

Hippocampus-dependent learning facilitated by a monoclonal antibody or D-cycloserine

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PERSISTENT neuronal plasticity, including that observed at some hippocampal synapses, requires N-methyl-D-aspartate (NMDA)-mediated transmission. NMDA receptor activation may be necessary for hippocampus-dependent learning as antagonists block acquisition in many such tasks. The behavioural effects of NMDA agonists are less well defined. We have shown that a monoclonal antibody (B6B21) displaced [³H]-glycine that was bound specifically to the NMDA receptor, and enhanced the opening of its integral cation channel in a glycine-like fashion, effects that were competitively antagonized by 7-chlorokynurenic acid¹. B6B21 also enhanced long-term potentiation in hippocampal slices¹. We report here that intraventricular infusions of B6B21 significantly enhances acquisition rates in hippocampus-dependent trace eye blink conditioning in rabbits, halving the number of trials required to reach a criterion of 80% conditioned responses. Peripheral injections of D-cycloserine, a partial agonist of the glycine site on the NMDA receptor which crosses the blood-brain barrier, also doubles rabbits' learning rates. Pseudoconditioning control experiments indicated a lack of nonspecific behavioural sensitization effects. Our data suggest that enhanced activation of the glycine coagonist site on the NMDA receptor/channel complex facilitates one form of associative learning and may be used in other learning tasks.

Our studies were designed to determine whether compounds with glycine-like activity *in vitro* can affect associative learning *in vivo*. Partial agonists were used to avoid induction of seizures and other pathological manifestations of excessive activation of NMDA receptors². A hippocampus-dependent learning task was used (that is, one abolished by hippocampal ablation³). The hippocampus is particularly rich in NMDA receptors⁴. Long-term potentiation of Schaffer collateral or perforant-path synap-

ses is blocked by competitive antagonists of the NMDA binding site⁵ or of its strychnine-insensitive glycine coagonist site⁶. Intracellular recordings from neocortical pyramidal cells indicate that exogenous glycine can enhance single NMDA-mediated excitatory postsynaptic potentials (e.p.s.ps)⁷. Long-term potentiation and other forms of neuronal plasticity may share common synaptic or subcellular mechanisms with associative learning⁸. NMDA antagonists (both competitive and non-competitive) inhibit acquisition in behavioural learning tasks at doses that do not impair performance^{9,10}, but few have demonstrated any beneficial effects of NMDA or glycine-site agonists. Most previous agonist studies have used simple tasks that are affected non-selectively by a wide range of neurotransmitter systems¹¹.

In the first set of experiments, lateral ventricular guide canulae were surgically implanted bilaterally in rabbits, which were then fitted with restraining head bolts. After recovery and environmental habituation, rabbits were trained in pairs in separate, darkened and sound-attenuated chambers. Training continued until the criterion level of 80% of paired stimulus presentations resulted in the conditioned response (80% CR).

Intraventricular infusion of B6B21 greatly accelerated acquisition of trace conditioning as compared with either IgG or artificial cerebrospinal fluid control treatments ($F_{2,5} = 15.14$, $P < 0.0003$; see Fig. 1a). In every case, B6B21-treated animals learned much more rapidly than their paired controls. Learning-curve slopes did not differ significantly between the control groups (Scheffe F -test, $F = 0.068$; $P > 0.5$). B6B21 treatment resulted in a 45% reduction in the number of trials required to reach 80% conditioned responses (one-way analysis of variance, $F_{2,15} = 5.601$; $P < 0.01$) and reductions in other operational measures of learning (Fig. 1b). No effects of B6B21 treatment on the unconditioned response amplitude during conditioning were observed ($P < 0.68$).

After conditioning, extinction with conditioned stimulus presentation alone was carried out for an additional three days. All rabbits returned to a low level of conditioned response performance at about the same rate, and there was no effect of B6B21 treatment on the percentage of conditioned responses on the three days of testing ($F_{1,12} = 0.51$; $P > 0.45$). But a more subtle result of B6B21 treatment was observed when extinction was analysed in 10-trial blocks. Rabbits commonly exhibit a phenomenon (shown by controls) termed 'spontaneous recovery'¹², in which the percentage of conditioned responses in early blocks of trials on each successive day return briefly to levels near 'criterion' (80% CR) performance, followed by a

TABLE 1 Results of pseudoconditioning experiments

Treatment group	Unpaired CS trials				Unpaired US trials			
	Responses to CS (CR)		Responses after trace interval no US presented (false UR)		Responses with no CS presented before US onset (false CR)		Responses to US (UR)	
	Responses (Per cent of trials)	Response amplitude (mV)	Responses (Per cent of trials)	Response amplitude (mV)	Responses (Per cent of trials)	Response amplitude (mV)	Responses (Per cent of trials)	Response amplitude (mV)
B6B21	11.0 ± 4.5	2,705 ± 810	8.3 ± 4.1	2,247 ± 428	6.3 ± 3.2	2,408 ± 497	99.2 ± 6.4	3,290 ± 798
Control	15.2 ± 3.6	2,716 ± 613	9.8 ± 5.2	2,291 ± 511	5.9 ± 2.8	2,372 ± 658	96.1 ± 8.3	3,244 ± 877
D-Cycloserine	11.4 ± 6.8	2,522 ± 708	9.1 ± 3.6	2,193 ± 693	7.9 ± 4.2	2,356 ± 528	97.2 ± 3.8	2,995 ± 254
Control	16.0 ± 10.2	2,344 ± 344	7.3 ± 3.7	2,310 ± 648	8.7 ± 2.9	2,513 ± 502	97.5 ± 4.2	2,878 ± 260

CS, US, conditioned and unconditioned stimulus, respectively; CR, UR, conditioned and unconditioned response, respectively. Experiments were done with different groups of rabbits (4 per group). The nictitating membrane response was measured with an optical detector as in earlier studies (refs 3, 24, 36). Treatment with glycine partial agonists had no non-associative effects on eye-blink responses (data shown are mean ± s.e.m.). The same stimuli used in conditioning were presented unpaired at pseudorandom intervals 80 times each per session for 10 daily sessions. Alterations in unconditioned nictitating membrane responses (far right columns) would be indicative of nonspecific motor effects that might alter performance. All unconditioned response measurements including number of responses, response amplitude, and response latency (not shown) were unaffected by treatment with B6B21 or D-cycloserine (2 factor repeated measures ANOVA, $P > 0.43$). Changes in behavioural responses to the unpaired tone (far left columns) would be indicative of altered sensitivity to the tone, and might alter the salience of the CS when paired with a US. No measurable changes in responses to the unpaired tone were observed ($P > 0.38$). Nonspecific changes in behavioural excitability would be indicated by alterations in spontaneous eye-blink responses during periods when no stimuli were presented (including those shown in the middle four columns). Again, no significant differences in these measures were observed ($P > 0.59$).

rapid decline in response to the conditioned stimulus alone. B6B21-treated rabbits failed to recover spontaneously (Fig. 2a). This may indicate a more robust 'memory' of the previous day's experience, though the overall impact on extinction, a process that occurs very rapidly, was minimal.

Other groups of rabbits underwent pseudoconditioning with unpaired stimuli presented for equal numbers of trials for ten days, to test whether treatment with B6B21 produced nonspecific sensitization of responses to either the unpaired tone conditioned stimulus or the airpuff unconditioned stimulus. No differences were found in comparisons between B6B21 and control treated rabbits during pseudoconditioning (Table 1). Antibody B6B21 facilitated learning without altering response rates to the unpaired conditioned or the unconditioned stimulus, and without changing the behavioural characteristics of the small number of eye-blink responses to the unpaired tone or of the consistent and large number of responses to the air-puff.

A second series of studies was carried out using D-cycloserine (D-4-amino-3-isoxazolidone) a rigid analogue of D-alanine which acts as a partial agonist of the glycine coagonist site on the NMDA receptor¹³. It readily crosses the blood-brain barrier and is excreted unmetabolized¹⁴. As a partial agonist competing with glycine to bind at the glycine site on the NMDA receptor, D-cycloserine is less effective than the native agonist, yet still demonstrates reasonable specificity and high affinity for the receptor¹⁵.

For these studies, rabbits were surgically fitted with restraining headbolts. After recovery and environmental adaptation, pairs were trained using the same trace eye-blink conditioning task. Intramuscular injections of D-cycloserine (6 mg per kg body mass, given daily immediately before training) greatly acceler-

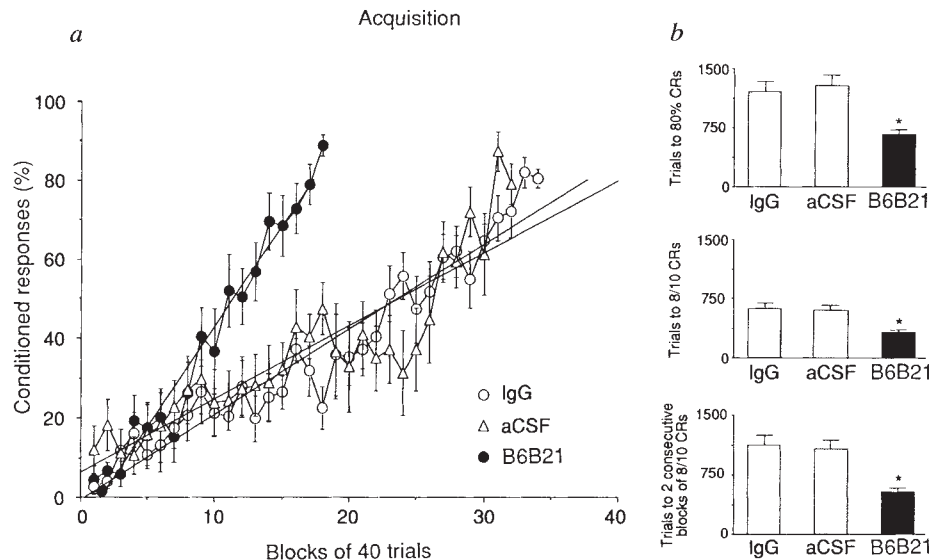
ated response acquisition. These rabbits exhibited learning curve slopes nearly twice as steep as those for controls (Fig. 3a; $F_{1,12} = 6.59$, $P < 0.02$) and the number of trials required to attain a level of performance of 80% CRs was reduced by 43% as compared with controls (one-way analysis of variance, $F_{1,12} = 6.04$, $P < 0.03$; Fig. 3b). Treatment with D-cycloserine had no effect on unconditioned response amplitude in conditioning ($P > 0.79$). Preliminary trials with a lower dose of D-cycloserine (3.0 mg per kg body mass) indicated a similar, though less effective enhancement of learning.

Treatment with D-cycloserine did not alter the percentage of CRs observed over three days of extinction of learned responses ($F_{2,15} = 0.002$; $P > 0.99$). But as in the earlier study, D-cycloserine-treated rabbits failed to recover spontaneously between successive days of extinction (Fig. 2b), demonstrating more uniform progressive extinction than controls. In pseudoconditioning studies, D-cycloserine-treated rabbits showed no evidence of sensitization or motor effects compared with saline-treated controls ($P > 0.38$; Table 1), which is similar to the results obtained with B6B21.

B6B21 and D-cycloserine greatly accelerated acquisition of trace eye-blink conditioning. Both act as partial agonists of the glycine site^{1,13,15}, and both facilitate NMDA-receptor mediated postsynaptic events^{1,16}. Although these molecules have quite different chemical structures, the epitope recognized by a monoclonal antibody may be equivalent to 3–6 amino acids¹⁷, thus it is probable that the glycine-like actions of both agents are mediated by interactions at the same recognition site. B6B21 competes for binding, in extensively washed hippocampal membrane preparations, with both the native agonist and with 7-chlorokynurenic acid, a competitive antagonist¹. B6B21

FIG. 1 Trace eye-blink conditioning was facilitated by daily intraventricular infusions of the monoclonal antibody B6B21. *a*, Mean learning curves (\pm s.e.m.) and least-squares fitted lines for each curve are shown, expressed as the percentage of conditioned responses per block of 40 training trials. Using the linear interpolation algorithms of Igor (WaveMetrics, Lake Oswego, Oregon), each curve was normalized to the mean number of trials required to reach the criterion (80% CR) level for that group, so that qualitative summaries of learning rates for animals requiring different numbers of trials to reach criterion could be made. For statistical purposes, the slopes of individual non-normalized learning curves were compared, indicating that B6B21 treated rabbits learned significantly faster ($P < 0.003$) than rabbits in either control group (whose learning curves overlapped). *b*, Comparisons of the number of trials required to reach the criterion level, number of trials to reach a simpler criterion of 8/10 conditioned responses, and number of trials to gain two consecutive blocks of 8/10 conditioned responses all indicated significant facilitation of learning in the trace conditioning task by B6B21 ($P < 0.01$)*.

METHODS. Monoclonal antibodies were made against freshly dissected 5 day postnatal rat dentate gyri and purified using Protein A-Sepharose chromatography^{1,35}. Eluates were immediately neutralized, dialysed, and concentrated. Concentrated antibody (typically 1–2 ml) was again dialysed against two changes (each 2 l) of HEPES buffer to remove endogenous glycine contamination. The antibodies were sterilized by filtration through a 0.22- μ m filter and frozen at -80°C in small aliquots. All antibody injections were given under sterile conditions. Neither SDS-polyacrylamide gel electrophoresis nor low molecular mass high-performance liquid chromatography (HPLC) of purified antibodies revealed any glycine-like contaminants (our unpublished data). Specifically, no glycine or other α -amino acid contaminants were detected either in fresh samples of the antibody or in aliquots of the solutions infused intraventricularly, even when samples were



subjected to several freeze-thaw cycles (minimal HPLC detection threshold of 0.1 pmol per injection). Commercial IgG antibodies were used which had no glycine-like activity, and gave similar negative results from HPLC. Cannulated rabbits were always paired with an animal from one of the other two groups, with six animals used in each group. For 5 min immediately before each day's training, rabbits simultaneously received 5 μ l infusions in each ventricle of either B6B21 (1 μ g μ l⁻¹) suspended in artificial cerebral spinal fluid (aCSF; composition, in mM: 124 NaCl; 26 NaHCO₃; 3 KCl; 2.4 CaCl₂; 1.3 MgSO₄; 1.24 NaH₂O₄; 10 D-glucose (pH 7.4)), of aCSF alone, or of mouse IgG (1 μ g μ l⁻¹ Sigma) in aCSF at a rate of 1 μ l ventricle⁻¹ min⁻¹. The treatment received by the subjects was unknown to the trainer. Trace nictitating membrane conditioning (using a 100 ms duration, 6 kHz, 85 db binaural tone conditioned stimulus (CS) followed, after a 500 ms interstimulus trace interval by a 150 msec corneal air-puff unconditioned stimulus (US)) began immediately after infusion with 80 trials per day. For details of the protocols used, see refs 3, 36).

enhances NMDA-stimulated noradrenaline release in hippocampal slices, an effect also competitively antagonized by 7-chlorokynurenic acid (P. E. Potter and J. R. Moskal, unpublished observations). *In vitro*, both B6B21 (ref. 1) and D-cycloserine^{13,15,17} functionally mimic the effects of glycine and other α -amino acids, with demonstrable specificity and partial agonist efficacy. Unfortunately, many antagonists (including HA-966 (ref. 18) and various kynurenines, such as 5,7-dichlorokynurenic acid; T. Lanthorn, personal communication) are extremely toxic *in vivo*, confounding efforts to demonstrate adverse effects of glycine site antagonists on learning or other functions. Although such demonstrations are lacking in *in vivo* preparations, evidence from *in vitro* studies are consistent with the working hypotheses presented here^{1,6,13,15,16}.

Our data support the hypothesis that glycine-like partial agonists enhance some learning processes^{11,19,20} and suggest that, under normal conditions, NMDA receptor glycine sites are not saturated. The mechanisms involved in the uptake, release and buffering of glycine in extracellular spaces within the central nervous system are unclear, but our data further suggest that activation of NMDA receptors *in vivo* can be enhanced exogenously. Treatment with partial agonists like B6B21 or D-cycloserine could moderately enhance submaximal postsynaptic events or recruit additional synapses. Because the net change would yield greater NMDA-mediated responses throughout the learning process, the rate of learning would be accelerated. This interpretation is consistent with the facilitation of hippocampal long-term potentiation by low concentrations of glycine-site agonists²¹ or of partial agonists including B6B21 (ref. 1). Persistent postsynaptic changes in the excitability of hippocampal pyramidal neurons are seen after delay²² and trace²³ eye-blink conditioning. Although direct evidence linking behavioural changes to specific synaptic mechanisms is scarce, numerous authors have postulated the involvement of NMDA receptor-mediated plasticity in the induction, if not the expression, of learning^{8,9,10,16,19,20}.

The hippocampus may be an important locus of the behavioural effects of the glycine-like partial agonists reported here. Our observations that both B6B21 and D-cycloserine produce similar facilitation in this hippocampus-dependent task is consistent with this hypothesis, but does not eliminate the possibility that other sites of action may also be involved. Trace conditioning requires the formation of a short-term 'memory trace' of the conditioned stimulus to bridge the interstimulus interval between conditioned and unconditioned stimulus to successfully form an association. The hippocampus may serve this and other mnemonic functions^{3,24,26}. Acquisition of the 500-ms trace eye-blink conditioning task used here is blocked by hippocampal lesions in rabbits^{3,24}. When B6B21 was injected into the lateral ventricles, this large glycoprotein (M_r 150K) was unlikely to penetrate distal structures within a reasonable time for action^{27,28}. But the ventricular system gave relatively direct access to the hippocampus. Similarly, although D-cycloserine given peripherally reaches many brain areas, NMDA receptors are most abundant in the hippocampus⁴. Though hippocampal function is not an absolute requirement for all forms of learning, enhancement of hippocampal function may be of benefit even in tasks that are not dependent on the hippocampus. The effects of glycine-site partial agonists on the persistence of memory are currently unknown, as are the critical periods when treatment is effective both on learning and memory. These and other questions warrant further study.

The monoclonal antibody B6B21 may be valuable in further characterizing the structure and function of the NMDA receptor-ionophore complex. Moreover, by obtaining sequence information on the variable region of B6B21, it may be possible to generate biologically active peptides or mimetics of greater potency and specificity, and thus greater clinical relevance^{27,29}. As eye-blink conditioning tasks in rabbits and humans have important behavioural and neurobiological parallels^{30,31}, our experiments should be relevant to the role of the NMDA receptor glycine coagonist site in some forms of human learning.

FIG. 2 Glycine site partial agonists had more subtle effects upon extinction than upon acquisition of learned responses. *a*, Conditioned eye-blinks by control and B6B21-treated rabbits seemed to extinguish at nearly identical rates, with subjects in all groups returning to naive performance levels within 3 daily sessions (80 trials per session) of CS presentation alone. An important difference is that B6B21-treated rabbits failed to show the spontaneous recovery¹² of CRs shown by control animals (arrows). After one day of extinction, in the next block of 10 trials, controls exhibited a recovery followed by a rapid decrease in the number of CRs, to a level that was successively lower than that observed on the previous day. Extinction for controls was not a simple linear process, but instead entailed behavioural curves resembling 'saw-tooth' functions. Thus, for the controls on the second and third day of extinction the number of CRs in the first block of 10 trials was significantly higher than for the last block of 10 trials in that same session (extinction: second day, one-way paired *t*-test, $t=2.84$, $P<0.02$; third day, $t=3.84$, $P<0.01$) and also compared to the last block in the preceding session (spontaneous recovery: second day, $t=3.13$, $P<0.02$; third day, $t=2.38$, $P<0.03$). But for B6B21-treated rabbits only the former comparison was true (second day, $t=3.31$, $P<0.01$; third day, $t=2.91$, $P<0.02$; extinction alone occurred). B6B21-treated rabbits exhibited no spontaneous recovery ($P>0.14$), but instead exhibited extinction curves that were linear functions of the number of non-paired trials. *b*, D-cycloserine treatment also resulted in a loss of spontaneous recovery during extinction of conditioned eye-blink responses (arrows). Control rabbits exhibited both extinction (second day, $t=2.47$, $P<0.03$; third day, $t=2.89$, $P<0.01$) and spontaneous recovery (second day, $t=2.91$, $P<0.01$; third day, $t=2.52$, $P<0.02$) for CS-alone trials whereas D-cycloserine-treated rabbits exhibited extinction alone (second day, $t=3.29$, $P<0.01$; third day, $t=3.36$, $P<0.01$) with no spontaneous recovery ($P>0.18$). Again, first order functions described the extinction curves of D-cycloserine-treated but not those of control rabbits.

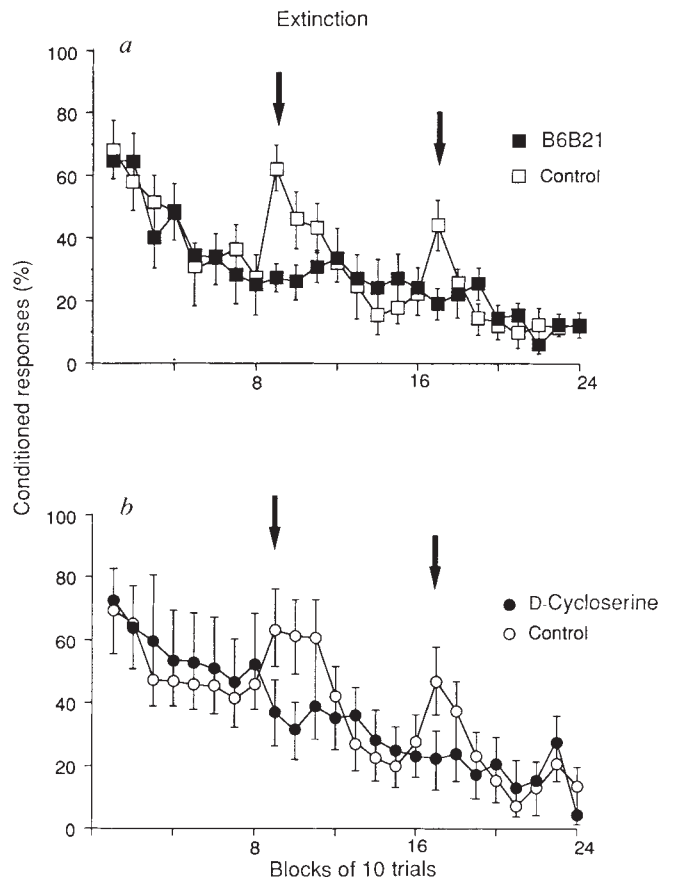
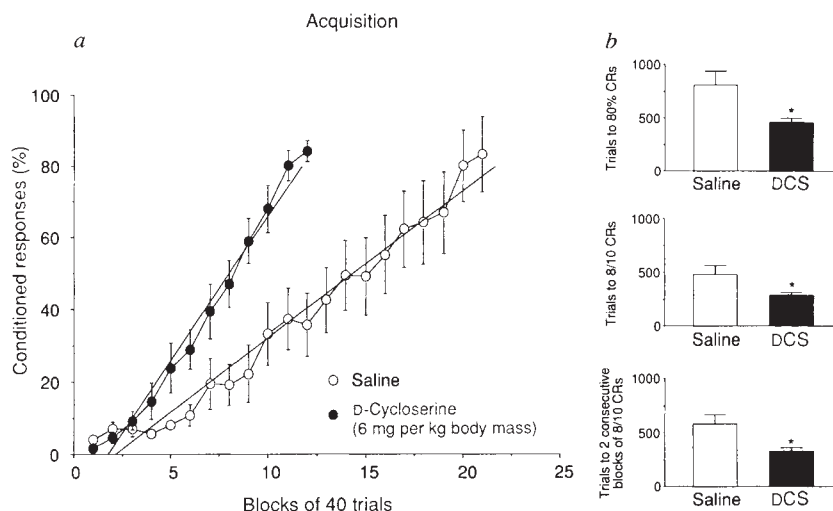


FIG. 3 Daily treatment with D-cycloserine, partial agonist of the glycine coagonist site on the NMDA receptor, also facilitated trace eye-blink conditioning in rabbits ($n=7$ per group). *a*, D-cycloserine significantly accelerated the learning rates of rabbits as compared with saline-treated controls ($P < 0.02$). The learning curves shown were normalized (as in Fig. 1) to the mean trials to the criterion level for each group, to allow qualitative comparisons between groups. *b*, D-cycloserine significantly reduced the number of trials required to acquire successfully the trace eye-blink conditioning task ($P < 0.01$)*. Note the similarities in behavioural facilitation obtained with these two chemically dissimilar compounds. Both glycine partial agonists reduced the training required to learn successfully the eye-blink task by nearly half, as compared with controls. Normal adaptive CRs were exhibited by the rapidly learning animals, with nictitating membrane extension beginning shortly before air-puff onset, to protect the cornea from the US. A large number of double blinks by B6B21 and D-cycloserine-treated but not control rabbits were observed within the trace interstimulus period (over 50% of all CRs by the final training session). The first of these double blinks typically occurred shortly after CS onset, the second with a normal response latency (≤ 150 ms before air-puff US onset). Other evidence (E. Akase and J. F. Disterhoft, unpublished data) suggests that these robust double blink responses may be driven by enhanced neural activity in the hippocampus during the trace interval. We have noted a novel short-latency burst of activity by rabbit hippocampal CA1 single-units during or shortly after CS



presentation in trace conditioning, followed by a long-latency burst of firing during the CR that matched earlier reports of behavioural modelling by hippocampal neurons during delay conditioning³⁷. The behavioural responses suggest that hippocampal function during the trace interval is enhanced by both compounds. The horizontal scale differs from Fig. 1, as rabbits without intraventricular cannulae (D-cycloserine studies) learned faster than those with cannulae.

B6B21, D-cycloserine and other glycine-like analogues should be useful in further study of the role of NMDA receptors in learning, as well as the functional deficits associated with ageing³², ischaemia^{2,33} and Alzheimer's dementia³⁴. □

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Deletion polymorphism in the gene for angiotensin-converting enzyme is a potent risk factor for myocardial infarction

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FACTORS involved in the pathogenesis of atherosclerosis, thrombosis and vasoconstriction^{1,2} contribute to the development of coronary heart disease. In a study comparing patients after myocardial infarction with controls, we have explored a possible association between coronary heart disease and a variation found in the gene encoding angiotensin-converting enzyme (ACE). The polymorphism ACE/ID is strongly associated with the level of circulating enzyme³. This enzyme plays a key role in the production of angiotensin II and in the catabolism of bradykinin, two peptides involved in the modulation of vascular tone and in the proliferation of smooth muscle cells. Here we report that the DD genotype, which is associated with higher levels of circulating ACE than the ID and II genotypes, is significantly more frequent in patients