to the second input. The HFS resulted in LTP in the second input [34% ± 8%, $n = 5$; $t(4) = 4.25$, $p < 0.05$; Figure 4], which did not differ from that previously established in input one (paired t test, $n = 5$, $t < 1$, $p > 0.05$). Thus, cholinergic antagonism affected neither the induction of LTP nor the maintenance of preestablished LTP. Accordingly, the effect of scopolamine is specific to LTD.

In sum, synaptic activation of muscarinic receptors is selectively required for the establishment of LTD but not LTP in perirhinal slices.

Discussion

To our knowledge, the findings establish for the first time for putative recognition memory mechanisms that interference with cholinergic transmission has consistent, selective effects at the behavioral, systems and cellular levels of analysis. This consistency of action provides experimental support to the hypotheses that (1) perirhinal neuronal response reductions underlie recognition memory, (2) these response reductions arise from a synaptic plastic mechanism used in LTD, and (3) cholinergic neurotransmission is essential to both these processes as well as familiarity discrimination behavior.

Thus, in particular, the Fos immunohistochemical results establish for the first time (to our knowledge) that the cholinergic muscarinic antagonist scopolamine can disrupt the differential activation of rat perirhinal neurons by novel and familiar stimuli when the period for which information must be retained is long (≥3 hr). This finding contrasts with the lack of effect found at short (<5 s) delays in monkeys (Miller and Desimone, 1993). Thus it is possible that the memory impairment at short delays is due to an effect of blockade of muscarinic receptors on another process, such as delay activity (Fuster and Jervey, 1981), or within another brain region, such as prefrontal cortex (Brown, 1996; Brown and Xiang, 1998). It should be noted that delay activity could not be expected to support memory retention across the long, distraction-containing delays that were employed in the present experiments. The Miller and Desimone (1993) finding suggests that the effect of scopolamine becomes apparent only at delay intervals longer than 5 s and hence, with the present results, could indicate a possible muscarinic effect on early consolidation rather than initial acquisition processes. The other possible neuronal recognition memory mechanism, response enhancement, described by Miller and Desimone (1994) is unlikely to be involved, as it has only been found after specialized training in monkeys. No such training was given, and the control Fos results indicated an overall reduction rather than enhancement of neuronal responses for familiar stimuli. Although the effect of scopolamine in perirhinal cortex might be disruptive to recollective mechanisms supporting recognition memory, given other evidence (Brown and Aggleton, 2001), it is parsimonious to presume its effect is on familiarity discrimination mechanisms.

A variety of effects of muscarinic antagonists on plasticity have been reported in other brain areas (e.g., Hasselmo, 1995; Hasselmo and Barkai, 1995; Auerbach and Segal, 1996; Hasselmo and Wyble, 1997; Rasmusson, 2000). In particular, in regard to LTD, Kirkwood et al. (1999) and Massey et al. (2001) have reported that pharmacological activation of muscarinic receptors results in LTD in visual cortex and perirhinal cortex, respectively. However, to our knowledge, our current results provide the first evidence that LTD in the perirhinal cortex is dependent on the synaptic activation of muscarinic receptors. Moreover, the effect of muscarinic antagonism was specific to LTD, being without effect on LTP. This finding thus provides the first evidence indicating that familiarity discrimination may be impaired by the selective impairment of mechanisms used in LTD.

There are good grounds for believing that the observed impairment of recognition memory arises from blockade of muscarinic receptors within the perirhinal and/or neighboring cortex. Thus, localized perfusion of scopolamine via a cannula implanted in rat perirhinal cortex was sufficient to impair recognition memory. This finding supports the impairment of recognition memory found with perirhinal scopolamine infusion in monkeys (Tang et al., 1997). Additionally, in the present experiments, an effect on differential Fos activation was found in perirhinal cortex and Te2, without a general effect in all brain regions. In particular, no effect of scopolamine was found in the hippocampus, though, as in previous

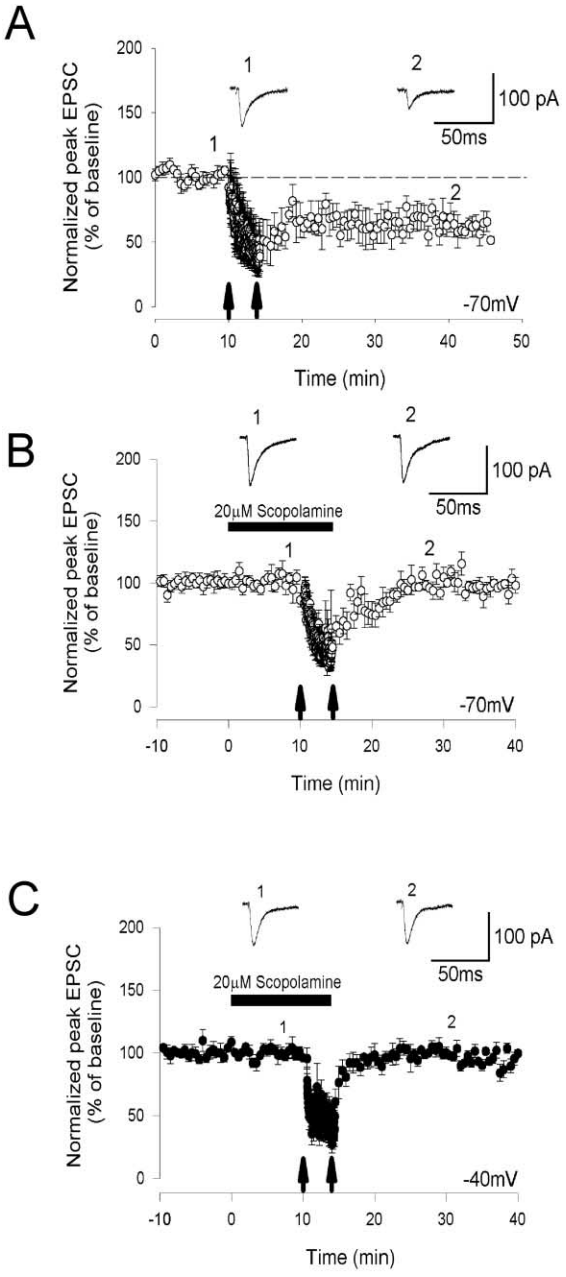

Figure 3. Muscarinic Antagonism Blocks LTD

(A) LTD can be induced by delivering 1 Hz stimulation (200 stimuli) while the neuron is voltage clamped at −70mV or at −40mV (data not shown).

(B and C) Scopolamine prevents the induction of LTD when 1 Hz stimulation is delivered either at −70mV (B) or at −40mV (C). Synaptic traces above each of the graphs are single EPSCs taken from the time points indicated (1, 2). Stimulation (1 Hz) is delivered during the time indicated by the two arrows.

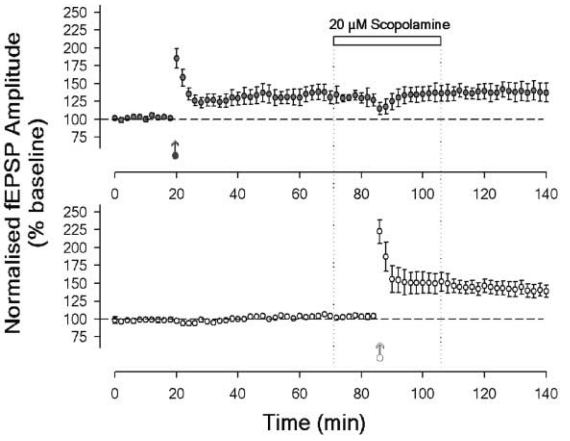

Figure 4. Muscarinic Antagonism Fails to Block LTP

In this two-pathway experiment, LTP is induced by HFS (four trains of 1 s, 100 Hz) in one input at the time indicated by the arrow (top panel). Scopolamine is subsequently bath applied and HFS delivered to the other input (bottom panel, HFS indicated by arrow). Scopolamine affects neither preestablished LTP nor the induction of LTP.

studies using individual stimuli (Zhu et al., 1996; Wan et al., 1999), hippocampal Fos counts were very low and did not differ for novel or familiar pictures. Together, these results rule out the possibility that the impairment of recognition memory could have been produced by an action of scopolamine within the hippocampus or prefrontal cortex (though it does not rule out that scopolamine might have actions on recognition memory were it to be infused into those areas). Further, the impairment of LTD in perirhinal slices as established here clearly demonstrates that cholinergic blockade has an effect within perirhinal cortex itself that could potentially explain the behavioral and neuronal activation results. In support of this suggestion, as mentioned above, activation of muscarinic acetylcholine receptors in perirhinal slices results in a long-lasting depression (Massey et al., 2001). Nevertheless, the present results in the rat, in contrast to the findings in the monkey (Tang et al., 1997), do not establish a difference in the actions of scopolamine on perirhinal cortex and adjacent visual association cortex (Te2): differential Fos activation was similarly disrupted by cholinergic antagonism in both regions. Thus, the present results leave open whether there may be important cholinergic actions in Te2 as well as in perirhinal cortex.

The results of the behavioral experiments demonstrate that the effect of muscarinic antagonism was selectively upon acquisition or early consolidation rather than retrieval, as impairment was only found when the scopolamine was present during acquisition and not when the drug was administered rapidly ($<$3 min) after acquisition and so present during the test (retrieval). This finding accords with impairments found in previous studies (Aigner and Mishkin, 1986; Huston and Aggleton, 1987; Hunter and Murray, 1989; Hasselmo, 1995; Steckler et al., 1998), though effects on retrieval have also been reported at higher doses (Norman et al., 2002). It was because of scopolamine's action on acquisition that in the Fos experiment cholinergic transmission was blocked by giving the drug prior to presentation of the repeatedly shown stimulus set but not during the critical final test comparison. Again this interference with acquisition resulted in disruption of the normal pattern of differential Fos expression for novel and familiar stimuli. It should be noted that, consistent with the behavioral results and immunohistochemical results indicating an

action of scopolamine on acquisition or early consolidation, the effect on LTD in perirhinal slices was apparent by shortly after the time stimulation ceased, i.e., it also was on induction or early consolidation processes.

Whether cholinergic antagonism affects mnemonic or attentive processes is disputed (Blokland, 1995; Hasselmo, 1995; Voytko, 1996; Everitt and Robbins, 1997; Steckler et al., 1998). The effects of such antagonism in the present experiments are capable of being explained by a mnemonic action. Thus, in particular, cholinergic antagonism blocked LTD in perirhinal slices, and the observed effect was on synaptic plasticity rather than transmission: this is not readily explained as impairment of a solely attentive mechanism. Further, this block, demonstrated within perirhinal cortex, could explain the behavioral and neuronal activation results without the need to invoke a general action on attentive systems. Also against an explanation of the observed effects as being due to an impairment of attention at the low dose used is the lack of evidence of a drug-associated change in explorative behavior in the recognition memory task. Moreover, for the final test comparison in the immunohistochemical experiment in the paired viewing apparatus, both the novel and repeatedly seen pictures were presented simultaneously and without scopolamine being present so as to minimize any potential attentive effects of the drug (although this procedure does not exclude any drug effect on attention during previous presentations). In fact, at short delays when purely attentive effects should be apparent, no impairment due to muscarinic antagonism of monkey perirhinal neuronal responses was found (Miller and Desimone, 1993). This finding also suggests that the effect of muscarinic blockade is not on visual perceptual processes in perirhinal cortex. Notwithstanding these considerations, it remains possible that, in the intact brain, attention results in activation of cholinergic afferents to the perirhinal cortex that are necessary for or facilitate plastic processes: in such a view, attentive and mnemonic cholinergic actions coalesce.

An unexpected finding was the reversal of the direction of normal difference in neuronal activation as measured by Fos, with familiar stimuli producing greater activation than novel in the scopolamine-treated animals. One possible explanation for the reversal is provided by the fact that scopolamine impairs familiarity discrimination but not reward association learning in monkeys (Mishkin, 1999). As juice is provided to the rats just before the stimulus pictures are turned off, it is possible that neuronal responsiveness to the repeatedly shown stimuli is enhanced, as has been shown previously in monkeys (Kobatake et al., 1998). It is also possible that this process is facilitated by enhanced excitatory feedback due to impairment of the normal cholinergic modulatory effects on synaptic transmission (Hasselmo, 1999). Thus, it is possible that scopolamine impairs the mechanism that results in a reduced response to a previously seen stimulus but not the mechanism that results in an associational enhancement of responsiveness for the repeatedly shown stimuli; in such case, activation for the previously shown stimuli would be greater than that for the novel stimuli, as was observed.

Thus the results establish the value of the perirhinal cortex as a site for investigating plastic processes involved in memory and, specifically, those involved in familiarity discrimination. They provide support to the idea that mechanisms involved in LTD may also be crucial to memory. Moreover, they provide additional support to indications that acetylcholine is important to memory functions and provide new evidence that acetylcholine may have a direct influence on plastic mechanisms.

Experimental Procedures

All experiments used adult male pigmented DA rats (Bantin and Kingman, UK) weighing between 170 and 220 g, which were maintained on a 14 hr light/10 hr dark cycle, with the dark phase during normal daylight. Animals were housed in groups of four and had 24 hr access to food and water except when used in the immunohistochemical experiments. All efforts were made to minimize animal suffering and the number of animals used. Experiments were performed in accordance with the UK Animals (Scientific Procedures) Act 1986. When given systemically in all the in vivo experiments, scopolamine hydrobromide or scopolamine methylbromide (Sigma Chemicals) was administered i.p. at 0.05 mg/kg in a volume of 1 ml/kg normal saline. Methyl scopolamine was used as a control, as this drug does not cross the blood-brain barrier. When given by cannula, scopolamine hydrobromide was given at a dose of 10 μg/μl per hemisphere.

Preferential Exploration of Novel over Familiar Objects

Apparatus

The experiments took place in an arena (50 cm $\times$ 90 cm $\times$ 100 cm) made of wood, the inside of which was painted gray and the floor covered with sawdust. The arena was surrounded by a black cloth to conceal the experimenter or any other external stimuli during testing. The rat's behavior was monitored and recorded using an overhead camera and video recorder. The stimuli to be discriminated were triplicate copies of objects that varied in size (between 10 to 15 cm high), shape, and color, made from glass or plastic.

Preferential Exploration Procedure

Each rat was habituated to the empty arena for 15 min on each of 2 days prior to the commencement of the recognition test. The object familiarity discrimination test was performed in two phases—acquisition/sample phase and test phase—separated by a delay. In the acquisition phase, two identical objects (A1 and A2) were placed in the far corners of the arena, 22 cm from each wall. The rat was placed in the arena and allowed to explore the objects until a total of either 25 s (experiments 1 and 3) or 40 s (experiment 2) of exploration had accrued or 3 min elapsed. Exploration of an object was defined as directing the nose to the object at a distance of less than 2 cm. Turning around or sitting on the object was not considered exploratory behavior. During the delay period, the rat was removed from the arena and placed in the home cage.

After a delay (experiment 1, 15 min; experiment 2, 30 min; experiment 3, 20 min), the rat was replaced in the arena for the test phase. The arena now contained a third identical copy of the familiar object (A3) and a novel object (B) in the same locations as the sample stimuli. The rat remained in the arena for 3 min, and the length of time spent exploring each object in each minute was recorded. The locations of the choice objects and the objects used as novel or familiar were counterbalanced across rats.

Subjects and Experimental Design

The subjects were 12 rats. A within-subjects crossover experimental design was used in both experiments, with the two experiments being separated by 48 hr.

Experiment 1: Administration of Scopolamine prior to Acquisition

Scopolamine (0.05 mg/kg, i.p.) was administered 30 min prior to the sample phase. Control rats received an equivalent volume of normal saline. Forty-eight hours later, the rats were tested again: this time, the control rats were given scopolamine and the drug group saline.

Experiment 2: Administration of Scopolamine after the Acquisition Phase

Scopolamine (0.05 mg/kg, i.p.) was injected immediately (<3 min) after the sample phase.

Data Analysis

All scoring of exploratory behavior was done without the drug status of the rat being known. For each test session, the total exploration times for the novel and familiar objects were calculated. From these times was calculated D1: the absolute difference in time spent exploring the novel and familiar objects. D1 was calculated for the first 2 min of the test session, as this period has been shown to be a more sensitive measure of discrimination than all 3 minutes (Dix and Aggleton, 1999). Group comparisons used a within-subjects ANOVA. One-sample t tests were used to determine whether the groups' D1 scores were above zero ($p < 0.05$): zero corresponds to a lack of discrimination between novel and familiar objects.

Cannulation Surgery

Rats were initially anaesthetised with Sagatal (0.25 ml at 60 mg/ml i.p.) and anesthesia was maintained with halothane. The rats were placed in a stereotaxic frame with the incisor bar set at −3.3 mm below the interaural line. Two stainless-steel guide cannulae (26 gauge, Plastics One, Roanoke, Virginia) were implanted at an angle of 20° to the vertical, through burr holes in the skull, according to the following coordinates: AP −5.6 mm, L ± 4.47 mm (relative to bregma), V −6.7 mm (relative to surface of the skull). The guide cannulae were anchored to the skull by two stainless-steel skull screws (Plastics One) and dental cement. Cannulae were obdurated by dummy cannula between infusions. Following surgery, fluid replacement therapy (5 ml saline s.c.) and analgesia (0.05 ml Temgesic i.m.) were administered and the animals housed individually. The animals were allowed to recover for at least 10 days before habituation began.

Histology

Following the completion of the experiment, subjects were anaesthetised with Euthatal and perfused transcardially with phosphate-buffered saline followed by 4% paraformaldehyde. The brain was postfixed in paraformaldehyde for a minimum of 2 hr before being transferred to 20% sucrose in 0.2 M phosphate buffer and left overnight. Coronal sections were cut at 50 μm on a cryostat and stained with cresyl violet (Figure 1B). Cannulae locations were mapped onto standardized sections of the rat brain (Swanson, 1998), as shown in Figure 1C.

Drug Delivery

Scopolamine was dissolved in sterile 0.9% saline solution. Intracerebral injections were made into the perirhinal cortex through a 33 gauge infusion cannula (Plastic One) attached to a 5 μl Hamilton syringe via a length of polyethylene tubing. A volume of 1.0 μl containing 10 μg of scopolamine was injected over a 2 min period, using an infusion pump (Harvard), and the infusion cannula was left in place for a further 5 min. Object recognition testing commenced 15 min after the start of drug infusion.

Preferential Neuronal Activation Produced by Novel Compared to Familiar Pictures as Measured by Fos Immunohistochemistry

Apparatus and Stimuli

Novel and familiar pictures were presented to each rat simultaneously under closely controlled conditions, so that the evoked neuronal activation as measured by Fos could be compared (Zhu et al., 1996; Wan et al., 1999). Hence, each rat was trained in a paired viewing chamber (30 × 30 × 35 cm); see Figure 2A. The top of the chamber was open, the bottom and the sides were black, the front was transparent (perspex) with a central observing hole 3 cm in diameter, 6 cm above the floor. In the chamber, 4.5 cm to either side of the observing hole ran two small barriers (12 cm long × 9 cm high); these kept the rat's body at 90° to the front screen when its head was in the observing hole. When the rat's head was positioned in the observing hole, an infrared beam was interrupted, signaling the computer (Viglen P5-100) to start a trial. After a variable interval of 3–4 s, provided the head remained in the hole, two pictures (each 15 × 12 cm) were shown simultaneously for 4.5 s, one on each of two computer monitors (AOC Spectrum Model 4Vlr) placed 30 cm from the observing hole. A black partition ensured that the rat's left eye could not see the right monitor screen, and his right eye could not see the left screen. After the pictures had been seen for 4 s, a drop of black currant juice was delivered by a metal tube that the rat could just reach and lick. This paired viewing procedure (Zhu et al., 1996; Wan et al., 1999) ensured that the rat's behavior (which was monitored by camera and videorecorded) was the same for the novel and familiar pictures. The visual pictures were 2D representations of single objects taken from Microsoft ClipArt. They were varied in color and shape.

Training

During training, the rats were allowed ad libitum access to water for 2 hr each day. Each rat was pretrained for 3 days without stimulus presentation to go to the observing hole for juice reward. The subsequent training period lasted 6 days, with two morning training sessions and one afternoon session per day. The second morning session followed the first without a delay, and in each of these morning sessions, two sets of 30 pictures were presented one to each eye. One of these sets was the "repeat set" (RPT set) that would be shown in the final test; the other would not be so tested. The eye that could see the RPT set alternated across days so each eye was exposed to these stimuli the same number of times. In the afternoon training session, 3 hr after the end of the morning session, one eye was exposed to the RPT set of pictures while the other eye was exposed to a set of 30 novel pictures. A different set of 30 novel pictures was shown each afternoon to familiarize the animal with seeing novel and familiar pictures simultaneously. Again the side of presentation of the novel and RPT sets was counterbalanced across days. Thus, the different sets of novel and the RPT set of stimuli were presented such that by the end of the experiment each eye had seen the same number of novel and repeated stimuli. The final set of novel pictures (the NOV set) was shown with the RPT set on the afternoon of day 6, one eye seeing each set. The RPT set for half the animals was the NOV set for the other half of the animals, so that the particular pictures used were counterbalanced across animals. Additionally, whether the left eye or the right eye was exposed to the NOV set was counterbalanced across animals (so that complete counterbalancing required a group of four animals). For there to be evidence of memory, information about the prior occurrence of the RPT set had to have been retained for a minimum of 3 hr (the time from the last morning session to the final test).

Subjects and Experimental Design

The subjects were 36 rats, 12 in each of three groups: saline control, methyl scopolamine control, and scopolamine. Scopolamine or methyl scopolamine control (0.05 mg/kg, i.p.) or saline alone was administered 30 min before the morning and afternoon training sessions on days 1–5 and the morning but not the afternoon session of day 6. Thus, the drug was present during acquisition but not during the final test.

Immunohistochemical Procedure

Each rat was deeply anaesthetised with pentobarbitone and perfused with 0.1 M phosphate buffer and 4% paraformaldehyde (pH 7.4) 1.5 hr after the final set of stimuli had been shown. After perfusion, the brain was removed and placed in 4% paraformaldehyde for 12 hr, followed by 24 hr in 30% sucrose. The immunohistochemical and counting procedures followed those of Zhu et al. (1995). In brief, coronal sections (25 μm) were cut on a cryostat, and floating sections were processed using a primary antibody and the avidin-biotin complex (ABC, Vector laboratories, Burlingame, CA) method. The primary antibody (kindly provided by Dr D. Hancock; Biochemistry of the Cell Nucleus Laboratory, Imperial Cancer Research Institute) was a rabbit polyclonal directed against the N-terminal region of the rat c-*fos* peptide and is c-*fos* specific (Brennan et al., 1992). The secondary antibody was biotinylated goat anti-rabbit (Vector Laboratories). DAB was used for visualization of Fos immunoreactivity.

Data Analysis

The automated counting of stained nuclei was performed using an image analysis system (Seescan Ltd.) (McCabe and Horn, 1994; Zhu et al., 1995). All processing and counting was done blind. Counts above threshold were obtained from the right and left hemispheres for rectangular areas (0.94 × 0.67 mm) from two sections for each brain region (Figure 2B). For statistical analysis, each count was normalized by dividing it by the mean of the total counts for the area across both hemispheres for each rat. The normalized counts

were then subjected to an ANOVA with factors rat, area, and stimulus repetition (NOV/RPT). Statistical tests (two-tailed) used a significance level of 0.05.

Measurement of LTD and LTP in Perirhinal Slices

Material

Slices of perirhinal cortex were prepared from 14 rats. Animals were anaesthetised with halothane, decapitated, and the brain rapidly removed and placed in ice-cold artificial cerebrospinal fluid (aCSF; bubbled with 95% O_2/5% CO_2), which was comprised of (in mM) NaCl, 124; KCl, 3; $NaHCO_3$, 26; NaH_2PO_4, 1.25; $CaCl_2$, 2; $MgSO_4$, 1; D-glucose, 10. A midsagittal section was made, the rostral and caudal parts of the brain were removed by single scalpel cuts made at approximately 45° to the dorsoventral axis, and each half was glued by its caudal end to a vibroslice stage (Campden Instruments, Sileby, UK). Slices (400 μm), which included perirhinal, entorhinal, and temporal cortices, were stored submerged in aCSF (20°C–25°C) for 1–2 hr before transferring to the recording chamber. A single slice was placed in a submerged recording chamber (28°C–30°C, flow rate ~2 ml min^{-1}) when required. For whole-cell experiments, picrotoxin (5 μM) was then perfused for the duration of the experiment. Scopolamine was made up as a stock solution of 10 mM and diluted to a final concentration of 20 μM in aCSF when required.

Recording

For LTD experiments, whole-cell recordings were obtained from perirhinal cortex. Pipette (4–7 MΩ) solutions (280 mosm, pH 7.2) were comprised of (in mM) $CsMeSO_4$, 130; NaCl, 8; Mg-ATP, 4; Na-GTP, 0.3; EGTA, 0.5; HEPES, 10; QX-314, 6. One stimulating electrode was placed dorsorostrally on the temporal cortex side (area 35/36) and one ventrocaudally on the entorhinal cortex side (area 35/entorhinal cortex) of the rhinal sulcus. Stimuli (constant voltage) were delivered alternately to the two electrodes (each electrode 0.033 Hz). Neurones recorded in layer II/III were voltage clamped at −70mV unless otherwise indicated. Low-frequency stimulation (LFS: 200 stimuli, 1 Hz) was delivered at −70mV or paired with depolarization to −40mV, as appropriate. Where the membrane potential was changed, this was done for the duration of LFS only. The amplitude of the EPSCS was measured and expressed relative to the normalized preconditioning baseline. Effects of LFS were measured at appropriate time points (averaged over a 5 min period) after delivering LFS. For LTP experiments, standard extracellular recording techniques were used to monitor evoked field responses from layer II/III. Stimulating electrode positions and stimulation parameters were as for whole-cell recording. LTP was induced by delivering four 1 s trains of 100 Hz stimulation (HFS) with a 10 s interval between each train, and effects of HFS were measured 60 min after HFS.

Data Analysis

Data were only analyzed from one slice per rat (number = n). Data were recorded using an Axopatch 200 (for whole cell) or Axoclamp 2B (for extracellular) amplifier (Axon Instruments, Foster City, CA), monitored and analyzed online and reanalyzed offline (Anderson and Collingridge, 2001). Data pooled across slices are expressed as the mean ± SEM, and effects of conditioning stimulation were measured between 20 to 25 min after induction of LTD or 60 min after induction of LTP. Significance ($p < 0.05$) from baseline was tested using two-tailed t tests.

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