Universidade Federal do Rio Grande do Sul
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Papel das Enzimas PKA e CaMKII na Formação da Memória

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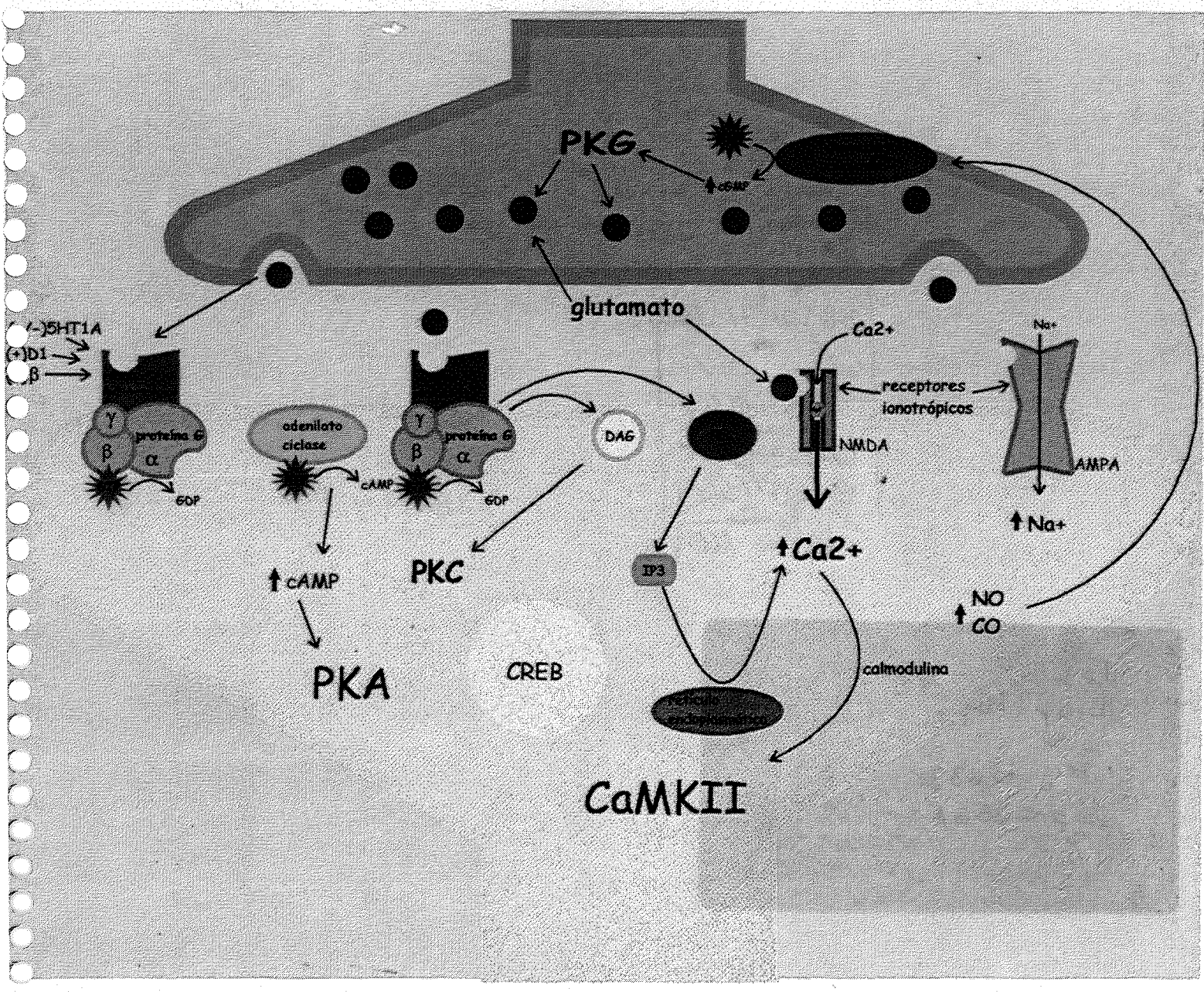
Dissertação de Mestrado



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Índice

Abreviaturas

ACTH - hormônio adrenocorticotrópico
AMI - amígdala
ATP - adenosina 5'-trifosfato
AMPA - receptor ionotrópico sensível ao ácido α-amino-3-hidroxi-5-metil-4-isoxazolone propiônico
8-Br - 8-Br-cAMP, análogo do cAMP
Ca^{2+} - íon cálcio
CA1 - região do hipocampo
CaMKII - proteína quinase dependente de Ca2+/calmodulina
cAMP - adenosina 3', 5'-monofosfato cíclico
CE - córtex entorrinal
CEC - choque eletroconvulsivo
cGMP - guanosina 3', 5'-monofosfato cíclico
CNQX - 6-ciano-7-nitroquinoxalina-2, 3-diona
CREB - elemento responsivo à união do cAMP
DAG - diacilglicerol
DPAT - 8-hidroxi-2-(di-η-propilamina) tetralina
EI - esquiva inibitória
β-end - β-endorfina
ERK - quinase regulada extracelularmente
ESTAU - estaurosporina
$GABA_A$ - ácido γ-aminobutírico tipo A
HAB - habituação
HAB 'WF" - habituação com "water finding"
HIPO - hipocampo
IP3 - "inositol 1, 4, 5- trifosfato
K^+- íon potássio
KN - 1-(*N*-*O*-bis(5-isoquinolinasulfonila-*N*-metil-L-tirosila)-4-fenil piperazina
KT - $C_{32}H_{31}N_3O_5$ hexilester de K252a
KT5823 - KT5823
LAV 0.1 - lavendustina 0,1 μg,lado
LAV 0.5 - lavendustina 0,5 μg,lado
LTM - memória de longa duração
LTP - potenciação de longa duração
LY - LY83,583

MAPK - proteína quinase ativada por mitogênio
Mg^{+} - íon magnésio
mGluR - receptor glutamatérgico metabotrópico
MUS - muscimol
NAN - 1-(2-metoxifenil)-4-(4-(2-ftalimido))butilpiperazina
NE - norepinefrina
NMDA - N-metil-D-aspartato
NO - oxido nítrico
PD - PD098059
PKA - proteina quinase dependente de cAMP
PKC - proteina quinase dependente de Ca^{2+}-diacilglicerol
PLC - fosfolipase C
SCH - 7-cloro-2,3,4,5-tetrahidro-3-metil-5-fenil-1-*H*-3-benzazepina 7-ol
SKF - 2, 3, 4, 5-tetrahidro-7, 8-dihidroxi-1-fenil-1 *H*-3-benzazepina hidroclorídio
STM - memoria de curta duração
TIM - timolol
VASO - vasopressina

Resumo

Ratos machos *Wistar* foram submetidos ao implante bilateral de cânulas na região CA1 do hipocampo, no córtex entorrinal e na amígdala.

Os animais foram expostos à uma sessão de treino em diversas tarefas comportamentais e receberam infusões bilaterais de diversas drogas nas estruturas citadas acima em horários diferentes ou i.p. 10 min. antes do treino.

As drogas usadas foram àquelas que atuam diretamente nas duas enzimas (KT5720, KN62) ou indiretamente através de agonistas e antagonistas de receptores que ativam as cascatas metabólicas iniciadas pela PKA e pela CaMKII (SKF38393, SCH23390, norepinefrina, timolol, muscimol, 8-HO-DPAT, NAN-190, CNQX) e afetam passos para a ativação destas enzimas (8-Br-cAMP, LY83583) ou também que atuam em outras cascatas metabólicas (lavendustina A 0,1, lavendustina A 0,5, KT5823, estaurosporina, PD098059).

Os testes foram feitos 24 h após o treino ou em duas etapas: 1,5 h e 24 h após o treino, afim de medir a Memória de Curta Duração (STM) e a Memória de Longa Duração (LTM).

Os resultados dos experimentos demonstraram que as enzimas PKA e CaMKII atuam em tempos e formas diferentes na formação da memória nas três estruturas cerebrais e que a STM é um processo separado da LTM.

Introdução

O hipocampo (HIPO), a amígdala (AMI) e o córtex entorrinal (CE) estão interconectados por duas vias diferentes e, provavelmente, o papel destas três estruturas na formação da memória, principalmente aversiva, ocorra de maneira coordenada (21,24). O HIPO processa informações espacial e contextual (17,27), a AMI faz o mesmo com as informações de caráter emocional ou aversivo (27) e o CE integra as informações procedentes do HIPO e da AMI após a aprendizagem (53).

A aprendizagem de uma tarefa de esquiva inibitória (EI), dispara no HIPO do rato uma série de cascatas bioquímicas, as quais são necessárias para a memorização da mesma. Estes processos bioquímicos são similares tanto para os descritos para as fases de indução e manutenção da Potenciação de Longa Duração (LTP), como para outros tipos de mecanismos de plasticidade neuronal (4,9,16,34,35). A LTP representa uma modificação duradoura da eficiência sináptica, a qual tem sido proposta como um modelo molecular para o estudo de certas formas de memória (10).

As cascatas bioquímicas mencionadas acima, dão início à fosforilação proteica, um processo chave que regula a função celular (44).

As proteína quinases são enzimas que usam adenosina 5'-trifosfato (ATP) como doador do grupo fosfato (32) e são classificadas como proteína quinase serina-treonina, que fosforila substratos proteicos nos resíduos serina e treonina e como proteína quinase tirosina, que fosforila substratos proteicos no resíduo de tirosina. O íon magnésio (Mg^{+}), é requerido para esta reação (41). A introdução de um grupo fosfato, carregado negativamente, pode alterar a

conformação de uma proteína para que esta modifique a função de um substrato (e.g. enzimas, proteínas do citoesqueleto, subunidade de um canal de íon, ativador transcripcional).

As consequências da ativação de proteína quinases são diversas e incluem: potenciação ou redução da corrente mediada por receptor e desensitização de receptores (43). A fosforilação de receptores parece ser necessária para a manutenção da integridade funcional de alguns receptores (41).

As proteína quinases melhores estudadas no cérebro são todas aquelas ativadas pelo sistema de segundo mensageiros como a proteína quinase dependente de cAMP (PKA), proteína quinase dependente de cálcio/calmodulina tipo II (CaMKII), proteína quinase dependente de cGMP (PKG), proteína quinase dependente de cálcio,diacilglicerol (PKC), proteína quinase ativada por mitogênio (MAPK) (41).

Portanto, proteína quinases dependentes de sistemas de segundos mensageiros, não somente modificam proteínas pré existentes, como também podem induzir a síntese de novas proteínas, alterando a expressão gênica, podendo levar a outras mudanças durando dias que, provavelmente, são importantes para o desenvolvimento neuronal e para a LTM (32).

Oportunidades de interação ou *crosstalk* ocorrem, porque enzimas individuais, canais de íon ou enzimas do citoesqueleto podem ser modificadas em mais de um sítio na molécula por proteína quinases dependentes de diferentes segundo mensageiros (32).

Ativação da PKA e da CaMKII

O aminoácido glutamato é o transmissor excitatório mais abundante no cérebro e espinha dorsal (23,25). Ao ser liberado na fenda sináptica pela despolarização da membrana neuronal pré-sináptica, o glutamato se liga aos receptores ionotrópicos que produzem uma resposta sináptica rápida e aos receptores metabotrópicos que produzem uma resposta sináptica mais lenta.

Estes receptores pós-sinápticos são:

•o receptor ionotrópico AMPA (sensível ao ácido α-amino-3-hidroxi-5-metil-4-isoxazolone-propiônico) cuja ativação permite a entrada de íons sódio na célula, causando despolarizão da membrana;

•o receptor ionotrópico NMDA (sensível à N-metil-D-aspartato) que, com a membrana despolarizada, expulsa o íon Mg^{+} que bloqueia seu canal, fazendo com que haja uma grande entrada de íons cálcio (Ca^{2+}) na célula;

•o receptor metabotrópico que, através da proteína G, estimula a adenilato ciclase, que aumenta os níveis de cAMP (adenosina 3', 5'-monofosfato cíclico) e, através da fosfolipase C (PLC), ativa o inositol 1, 4, 5-trifosfato (IP3), que mobiliza as reservas de Ca^{2+} do retículo endoplasmatico, contribuindo no aumento dos níveis de Ca^{2+} intracelular.

O aumento do cAMP ativa a PKA e o Ca^{2+} se liga à calmodulina ativando a CaMKII.

Atividade da PKA

A PKA é composta por subunidades catalítica e regulatória (onde se ligará o cAMP). Esta enzima fosforila os aminoácidos serina ou treonina em sítios específicos em várias proteínas (41), entre as quais, canais de Ca^{2+}, fosfolipase C (PLC) (que modulam a concentração de cálcio intracelular), proteína quinase quinase ativada por mitogênio (MAPKK) (2), elemento responsivo à união do cAMP (CREB), receptores glutamatérgicos AMPA (43), receptores noradrenérgicos β (atuando como *feedback* negativo).

A atividade da PKA tem dois picos. Um à 0 h e outro de 3 a 6 h após o treino em tarefa de EI (26).

Os sistemas modulatórios que têm receptores ligados à proteína G, que regulam a atividade da PKA através da estimulação ou inibição da adenilato ciclase são os noradrenérgico (receptores β) e dopaminérgico (receptores D1) que estimulam e o serotoninérgico (receptores 5HT1A) que inibe sua atividade entre 3 e 6 h após o treino em EI (5,8). Em *Aplisia*, a serotonina produz uma excitação sináptica lenta, fechando os canais de K+, ativando a produção de cAMP (41).

A PKA pode ser ativada indiretamente, através da estimulação da adenilato ciclase tipo II, abundante no hipocampo e córtex, pela PKC (54).

Atividade da CaMKII

A enzima CaMKII tem um domínio regulatório que, em estado basal, se liga ao domínio catalítico, inibindo-o. Esta inibição é desfeita, quando o Ca2+/calmodulina liga-se ao domínio regulatório/ ativando a enzima (41).

A CaMKII é a proteína quinase dependente de Ca2+/calmodulina melhor caracterizada de sua família. Esta enzima fosforila tirosina hidroxilase, sinapsina I, canais de Ca2+, fatores de transcrição e receptores glutamatérgicos, entre outras proteínas (44). O receptor Ras é fosforilado pela CaMKII, ativando a cascata da MAPK (41).

A atividade da CaMKII mantém-se alta nas 2 h seguintes ao treino em tarefa de EI e seu pico é visto imediatamente após o treino (6).

Objetivos

Este trabalho tem por objetivo analisar o papel das enzimas PKA e CaMKII na formação da memória, através de infusão intracerebral em vários tempos, no hipocampo, córtex entorrinal e amígdala, de agonistas e antagonistas de receptores envolvidos, direta ou indiretamente, em suas cascatas; bem como de ativadores e inibidores destas enzimas na memória de tarefas diversas.

O curso dos experimentos 1, 2 e 3, nos levou a estudar a divisão da memória STM e LTM. Define-se a STM, como àquela que se encarrega da função mnemônica durante o longo período de formação da LTM, que é de 3 a 6 h após o treino em tarefa de EI (26,37).

Conseguimos demonstrar que a STM e a LTM obedecem a processos separados, respondendo, assim, uma pergunta de mais de 100 anos (31,37).

Material e Métodos

Animais de Experimentação

Foram usados ratos machos Wistar, de 2,5 a 3 meses de idade, pesando entre 220 e 340 g, provenientes dos Biotérios do Instituto de Ciências Básicas da Saúde da UFRGS e do Departamento de Bioquímica da UFRGS. Os animais receberam água e ração padronizada "ad libitum" e foram submetidos a um ciclo claro,escuro de 12 horas. A temperatura foi mantida constante em torno de 23° C.

Implantação de Cânulas

Os animais foram anestesiados com tionembutal e submetidos ao implante bilateral de cânulas-guia de aço inoxidável calibre 30-gauge, 1 mm acima das seguintes estruturas:

- junção entre o núcleo central e basolateral da amígdala, AMI (A-2.3, L+4.5, V5>4);
- área CA1 do hipocampo dorsal, CA1 (A-4.3, L+4.0, V3.4);
- córtex entorrinal, CE (A-7.0, I5.0, V8.4).
- As coordenadas estão em mm, de acordo com o Atlas de Paxinos e Watson (42) e são idênticas às empregadas em estudos anteriores (27).

Tarefas

Tarefa de Esquiva Inibitória (EI). O aparato da EI consiste em uma caixa de madeira com a parte frontal de acrílico transparente, medindo 50x25x25 cm. O assoalho da caixa é uma grade de barras de bronze paralelas de 0,1 cm de calibre, separadas entre si por 1 cm. Na parte esquerda, sobre a grade, há uma plataforma de fórmica com 7 cm de largura e 2,5 cm de altura.
A tarefa consiste em colocar o animal na plataforma, virado para o fundo da caixa até que ele desça para a grade. Neste instante, o animal recebe um choque de 0,3 ou 0,4 mA por 2 seg. O tempo da descida do animal com as quatro patas da plataforma na grade é chamado de latência. Na sessão de teste, 24 h após o treino, o choque é omitido e o teto da latência é de 180 seg. O aumento da latência entre as sessões de treino e teste, é usado como uma medida de memória (Fig. 1).

Tarefa de Habituação ao Campo Aberto (Hab). O aparato da Hab é uma caixa de madeira com a parte frontal de vidro transparente, medindo 60 cm de largura, 40 cm de profundidade e 50 cm de altura. O chão é dividido em 12 quadrados com linhas pretas.
A tarefa consiste em colocar o animal no quadrante esquerdo do fundo virado para o canto e deixar que ele explore a caixa livremente por 2 min. O tempo para sair do primeiro quadrante, o nº de cruzamentos das linhas pretas no chão e o nº de respostas de *rearing* são registrados. A diminuição do nº de cruzamentos e respostas de *rearing* entre as sessões de treino e teste, é usada como uma medida de habituação (Fig. 2).

Tarefa de Habituação ao Campo Aberto com *water finding* (Hab WF). O aparato da Hab WF é o mesmo descrito acima, porém com um tubo de metal adaptado na parede do fundo da caixa, a 10 cm do chão. Este tubo está acoplado a uma garrafa de vidro com água, colocada fora da caixa, idêntica àquelas usadas pelos animais diariamente.

Nesta tarefa, é medida a habituação e o tempo gasto para achar o tubo de água. Após o treino, os animais são deprivados de água até a sessão de teste, 24 h após. A diminuição da latência de achar o tubo de água entre as sessões, é usada como uma medida de memória desta tarefa (Fig. 3).

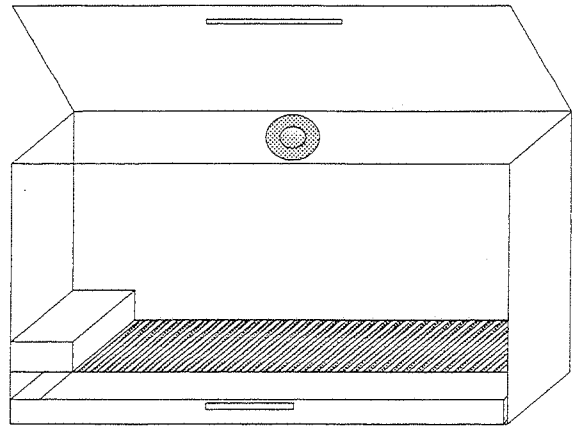

Fig. 1: Aparato de Esquiva Inibitória

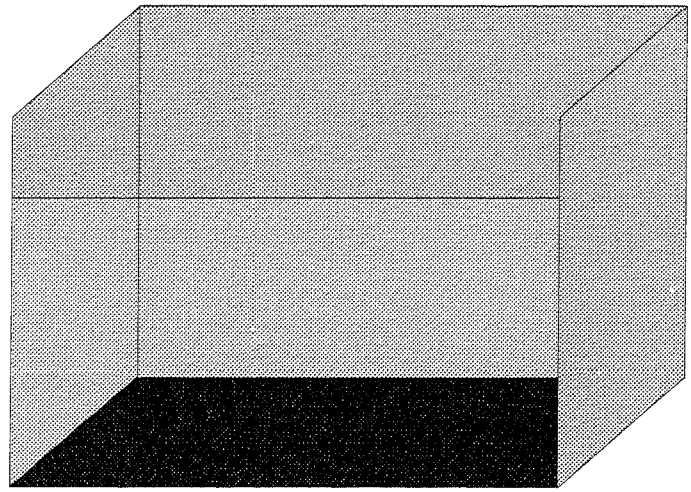

Fig. 2: Aparato de Habituação

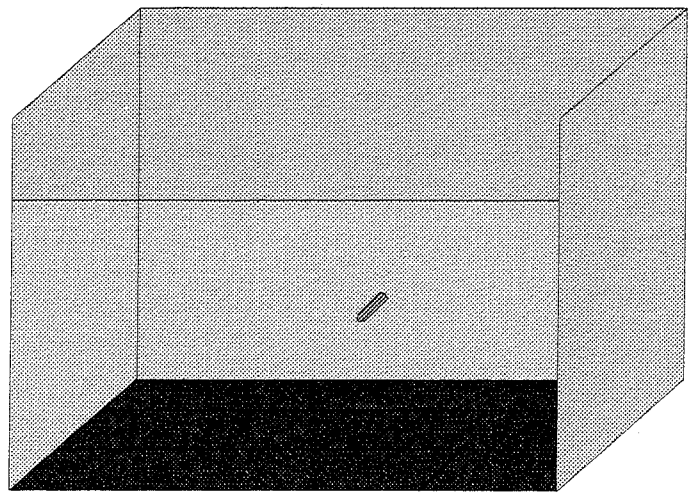

Fig. 3: Aparato de Habituação com "water finding"

Drogas

O volume de infusão de todas as drogas infundidas bilateralmente em qualquer das regiões acima citadas foi de 0,5 μl/lado e as injetadas i.p. foi de 1 ml/kg.

Drogas diluidas em salina (SAL)

ACTH - hormônio adrenocorticotrópico, $ACTH_{1-24}$ (0,2 μg/kg)

VASO - peptídeo neurohiposifário, lisina-vasopressina (10 μg/kg)

β-end - peptídeo opióide, β-endorfina (1 mg/kg)

SKF - SKF38393 - agonista de receptores dopaminérgicos tipo D1 (7,5 μg/lado)

SCH -SCH23390- antagonista de receptores dopaminérgicos tipo D1 (0,5 μg/lado)

NE - Norepinefrina C1H (0,3 μg/lado)

TIM - Timolol C1H - bloqueador de receptores noradrenérgicos tipo β (0,3 μg/lado)

PD - PD098059 - inibidor da proteína quinase ativada por mitogênio, MAPKK (0,05 μg/lado)

MUS - Muscimol - agonista de receptores gabaérgicos $GABA_A$ (ácido γ-aminobutírico tipo A) (0,5 μg/lado)

Drogas diluidas em veículo (VEI) (20% de dimetilsulfoxido em salina)

8-Br - 8-Br-cAMP - análogo do cAMP (adenosina 3',5' monofosfato) (1,25 μg/lado)

KT - KT5720 - inibidor da proteina quinase dependente de cAMP, PKA (0,5 μg/lado)

KN - KN62 - inibidor da proteina quinase dependente de Ca2+/calmodulina, CaMKII (3,5 μg/lado)

DPAT - 8-HO-DPAT (8-hidroxi-2-(di-η-propilamino) tetralina) agonista de receptores serotoninérgicos tipo $5HT1_A$ (5-hidroxi triptofano tipo A) (2,5 μg/lado)

NAN - NAN-190 (1-(2-metoxifenil)-4-(4-(2-fetalimido)) butilpiperazina) antagonista de receptores serotoninérgicos tipo $5HT1_A$ (2,5 μg/lado)

CNQX - (6-ciana-7-nitroquinoxalina-2, 3-diona), antagonista de receptores glutamatérgicos AMPA (sensível ao ácido α-amino-3-hidroxi-5-metil-4-isoxazolone propiônico) (0,5 μg/lado)

LY - LY83583 - inibidor da enzima guanilil ciclase (2,5 μg/lado)

LAV 0,1 - Lavendustina A 0,1 - inibidor da tirosina proteina quinase (0,1 μg/lado)

LAV 0,5 - Lavendustina A 0,5 - inibidor da proteina quinase dependente de cGMP (guanosina 3', 5'-monofosfato cíclico), PKG (0,5 μg/lado)

KT5823 - KT5823 - inibidor da proteina quinase dependente de cGMP, PKG (2 μg/lado)

ESTAU - Estaurosporina - inibidor da proteina quinase dependente de Ca^{2+},diacilglicerol, PKC (2,5 μg/lado)

Procedimento de Infusão

Uma cânula de infusão, calibre 30-gauge, acoplada a uma seringa *Hamilton*, foi introduzida primeiro em uma cânula-guia por 30 s, afim de que a droga difundisse no sítio de infusão e, logo após, na outra cânula.

Experimentos

Experimento 1

Os animais foram treinados e testados 24 h após em tarefa de EI. Os ratos de todos os grupos receberam, 10 min antes do teste, injeção i.p. de SAL ou ACTH ou VASO, dissolvidas em salina.

Grupo 1: os animais receberam injeção i.p. de SAL ou β-end imediatamente após o treino.

Grupo 2: os animais receberam choque eletroconvulsivo, CEC (15 mA, 60 Hz, 2 seg), transmitido através de eletrodos transcorneais, imediatamente após o treino. Nos ratos controles, foram colocados os eletrodos, porém o choque foi omitido.

Grupo 3: os animais receberam infusão bilateral no CA1 de VEI ou KN, imediatamente após o treino.

Grupo 4: os animais receberam infusão bilateral no CA1 de VEI ou KT, 3 h após o treino.

Experimento 2

Os animais treinados e testados 24 h após em tarefa de EI. No grupo 1, os animais foram implantados bilateralmente no CA1 e na AMI.

Grupo 1: os animais receberam infusão bilateral imediatamente após o treino, na AMI, de VEI ou KN e, 3 h após o treino, no CA1, de 8-Br, SKF, NE ou VEI.

Experimento 3

Os animais foram submetidos às tarefas de Hab WF e Hab, com duração de 2 min. cada e com intervalo de 24 h entre treino e teste em cada tarefa.

Grupo 1: os animais foram colocados na caixa de Hab WF e deixados explorando a área por 2 min. mais o tempo gasto no tubo de água. Imediatamente após o treino, os animais receberam infusão intrahipocampal de KN ou VEI e foram privados de água até a hora da sessão de teste.

Grupo 2: idem ao grupo 1, porém sem o tubo de água.

Grupo 3: os animais receberam infusão bilateral de KN ou VEI no CA1, foram privados de água por 24 h e expostos à uma sessão de Hab.

Experimento 4

Os animais foram submetidos à tarefa de EI.

Grupo 1: os animais receberam infusão bilateral imediatamente após o treino, no CA1 e CE, de SAL ou VEI ou CNQX ou MUS ou DPAT ou NAN e foram testados 1,5 h e 24 h após o treino, para medir a STM e a LTM, respectivamente.

Grupo 2: os animais receberam infusão bilateral imediatamente após o treino das drogas que deprimiram a STM nas duas estruturas (DPAT no CA1 e MUS, CNQX e NAN no CE) e foram testados 1,5, 3 e 4,5 h após o treino.

Experimento 5

Os animais foram submetidos à tarefa de EI.

Grupo 1: os animais receberam infusão bilateral imediatamente após o treino, no CA1 e no CE, de SAL ou VEI ou SKF ou SCH ou TIM ou DPAT ou NAN ou NE e foram testados 1,5 h e 24 h após o treino, para medir a STM e a LTM, respectivamente.

Grupo 2: os animais receberam infusão bilateral das drogas que deprimiram a STM nas duas estruturas (DPAT no CA1 e MUS, CNQX e NAN no CE), 6 min. antes do teste de STM e receberam infusão bilateral das drogas que tiveram algum efeito na STM nas duas estruturas (SKF, SCH e 8-HO-DPAT no CA1 e SKF, SCH, NE, TIM, DPAT e NAN no CE) 1,5 h antes do teste de LTM.

Experimento 6

Os animais foram submetidos à tarefa de EI.

Grupo 1: os animais receberam infusão bilateral imediatamente após o treino no CA1 e no CE de LY ou LAV 0,1 ou LAV 0,5 ou KT5823 ou ESTAU ou KN ou KT ou PD ou SAL ou VEI e foram testados 1,5 h e 24 h após o treino, para medir a STM e a LTM, respectivamente.

Grupo 2: os animais receberam infusão bilateral 10 min. antes do teste de STM, da droga que inibiu a STM sem afetar a LTM.

Experimento 7

Os animais foram submetidos à tarefa de EI.

Grupo 1: os animais receberam infusão bilateral de KT ou VEI a 0, 22 ou 45 min após o treino no CA1 e foram testados 1,5 e 24 h após o treino, para medir a STM e a LTM, respectivamente.

Grupo 2: os animais receberam infusão bilateral de KT ou VEI aos 90, 135 ou 175 min após o treino no CA1 e foram testados 3 e 24 h após o treino, para medir a STM e a LTM, respectivamente.

Resultados

Experimento 1

Grupo 1: a injeção pré-teste de ACTH ou VASO, reverteu o efeito amnésico causado pela administração de β-end imediatamente após o treino (Anexo 1, fig. 1).

Grupo 2: a injeção pré-teste de ACTH ou VASO, reverteu o efeito amnésico causado pelo CEC dado imediatamente após o treino (Anexo 1, fig. 1).

Grupo 3: a injeção pré-teste de ACTH ou VASO não reverteu o efeito amnésico causado pela infusão de KN, imediatamente após o treino (Anexo 1, fig. 2).

Grupo 4: a injeção pré-teste de ACTH ou VASO não reverteu o efeito amnésico causado pela infusão de KT, 3 h após o treino (Anexo 1, fig. 2).

Experimento 2

Grupo 1: a infusão no CA1 de 8-Br, NE e SKF, 3 h após o treino, causou um aumento da memória dos animais que receberam infusão na AMI de KN, imediatamente após o treino (Anexo 2, fig. 2).

Grupo 2: a infusão no CA1 de 8-Br, NE e SKF, 3 h após o treino, não alterou o efeito amnésico causado pela infusão de KN no CA1, imediatamente após o treino (Anexo 2, fig. 3).

Experimento 3

Grupo 1: a infusão bilateral no CA1 de KN, prejudicou a tarefa de *water finding*, mas não a tarefa de Hab (Anexo 3, fig. 1 e 2a).

Grupo 2: a infusão bilateral no CA1 de KN, prejudicou a tarefa de Hab (Anexo 3, fig. 2b).

Grupo 3: a performance de respostas de rearing e cruzamentos nesta sessão não foi diferente entre os grupos, mas foi mais baixa que na sessão de treino dos grupos 1 e 2 (Anexo 3, tab. II).

Experimento 4

Grupo 1: a infusão no CA1 de CNQX e MUS prejudicou a STM e a LTM; NAN não teve efeito e DPAT bloqueou a STM sem afetar a LTM (Anexo 4, fig. 1a). A infusão na CE de CNQX, MUS e NAN bloqueou a STM sem alterar a LTM e DPAT aumentou a STM e bloqueou a LTM (Anexo 4, fig. 1b).

Grupo 2: o efeito, sobre a STM, da infusão de DPAT no CA1, não foi mais visto às 3 h depois do treino (Anexo 4, fig. 1c) e o efeito sobre a STM da infusão CNQX, MUS e NAN no CE durou 3 h após o treino (Anexo 4, fig. 1d).

Experimento 5

Grupo 1: a infusão no CA1 de SKF e DPAT inibiu a STM sem afetar a LTM; SCH aumentou a STM sem alterar a LTM; de TIM e NAN não teve efeito e de

NE não alterou a STM mas aumentou a LTM (Anexo 5, fig. 1A). A infusão no CE de SKF deprimiu a STM e aumentou a LTM; de NAN e TIM deprimiu a STM e não alterou a LTM e de DPAT e SCH aumentou a STM e deprimiu a LTM (Anexo 5, fig. 1B).

Grupo 2: nenhuma das drogas que inibiram a STM tiveram efeito na performance da sessão de teste da STM, dadas 6 min. antes deste (Anexo 5, fig. 2A e 2B). Nenhuma das drogas que afetaram a STM tiveram efeito na performance da sessão de teste de LTM, dadas 1,5 h antes deste (Anexo 5, fig. 2C e 2D).

Experimento 6

Grupo 1: a infusão no CA1 de ESTAU, KN, KT5823 e LAV 0,5 aumentou a LTM sem afetar a STM; de PD bloqueou a STM sem afetar a LTM; de LY e KT inibiu a STM e a LTM; de LAV 0,1 não teve efeito sobre as duas formas de memória (Anexo 6, fig. 1). Já no CE, a infusão de ESTAU e KT inibiu a STM e a LTM e de PD aumentou a STM e deprimiu a LTM. Todas os outros tratamentos não tiveram efeito nas duas formas de memória (Anexo 6, fig. 1).

Grupo 2: a infusão de PD, que inibiu a STM sem afetar a LTM, 10 min. antes do teste de STM, mostrou que não existem evidências para um efeito desta droga na recuperação da STM.

Experimento 7

Grupo 1: a infusão de KT no CA1 à 0 h após o treino, prejudicou a STM e a LTM (Anexo 7, fig. 2a); aos 22 (Anexo 7, fig. 2b) e 45 min. (Anexo 7, fig. 2c) após o treino, prejudicou a STM sem afetar a LTM.

Grupo 2: a infusão de KT no CA1 aos 90 min após o treino (Anexo 7, fig. 2d), prejudicou a STM sem afetar a LTM; aos 135 min após o treino (Anexo 7, fig. 2e), não teve efeito sobre as duas formas de memória e 175 min após o treino(Anexo 7, fig. 2f), não teve efeito na STM e bloqueou a LTM.

Localização Histológica dos Sítios de Infusão

De duas a 24 h após o término dos experimentos, os animais operados receberam infusão bilateral de uma solução de 4% de azul de metileno em salina. Após 1 h, foram sacrificados por decapitação e seus cérebros retirados e armazenados em solução de 10% de formol em água destilada para posterior localização histológica dos sítios de infusão. As infusões foram consideradas corretas, quando o local corado não excedeu 1 mm^2 da área pretendida.

Somente os dados comportamentais dos animais com localização correta das cânulas foram considerados.

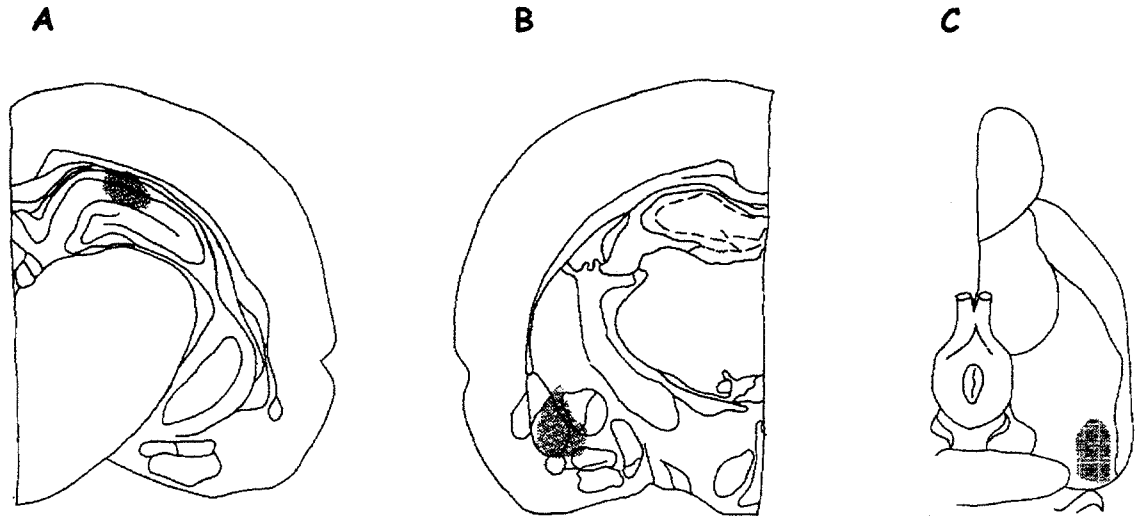

Fig. 4: Localização dos sítios de infusão A) na região CA1 do hipocampo; B) na amígdala e C) no córtex entorrinal.

Discussão

Experimento 1

Os resultados sugerem que a amnésia induzida por inibidores enzimáticos foi mais forte que a causada por CEC ou e β-endorfina, apoiando a idéia que a CaMKII e a PKA estão diretamente envolvidas no mecanismo central da consolidação da memória no hipocampo (6,25), como estão numa variedade de fenômenos plásticos nesta estrutura e em outras (14). O CEC e a e β-end, ao contrário, são conhecidos por exercerem seus efeitos amnésicos principalmente através de influências nos sistemas modulatórios da memória (22,39).

Experimento 2

Têm sido sugerido que tanto a cascata da CaMKII quanto a do cAMP/PKA participam na formação da memória (20,33,36,48,49,52).

Existem, entretanto, diferenças importantes entre as duas cascatas, tanto com respeito ao seu tempo de operação, quanto às áreas cerebrais envolvidas. A CaMKII, pela fosforilação do Glur1, modula uma fase de curta duração de transmissão sináptica excitatória aumentada, que é necessária nos primeiros estágios da memória no CA1 e na modulação desta na amígdala (26,33). Porém a cascata do cAMP/PKA é diferente, já que há dois picos de atividade, um muito breve no período pós-treino de EI (6,7) e outro maior de 3-6 h após o treino em EI, simultaneamente com o crescimento dos níveis de

cAMP endógeno, seguido de um grande aumento da fosforilação de CREB e de c-fos (6,7,26).

No hipocampo, o bloqueio precoce da CaMKII pelo KN, impede a formação da memória de longa duração que depende de uma intervenção tardia da cadeia do cAMP/PKA. Na amigdala, o bloqueio da CaMKII não previne a formação da memória porque esta depende de sistemas que não estão localizados nesta estrutura.

Os presentes dados estão de acordo com o de vários experimentos anteriores, sugerindo que a amígdala participa na modulação precoce da memória (11,40) e o hipocampo está envolvido no armazenamento de várias formas de memória declarativa (17,26,46,50) no mínimo por vários dias (27,28).

Experimento 3

Claramente, a formação da memória da tarefa de habituação concomitante com a tarefa de *water finding*, pode ocorrer na presença de um inibidor da enzima. Diferente de outras tarefas, a amnésia retrógrada causada pelo KN na habituação, não foi completa, o que sugere que mesmo quando adquirida sozinha, esta tarefa não é grandemente dependente da CaMKII como outras tarefas (36,54).

Existe a possibilidade de que, quando a tarefa de *water finding* está presente, o CA1 usa diferentes células ou sinapses para processar a tarefa de habituação do que quando a tarefa de habituação é processada sozinha. A primeira vista, não há razão para presumir que diferentes células serão usadas

para processar exatamente a mesma tarefa, no mesmo aparato, durante um intervalo de treino e teste e à uma similar responsividade em cada caso (45). Existe, entretanto, uma diferença de procedimento entre a habituação do grupo 1 e 2, onde o primeiro ocorreu com interrupções causadas pela sede, que foram mais longas na sessão de teste, o que não ocorreu com o grupo 2. Estas interrupções requeriram um reinício do curso de habituação. Isto poderia implicar num envolvimento de diferentes células nos dois experimentos. Entretanto, a CaMKII parece ser indispensável (36), particularmente no período pós-treino (48) para tarefas espaciais de provas variadas que também usam células detectoras de lugar e que envolvem, por definição, múltiplas interrupções e reinícios.

A saturação da via sinalizante da CaMKII nas mesmas células pela concomitância de duas tarefas simultâneas, é uma possibilidade (3,12).

Experimento 4

O efeito do tratamento com DPAT na STM no CE durou mais que no CA1, sugerindo que os receptores entorrinais lidam com a STM por um tempo maior. Receptores similares participam diferentemente nas duas estruturas cerebrais no mesmo tempo, durante o período imediatamente depois do treino.

STM e LTM envolvem, essencialmente, mecanismos separados. Alguns processos no CA1 vistos serem comuns para os dois sistemas de memória, não é surpreendente, pois os dois sistemas lidam com aproximadamente as mesmas representações sensorio-motoras.

Experimento 5

Baseado em experimentos usando inibidores de síntese proteica e outros tratamentos que, dependendo do tempo de administração, prejudica as duas formas de memória ou somente a LTM (38), tornou-se tradicional considerar que a STM é um passo em direção à LTM (41,47). Os presentes achados não suportam esta idéia, pois DPAT e NAN já demonstraram previamente (29) e outros tratamentos estão descritos aqui pela primeira vez.

Os presentes dados concordam com a idéia do traço único, dois processos (19). Existem mecanismos em comum para a STM e a LTM (29). Isto sugere que a STM e a LTM compartilham alguns mecanismos bioquímicos no CA1 e CE e concorda com a idéia do traço único além do que, os animais lidam com um conjunto de informações aprendidas e respondem da mesma forma, quando testados 1,5 e 24 h após o treino. A idéia de dois processos é substanciada pelas diferenças farmacológicas entre a STM e a LTM, que implica diferenças bioquímicas.

A influência de vários dos tratamentos dados no CA1 ou CE na STM e LTM foram diferentes, em alguns casos até opostos. É tentador formular hipóteses de como as várias vias monoaninérgicas podem ser ativadas pela experiência de treino e influenciar diferentemente a STM e a LTM.

Experimento 6

Foram encontradas recentemente drogas que bloqueiam a formação da STM sem alterar a LTM, quando infundidas imediatamente após o treino no CA1, CE (13,15) e CP (30). Portanto, claramente a STM e a LTM envolvem sistemas separados, apesar delas deverem ser conectadas e ambas apresentarem as mesmas representações sensorio-motoras (13). As ligações são mostradas pelo fato que vários antagonistas de receptores sinápticos influenciam a STM e a LTM similarmente (13,15). Isto sugere que a diferenciação entre os processos de STM e LTM podem ocorrer dentro do mesmo conjunto de células, em um nível pós receptor.

Experimento 7

A LTM depende da PKA no CA1, em tempos nos quais a sua atividade e os níveis de seu substrato, CREB, estão no pico: 0 e 3 h após o treino (6). Na metade do tempo, a PKA controla a STM. O primeiro pico tem uma influência primária, tanto no segundo pico, quanto na regulação da STM: sem isso nenhuma das duas ocorreria. A regulação da STM pela PKA pode envolver outros substratos além do CREB, tais como proteínas de membrana que podem marcar ou sinalizar sinapses estimuladas recentemente (18). A distinção entre a STM e a LTM pode ocorrer principalmente a nível pós-sináptico, desde que as sinapses envolvidas na geração dos dois tipos de memória devem, em uma extensão maior, serem as mesmas, já que as duas lidam com o mesmo conjunto sensorio-motor de eventos.

Conclusões

✔ A amnésia causada por agentes que bloquearam a PKA e a CaMKII foi mais forte, o que é compatível com a idéia de que estas enzimas estão diretamente envolvidas no mecanismo principal da consolidação da memória.

✔ O bloqueio da CaMKII na amígdala, não impediu a formação da memória, pois isto depende de sistemas que não estão localizados nesta estrutura.

✔ A tarefa de HAB não depende da CaMKII, mas esta mesma tarefa adquirida junto com a de *water finding*, que dependente desta, tem uma dependência parcial da CaMKII.

✔ A STM e a LTM são, essencialmente, processos separados, mas têm alguns mecanismos bioquímicos em comum no CA1 e CE.

✔ No HIPO, o mecanismo de STM requer PKA, MAPKK e guanilato ciclase, mas não PKC, PKG, tirosina quinase e CaMKII. O mecanismo da LTM requer guanilato ciclase, PKG, PKC, CaMKII e PKA, mas não MAPKK e tirosina quinase. No CE, A STM requer PKC e PKA, mas não guanilato ciclase, PKG, CaMKII, tirosina quinase e MAPKK. A LTM requer PKC, PKA e MAPKK, mas não guanilato ciclase, PKG, tirosina quinase e CaMKII.

✔ A enzima PKA é necessária para a formação da STM nos primeiros 90 min. após o treino em EI. Na LTM, é necessária imediatamente e, novamente, aos 180 min. após o treino em EI.

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Trabalhos que fazem parte desta tese

1. Systemic Administration of ACTH or Vasopressin Reverses the Effect of Postraining β-Endorphin or Eletroconvulsive Shock but Not That of Intrahippocampal Infusion of Protein Kinase Inhibitors (1997). Luciana A. Izquierdo, Nadja Schröder, Patrícia Ardenghi, João Quevedo, Carlos A. Netto, Jorge H. Medina e Iván A. Izquierdo. *Neurobiology of Learning and Memory*, **68**, 197-202.

2. Stimulators of the Cascade Reverse Amnesia Induced by Intra-amygdala but Not Intrahippocampal KN-62 Administration (1999). Daniela M. Barros, Luciana A. Izquierdo, Márcia K. Sant'Anna, João Quevedo, Jorge H. Medina, James L. McGaugh e Iván A. Izquierdo. *Neurobiology of Learning and Memory*, **71**, 94-103.

3. Intrahippocampal KN-62 Hinders the Memory of Habituation Acquired Alone, but Not Simultaneously with a Water Finding Task (1999). Claudia Wolfman, Luciana A. Izquierdo, Nadja Schröder e Iván Izquierdo. *Behavioural Pharmacology*, **10**, *in press*.

4. Mechanisms for memory types differ (1998). Iván Izquierdo, Daniela M. Barros, Tadeu Mello e Souza, Márcia M. Souza e Luciana A. Izquierdo. *Nature*, **393**, 635-636.

5. Short- and long-term memory are differentially regulated by monoaminergic systems in the rat brain (1998). Iván Izquierdo, Jorge H. Medina, Luciana A. Izquierdo, Daniela M. Barros, Márcia M. Souza e Tadeu Mello e Souza. *Neurobiology of Learning and Memory*, **69**, 219-224.

6. Short- and Long-Term Memory are Differentially Affected by Metabolic Inhibitors Given into Hippocampus and Entorhinal Cortex (1999). Luciana A. Izquierdo, Mônica Vianna, Daniela M. Barros, Tadeu Mello e Souza, Patrícia Ardenghi, Márcia K. Sant'Anna, Cleverson Rodrigues, Jorge H. Medina e Iván Izquierdo. *Behavioural Pharmacology, in press.*

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Anexos

Anexo 1

NEUROBIOLOGY OF LEARNING AND MEMORY **68,** 197–202 (1997)
ARTICLE NO. NL973794

RAPID COMMUNICATION

Systemic Administration of ACTH or Vasopressin Reverses the Amnestic Effect of Posttraining β-Endorphin or Electroconvulsive Shock but Not That of Intrahippocampal Infusion of Protein Kinase Inhibitors

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Retrograde amnesia was induced in rats trained in step-down inhibitory avoidance by four different treatments: an ip injection of β-endorphin (1.0 μg kg), an electroconvulsive shock (ECS), an intrahippocampal infusion of the calcium/calmodulin protein kinase II inhibitor, KN62 (0.08 μg/side), given 0 h after training, or an intrahippocampal infusion of the protein kinase A inhibitor, KT5720 (0.5 μg/side), given 3 h after training. Pretest ip injections of ACTH (0.2 μg/kg) or vasopressin (10.0 μg/kg), but not saline, reversed the amnesia caused by β-endorphin and ECS but not that caused by the enzyme inhibitors. This suggests that the amnesia produced by intrahippocampal KN62 and KT5720 administration is stronger than that caused by ECS and β-endorphin, possibly because the former interfere directly with specific steps of the core biochemical chain of events that underlies memory consolidation.

Pretest administration of $ACTH_{1-24}$, lysine–vasopressin, or their chemical analogs reverses the retrograde amnesia induced by CO_2 and electroconvulsive shock (ECS) (Riccio & Concannon, 1981; Rigter, Elbertse, & Van Riezen, 1975; Rigter, van Riezen, & De Wied, 1974). The effects are obtained within a narrow dose range. ECS-induced amnesia is accompanied by a massive release of various modulatory substances, including β-endorphin and other opioids (Izquierdo, 1991). Systemic, intracerebroventricular, intra-amygdala, or intraseptal administration of β-endorphin or other opioids causes retrograde amnesia, which is explained by an interference with neurohumoral mechanisms involved in memory modulation (for reviews, see Izquierdo, 1991; McGaugh, 1989). The effects of ACTH, vasopressin, ECS, and β-endorphin have been best studied using inhibitory avoidance tasks in rats.

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Biochemical pathways necessary for memory formation of this task have been recently described in the CA1 region of rat hippocampus (Bernabeu, Bevilaqua, Ardenghi, Bromberg, Schmitz, Bianchin, Izquierdo, & Medina, 1997; Carew, 1996). They involve the participation of calcium/calmodulin-dependent protein kinase II (CaMKII) early after training and of protein kinase A (PKA) 3 to 6 h after training (Bernabeu et al., 1997; Bevilaqua, Ardenghi, Schröder, Bromberg, Schaeffer, Schmitz, Quevedo, Bianchin, Walz, Medina, & Izquierdo, 1997; Izquierdo & Medina, 1997). CaMKII regulates the function of the AMPA glutamate receptor, which is essential for both memory formation and retrieval (Izquierdo & Medina, 1997). PKA regulates the activation of constitutive gene transcription factors that are essential for long-term memory formation (Bernabeu et al., 1997; Carew, 1996). The biochemical chain of events in CA1 is modulated by a variety of mechanisms, among which are those that may underlie the effects of β-endorphin, ECS, ACTH, or vasopressin (Izquierdo & Medina, 1997).

Here we study whether the pretest administration of $ACTH_{1-24}$ or lysine–vasopressin is capable of counteracting the retrograde amnestic effect of ECS, systemic β-endorphin administration, and intrahippocampal infusion of the CaMKII inhibitor, KN62 (Wolfman, Fin, Dias, Bianchin, Da Silva, Schmitz, Medina, & Izquierdo, 1994) or of the PKA inhibitor, KT5720 (Bevilaqua et al., 1997).

Two hundred male Wistar rats (age 2.5–3.0 months, weight 220–280 g) were used. They were trained in step-down inhibitory avoidance using a 50.0 × 25.0 × 25.0-cm acrylic box with a frontal glass panel. The floor of the box was a series of parallel 1.0-mm-caliber bronze bars spaced 1.0 cm apart. The left extreme of the grid was covered by a 7.0-cm-wide, 2.5-cm-high formica platform. Rats were placed on the platform facing the rear left corner and their latency to a step down, placing their four paws on the grid, was measured with an automatic device, upon which they received a 0.3-mA, 2-s scrambled footshock and were immediately withdrawn from the box. The test session was 24 h later. It was similar to the training session, except that the footshock was omitted. No ceiling was imposed on training session latencies. Mean overall training latency was 5.3 s (N = 198); differences among groups were not significant at a $p < 0.1$ level either in a one-way ANOVA comprising all groups (df = 21) or in separate one-way ANOVAS for each experiment (see below). A ceiling of 180 s was imposed on the measurement of test session latency. Significant training–test latency differences were taken as a measure of memory (Bevilaqua et al., 1997; Wolfman et al., 1994).

Four separate experiments were carried out. In Experiment 1 (N = 10 per group), the animals received ip, 0 h posttraining, saline or β-endorphin (1.0 mg/kg). In Experiment 2 (N = 10 per group), the animals received, immediately posttraining, an ECS (15 mA, 60 Hz, 2 s, delivered through transcorneal electrodes); in this experiment there was a sham-ECS control group in which the electrodes were placed on the corneae for 2 s but no current was delivered. In Experiment 3 (N = 8 per group), the animals received, 0 h after training, a bilateral intrahippocampal infusion of a vehicle or of the CaMKII inhibitor, KN62 (Wolfman et al., 1994). In Experiment 4 (N = 8 per group), the animals received, 3 h after training, a bilateral intrahippocampal infusion of vehicle or of the PKA inhibitor, KT5720 (Bevilaqua et al., 1997). In all experiments the rats received, 10 min prior to testing, an ip injection of saline, $ACTH_{1-24}$

(0.2 μg/kg), or lysine vasopressin (10.0 μg/kg). Drugs given ip were dissolved in saline to an injection volume of 1.0 ml/kg. The doses chosen for ACTH and vasopressin were within those that do not increase test session latencies on their own to avoid possible confounds between performance and memory effects (see Izquierdo, 1991; Riccio & Concannon, 1981, for references).

Animals in Experiments 3 and 4 were bilaterally implanted under deep thionembutal anesthesia with 30-g guide cannulae in the dorsal CA1 region of the hippocampus (A −4.3, L ±4.0, V 3.4; coordinates according to Paxinos & Watson, 1986). Behavioral training was carried out after full recovery from surgery. In Experiments 3 and 4, a 27-g infusion cannulae was fitted into the guide cannulae on each side. In Experiment 3, vehicle (20% dimethyl sulfoxide in saline) or KN62 (0.08 μg) was slowly infused over 30 s, first on one side and

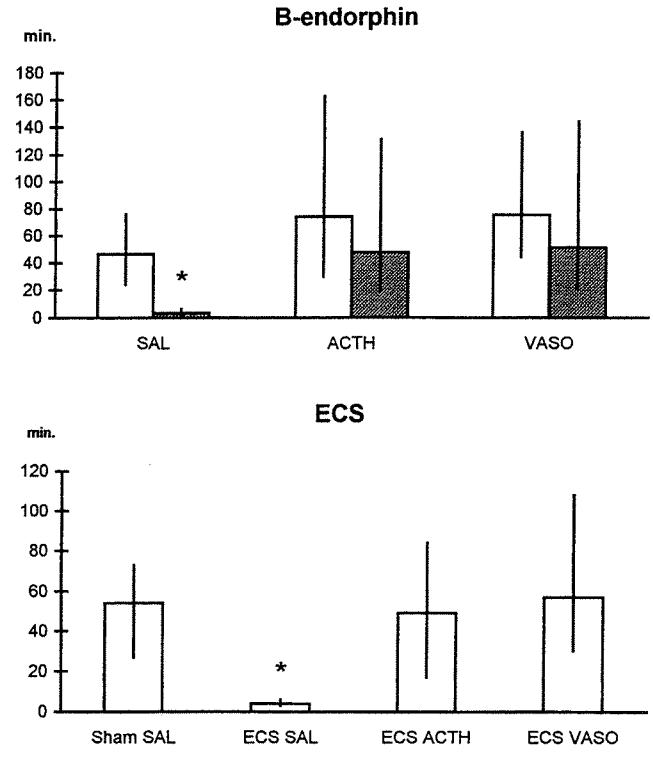

FIG. 1. In this and the following figure, data are expressed as median (interquartile range) test session step-down latency (ordinates). In the two experiments shown $N = 10$ per group. Top (β-endorphin): Effect of saline (SAL), ACTH (0.2 μg/kg), or lysine–vasopressin (VASO, 10.0 μg/kg) given ip 10 min prior to testing on retention test performance of rats trained in step-down inhibitory avoidance (0.3-mA footshock) 24 h before. The animals received, immediately posttraining, ip injections of saline (white columns) or β-endorphin (1.0 μg/kg) (grey columns). β-Endorphin was amnestic and ACTH and VASO reversed this effect. Bottom (ECS): Effect of saline, ACTH, or vasopressin given ip 10 min prior to testing in animals that received an ECS (15 mA, 60 Hz, 2 s, transcorneal) immediately posttraining; in the sham-ECS group the electrodes were placed on the corneae but current was not delivered. ECS was amnestic and ACTH and VASO reversed this effect. In this figure and Fig. 2 an asterisk indicates a significant difference with respect to any of the other groups at the $p < 0.002$ level in Mann–Whitney U tests, two-tailed. In all groups except for those marked by an asterisk, training–test latency differences were significant at $p < 0.002$ level in Mann–Whitney U tests, two-tailed.

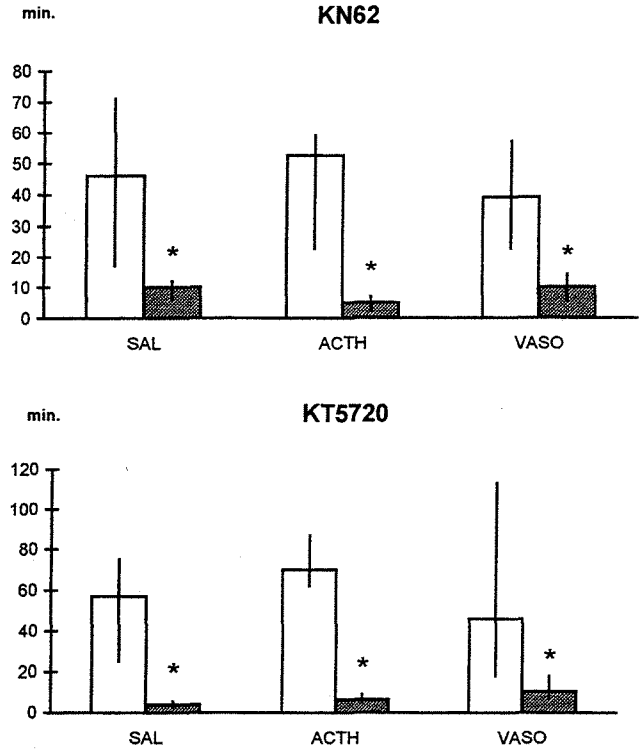

FIG. 2. In the two experiments shown $N = 8$ per group. Top (KN62): Effect of saline (SAL), ACTH (0.2 μg/kg), or lysine–vasopressin (VASO, 10.0 μg/kg) given ip 10 min prior to testing on retention test performance of rats trained in step-down inhibitory avoidance (0.3-mA footshock) 24 h before. The animals received, 0 h after training, a bilateral infusion of vehicle (white columns) or KN62 (0.08 μg/side, grey columns. Neither ACTH nor VASO was able to reverse the amnesia induced by KN62. Bottom (KT5720): Same as above, except that the amnestic treatment here was KT5720 (0.5 μg/side) given 3 h posttraining. The amnesia induced by this drug was also insensitive to pretest ACTH or VASO.

then on the other, starting 0 h after training. In Experiment 4, vehicle or KT5720 (0.5 μg) was infused 3 h after training. In all cases, the infusion cannula was left in place for an additional 15 s after infusion had been completed; therefore, the entire procedure took about 90 s. Infusion volume was 0.5 μl in all cases (Bevilaqua et al., 1997; Wolfman et al., 1994). Two to 24 h after the end of the behavioral procedures, 0.5 μl of a solution of 4% methylene blue in saline was infused as above, animals were sacrificed by decapitation 1 h later, and their brains were withdrawn and stored in formalin for histological localization of infusion sites as explained elsewhere (Bevilaqua et al., 1997). Infusions were considered correct when the spread of the dye did not exceed 1 mm^2 from the intended injection site in the CA1 area of the dorsal hippocampus (Bevilaqua et al., 1997; Wolfman et al., 1994). This occurred in 48 of 49 animals in Experiment 2, and in 48 of 49 animals in Experiment 3. Infusion placements will not be illustrated here since they were exactly like those reported in other recent papers (Bevilaqua et al., 1997; Wolfman et al., 1994). Only behavioral data from animals with correct cannula placements were considered.

Results of Experiment 1 are shown in Fig. 1, top graph. β-Endorphin caused

retrograde amnesia. Pretest ACTH and vasopressin administration reverted the effect.

Results of Experiment 2 are shown in Fig. 1, bottom graph. ECS caused retrograde amnesia. The effect was reverted by ACTH and vasopressin given prior to testing.

Results of Experiment 3 are shown in Fig. 2, top graph. As reported previously (Wolfman et al., 1994), KN62 given intrahippocampally 0 h after training caused retrograde amnesia. This was not reversed by the pretest administration of either ACTH or vasopressin.

Results of Experiment 4 are shown in Fig. 2, bottom graph. As shown elsewhere (Bevilaqua et al., 1997), KT5720 given into the hippocampus 3 h after training caused retrograde amnesia. This was not reversed by pretest ACTH or vasopressin.

The data show that the retrograde amnesia caused by β-endorphin or ECS was reversed by pretest ACTH or vasopressin, whereas that caused by agents that block CaMKII or PKA was not. This suggests that the amnesia induced by the enzyme inhibitors was stronger than that caused by ECS or β-endorphin, despite the fact that in terms of reduction of the test session latency all these agents appeared to be equally effective. This is compatible with the idea that CaMKII and PKA are directly involved in the core mechanism of memory consolidation in the hippocampus (Bernabeu et al., 1997; Izquierdo & Medina, 1997), as they are in a variety of plastic phenomena in this structure and elsewhere (Carew, 1996). ECS and β-endorphin, instead, are believed to exert their amnestic effects mainly through influences on memory modulatory systems (Izquierdo, 1991; McGaugh, 1989).

The present conclusions must be regarded as based on qualitative observations, inasmuch as they reflect findings using one single dose of the enzyme inhibitors or the hormones. A study on the reversal of the amnesia produced by intrahippocampal infusion of inhibitors of other protein kinases believed to play a key role in posttraining memory processing, such as protein kinases C and G, or perhaps tyrosine kinases (Izquierdo & Medina, 1997), would be desirable.

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Anexo 2
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Stimulators of the cAMP Cascade Reverse Amnesia Induced by Intra-amygdala but Not Intrahippocampal KN-62 Administration

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Infusion of the calcium–calmodulin-dependent protein kinase II (CaMKII) inhibitor KN-62 (3.5 ng/side) 0 h after training into rat hippocampus CA1 or amygdala has been known for years to cause retrograde amnesia for step-down inhibitory avoidance. On the other hand, drugs that indirectly stimulate protein kinase A (PKA) (8-Br-cAMP, 1.25 μg/side; norepinephrine, 0.3 μg/side; the dopamine D1 receptor agonist, SKF38393, 7.5 μg/side) infused 3 h posttraining into CA1 but not amygdala markedly facilitate retention of this task. Here we find that 8-Br-cAMP, norepinephrine, or SKF38393 given 3 h posttraining into rat CA1 reverses the amnestic effect of KN-62 given into the amygdala 0 h after training, but not that of KN-62 given into CA1 0 h posttraining. The findings bear on the participation of CaMKII and of the cAMP/PKA cascade in memory processes in the hippocampus and the amygdala. Both cascades have been proposed to play a role in memory: CaMKII in the early phase and PKA in the transition between the early phase and long-term memory. Clearly, in CA1, both cascades are involved and are crucial, and the CaMKII cascade must precede the PKA cascade. In contrast, in the amygdala, only the CaMKII cascade is active, and it does not play a central role in memory, inasmuch as its deleterious effect may be fully recovered by stimulation of the PKA cascade in the hippocampus. This further supports the contention that the hippocampus is essential for memory formation of this task, as it is for many others, whereas the amygdala appears to play instead an early modulatory role.

Infusion of the specific inhibitor of calcium–calmodulin protein kinase II (CaMKII), KN-62, into the CA1 region of the hippocampus or into the amygdala 0 h but not 2 h posttraining causes amnesia for one-trial step-down inhibitory avoidance learning (Wolfman, Fin, Dias, Bianchin, Da Silva, Schmitz, Medina, & Izquierdo, 1994; see also Izquierdo, L. A., Schröder, Ardenghi, Quevedo, Netto, Medina, & Izquierdo, 1997). Similar effects have

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1074-7427/99 $30.00

been obtained using pretraining infusions of KN-62 in the hippocampus in a spatial task (Tan & Liang, 1996) or, in the amygdala, in step-through inhibitory avoidance (Tan & Liang, 1997). KN-62 also blocks CA1 LTP (Ito, Hidaka & Sugiyama, 1991; Barria, Muller, Derkach, Griffith, & Soderling, 1997). Transgenic mice expressing an abnormal isoform of CaMKII in the hippocampus (Mayford, Bach, Huang, Wang, Hawkins, & Kandel, 1996) or animals lacking the gene that encodes for CaMKII in the whole body (Grant & Silva, 1994) show an impairment both of spatial learning and LTP.

Total and Ca-independent CaMKII increases in rat CA1 in the first 30 min after LTP induction (Barria et al., 1997) and after step-down avoidance training (Cammarota, Bernabeu, Levi de Stein, Izquierdo, & Medina, 1997a, 1997b). In both cases, the effect is accompanied by increased autophosphorylation of the enzyme and followed by enhanced phosphorylation of the GluR1 subunit of the glutamate AMPA receptor (Barria et al., 1997; Cammarota et al., 1997a, 1997b). The total amount of CaMKII and of GluR1 increases after the CaMKII activity peak, following both LTP (Ouyang, Kantor, Harris, Schuman, & Kennedy, 1997) and inhibitory avoidance training (Cammarota et al., 1997b). The data fit with others showing that in the first 1–3 h after LTP induction (Tocco, Maren, Shors, Baudry, & Thompson, 1992) or step-down avoidance (Cammarota, Bernabeu, Izquierdo, & Medina, 1996; Cammarota, Izquierdo, Wolfman, Levi de Stein, Bernabeu, Jerusalinsky, & Medina, 1995) there is increased [^{3}H]AMPA binding to its receptor in CA1. This increased binding correlates with the CaMKII-mediated phosphorylation and/or increased amount of GluR1 (Cammarota et al., 1997a, 1997b). In the case of avoidance training, this appears necessary for memory formation: Blockade of AMPA receptors in CA1 or in the amygdala with CNQX 0–3 h but not 6 h after training causes full retrograde amnesia in this task (Jerusalinsky, Ferreira, Da Silva, Bianchin, Ruschel, Medina, & Izquierdo, 1992). An increase of CaMKII has also been reported in the hippocampus of rats submitted to a spatial task (Tan & Liang, 1996) or in the amygdala of rats submitted to step-through inhibitory avoidance (Tan & Liang, 1997).

The persistence of memory or of LTP for periods longer than 3–6 h, however, does not seem to depend on the initial intervention of CaMKII, but rather on the activation, at about 3 h, of a cascade of events involving activation of cAMP-dependent protein kinase (PKA) and the resulting phosphorylation of the constitutive transcription factor CREB (cAMP-responsive element-binding protein) (Bernabeu, Bevilaqua, Ardenghi, Bromberg, Schmitz, Bianchin, Izquierdo, & Medina, 1997; Bourchuladze, Frenguelli, Blendy, Cioffi, Schutz, & Silva, 1994; Carew, 1996; Guzowski & McGaugh, 1997; Huang, Li, & Kandel, 1994). The link between the CaMKII-dependent phase and the cAMP/PKA-dependent phase of LTP is not known (Barria et al., 1997).

Evidence of various sorts indicates that, despite the fact that early after different forms of training the amygdala presents biochemical changes and pharmacological sensitivities similar to those seen in the hippocampus (Davis, 1992; Izquierdo & Medina, 1997), it plays largely a modulatory role in the initial stage of memory formation (Cahill & McGaugh, 1996; McGaugh et al., 1995), rather than a role in storage. The hippocampus, instead, is widely believed to be essential for the storage of numerous declarative memories, including those of spatial learning and of inhibitory avoidance (Bourchouladze

et al., 1994; Eichenbaum, Schoenbaum, Young, & Bunsey, 1996; Izquierdo & Medina, 1997; Mayford et al., 1996; Squire, 1992; Vnek & Rothblat, 1996).

The infusion of 8-Br-cAMP, of various activators of adenylyl cyclase (forskolin, the dopamine D1 agonist SKF38393, norepinephrine) in rat CA1 but not amygdala, 3–6 h after step-down avoidance training, markedly enhances retention of this task measured 1 day later; infusion into CA1 of the dopamine D1 antagonist SCH23390 of the β-adrenoceptor antagonist timolol or of the PKA inhibitor KT5720 is, instead, deeply amnestic (Bernabeu et al., 1997a, 1997b; Bevilaqua, Ardenghi, Schröder, Bromberg, Schmitz, Schaeffer, Quevedo, Bianchin, Walz, Medina, & Izquierdo, 1997).

Here we study, in the step-down avoidance task, whether the retrograde amnesia induced by the infusion of KN-62 0 h posttraining in the amygdala or in the CA1 region of the rat hippocampus can be attenuated or reversed by 8-Br-cAMP, SKF38393, or norepinephrine given 3 h after training into CA1.

MATERIALS AND METHODS

One hundred fifty-four male Wistar rats (age, 2.5–3 months; weight, 220–340 g) from our own breeding colony were used. The animals were housed in plastic cages, five to a cage, with water and food ad libitum, under a 12-h light/dark cycle (lights on at 7:00 AM) at a constant temperature of 23 ± 1°C. Animals were subdivided into two major groups. Eighty-eight rats were bilaterally implanted under deep thionembutal anesthesia with 30-gauge guides 1 mm above the junction between the central and the basolateral nucleus of the amygdala (A −2.3, L +4.5, V 5.4) and 1 mm above the CA1 area of the dorsal hippocampus (A −4.3, L +4.0, V 3.4). Sixty-six rats were implanted only with cannulae aimed at CA1. Coordinates are in millimeters, according to the atlas by Paxinos and Watson (1986).

After recovery from surgery, the animals were submitted to one-trial step-down inhibitory avoidance (Bernabeu et al., 1997a, 1997b; Bevilaqua et al., 1997; Izquierdo, L. A., et al., 1997; Wolfman et al., 1994) carried out as follows. Animals were placed on a 2.5-cm-high, 7.0-cm-wide platform at the left of a 50.0 × 25.0 × 25.0-cm box whose floor was a series of parallel 0.1-cm-caliber stainless-steel bars spaced 1.0 cm apart. Latency to step down placing the four paws on the grid was measured. Training–test interval was 24 h. In the training session, immediately upon stepping down, the animals received a 0.3-mA, 2.0-s scrambled foot shock. In the test session, no foot shock was given and the step-down latency was cut off at 180 s; i.e., values equal to or higher than 180 s were counted as 180 s. This required the use of nonparametric statistics (Kruskal-Wallis analysis of variances followed by Mann–Whitney *U* tests, two-tailed).

Infusion cannulae (27 gauge) were fitted into the guide cannulae 0 and 3 h after training. Drug or vehicle infusions were performed manually using a microsyringe attached to the cannulae with a polyethylene tube. The tip of the infusion cannulae protruded 1 mm beyond that of the guide cannulae. Infusions were carried out slowly over 30 s, after which the infusion cannula was left in place for an additional 15 s, first on one side and then on the other. Thus, the entire procedure took slightly over 90 s for each time of infusion (Bevilaqua et al., 1997; Izquierdo, L. A., et al., 1997). The animals implanted into both amygdala and hippocampus received, at 0 h posttraining, either KN-62 (3.5

ng/side) or a vehicle (2% dimethyl sulfoxide in saline) into the amygdala, and, 3 h after training, 8-Br-cAMP (1.25 μg/side), SKF38393 (7.5 μg/side), noradrenaline CIH (0.3 μg/side), or the vehicle in the hippocampus. The animals implanted bilaterally in CA1 received, 0 h posttraining, either KN-62 or the vehicle, and, at 3 h, 9-Br-cAMP, SKF38393, or norepinephrine, in all cases in the same doses stated above. All drugs were dissolved in the vehicle; infusion volume was 0.5 μl in all cases. The doses chosen were found to have strong effects on memory when given into the hippocampus and/or amygdala in previous experiments (Bernabeu et al., 1997a, 1997b; Bevilaqua et al., 1997; Izquierdo, L. A., et al., 1997).

Two to 24 h after the end of the behavioural procedures, 0.5 μl of a solution of 4% methylene blue in saline was infused as indicated above into the implanted sites. Animals were sacrificed by decapitation 1 h later and their brains withdrawn and stored in formalin for histological localization of infusion sites as explained elsewhere (Bevilaqua et al., 1997; Izquierdo, L. A., et al., 1997). Results of the anatomical verifications are summarized in Fig. 1. Infusion placements were correct (i.e., within 1.5 mm^3 of the intended site) in all cases.

RESULTS

Training session step-down latency differences were not significant among groups in a Kruskal–Wallis analysis of variance ($H = 3.9$; df = 15). Median (interquartile range) training step-down latency was 5.0 (4.1/7.7) s.

Test session latency data from the animals implanted in amygdala and in CA1 are shown in Fig. 2. The Kruskal–Wallis analysis showed a significant group effect in these animals ($H = 48.2$, $p < .002$). As shown previously (Wolfman et al., 1994), the intra-amygdala infusion of KN-62 caused complete retrograde amnesia. However, both in the animals that received KN-62 into the amygdala and in those that received saline 0 h after training, the infusion into CA1 of 8-Br-cAMP, SKF 38393, or norepinephrine 3 h after training caused a major memory enhancement. This was not statistically significant from that seen in animals treated first with the vehicle and then with these drugs in CA1 (Mann–Whitney U tests between any of the KN-62 or saline drug groups in Fig. 2 and the corresponding saline drug groups of Fig. 1, $p > .1$, two-tailed).

Test session latency data from the animals implanted only in the hippocampus are shown in Fig. 3. The Kruskal–Wallis analysis revealed a significant groups effect in these animals ($H = 59.5, p < .002$). In agreement with previous findings (Wolfman et al., 1994; Izquierdo, L. A., et al., 1997), KN-62 given into CA1 caused full retrograde amnesia. This amnesia was unaltered by the administration of 8-Br-cAMP, SKF 38393, or norepinephrine into this area 3 h after training. In contrast, when these drugs were infused into CA1 3 h after training in the animals treated with saline at 0 h, there was a major memory enhancement, also in agreement with previous data (Bevilaqua et al., 1997).

DISCUSSION

KN-62 given 0 h posttraining into either the amygdala or the CA1 area of the hippocampus caused full retrograde amnesia for this task, as shown previously

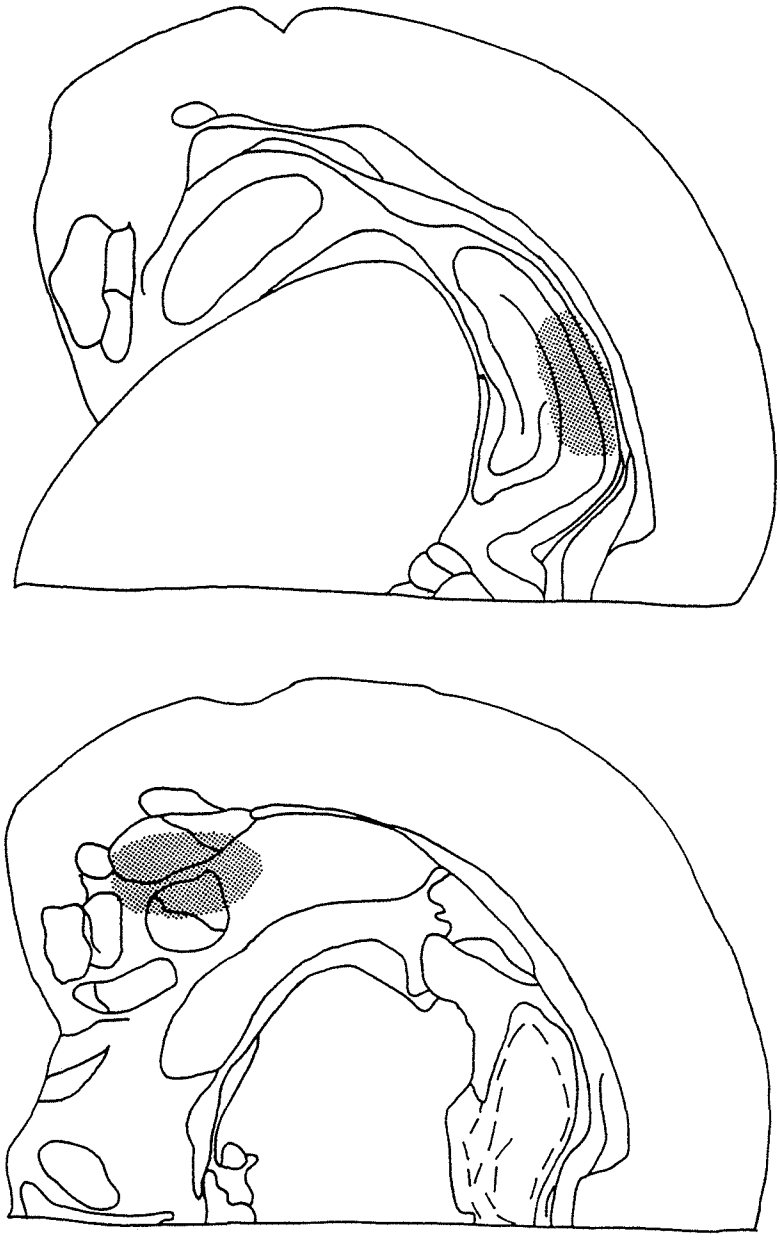

FIG. 1. (Top) schematic drawing of rat brain section at plane A −4.3 of the atlas by Paxinos and Watson (1986) showing, in stippling, the extension of the area reached by the infusions in the hippocampus. (Bottom) Same, at plane A −2.3, showing the extension of the area reached by the infusions in the amygdala. In all cases, maximum extension of the site(s) reached by the infusions was less than 1 mm^2 as ascertained by the spread of a 0.5-μl infusion of 4% methylene blue into each of the structures, 24 h after the test session.

(Wolfman et al., 1994; Izquierdo, L. A., et al., 1997) and as shown using pretraining infusions in other tasks (Tan & Liang, 1996, 1997). There was a major difference between the two routes of administration of the drug. The amnesia caused by its intraamygdala administration was fully reversed by

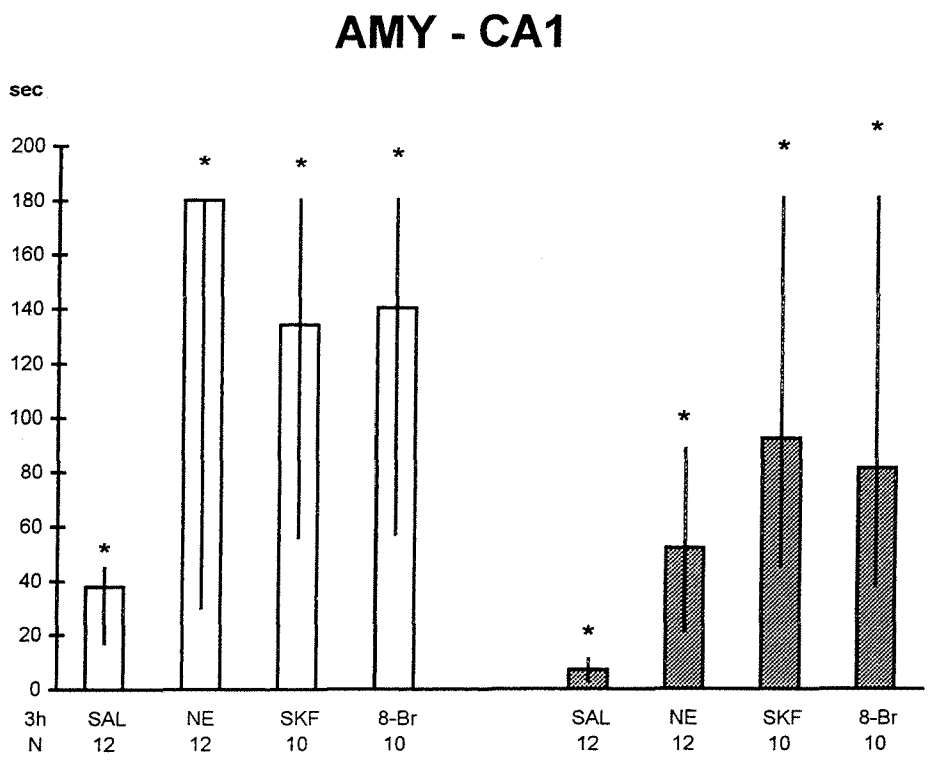

FIG. 2. Median (interquartile range) test session latency in groups infused bilaterally in the amygdala 0 h after training with 0.5 μl of vehicle (2% dimethyl sulfoxide in saline, SAL, white columns) or KN-62 (3.5 ng/side, gray columns) and, 3 h after training, with SAL or with noradrenaline C1H (NE, 0.3 μg/side), SKF38393 (SKF, 7.5 μg/side), or 8-Br-cAMP (8-Br, 1.25 μg/side) in the CA1 region of the dorsal hippocampus. *N* per group is given below each column. Asterisks indicate a significant difference from control group (SAL at 0 h in amygdala and SAL at 3 h in CA1) at $p <$ .002 level in Mann–Whitney *U* tests, two-tailed. Differences among asterisked groups are not significant at a $p = .1$ level. NE, SKF, and 8-Br given into CA1 3 h posttraining caused retrograde memory facilitation regardless of whether the animals had received SAL or KN-62 in the amygdala 0 h posttraining. Intraamygdala KN-62 was amnestic, and that effect was reversed by the intrahippocampal treatments.

8-Br-cAMP, SKF38393, or norepinephrine given into CA1 3 h later; the amnesia caused by intrahippocampal KN-62 was not reversed by these treatments.

Both the CaMKII cascade and the cAMP/PKA cascade have been suggested to play a role in memory formation (Lisman, Malenka, Nicoll, & Malinow, 1997; Mayford et al., 1996; Grant & Silva, 1994; Tan & Liang, 1996, 1997; Wolfman et al., 1994). There are, however, important differences between the two cascades, with regard both to their time of operation and to the brain areas involved. The differences point to a very different role for each of them in memory.

In CA1, both after LTP induction and after step-down inhibitory avoidance learning, there is an increase of CaMKII activity early on (Barria et al., 1997; Cammarota et al., 1997a, 1997b) followed by increased amount of the enzyme, which suggests that it is rapidly synthesized locally, at the dendrites, following its phosphorylation (Cammarota et al., 1997b; Ouyang et al., 1997). KN-62

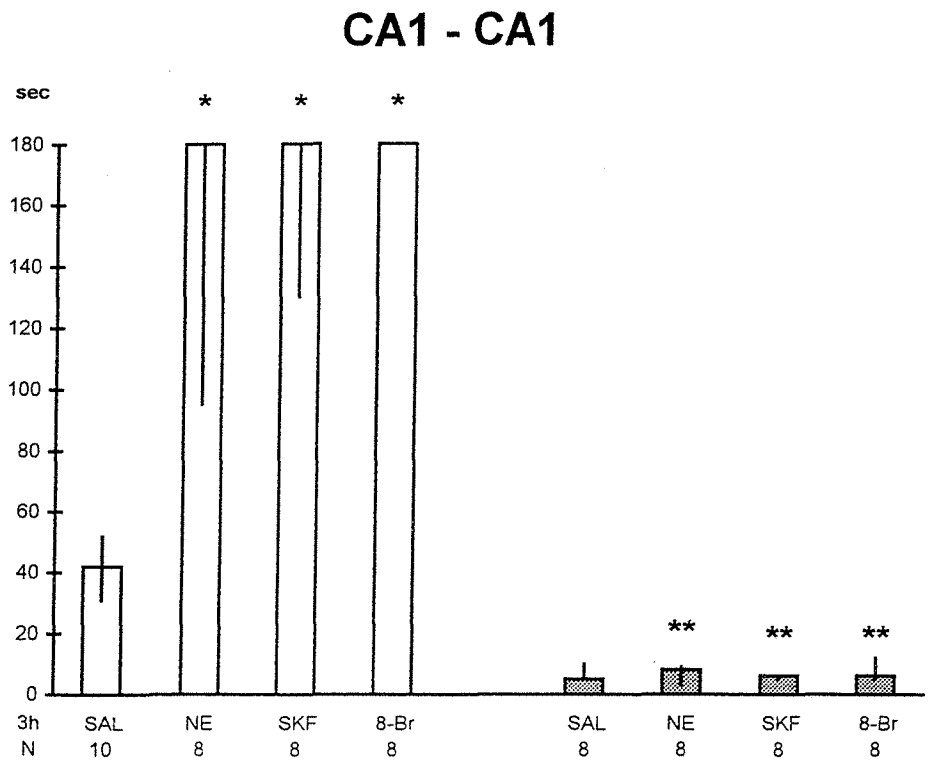

FIG. 3. Median (interquartile range) test session latency in groups infused bilaterally in the CA1 region of the dorsal hippocampus 0 h after training with 0.5 μl of vehicle (2% dimethyl sulfoxide in saline, SAL, white columns) or KN-62 (3.5 ng/side, gray columns) and, 3 h after training, with SAL or with noradrenaline C1H (NE, 0.3 μg/side), SKF38393 (SKF, 7.5 μg/side), or 8-Br-cAMP (8-Br, 1.25 μg/side). *N* per group is given below each column. Single asterisks indicate a significant difference from control group (SAL at 0 h in amygdala and SAL at 3 h in CA1) at $p <$ .002 level in Mann–Whitney *U* tests, two-tailed. Double asterisks indicate both a a significant difference both from absolute control group (SAL at 0 h in amygdala and SAL at 3 h in CA1) and from all SAL-drug groups at $p <$ .002 level in Mann–Whitney *U* tests, two-tailed. NE, SKF, and 8-Br given into CA1 3 h posttraining caused retrograde memory facilitation in animals treated with SAL in the same area 0 h posttraining, but were unable to reverse the amnestic effect of KN-62 given into CA1 0 h posttraining.

inhibits memory formation (Izquierdo, L. A., et al., 1997; Wolfman et al., 1994) or LTP (Ito et al., 1991; Barria et al., 1997) when given into CA1 before or immediately after the onset of both processes, but not later. The CaMKII activation that occurs after LTP induction or avoidance learning in CA1 is followed by phosphorylation of the GluR1 subunit of the AMPA receptor during 1–3 h (Barria et al., 1997; Cammarota et al., 1997a, 1997b) and, in the case of avoidance, by increased GluR1 immunoreactivity and increased B_{max} of this subunit for [^{3}H]AMPA, which is reversed by KN-62 (Cammarota et al., 1997a, 1997b). Both LTP and step-down training are indeed followed by increased [^{3}H]AMPA binding to its receptor in CA1 and other areas which lasts 1–3 h (Cammarota et al., 1995, 1996; Tocco et al., 1992). Blockade of this receptor with CNQX in amygdala or CA1 1–3 but not 6 h posttraining causes full amnesia for this (Jerusalinsky et al., 1992). Increased CaMKII activity has also been reported in the hippocampus following spatial training (Tan & Liang,

1996) and in the amygdala following step-through inhibitory avoidance training (Tan & Liang, 1997).

When all these data are put together, a clear role for CaMKII emerges, both in hippocampus and amygdala. The enzyme, by phosphorylation of GluR1, mediates a short-lasting phase of enhanced excitatory synaptic transmission which is necessary for the early instances of LTP and of memory processing in CA1 and of memory processing or modulation in the amygdala (Izquierdo & Medina, 1997; Lisman et al., 1997).

The picture is quite different for the cAMP/PKA cascade. To begin with, although there is a brief early peak of enhanced PKA activity in rat CA1 0 h after step-down avoidance training (Bernabeu et al., 1997a, 1997b), the major increase occurs 3–6 h later, simultaneously with a slow buildup of endogenous cAMP levels, with a large increase of CREB phosphorylation and with an increase of c-*fos* (Bernabeu et al., 1997a, 1997b; Izquierdo & Medina, 1997). The second, late peak of PKA, but not the first, correlates with a great sensitivity of memory processes to the facilitatory effect of infusions into CA1 of 8-Br-cAMP, forskolin, SKF38393, norepinephrine, or the 5HT1A antagonist NAN-190 and with the amnestic effect of a PKA inhibitor (KT5720), SCH23390, timolol, and the 5HT1A agonist 8-HO-DPAT (Bernabeu et al., 1997a, 1997b; Bevilaqua et al., 1997). This fits with data showing that 2–6 h after training in various tasks there is a need both for protein synthesis (Matthies, 1989) and CREB-P activity (Guzowski & McGaugh, 1997) and with findings showing that 5–7 h after training there are changes in cell adhesion properties and synaptic morphology in the hippocampus and its connections (Matthies, 1989; O'Connell, O'Malley, & Regan, 1997). Very conspicuously, the pharmacological evidence listed above for a participation of the cAMP/PKA cascade 3–6 h after training in the hippocampus is lacking in the amygdala (Bevilaqua et al., 1997). The linkage between the CaMKII cascade and the cAMP/PKA cascade is not known; it might depend on the hyperactivation of AMPA receptors brought about by CaMKII, which lasts for several hours, both in LTP experiments (Barria et al., 1997) and in memory studies (Cammarota et al., 1996). The persistence of LTP beyond 3 h in hippocampus depends on a cAMP/PKA/CREB-P phase followed by protein synthesis (Carew, 1996; Huang et al., 1996), as does memory processing in the hippocampus (see Izquierdo & Medina, 1997).

Thus, the cascade that is initiated by cAMP-dependent processes begins about 3 h after the CaMKII cascade is active in memory processes in the hippocampus but not the amygdala and is necessary in the former for the persistence of both LTP and memory beyond 3–6 h (Bernabeu et al., 1997b; Guzowski & McGaugh, 1997; Izquierdo & Medina, 1997).

To conclude, in the hippocampus, early blockade of CaMKII by KN-62 impedes the formation of long-term memory, which depends on the later intervention of a cAMP/PKA chain. In the amygdala, CaMKII blockade does not prevent memory formation because this hinges upon systems that are not located in the amygdala. The cAMP/PKA/CREB-P cascade in the CA1 area of the hippocampus appears to be a major such system.

The present data fit with many experiments suggesting that the amygdala plays a role in early posttraining memory modulation, rather than storage (Cahill & McGaugh, 1996; McGaugh et al., 1995), whereas the hippocampus is involved in the storage of this as well as for many other forms of declarative

memory (Eichenbaum, Schoenbaum, Young, & Bunsey, 1996; Izquierdo & Medina, 1997; Squire, 1992; Vnek & Rothblat, 1996), at least for several days (Izquierdo, L. A., et al., 1997).

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Intra-hippocampal KN-62 hinders the memory of habituation acquired alone, but not simultaneously with a water-finding task

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Rats were implanted bilaterally with cannulae in the CA1 region of the dorsal hippocampus. After recovery from surgery, they were submitted to a water-finding task, which required detecting a water tube in an open field, and then remembering its location under conditions of thirst. This task was acquired simultaneously with habituation to the open field. Training and test sessions lasted 2 min, in addition to the time spent at the water tube. The training-test interval was 24 h, during which the animals were deprived of water. Immediate post-training intra-hippocampal administration of the calcium/calmodulin-dependent protein kinase II (CaMKII) inhibitor KN-62 (3.6 ng/side) attenuated memory of the water-finding task, but not that of the habituation acquired concomitantly. However, when the habituation was carried out alone in the absence of the water-finding task, its retention was inhibited by KN-62. Thus, depending on circumstances, habituation can be memorized with or without hippocampal CaMKII activity. In the post-training period, CA1 neurones appear to 'choose' which task will be processed by a metabolic pathway that includes CaMKII.

Keywords: habituation learning, water-finding learning, calcium/calmodulin-dependent protein kinase II, KN-62, rat

INTRODUCTION

Tenen (1965) and Le Moal *et al.* (1984) described a water-finding paradigm, in which rats detect a water tube in the course of the exploration of an open field; when tested the next day under water deprivation, animals reach the water tube faster than on the training session. At the same time, they acquire habituation of exploration of the open field, as revealed by the decrease in rearings between the two sessions (Netto *et al.*, 1986). Post-training electroconvulsive shock, leuenkephalin, β-endorphin, and naloxone modulate the habituation, but not the simultaneously acquired water-finding task (Netto *et al.* 1986), which suggests that the two tasks are somehow acquired independently of each other.

Both water finding, which involves the detection of a visual cue (the tube) within a restricted environment, and habituation, which implies perception of where the animals are at any given time, should involve place cell activity in the CA1 region of their hippocampus (Skaggs and McNaughton, 1996). Calcium/calmodulin protein kinase II (CaMKII) is necessary for the activity of CA1 place cells (Mayford *et al.*, 1996). It is also necessary for the early phases of long-term potentiation (Barria *et al.*, 1997), and for the early phases of long-term memory formation in CA1 (Tan and Liang, 1996; Cammarota *et al.*, 1998). Immediate post-training infusion of a specific inhibitor of this enzyme, KN-62, into CA1, hinders the persistence of LTP (Barria *et al.*, 1997), and the retention of one-trial inhibitory avoidance (Wolfman *et al.*, 1994; Izquierdo *et al.*, 1997) and multi-trial spatial tasks (Tan and Liang, 1996). CaMKII activity increases in CA1 after training in these tasks (Cammarota *et al.* 1998; Tan and Liang, 1996), or after LTP induction (Barria *et al.*, 1997). This increase correlates with increased phosphorylation and binding properties of the GluR1 subunit of the AMPA glutamate receptor (Cammarota *et al.*, 1998).

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In these experiments, we studied the effect of the immediate post-training infusion of KN-62, at the same dose previously shown to be amnestic for other tasks (Wolfman *et al.*, 1994; Izquierdo *et al.*, 1997), on the memory of water finding and habituation to the open field acquired simultaneously (Netto *et al.*, 1986), and on that of habituation acquired alone.

METHODS

Subjects and surgery

Seventy male Wistar rats (aged 2.5–3 months; weight, 220–340 g) from our own breeding colony were used. They were implanted, under deep thionembutal anaesthesia, with 30-gauge guides 1.0 mm above the CA1 area of the dorsal hippocampus (A −4.3, L +4.0, V +3.4; coordinates are in mm, according to the atlas of Paxinos and Watson (1986)).

Behavioural procedures

After recovery from surgery, the animals were placed in a 40 × 60 cm open field, surrounded by 50 cm high walls, made of brown plywood with a frontal glass wall. The floor of the open field was divided into 12 equal rectangles by black lines. Three separate experiments were carried out. In Experiment 1, a metal water tube protruded into the box through a hole 10 cm above floor level, in the middle of the rear wall. The tube was attached to a water container placed on the outside, and was identical to the tubes from which the rats normally drank water in their home cages (Le Moal *et al.*, 1984; Netto *et al.*, 1986). In Experiments 2 and 3, the water tube was absent.

In Experiment 1 (n = 18), the animals were trained and tested simultaneously for water finding and habituation (Netto *et al.*, 1986). Rats were taken directly from their home cages to the apparatus, placed on its left rear quadrant, and left to explore the arena for 2 min, plus the time spent at the water tube. All animals eventually sniffed and/or licked from the water tube within this time (Table 1). Immediately after training, the animals received an intra-hippocampal infusion of either KN-62 or its vehicle (see below). After this, the animals were deprived of water in their home cages for 24 h, and then taken back to the open field for testing. The test session lasted 2 min, in addition to the time spent at the water tube by each animal, which was much larger than in the training session (Table 1). Crossing of the black lines and rearings performed in both sessions were counted. The decrease of the latency to reach the water tube between the two sessions was used as a measure of retention of the water-finding task (Tenen, 1965; Le Moal *et al.*, 1984; Netto *et al.*, 1986). The decrease of the number of crossings and rearings between the two sessions was taken as a measure of the retention of habituation (Netto *et al.*, 1986; Novas *et al.*, 1988).

TABLE 1. Mean ± SEM time, in seconds, spent sniffing or licking from the water tube in the training and test session, in rats receiving post-training infusion into CA1 of vehicle or of KN-62 (3 ng/side)

Treatment	n	Time at water tube[a] Training session	Test session
Vehicle	9	6 ± 2	71 ± 19
KN-62	9	6 ± 1	74 ± 23

[a]Training-test differences were significant in both groups at $P < 0.05$ level (t-tests). Differences between groups in either training or test session scores were not significant.

In Experiment 2 (n = 28), the water tube was not present, and only habituation was measured. The animals were simply left to explore the box freely twice for 2 min, with a 24 h interval between sessions (Netto *et al.*, 1986). As in Experiment 1, the animals were deprived of water, starting immediately after the post-training infusions, and for the entire training-test interval.

In Experiment 3 (n = 24), animals received an infusion of either KN-62 or the vehicle into CA1 (see below), were subsequently deprived of water for 24 h, and were then submitted to one session of habituation in the apparatus without the water tube. This experiment was carried out as a control for the possibility that thirst may have interfered with performance in Experiments 1 and 2.

Data were analysed by Student's t-tests in the water-finding task (Figure 1), and by a 3-way analysis of variance (ANOVA – sessions, Experiments 1 and 2, treatments), followed by Duncan multiple range tests for all behavioural measures of the habituation task (Figure 2).

Infusion procedures, treatments and control for cannula placements

Infusion cannulae (27 gauge) were fitted into the guide cannulae at the appropriate times (immediately after training in Experiments 1 and 2; upon withdrawal from their home cage in Experiment 3). Drug or vehicle infusions were performed using a microsyringe, attached to the cannulae with a polyethylene tube. The tip of the infusion cannulae protruded 1 mm beyond that of the guide cannulae. Infusions were carried out slowly over 30 s, after which the infusion cannula was left in place for another 15 s, first on one side and then on the other. Thus, the

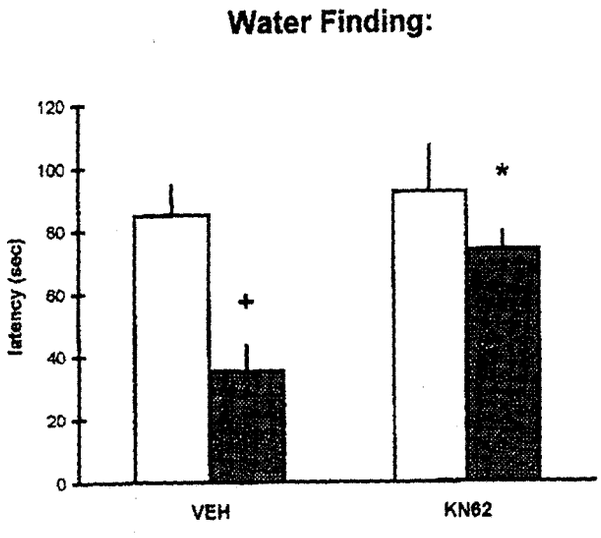

FIGURE 1. Effect of the vehicle and of KN-62 (3 ng/side) given into the dorsal CA1 region, immediately after training in the water-finding task, on the latency (in s) to reach the water tube. In this and following figures mean ± SEM training session performance is shown in white columns, and test session performance is shown in grey columns. The cross indicates a significant difference between training and test session performance at the $P < 0.01$ level in a *t*-test. The asterisk indicates a significant difference from the test session performance of the vehicle-treated group at the $P < 0.02$ level. $n = 9$ per group.

entire procedure took slightly over 90 s for each time of infusion (Wolfman *et al.*, 1994; Izquierdo *et al.*, 1997; L.A. Izquierdo *et al.*, 1998). The treatments were KN-62 (3.5 ng/side) or its vehicle (2% dimethylsulfoxide in saline), given bilaterally into the CA1 area. Infusion volume was 0.5 μl in all cases. The dose used is amnestic for several other tasks when given into CA1 (Wolfman *et al.*, 1994; Izquierdo *et al.*, 1997; Izquierdo and Medina, 1997).

Two to 24 hours after the end of the behavioural procedures, 0.5 μl of a solution of 4% methylene blue in saline was infused, as indicated above, into the implanted sites. Animals were sacrificed by decapitation 1 h later, and their brains withdrawn and stored in foralin for histological localization of infusion sites, as explained elsewhere (Wolfman *et al.*, 1994; Izquierdo *et al.*, 1998). Infusion placements were correct (i.e., within 1.5 mm^3 of the intended site) in all cases.

RESULTS

The results of Experiment 1 are shown in Figure 1 (water finding) and the left panels of Figure 2 (concomitant habituation), and results of Experiment 2 (habituation alone) are shown in the right panels of Figure 2.

Memory of the water-finding task was impaired in the animals treated with KN-62 (Figure 1). The time spent at the water tube was, as expected, much higher, and much more variable in the test session (Table 1). This measure did not differ significantly between groups. All animals in both groups spent time at the water tube in a single period in the training session. Except for two animals in each group, which went to the water tube twice, this was also true for the test session.

In the habituation task, there was a significant Sessions × Experiments × Treatment interaction for both rearings ($F = 3.56$, $P < 0.05$) and crossings ($F = 2.94$, $P < 0.05$) (Figure 2, left panels). In addition, there was a significant Sessions effect for rearings ($F = 101.51$, $P < 0.001$) and crossings ($F = 142.71$, $P < 0.001$). In Experiment 1 (Figure 2, left panels), training-test differences in rearings and crossings were significant in the Duncan test at the $P < 0.02$ level in both the vehicle- and the drug-treated group. In Experiment 2 (Figure 2, right panels) this was also the case, except for the training-test difference in crossings, which was not significant in the drug-treated group ($P > 0.1$).

There was no significant Experiments effect for rearings ($F = 0.14$) or crossings ($F = 0.69$).

There were significant Treatments effects for rearings ($F = 10.62$, $P < 0.01$) and crossings ($F = 2.59$, $P < 0.05$). Test session performance of rearings and crossings in the drug-treated group of Experiment 2 (Figure 2, right panels) was significantly higher than those of the drug-treated groups of Experiment 1 (Figure 2, left panels).

The Sessions × Experiments and Sessions × Treatments interactions were significant both for rearings ($F = 3.56$ and 3.06 respectively), and for crossings ($F = 2.58$ and 2.94 respectively) ($P < 0.05$ in all cases).

There were no significant Sessions, Experiments or group effects in the latency to leave the first quadrant (Figure 2; $F < 1.00$ in all cases), or in the number of faecal boluses (overall mean, 0.5; median, 0; range, 0-6, $F < 0.80$ in all cases).

In Experiment 3, rats were submitted to an intrahippocampal infusion of KN-62 or its vehicle, and then to 24 h of water deprivation, prior to a single (training) session of habituation. The performance of rearings and crossings in this session was not different between groups, but was lower than that of the training session of the animals of Experiments 1 and 2 (Table 2). Time taken to leave the first quadrant was not measured in this experiment. The number of faecal boluses was similar in the two groups of Experiment 3 (means 1.0 and 1.5 [illegible]

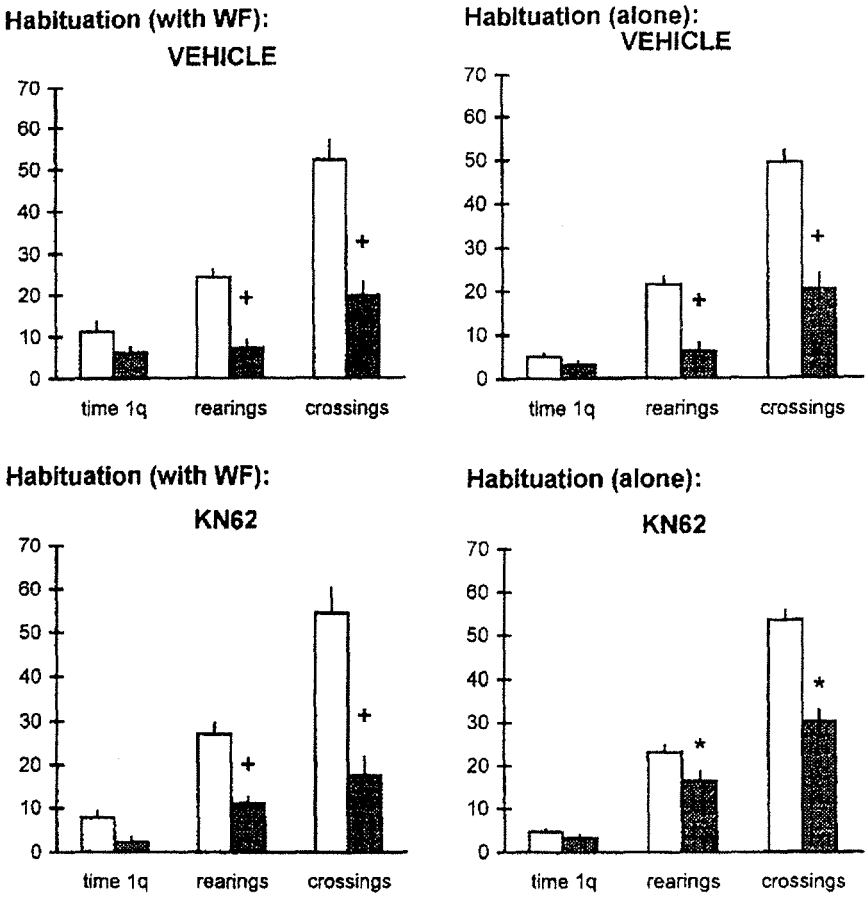

FIGURE 2. Left panels: habituation (with water finding, WF). Effect of the vehicle (upper graph) and of KN-62 (3 ng/side, lower graph) given into the dorsal CA1 region immediately after training, on the habituation that takes place concomitantly with the WF task whose data were shown in Figure 1. Right panels: habituation (alone). Same, but in animals not exposed to the water-finding task. No difference between sessions or treatments in the mean ± SEM number of seconds spent in the first quadrant (time 1q) was detectable at the $P < 0.1$ level in any group. In habituation with WF (left panels), training-test differences in the mean ± SEM number of rearings and crossings were significant at the $P < 0.05$ level in Duncan multiple range test, in both vehicle- and KN-62-treated groups, as indicated by the crosses. All differences in performance between control and drug-treated groups were not significant at the $P < 0.1$ level. $n = 9$ per group. In habituation alone (right panels), training-test differences in the mean ± SEM number of rearings and crossings were significant at the $P < 0.05$ level in Duncan multiple range tests in the vehicle groups, as indicated by the crosses, but only those in rearings were significant in the KN-62 animals. Differences in both rearings and crossings in the KN-62-treated rats in this experiment were significantly different from those of habituation with WF (left panels) at the $P < 0.05$ level in a Duncan test. $n = 14$ per group.

vehicle- and drug-treated animals; spread, 0–21 and 0–10 respectively; differences not significant).

TABLE 2. Mean ± SEM number of rearings and crossings performed in a training session of habituation in an open field, in animals submitted to 24 h of water deprivation immediately following an intra-hippocampal infusion of either KN-62 (3 ng/side) or its vehicle[a]

Treatment	*n*	Rearings	Crossings
Vehicle	12	8.6 ± 0.7	32.6 ± 3.6
KN-62	12	8.5 ± 1.1	28.1 ± 4.0

[a]Within-session differences were not significant. Between-session differences were significant at $P < 0.05$ level in both groups.

DISCUSSION

KN-62 given post-training bilaterally into CA1, at a dose that is amnestic for other tasks (Wolfman *et al.*, 1994; Tan and Liang, 1996; Izquierdo *et al.*, 1997), was also amnestic for water finding, but not for habituation to the open field, when both tasks were acquired together. KN-62 did, however, cause amnesia for habituation when this was acquired alone in the same apparatus. Thus, CA1 cells can apparently 'choose' which immediately preceding task will be processed for memory formation, by metabolic pathways that include CaMKII. In addition, memory formation of the habituation task in Experiment 1 can clearly occur in the presence of an inhibitor of the

enzyme. Therefore, CA1 CaMKII activity is not invariably necessary for long-term memory formation, as previous findings on other tasks have suggested (Wolfman *et al.*, 1994; Mayford *et al.*, 1996; Tan and Liang, 1996; Barria *et al.*, 1997; Izquierdo and Medina, 1997; Izquierdo *et al.*, 1997; Cammarota *et al.*, 1998). It may be noted here that, unlike in the other tasks, the retrograde amnesia caused by KN-62 on habituation in Experiment 2 was not complete, which suggests that even when acquired alone, this task is not as greatly dependent on CaMKII as others seem to be (Wolfman *et al.*, 1994; Mayford *et al.*, 1996).

KN-62 had no effect on thirst. The time spent at the water tube in the test session was similar in the drug-treated and control animals of Experiment 1 (Table 1). In Experiment 3, however, training rearing and crossing scores were much lower than in Experiments 1 or 2 (compare the data of Table 2 with those of the training sessions of Figure 2). Thus, thirst attenuated habituation performance, regardless of whether the animals received an intra-hippocampal infusion of vehicle or the drug. Thirst, then, while possibly not a factor in the lack of effect of KN-62 on memory of habituation in Experiment 1, could somehow have helped to keep test session scores low in that particular experiment.

Anxiety was not a factor. Latency to leave the first quadrant and the number of faecal boluses, two time-honoured measures of anxiety levels (Novas *et al.*, 1988), were similar in vehicle- and drug-treated animals in both sessions in Experiments 1 and 2.

The 'decision' by CA1 whether the habituation that had just been acquired would be processed for retention via CaMKII (Experiment 2) or not (Experiment 1), may in principle be viewed as largely dependent on the concomitant absence or presence of water-finding learning respectively.

One possibility is that, when water finding is present, the CA1 region uses different cells and/or synapses to process habituation than it would when the latter is processed alone. At first sight, there is no reason to presume that different cells will be used to process exactly the same task, in the same apparatus, during a similar period of time, using the same training-test interval, and to a similar degree of responsiveness in each case (Skaggs and McNaughton, 1996). There is, however, one procedural difference between the habituation of Experiment 1 and that of Experiment 2. The former occurred with interruptions caused by drinking, which were much longer in the test session (Table 1); the latter took place uninterruptedly over the 2 min period. The interruptions of Experiment 1 required a 'reset' of the course of habituation in Experiment 1. This could imply an involvement of different groups of cells in the two experiments. However, CaMKII appears to be indispensable (Mayford *et al.*, 1996), particularly in the post-training period (Tan and Liang, 1996), for multi-trial spatial tasks that also use place cells, and which involve, by definition, multiple interruptions and 'resets'.

Another possibility is that, when simultaneously confronted with the two tasks, the same CA1 cells 'choose' which of the two will be processed, via a CaMKII signalling pathway (see Wolfman *et al.*, 1994; Tan and Liang, 1996; Cammarota *et al.*, 1998). 'Saturation' of the CaMKII signalling pathway in the same cells by the concomitance of two simultaneous tasks is a possibility (Barria *et al.*, 1998; Cammarota *et al.*, 1998). For biochemical pathways this may be an alternative to the use of CaMKII, see Izquierdo and Medina (1997). The post-training treatment design suggests that the 'choice' should be at a post-receptor level. At the time of training, when synapses carry ongoing sensorimotor information (Izquierdo and Medina, 1997; Izquierdo *et al.*, 1998), the rats do not know that they will be deprived of water later on. The choice might involve an interaction with working memory (Baddeley, 1986; Izquierdo *et al.*, 1998), or with other short-term stores (Izquierdo *et al.*, 1998). Clearly, once thirst sets in, whether a a water tube has been seen or not in the course of habituation becomes an important piece of information.

Whatever the interpretation, the present findings do suggest that the biochemistry of memory formation of habituation to an open field may be different from that of other tasks, and therefore deserves further study.

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Mechanisms for memory types differ

The formation of long-term memory takes several hours[1–3], during which time memories rely on short-term systems[1,2,4,5]. For over 100 years[1], the main unanswered question of memory research has been whether short-term memory is a necessary step towards long-term memory[4,5], or whether they are separate processes[1,2]. Here we report four treatments that block short-term memory while leaving long-term memory intact, showing that these memory systems are separate to some degree.

The treatments we used here all alter long-term memory when infused into the CA1 subregion of the hippocampus or the entorhinal cortex of rats that have been trained to perform certain behaviours[6–8]. These treatments were the glutamate AMPA (α-amino-3-hydroxy-5-methyl-4-isoxazole propionic acid) receptor antagonist 6-cyano-7-nitroquinoxaline-2,3-dione (CNQX, 0.5 μg), the $GABA_A$ (γ-aminobutyric acid type A) receptor agonist muscimol (MUS, 0.5 μg), the serotonin 1A receptor agonist 8-hydroxy-2-(di-*n*-propylamino) tetralin (DPAT, 2.5 μg) and the serotonin 1A receptor antagonist 1-(2-methoxyphenyl)-4-(4-(2-phthalimido)) butylpiperazine (NAN, 2.5 μg).

We implanted 30-gauge guides bilaterally 1 mm above the dorsal CA1 region of the hippocampus (A −4.3, L ±4.0, V 3.4) or 1 mm above the surface of the entorhinal cortex (A −7.0, L ±5.0, V 8.4) of Wistar rats (240–300 g) under deep thionembutal anaesthesia; stereotaxic coordinates are given in millimetres according to ref. 9. After recovery, the rats were placed on a platform that was 25 cm high and 7 cm wide. This platform faced a 43 × 25 cm grid of stainless steel bars, spaced 1.0 cm apart and of 0.1 cm in width. The platform was used for inhibitory avoidance training. We measured how quickly the rats stepped down onto the grid with all four paws. Once on the grid, the rats received a scrambled electric shock to their paws of 0.3 mA for 1 second. They immediately received bilateral infusions of 0.5 μl saline, a vehicle (20% dimethylsulphoxide) or a drug. MUS and DPAT were dissolved in saline, and CNQX and NAN were dissolved in the vehicle. Infusion cannulae protruded 1.0 mm beyond the guides. Infusion procedures and verification of cannula placement were performed as described[6–8].

There were two main experiments. In the first, animals were tested twice to see whether they had retained the memory of the electric shock: once at 1.5 hours after training, to measure short-term memory, and once at 24 hours after training, to measure long-term memory (Fig. 1a, b). In the second experiment, we tested the animals 1.5, 3.0 and 4.5 hours after training (Fig. 1c, d). Test

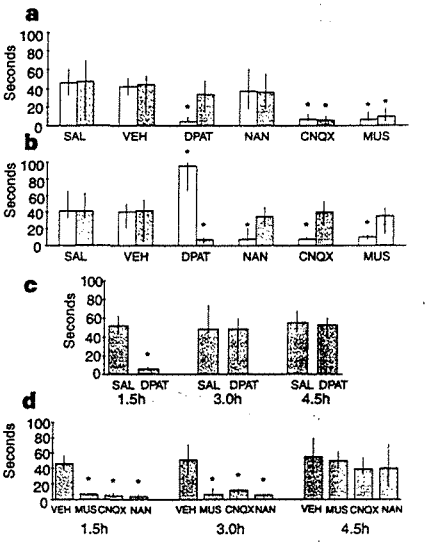

Figure 1 Data are expressed as median (interquartile range) test session latency in the step-down inhibitory avoidance task. **a, b,** Test latency was measured at 1.5 h after training (short-term memory; white columns) and at 24 h after training (long-term memory; shaded columns). Each animal was tested twice, first for short-term and then for long-term memory. Rats received immediate post-training bilateral infusions of the named compounds in the dorsal CA1 subregion of the hippocampus (**a**) or in the entorhinal cortex (**b**). SAL, saline; VEH, vehicle. **c,** The effect of intrahippocampal DPAT on short-term memory was seen when the animals were tested 1.5 h after training, but not later. **d,** The effect of intra-entorhinal CNQX, MUS and NAN on short-term memory was seen when the animals were tested 1.5 or 3.0 h, but not 4.5 h, after training. In this last experiment, MUS was dissolved in the vehicle. $N = 10$ for all groups. Asterisks, significant differences from both controls at $P < 0.02$ in a two-tailed Mann–Whitney U-test. Bars are interquartile bars.

sessions for both experiments were as above, except that the foot-shock was omitted (so we were measuring how long it took for rats to step down to the grid, and we used this time as a measurement of their memory of the shock). We stopped measuring the time taken to step down to the grid after 180 seconds[6–8]. This required the use of non-parametric statistics[7,8].

When given into the CA1 subregion of the hippocampus, CNQX and MUS impaired both short-term and long-term memory; NAN had no effect on either; and DPAT blocked short-term without affecting long-term memory (Fig. 1a). When given into the entorhinal cortex, CNQX, MUS and NAN blocked short-term memory without altering long-term memory, and DPAT enhanced short-term and blocked long-term memory (Fig. 1b). The effect of intrahippocampal DPAT on short-term memory was no longer seen at 3 hours after treatment (Fig. 1c). The effect of intra-entorhinal CNQX, MUS and NAN on short-term memory lasted 3 hours (Fig. 1d).

We draw three conclusions. First, and foremost, four treatments (intrahippocampal DPAT and intra-entorhinal CNQX, MUS and NAN) block short-term memory without altering long-term memory. This shows for the first time, to our knowledge, that short-term and long-term memory mechanisms are separated. Second, the effects of the intra-entorhinal treatments on short-term memory lasted longer than that of intrahippocampal DPAT, suggesting that the intra-entorhinal receptors 'handle' short-term memory for a longer time. Third, similar receptors play different roles in the two brain structures at the same time during the period immediately after training. In the CA1 subregion, AMPA receptors are necessary for both short-term and long-term memory[3,6]; $GABA_A$ receptors inhibit both types of memory; and serotonin 1A receptors inhibit short-term but not long-term memory. In the entorhinal cortex, AMPA and serotonin 1A receptors are needed for short-term memory; serotonin 1A receptors inhibit long-term memory; and $GABA_A$ receptors inhibit short-term memory.

We have shown previously that none of these treatments has any effect on short-term memory when given 0.1 hours before testing, or on long-term memory when given 1.5 hours before testing[10]. This rules out the possibility that our findings (Fig. 1) could be interpreted in terms of influences on retrieval or performance. The use of post-training infusions precluded the study of forms of short-term memory lasting less than 1.5 hours[4,5]: drugs such as those used here take 1 hour to diffuse away from brain infusion sites[11].

So short-term and long-term memory involve essentially separate mechanisms. Some processes in the CA1 subregion (such as the involvement of AMPA receptors and the susceptibility to MUS) seem to be common to both memory systems, which should not be surprising as they both deal with nearly the same sensorimotor representations[10].

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Walking on Mars

Sometime in the near future humans may walk in the reduced gravity of Mars. Gravity plays an essential role in walking. On Earth, the body uses gravity to 'fall forwards' at each step and then the forward speed is used to restore the initial height in a pendulum-like mechanism. When gravity is reduced, as on the Moon or Mars, the mechanism of walking must change[1]. Here we investigate the mechanics of walking on Mars onboard an aircraft undergoing gravity-reducing flight profiles. The optimal walking speed on Mars will be 3.4 km h^{-1} (down from 5.5 km h^{-1} on Earth) and the work done per unit distance to move the centre of mass will be half that on Earth.

In contrast to swimming or flying, during which the fins or wings can slide against the surrounding medium, locomotion on a solid surface is constrained by the link between the centre of gravity of the body and the fixed point of contact of the foot on the ground. After foot contact, this link leads to a forward deceleration which must be compensated for by a subsequent forward acceleration in order to maintain a constant average speed of locomotion. This increases the cost of terrestrial locomotion.

However, this cost is contained by the transfer of kinetic energy into gravitational potential energy during the deceleration (when the body rides upwards on the leg after heel strike) and the subsequent recovery of kinetic energy from the potential energy during acceleration. The recovery of mechanical energy by this mechanism is described by

$$R = (W_f + W_v - W_{cm})/(W_f + W_v)$$

where W_f is the work needed to increase the kinetic energy, W_v is the work to increase the potential energy, and W_{cm} is the work to increase the total mechanical energy of the centre of mass. With an ideal, frictionless pendulum, W_{cm} would be nil and R equal to 1. With walking on Earth, R attains a maximum of 0.7 at about 5.5 km h^{-1} (Fig. 1) near the speed at which the energy expenditure per unit distance is at a minimum[2]. At higher and lower speeds, R decreases and the energy cost increases.

We investigate the mechanics of locomotion on Mars in three male subjects (of weight and height, respectively, of 77 kg, 1.79 m; 92 kg, 1.93 m; 86 kg, 1.79 m) walking at different speeds in a Martian gravity (0.4*g*, maintained for about 30 s) on a force platform (3 m × 0.4 m) sensitive to the force exerted in both forward and vertical directions[3]. The platform was fixed to the floor of a KC-135 and an A300 Airbus aeroplane during the 23rd and 24th European Space Agency parabolic flight campaigns. The force signals from the plate (also measured in ref. 4) were analysed[5] to determine W_{cm} and R.

Figure 1 shows that on Mars the maximum pendular recovery of mechanical energy R is reduced to 0.6 and occurs at the lower speed of 3.4 km h^{-1}. In spite of the lower R, the minimum mechanical work done per unit distance to maintain the motion of the centre of mass on Mars is about one half of that on Earth. In general, walking a given distance at any absolute speed will be cheaper on Mars than on Earth. In fact, the energy consumption measured during locomotion in simulated partial gravity is less than that at 1*g* (refs 6,7). A decrease in the maximum speed of walking was also observed in partial gravity simulators[7,8].

On Mars, then, both the optimal walking speed and the range of possible walking speeds will be about half those on Earth. The walk–run transition on Mars will occur near the optimal walking speed on Earth, and the mechanical work done to walk a given distance on Mars will be about half of what it would be on Earth. So, energy expenditure will probably be lower and locomotion smoother on Mars.

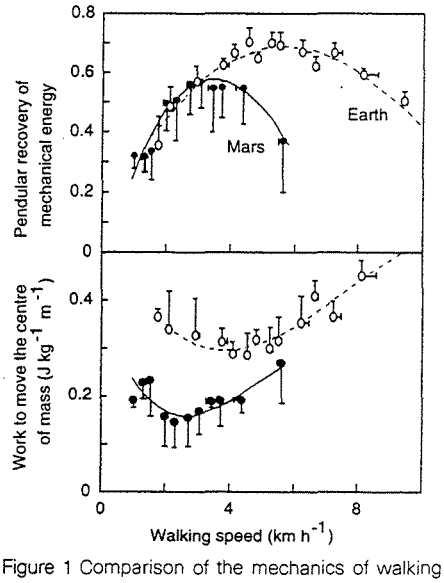

Figure 1 Comparison of the mechanics of walking on the Earth and on Mars. Top, mechanical energy recovered during walking by the pendular transfer between potential energy and kinetic energy of the centre of mass of the body on Mars and on Earth. Bottom, the work required to maintain the movement of the centre of mass in a sagittal plane. Note that the range of walking speeds on Mars is about half that on Earth (data on Earth were obtained from earlier studies on the same subjects[9,10]). Mars, filled circles; Earth, open circles.

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Relations of the new phylum Cycliophora

The Cycliophora is the most recently described animal phylum and is based on a single species, *Symbion pandora*, which was discovered on the mouthparts of the Norway lobster *Nephrops norvegicus*[1]. Because only a few morphological data[1–3] are available for *Symbion*, the precise nature of its phylogenetic relationships is highly controversial[2,4,5]. Here we present a phylogenetic analysis of 18S ribosomal RNA sequence data, including a new *Symbion* sequence, which places *Symbion* in a lophophorate–aschelminth–protostome clade and which suggests a sister-group relationship between Cycliophora and a Rotifera–Acanthocephala clade.

N. norvegicus with attached *S. pandora* were collected in Kosterfjord, Sweden. We used the polymerase chain reaction to amplify 18S rRNA gene sequences from alcohol-preserved animals, and then used standard sequencing techniques to obtain 1,542 base pairs (bp) and 1,858 bp of the 18S rRNA gene sequences from *S. pandora* (EBI number Y14811) and *N. norvegicus* (Y14812), respectively. We added the sequences to an 18S rRNA database[6]. We used four tree-construction methods and

Anexo 5

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RAPID COMMUNICATION

Short- and Long-Term Memory Are Differentially Regulated by Monoaminergic Systems in the Rat Brain

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Rats with cannulae implanted in the dorsal CA1 region of the hippocampus or in the entorhinal cortex (EC) were trained in one-trial step-down inhibitory avoidance and tested 1.5 or 24 h later, in order to measure short-term memory (STM) and long-term memory (LTM) respectively. Several drugs infused immediately post-training inhibited STM without altering LTM: the D1 receptor agonist SKF38393 (7.5 μg) given into either CA1 or EC, the β blocker timolol (0.3 μg) given into EC, the 5HT1A receptor agonist 8-HO-DPAT (2.5 μg) given into CA1, and the 5HT1A antagonist NAN-190 (2.5 μg) given into EC. These findings indicate that STM is not a necessary step toward LTM. Intraentorhinal 8-HO-DPAT enhanced STM and depressed LTM. The D1 antagonist SCH23390 (0.5 μg) enhanced STM without affecting LTM when given into CA1, and blocked LTM without affecting STM when given into EC. Intraentorhinal norepinephrine (0.3 μg) enhanced both STM and LTM, and the same drug when given into CA1 enhanced LTM selectively. None of the drugs had any effect on retrieval of either STM or LTM when given prior to testing. The data indicate that STM and LTM are differentially modulated by D1, β, and 5HT1A receptors in CA1 and EC.

Long-term memory (LTM) becomes fully consolidated only several hours after acquisition (McGaugh, 1966). This involves interactions between the CA1 area of the hippocampus and the entorhinal cortex (EC) (Izquierdo, Quillfeldt, Zanatta, Quevedo, Schmitz, & Medina, 1997), including modulation by dopaminergic D1, β-noradrenergic, and serotoninergic-1A (5HT1A) receptors in both areas (Ardenghi, Barros, Izquierdo, Bevilaqua, Schröder, Quevedo, Rodrigues, Madruga, Medina, & Izquierdo, 1997; Bevilaqua, Ar-

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denghi, Schröder, Bromberg, Schmitz, Schaeffer, Quevedo, Bianchin, Walz, Medina, & Izquierdo, 1997).

During LTM consolidation, it is believed that a short-term memory (STM) system acting during at the most a few hours is in charge of maintenance of the trace (McGaugh, 1966; Rosenzweig, Bennett, Colombo, Lee, & Serrano, 1993; Squire, Knowlton, & Musen, 1993). Immediate memory, which is an extension of working memory (Goldman-Rakic, 1991), and putative subdivisions of STM (Rosenzweig et al., 1993) will not be dealt with here.

The fundamental question concerning STM and LTM was raised by McGaugh in 1966: Are they separate (e.g., Gold & McGaugh, 1975), or is STM a necessary step toward LTM (e.g., Rosenzweig et al., 1993; Squire et al., 1993)? The crucial evidence must be provided either by an experiment showing LTM without detectable STM for the same task in the same animal, or by a clearcut demonstration that this is impossible. Recently, we described several treatments that block STM without affecting LTM of step-down inhibitory avoidance in rats (Izquierdo, Barros, Izquierdo, Mello e Souza, Souza & Medina, 1998). Here we report on the effect on STM and LTM of specific D1, β, and 5HT1A receptor agonists and antagonists given immediately post-training into CA1 or the EC.

Three hundred and forty-seven male Wistar rats (age, 3 months; weight, 240–290 g) were bilaterally implanted under thionembutal anaesthesia with 30-gauge guides 1.0 mm above dorsal CA1 (A −4.3, L ±4.0, V 8.4) or 1.0 mm above the surface of the EC (A −7.0, L 5.0, V 8.4) (Paxinos & Watson, 1986). For procedural details, see Ardenghi et al. (1997), Bevilaqua et al. (1997), and Izquierdo et al. (1997).

After recovery, animals were trained for step-down inhibitory avoidance. They were placed on a 2.5 cm high × 7.0 cm wide platform facing a 43.0 × 25.0 cm floor made of 0.1 cm caliber stainless steel bars spaced 1.0 cm apart. Latency to step-down placing the four paws on the grid was measured. In the training session, upon stepping down the animals received a 0.3 mA, 2.0-s scrambled footshock. In test sessions (see below), no footshock was given and step-down latency was cut off at 180 s; i.e., values equal to or higher than 180 s were counted as 180 s. This required nonparametric statistics (Kruskal–Wallis analysis of variances followed by individual Mann–Whitney U tests, two-tailed).

Infusion cannulae (27 gauge) were fitted into the guides either post-training (Fig. 1) or prior to testing (Fig. 2). The cannula tips protruded 1.0 mm beyond that of the guide. Infusions were carried out with a microsyringe attached to the cannulae over 30 s on each side, after which the cannula was left in place for another 15 s. Correct infusion placement was determined by the administration of 0.5 μl of 4% methylene blue in saline into the implanted sites, 2–24 h after testing. Placements were correct (i.e., within 1.5 mm^3 of the intended site) in 344 of the 347 animals. They were as shown elsewhere (Ardenghi et al., 1997; Bevilaqua et al., 1997; Izquierdo et al., 1997, 1998) and are not illustrated here.

The drugs used were SKF38393 (SKF, dopamine D_1 receptor agonist, 7.5 μg/side), SCH23390 (SCH, D_1 antagonist, 0.5 μg/side), norepinephrine ClH (NE, 0.3 μg/side), timolol ClH (TIM, β blocker, 0.3 μg/side), 8-HO-DPAT (DPAT, 5HT1A receptor agonist, 2.5 μg/side), and NAN-190 (NAN, 5HT1A antagonist, 2.5 μg/side). These substances, at the same doses that were used

here, were recently found to exert profound effects on LTM of the inhibitory avoidance task when infused into CA1, the entorhinal cortex, or the posterior parietal cortex, but not the amygdala (Ardenghi et al., 1997; Izquierdo et al., 1998). SKF, SCH, NE, and TIM were here dissolved in saline and DPAT and NAN were dissolved in 20% dimethyl sulfoxide in saline. Control animals received either saline or the vehicle. Infusion volume was 0.5 μl in all cases. In the experiments shown in Fig. 1, treatments were given immediately post-training and animals were tested for retention twice, 1.5 and 24 h later, in order to measure STM and LTM, respectively. In the experiments shown in Figs. 2A and 2B, treatments were given 6 min prior to testing and animals were tested for STM 1.5 h after training. In the experiments shown in Figs. 2C and 2D, animals were tested at 24 h and received the treatments 1.5 h before testing.

Training session latencies (median 4.3 s, range 0.9 to 22.7 s, $N = 344$) were not significantly different among groups ($\chi^2 = 2.3$ and 2.6 for the experiments of Figs. 1A and 1B, respectively, and 1.0, 3.1, 2.7, and 3.0 for those of Figs. 2A, 2B, 2C, and 2D, respectively).

Figure 1A shows the effects of post-training drug infusions into CA1 on STM and LTM. There were significant groups effects for both STM ($\chi^2 = 27.9$) and LTM ($\chi^2 = 34.6$). SKF and DPAT inhibited STM but left LTM intact; SCH enhanced STM but left LTM unaffected; TIM and NAN had no effect on either; and NE left STM intact but enhanced LTM.

Figure 1B shows the effects of post-training drug infusions into EC on STM and LTM. There were significant groups effects for STM ($\chi^2 = 40.1$) and LTM ($\chi^2 = 33.9$) in these animals. SKF depressed STM but enhanced LTM; NAN and TIM depressed STM but left LTM intact; and DPAT and SCH enhanced STM and depressed LTM (Fig. 1B).

The five treatments that inhibited STM in the preceding experiments were administered 6 min prior to STM testing into CA1 (Fig. 2A) or EC (Fig. 2B). None of them had any effect on test session performance ($\chi^2 = 2.0$ and 2.3, respectively). All the treatments that had any effect on STM in the experiments of Fig. 1 were infused into CA1 (Fig. 2C) or EC (Fig. 2D) 1.5 h prior to a 24-h LTM test. None of them had any effect on test session performance ($\chi^2 = 2.1$ and 3.2, respectively). Therefore, there was no inkling that any of the effects described in Fig. 1 for these drugs on STM could have been due to influences on retrieval or performance, and the most parsimonious explanation indeed is that they do affect STM *formation* rather specifically.

The findings raise two major points. The first is that five different treatments were found here to block STM but not LTM *for the same task in the same animal.* When the present data are put together with those of a previous report (Izquierdo et al., 1998), there are seven treatments in total having this property. The findings indicate that STM involves mechanisms that are different from those of LTM and thus provide an answer to McGaugh's (1966) question. On the basis of experiments using protein synthesis inhibitors and other treatments that, depending on the time of administration, disrupt either both STM and LTM or only the latter (see McGaugh & Herz, 1972, for references), it had become somewhat traditional to consider that STM is a step toward LTM (Rosenzweig et al., 1993; Squire et al., 1993). The present findings do not support that view. The present data on DPAT and NAN confirm a

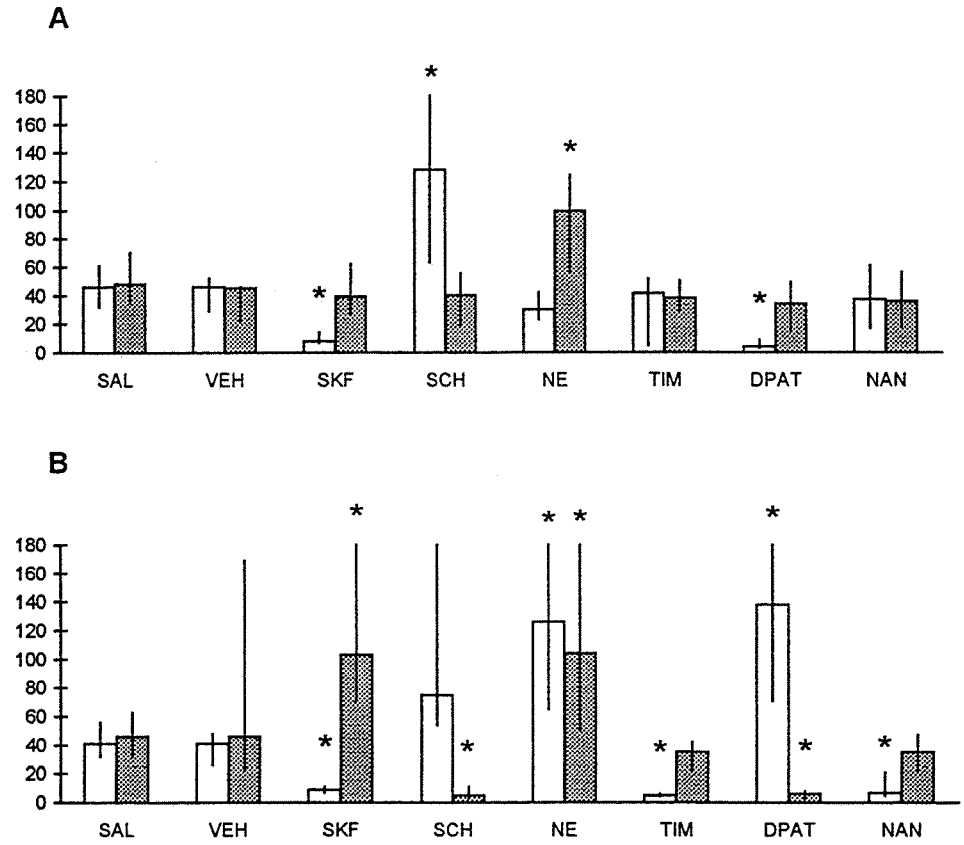

FIG. 1. In this and Fig. 2, data are expressed as median (interquartile range) test session step-down latency (in seconds, ordinates). In the two experiments, $N = 10$ per group. Effect of various treatments infused into the dorsal CA1 region of the hippocampos (A) or the entorhinal cortex (B) immediately after training in one-trial step-down inhibitory avoidance training on memory measured 1.5 h later (white columns, short-term memory) and again 24 h later (grey columns, long-term memory). The treatments were saline (SAL); 20% dimethyl sulfoxide in saline (VEH); SKF 38393, 7.5 μg/side (SKF); SCH23390, 0.5 μg/side (SCH), norepinephrine ClH, 0.3 μg/side (NE), timolol ClH, 0.3 μg/side (TIM), 8-HO-DPAT, 2.5 μg/side (DPAT), and NAN-190, 2.5 μg/side (NAN). When given into CA1, SKF and DPAT hindered and SCH enhanced STM while leaving LTM intact; NE had no effect on STM but enhanced LTM; TIM and NAN had no effect on either. When given into the entorhinal cortex, TIM and NAN inhibited STM while leaving LTM intact; SKF reduced STM and enhanced LTM; DPAT enhanced STM and reduced LTM; SCH inhibited LTM; and NE enhanced both types of memory. Asterisks indicate significant differences from both control groups at $p < .02$ level in Mann–Whitney U tests, two-tailed. In all groups except those indicated as amnestic, training–test differences were significant at $p < .02$ level in Mann–Whitney U tests, two-tailed, at both test intervals.

previous report (Izquierdo et al., 1998). The others are described here for the first time.

The second point concerns early post-training modulatory influences of D1, β, and 5HT1A receptors on memory processing. The findings on LTM confirm those of previous papers (Ardenghi et al., 1997; Bevilaqua et al., 1997). The influence of several of the treatments given into CA1 or EC on STM and LTM was dissimilar, in some cases (SKF and DPAT given into EC) even opposite. It is tempting to formulate hypotheses on how the various monoaminergic

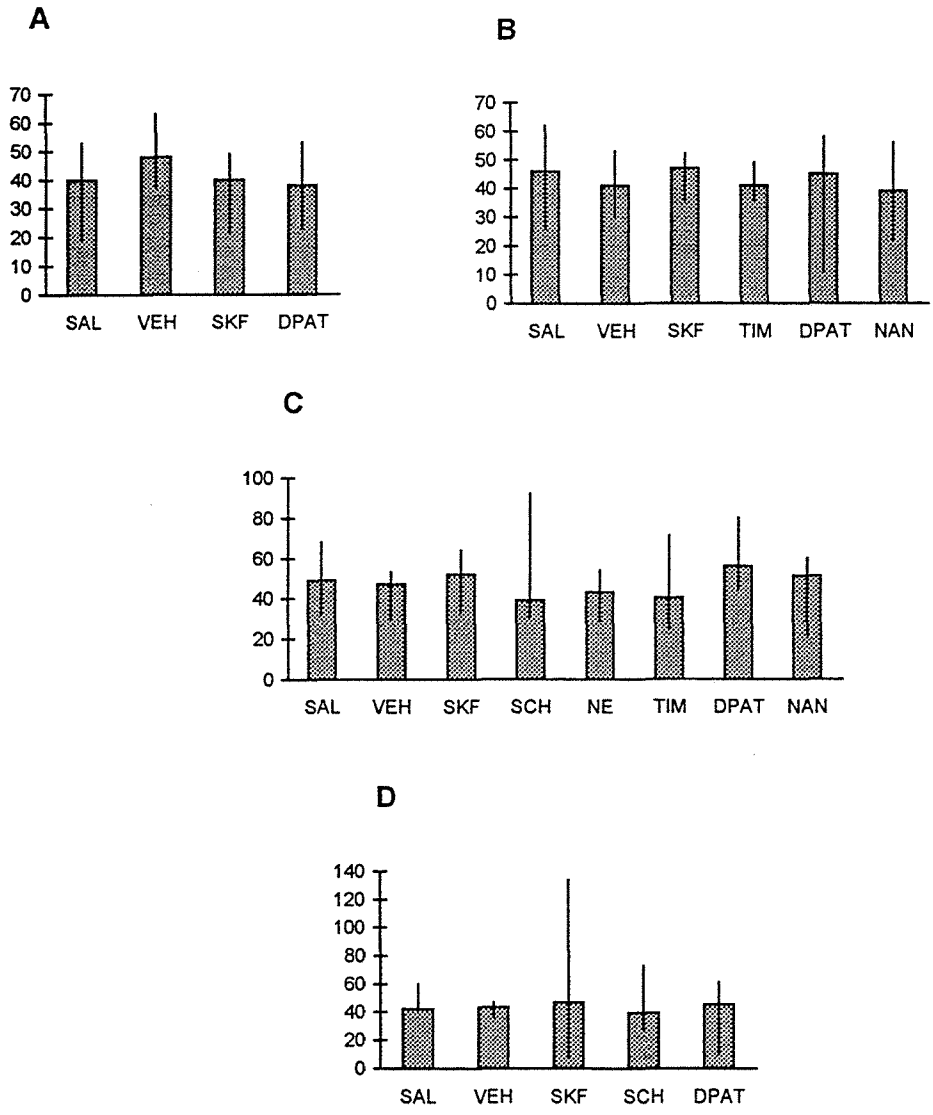

FIG. 2. (A) Effect of SAL, VEH, SKF, and DPAT, given into CA1 6 min prior to testing at 1.5 h from training, on STM retention test performance. $N = 9$ per group. (B) Effect of SAL, VEH, SKF, TIM, DPAT, and NAN, given into EC 6 min prior to testing at 1.5 h from training, on STM retention test performance. $N = 9$ per group. (C) Effect of SAL, VEH, SKF, SCH, NE, TIM, DPAT, and NAN given into EC 1.5 h prior to testing at 24 h from training, on retention test performance of LTM. $N = 10$ in all groups except TIM ($N = 9$) and DPAT ($N = 8$). (D) Effect of SAL, VEH, SKF, SCH, and DPAT given into CA1 1.5 h prior to testing at 24 h from training, on retention test performance of LTM. There were no significant differences among groups at $p = .1$ level in Mann–Whitney U tests, two-tailed, in any of the four experiments.

pathways may be activated by the training experience and influence STM and LTM processing differentially, in some cases seemingly at the expense of each other. The data are complex, and further evidence is needed in order to formally propose any such interpretation.

The present data endorse the "single-trace, dual-process" view of Gold

and McGaugh (1975). There are mechanisms in common to STM and LTM (role of glutamate receptors and of several protein kinases; see Izquierdo et al., 1998). This suggests that STM and LTM share some biochemical mechanisms in CA1 and EC and agrees with the single-trace idea. After all, the animals deal with *one set* of learned information and respond *in the same way* when tested 1.5 or 24 h after training. The dual-process idea is substantiated by the pharmacological differences between STM and LTM, which imply biochemical differences.

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Short- and Long-term Memory are Differentially Affected by Metabolic Inhibitors Given into Hippocampus and Entorhinal Cortex

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Running title:

Short- and long-term memory

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Rats were implanted with cannulae in the CA1 area of the dorsal hippocampus or in the entorhinal cortex and trained in one-trial step-down inhibitory avoidance. Two retention tests were carried out in each animal, one at 1.5 h to measure short-term memory (STM) and another at 24 h to measure long-term memory (LTM). The purpose of the present study was to screen the effect on STM of various drugs previously shown to affect LTM of this task when given posttraining at the same doses that were used here. The drugs and doses were: The guanylyl cyclase inhibitor, LY83583 (LY, 2.5 μg), the inhibitor of Tyr-protein kinase at low concentrations and of protein kinase G (PKG) at higher concentrations, lavendustin A (LAV, 0.1 and 0.5 μg), the PKG inhibitor, KT5823 (2.0 μg), the protein kinase C (PKC) inhibitor, staurosporin (STAU, 2.5 μg), the inhibitor of calcium/ calmodulin protein kinase II (CaMKII), KN62 (3.6 μg), the protein kinase A (PKA) inhibitor, KT5720 (0.5 μg), and the mitogen-activated protein kinase kinase (MAPKK) inhibitor, PD098059 (PD, 0.05 μg). PD was dissolved in saline; all the other drugs were dissolved in 20% dimethylsulfoxide. In all cases the drugs affected LTM as had been described in previous papers. The drugs affected STM and LTM differentially depending on the brain structure into which they were infused. STM was inhibited by KT5720, LY and PD given into CA1, and by STAU and KT5720 given into the entorhinal cortex. PD given into the entorhinal cortex enhanced STM. LTM was inhibited by STAU, KN62, KT5720, KT5823 and LAV 0.5 μg given into CA1, and by STAU, KT5720 and PD given into the entorhinal cortex. The results suggest that STM and LTM involve different physiological mechanisms but are to an extent linked. STM appears to require PKA, guanylyl cyclase and MAPKK activity in CA1, and PKA and PKC activity in the entorhinal cortex; MAPKK seems to play an inhibitory role in STM in the entorhinal cortex. In contrast, LTM appears to require PKA and PKC activity in both structures, guanylyl cyclase, PKG and CaMKII activity in CA1, and MAPKK activity in the entorhinal cortex.

The task in which the biochemical substrates of long-term memory (LTM) formation have been studied most extensively is, by far, one-trial step-down inhibitory avoidance (Izquierdo & Medina, 1995, 1997). There are several reasons for this: This task is acquired in seconds so that the time of the initiation of LTM formation can be determined very accurately, its memory may last for months, and it is by far the task whose pharmacology has been best studied (Gold, 1986; McGaugh, 1966). Further, it crucially involves the CA1 region of the dorsal hippocampus (Izquierdo & Medina, 1997). These characteristics make it similar in many respects to CA1 long-term potentiation (LTP) (Bliss & Collingridge, 1993); therefore, it is not surprising that the molecular steps underlying LTM formation in CA1 and their timing is quite similar to that of LTP (Izquierdo & Medina, 1995, 1997). However, unlike CA1 LTP, inhibitory avoidance involves the sequential intervention of the entorhinal cortex and other brain areas (Izquierdo, Quillfeldt, Zanatta, Quevedo, Schaeffer, Schmitz & Medina, 1997), some in the formation of LTM, and others in its modulation (Cahill & McGaugh, 1998; Izquierdo & Medina, 1997).

LTM formation of this task requires an early posttraining participation first of protein kinases A (PKA) (Bernabeu, Bevilaqua, Ardenghi, Bromberg, Schmitz, Bianchin, Izquierdo & Medina, 1997 a) and G (PKG), Bernabeu, Schröder, Quevedo, Cammarota, Izquierdo & Medina (1997 b) and then of protein kinase C (PKC) (Cammarota, Paratcha, Levi de Stein, Bernabeu, Izquierdo & Medina, 1997), and of the calcium/calmodulin dependent protein kinase II (CaMKII) (Cammarota, Bernabeu, Levi de Stein, Izquierdo & Medina, 1998) in CA1. This was ascertained both by measuring the activity of these enzymes, and the effect of the timed infusion of specific inhibitors into this area (Bernabeu et al., 1997 b; Izquierdo & Medina, 1997; Jerusalinsky, Ferreira, Da Silva, Bianchin, Ruschel, Medina & Izquierdo, 1994; Wolfman, Fin, Dias, Bianchin, Da Silva, Schmitz, Medina & Izquierdo, 1994). A participation of the mitogen-activated protein kinase kinase (MAPKK) in CA1 in LTM formation was recently established using an inhibitor of this enzyme (Walz, Roesler, Quevedo, Sant'Anna, Madruga, Rodrigues, Gottfried, Medina & Izquierdo, 1998). Evidence suggests that the PKC, CaMKII and PKA cascades also play a role in LTM formation in the entorhinal cortex (see Izquierdo & Medina, 1997). The biochemical and some of the pharmacological findings on the role of CA1 in LTM of the step-down task have been confirmed in other tasks, notably multi-trial spatial learning tasks (see Izquierdo & Medina, 1997); in these, however, the timing of the intervention of each cascade in LTM formation can not be determined (Gold, 1986; Izquierdo & Medina, 1997).

Recently it was shown that a short-term memory (STM) system or systems operate in parallel to LTM formation both in CA1 and in the entorhinal cortex in the first 1.5 - 3 h after inhibitory avoidance training (I. Izquierdo, Barros, Mello e Souza, de Souza, L.A. Izquierdo & Medina, 1998 a; I. Izquierdo, Medina, L.A. Izquierdo, Barros, de Souza MM & Mello e Souza, 1998 c). STM was defined as the system or systems that maintain memory functional while the consolidation of LTM is not yet completed; a process that, in CA1 and the entorhinal cortex, takes 3-6 h (Bernabeu et al., 1997; Izquierdo & Medina, 1997; Izquierdo et al., 1997). STM was found to be in many ways independent from LTM: Several receptor antagonists given into these brain structures can block STM without altering LTM measured in the same animals for the same task. In addition, the participation of other brain areas in STM and LTM also differs (Bianchin, Mello e Souza, Medina & Izquierdo, 1998; Izquierdo, I., Izquierdo, L.A., Barros, Mello e Souza, de Souza, Quevedo, Rodrigues, Sant'Anna, Madruga & Medina, 1998 b). This introduces a complication in the analysis of the role of the various metabolic pathways mentioned above in memory (Maren and Baudry, 1995; Izquierdo and Medina, 1997): do they participate in STM, in LTM, or in both? Biochemical observations in the first several h after training (see Izquierdo & Medina, 1997) are by themselves uninformative as to whether they reveal changes in mechanisms of STM or LTM; the use of enzyme inhibitors or receptor blockers becomes, thus, necessary for this purpose (Izquierdo et al., 1998 a,b,c,d).

Here we study the effect on STM and LTM of well-known inhibitors of PKA, PKC, PKG, guanylyl cyclase and MAPKK, at doses previously shown to affect LTM and LTP and to effectively inhibit the enzymes (see Bliss and Collingridge, 1993; Izquierdo and Medina, 1995, 1997; Walz et al., 1998). The drugs were infused into CA1 or into the entorhinal cortex of rats immediately after training in one trial step-down inhibitory avoidance, and the animals were tested for retention twice: at 1.5 h and at 24 h from training, in order to evaluate STM and LTM respectively.

METHODS

Animals. Two-hundred-and-twenty-one male Wistar rats (age, 2- 2.5 months; weight, 220-280 g) from our own breeding colony were used. The animals were housed in plastic cages, 5 to a cage, with water and food ad libitum, under a 12 h light/dark cycle (lights on at 7.00 A.M.) at a constant temperature of 23.0°C. One-hundred-and-fourteen rats were bilaterally implanted under deep thionembutal anesthesia with 30-gauge guides 1 mm above the surface of the entorhinal cortex

(A -7.0, L 5.0, V 8.4). Another 100 rats were bilaterally implanted with guides aimed 1.0 mm above the CA1 area of the dorsal hippocampus (A -4.3, L 4.0, V 3.4). Coordinates are in mm, according to the atlas by Paxinos and Watson (1986).

Training and testing. After recovery from surgery, the animals were submitted to one-trial step-down inhibitory avoidance (Bernabeu et al., 1997 a,b; Bianchin et al., 1998; Izquierdo et al., 1998 a,b,c). The rats were placed on a 2.5 cm high, 7.0 cm wide platform at the left of a 50.0 x 25.0 x 25.0 cm box whose floor was a series of parallel 0.1 cm caliber stainless steel bars spaced 1.0 cm apart. Latency to step down placing the four paws on the grid was measured. Immediately upon this, the animals received a 0.4 mA, 2.0-sec scrambled footshock. Two-hundred animals (those in Fig. 1 A and B) were tested twice: at 1.5 h from training, in order to measure STM, and at 24 h from training, in order to measure LTM (Izquierdo et al., 1998 a,b,c). Twenty-one animals (those in Table 1) were tested only for STM. In the test sessions no footshock was given and the step-down latency was cut off at 180 sec: i.e., values equal to or higher than 180 sec were counted as 180 sec. This required the use of non-parametric statistics (Kruskal-Wallis analysis of variance followed by Mann-Whitney U tests, two-tailed).

Infusion procedures. Infusion cannulae (27 gauge) were fitted into the guide cannulae at the time of infusion. This was immediately after training in the 100 animals implanted in the hippocampus and in 100 of those implanted in the entorhinal cortex, and 6 min prior to STM testing in the remaining 21 animals implanted in the entorhinal cortex. Infusions were performed manually using a 1 μl microsyringe attached to the cannulae with a polyethylene tube. The tip of the infusion cannulae protruded 1 mm beyond that of the guide cannulae. Infusions were carried out slowly by gently rotating the embolus over 30 sec, after which the infusion cannula was left in place for another 15 sec, first on one side and then on the other. Thus, the entire procedure took slightly over 90 sec for each time of infusion (Ardenghi et al., 1997; Bernabeu et al., 1997; Bevilaqua et al., 1997; Izquierdo et al., 1997, 1998 a,b). Infusion volume was 0.5 μl in all cases. The drugs used were the guanylyl cyclase inhibitor, LY83583 (LY, 2.5 μg/side), the inhibitor of Tyr-protein kinase at low doses and of and protein kinase G (PKG) at high doses, lavendustin A (LAV, 0.1 and 0.5 μg/side), the PKG inhibitor, KT5823 (2.0 μg/side), the protein kinase C (PKC) inhibitor, staurosporin (STAU, 2.5 μg/side), the calcium/calmodulin protein kinase II (CaMKII) inhibitor, KN62 (3.6 μg/side), the protein kinase A (PKA) inhibitor, KT5720 (0.5 μg/side), and the MAPKK inhibitor, PD098059 (PD, 0.05 μg/side). The latter was dissolved in saline; all the other drugs were dissolved in 20% dimethylsulfoxide. The doses chosen were calculated so as to reach a con-

centration at the infusion sites higher than those known to effectively cause maximum inhibition of the corresponding enzymes and/or of LTP (see Bliss & Collingridge, 1993; Izquierdo & Medina, 1995, 1997; Walz et al., 1998 for references), assuming a diffusion volume of 1.5 mm^3. This is the maximum volume to which 0.5 µl infusions of lidocaine or muscimol into brain tissue extend, and exert their effects upon (Martin, 1991), and the maximum volume reached by methylene blue solutions given through the same cannulae used in the present experiments (see Izquierdo & Medina, 1997; Izquierdo et al., 1997; Walz et al., 1998 for references).

Cannula placement control. Two to 24 hours after the end of the behavioral procedures, 0.5 µl of 4% methylene blue in saline was infused as indicated above into each site. Animals were sacrificed by decapitation 1 h later and their brains withdrawn and stored in formalin for histological localization of infusion sites (Izquierdo et al., 1997). Infusion placements were correct (i.e., within 1.5 mm^3 of the intended site) in all cases and were as shown in numerous previous papers (see Izquierdo & Medina, 1997; Izquierdo et al., 1997 for references).

RESULTS

Training session step-down latency differences were not significant among either the entorhinal or the CA1 groups in Kruskal-Wallis analyses of variance ($H = 4.0$ and 2.9 respectively, $df = 9$ in both cases). Over-all median (interquartile range) training step-down latency was 4.3 (3.5/7.2) and 4.6 (3.6/7.1) sec respectively.

STM and LTM test session latency data from the animals that received posttraining treatments into the CA1 region are shown in Fig. 1 (top), and those from the animals that received treatments into the entorhinal cortex are shown in Fig. 1 (bottom). The Kruskal-Wallis analysis showed a significant groups effect in both cases ($H = 44.5$ and 29.3 respectively, $p < 0.002$ in both cases. There was no visible effect of the STM test on performance in the LTM test carried out on the next day: in all control groups both test session values were remarkably similar and not statistically different.

The spectrum of drug effects was different in CA1 and the entorhinal cortex.

When given into CA1, STAU, KN62, KT5823 and the higher dose of LAV hindered LTM without affecting STM, and PD blocked STM without affecting LTM. Two substances, LY and KT5720, inhibited both STM and LTM. Only one treatment, the lower dose of LAV, had no effect on either form of memory (Fig. 1, top). In order to investigate the possibility that the inhibitory

effect of PD on STM may have been due to an influence on retrieval rather than on memory formation (Izquierdo et al., 1998 a,b,c), an additional experiment was performed. Three groups of 7 rats were trained, infused with saline, vehicle or PD into CA1 1.4 h after training, and tested for STM at 1.5 h. Median training session was 3.9 sec (range 1.6 to 24.0) in these groups. Median (interquartile range) test latency values were 51.2 (38.6/55.5) for the saline group, 42.9 (12.1/54.5) for the vehicle group, and 41.9 (33.6/54.9) for the group treated with PD. In all cases, training-test differences were significant at a $p < 0.02$ level in Mann-Whitney U tests, two-tailed. Test session latency differences among these groups were not significant; therefore, there was no evidence for an effect of PD on the retrieval of STM.

When given into the entorhinal cortex, STAU and KT5720 inhibited both STM and LTM, and PD actually enhanced STM and simultaneously depressed STM. All other intra-entorhinal treatments were ineffective on the two forms of memory (Fig. 1, bottom).

DISCUSSION

The possibility that STM may involve systems separate from those of LTM was raised by James (1890), McGaugh (1966, 1968) and Gold and McGaugh (1975). Due to the lack of an experiment showing LTM in the absence of STM for the same task in the same animal, or of evidence that this is impossible, it became traditional to view STM as a step towards LTM (see McGaugh, 1968; Gold & McGaugh, 1975; Izquierdo et al., 1998 a,c for references).

Recently we found that several different drugs infused posttraining into CA1, the entorhinal cortex (Izquierdo et al., 1998 a,b), or the parietal cortex (Izquierdo et al., 1998 c) block STM formation without altering LTM. One more treatment should now be added to the list: PD098059 given into CA1 (Figure 1 A). Thus, clearly STM and LTM involve separate systems, even though they must obviously be linked since, after all, both deal with the same sensorimotor representations (Izquierdo et al., 1998 a). The linkages are shown by the fact that several synaptic receptor antagonists do influence STM and LTM similarly (Izquierdo et al., 1998 a,b). In the present experiment, LY and KT5720 given into CA1, and STAU and KT5720 given into the entorhinal cortex. This suggests that the differentiation between STM and LTM processes may occur within the same sets of cells, at a post-receptor level.

The data suggest the following differences in the intracellular cascades involved in STM and LTM: 1) In CA1, the STM mechanism requires PKA, MAPKK and GC, but not PKC, PKG or

CaMKII, as does LTM (Bernabeu et al., 1997 a,b; Cammarota et al., 1997, 1998); 2) In the entorhinal cortex, STM requires PKC and PKA and is normally inhibited by MAPKK; LTM requires PKC, PKA and MAPKK. The data do not suggest that enzymatic systems affected by the lower dose of LAV (eg., Tyr-kinases, O'Dell, Kandel & Grant,, 1994) are involved in STM or LTM in either structure. The data on the effect of LY and KT5823 and of the higher dose of LAV in CA1 endorse a previous suggestion that cGMP-dependent processes are involved in LTM formation (Bernabeu, Schmitz, Faillace, Izquierdo & Medina, 1996).

In spite of all these rather obvious suggestions, the present findings should be taken merely as a possible starting point for future research on the biochemical basis of STM and LTM in CA1 and the entorhinal cortex. Studies using dose-response curves for each inhibitor, and comparing their effect on memory to that on the activity of each corresponding enzyme, should provide more definite evidence as to the nature of the possible interactions among the various protein kinase cascades in the processing of either STM or LTM. One point, however, is quite striking and deserves attention: The biochemical similarity between STM and the similarly lasting phenomenon known as short-term potentiation (STP). Like STM, STP is not blocked by inhibitors of PKC or CaMKII (Bliss & Collingridge, 1993; Colley & Routtenberg, 1993) or CaMKII (Barria, Muller, Derkach, Griffith & Soderling, 1997). The similarities between LTM and LTP are many, and have been extensively commented upon elsewhere (Bliss & Collingridge, 1993; Izquierdo & Medina, 1997; Maren & Baudry, 1995).

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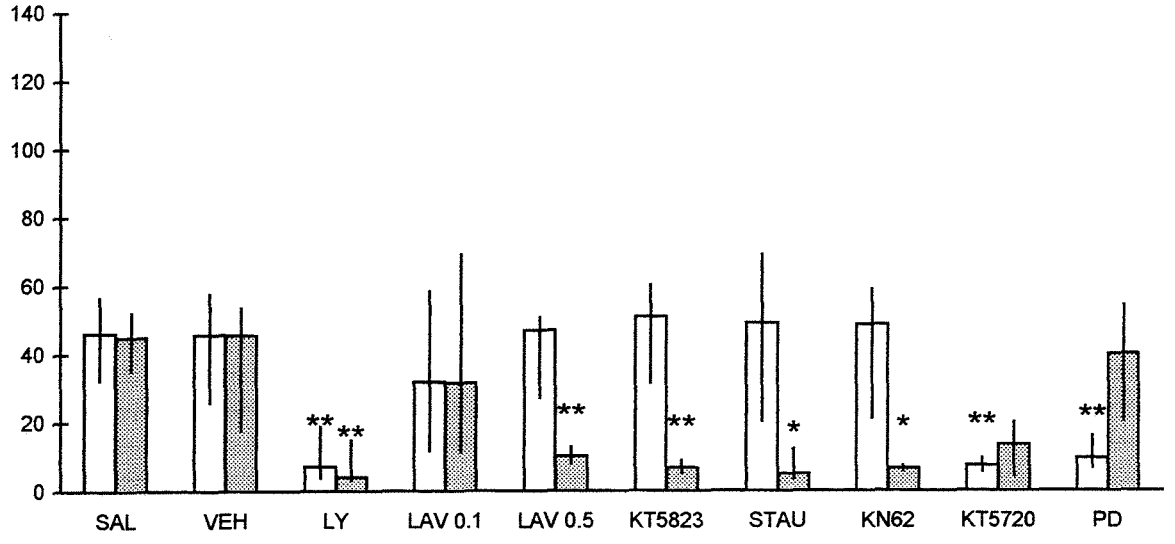
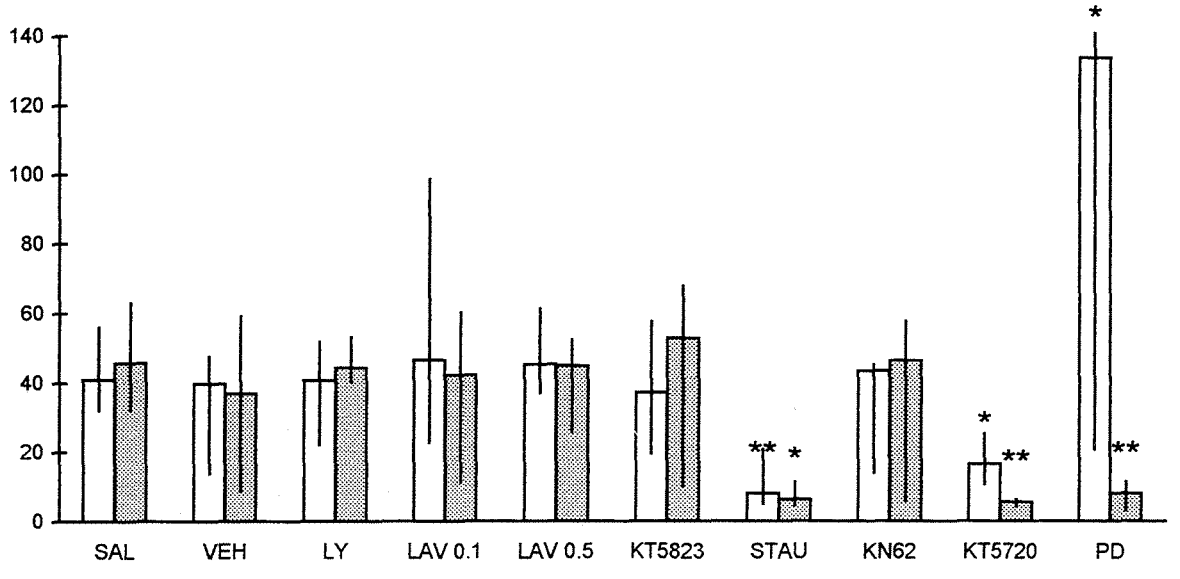
LEGEND TO FIGURE

Fig. 1: Ordinates: test session step-down latencies expressed as medians (interquartile ranges), in sec. The animals were tested for retention twice: at 1.5 h from training (STM, white columns) and at 24 h (LTM, gray columns). Effect of the immediate posttraining infusion into the CA1 area of the dorsal hippocampus (top) or into the entorhinal cortex (bottom), of saline (SAL), vehicle (VEH), LY83583 (LY, 2.5 μg/side), lavendustin A (LAV, 0.1 and 0.5 μg/side), KT5823 (2.0 μg/side), staurosporin (STAU, 2.5 μg/side), KN62 (3.6 μg/side), KT5720 (0.5 μg/side), and PD098059 (PD, 0.05 μg/side). When given into CA1, LY and KT5720 inhibited both STM and LTM, LAV had no effect at the lower dose and at the higher dose it selectively inhibited LTM, like KT5823, STAU and KN62. PD given into CA1 depressed STM without affecting LTM. When given into the entorhinal cortex, STAU and KT5720 inhibited both STM and LTM, and PD enhanced STM and depressed LTM; the other treatments had no effect. N = 10 per group. Single asterisks denote significant differences from both control groups at $p < 0.002$ level in Mann-Whitney U tests (two-tailed); double asterisks indicate significant differences from both control groups at $p < 0.05$ level.

CA1



ENTORHINAL



Anexo 7

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SHORT REPORT

Intrahippocampal infusion of an inhibitor of protein kinase *A* separates short- from long-term memory

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Rats implanted bilaterally with cannulae in the CA1 region of the dorsal hippocampus were trained in one-trial step-down inhibitory (passive) avoidance, and tested for short- and long-term memory of this task at 1.5–3.0 and at 24 h from training, respectively. At various times after training (0, 22, 45, 90, 135 or 175 min) they received a 0.5 μl infusion of the protein kinase *A* (PKA) inhibitor, KT5720 (0.1 or 0.5 μg), or of its vehicle (20% dimethylsulfoxide in saline). At the higher dose, KT5720 inhibited PKA activity by 90%. KT5720 blocked long-term memory (LTM) when given either 0 or 175 min post-training, and short-term memory (STM) when given 0, 22, 45 or 90 min post-training. Therefore, PKA plays a different role in the process of formation of the two types of memory. Its role in LTM may be related to the peak of PKA activity, and to the levels of its substrate, nuclear P-CREB, that have been described in a previous paper to occur at 0 and again at 3 h after training. The role of PKA in STM may well involve other substrates of the enzyme. This finding points to a cleavage between the mechanisms of STM and LTM formation. © **1999 Lippincott Williams & Wilkins.**

Keywords: PKA, short-term memory formation, long-term memory formation, KT-5720, rat

INTRODUCTION

Long-term memory (LTM) formation of one-trial inhibitory avoidance requires cyclic adenylyl monophosphate (cAMP)-dependent protein kinase (PKA) in the CA1 region of rat hippocampus twice: immediately, and 3–6 h after training (Bernabeu *et al.*, 1997; Bevilaqua *et al.*, 1997). Infusion into CA1 of the PKA inhibitor KT5720 immediately, 3 or 6 h but not 1.5 h after training, causes full retrograde amnesia for the avoidance task (Bernabeu *et al.*, 1997; Bevilaqua *et al.*, 1997). At 0, and again at 3–6 h after training, there are peaks of PKA activity and of the presence of nuclear P-^{133}-Ser-CREB$_1$ (cAMP response element binding protein) in CA1 cells (Bernabeu *et al.*, 1997). Memory-related phosphorylation of CREB$_1$ at ^{133}Ser in CA1 is mediated mainly by PKA (see Silva *et al.*, 1998). The second peak of PKA activity and P-CREB levels is accompanied by a concomitant rise in endogenous cAMP levels, presumably resulting from increased synthesis of this compound (Bernabeu *et al.*, 1996). Intrahippocampal infusion of forskolin or of other stimulants of adenylyl cyclase at 3 or 6 h from training, but not before, enhances LTM of the avoidance task (Bevilaqua *et al.*, 1997).

Between 0 and 3 h after training, while LTM is being formed (Izquierdo and Medina, 1997), a short-term memory (STM) system is in charge (Izquierdo *et al.*, 1998a). The STM system operates in CA1, the entorhinal cortex and the posterior parietal cortex (Izquierdo *et al.*, 1998a–c), and is parallel to, and to a large extent independent of, LTM. These claims are based on the evidence that a variety of neurotransmitter receptor agonists, and antagonists applied immediately post-training, into those regions of the brain can effectively block STM, measured 1.5 or 3 h after training, without affecting LTM for the avoidance task measured 24 h later (Izquierdo *et al.*, 1998a–c). Their effect was shown to be upon STM formation, and not on retrieval or performance: they have no influence when given 5 min prior to STM testing, or 1.5 h prior to LTM testing (Izquierdo *et al.*, 1998 b, c). Influences of repeated testing on STM or LTM test-session performance were ruled out by a variety of experiments (Izquierdo *et al.*, 1998a).

Here we investigate whether the infusion into CA1 of KT5720 has any effect on STM and LTM, measured in the same animals. Two doses of the compound were studied: 0.1 and 0.5 μg/side. The higher dose had been previously reported to cause retrograde amnesia for LTM when given into CA1 immediately or 3–6 h after training (Bernabeu *et al.*, 1997; Bevilaqua *et al.*, 1997).

METHODS

Subjects and surgery

The subjects were 124 Wistar rats aged 2.5 months, and weighing 215–300 g. They were implanted under deep thionembutal anaesthesia with 30-gauge guides, 1.0 mm above the pyramidal cell layer of the CA1 area of the dorsal hippocampus, at coordinates A 4.3 mm, L 4.0 mm, V 3.4 mm, according to the atlas by Paxinos and Watson (1986).

Behavioural procedures

After recovery from surgery, the rats were trained in one-trial step-down inhibitory avoidance, as described elsewhere (Ardenghi *et al.*, 1997; Bernabeu *et al.*, 1997; Bevilaqua *et al.*, 1997). Briefly, the animals were placed on a 2.5 cm-high and 7 cm-wide platform facing a 43 × 25 cm grid of bronze bars, spaced 1 cm apart. Latency to step down with their four paws onto the grid was measured. Immediately, a 0.4 mA, 1-s scrambled foot-shock was delivered to the grid. Rats then received bilateral 0.5 μl infusions of a vehicle (20% dimethylsulfoxide in saline) or of KT5720 (RBI) 0, 22, 45, 90, 135 or 175 min after training. At the 0, 90 and 175 min training-infusion intervals, two doses of KT5720 (0.1 and 0.5 μg) were studied. At the other intervals, only the higher dose of the compound was used. Infusion cannulae (27-gauge) were fitted into the guide cannulae immediately after training. Infusions were performed manually using a microsyringe, attached to the cannulae with a polyethylene tube. The tip of the infusion cannulae protruded 1 mm beyond that of the guide, so that it reached exactly the pyramidal cell layer of CA1. Infusions were carried out over 30 s, after which the cannulae were left in place for another 15 s, first on one side and then on the other. Thus, the infusion procedure took slightly over 90 s (Ardenghi *et al.*, 1997; Bernabeu *et al.*, 1997; Bevilaqua *et al.*, 1997; Izquierdo *et al.*, 1998b, c). Infusions such as these reach an area of approximately 1 mm^3, and diffuse away from the infusion site in less than 60 min (Figure 1) (Ardenghi *et al.*, 1997; Bevilaqua *et al.*, 1997; Martin, 1991).

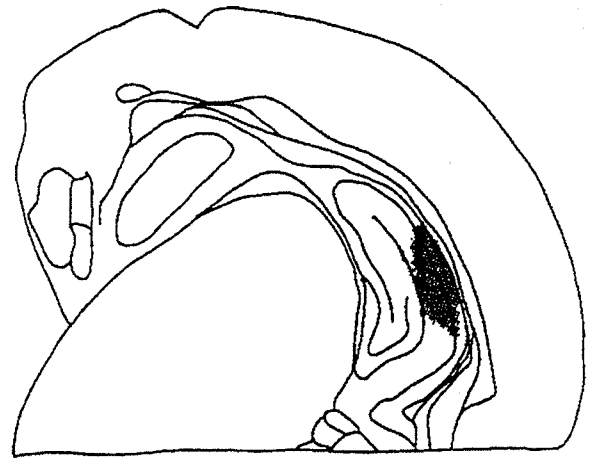

FIGURE 1. Schematic drawing of a rat brain section at plane A −4.3 of the atlas by Paxinos and Watson (1986) showing the areas reached by methylene blue infusions into the dorsal hippocampus (stippled). The figure illustrates a composite of all infusions given into both sides. In all cases, the maximum extension of the sites reached by the infusions was less than 1 mm^2, as measured in planes A −3.8 to A −4.8; therefore, it was 1 mm^3 or less in each animal, and reached the CA1 pyramidal layer in all of them.

The animals were tested for retention at 1.5–3 h, and again at 24 h (Izquierdo *et al.*, 1998a). The first test measured STM and the second test measured LTM (Izquierdo *et al.*, 1998a–c). Rats infused 0, 22 or 45 min post-training were tested for STM 90 min later; rats infused 90, 135 or 175 min post-training were tested for STM at 180 min.

In the three training-infusion intervals in which two doses of the drug were studied (0, 90 and 180 min), differences between groups were examined by a Dunnett *t*-test. In the remaining groups they were examined by a Student *t*-test.

Inhibition of PKA activity by KT5720

The higher dose of KT5720 used here or in previous studies (Bernabeu *et al.*, 1997; Bevilaqua *et al.*, 1997) inhibited PKA activity. PKA assays were carried out as described by Bernabeu *et al* (1997), by quadruplicate in a pool of ten pieces of approximately 2 mm^3 from CA1 tissue of implanted animals in which KT5720 or the vehicle had been infused 15 min before. PKA activity was 48 nmol of ^{32}P-kemptide/min/mg in vehicle-treated tissue and 5 nmol/min/mg in tissue treated with KT5720.

Verification of cannula placements

Two to 48 h after the end of the behavioural procedures, 0.5 μl of a 4% solution of methylene blue in saline were infused, as indicated above, into

the implanted sites. Animals were decapitated 1 h later, and their brains were withdrawn and stored in formalin for histological localization of the extent of the infusions (Ardenghi *et al.*, 1997; Bevilaqua *et al.*, 1997). Infusion placements were correct in 122 of the 124 animals. The maximum spread of the dye in all animals is shown in Figure 1. Only behavioural data from these animals were considered.

RESULTS

Training session latencies ranged between 0.9 and 22.9 s (mean, 5.6 s; median, 8.0 s, $n = 98$), and were not significantly different among groups ($F[14,110] = 1.04$, n.s.).

The effects of KT5720 on STM and LTM are shown in Figure 2. When it was given 0 min after training into CA1, KT5720 impaired both STM and LTM; the effect was dose-dependent. When given 22, 45 or 90 min after training, KT5720 impaired STM but not LTM; at the 90 min interval, the effect was dose-dependent. When infused into CA1 135 min post-training, KT5720 had no effect on either STM or LTM (Figure 2e). When infused 175 min after training, KT5720 did not affect STM, but blocked LTM in a dose-dependent fashion.

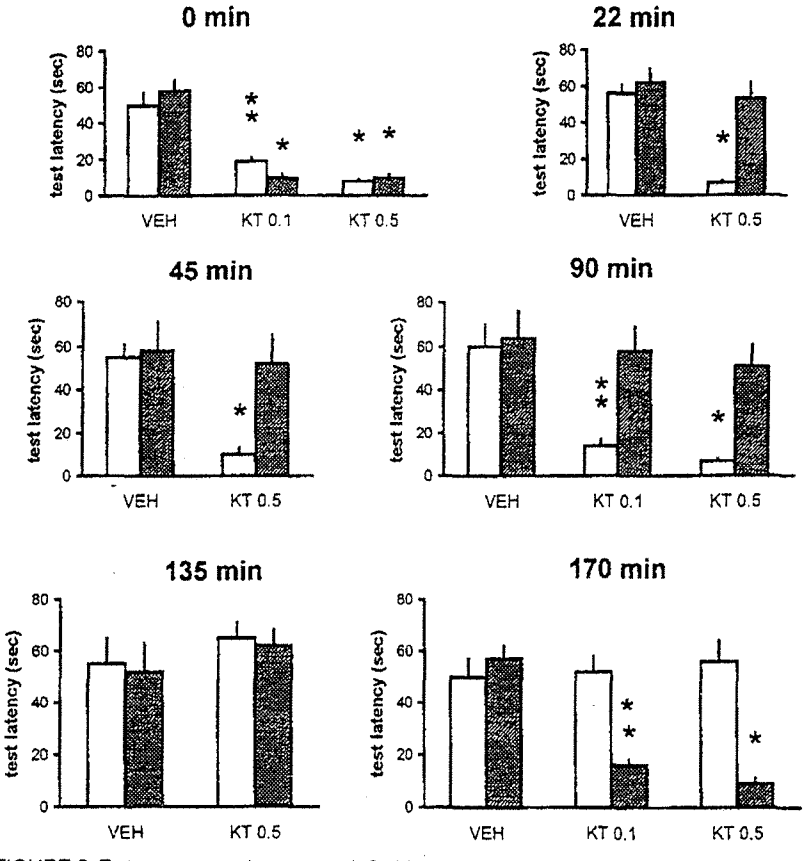

FIGURE 2. Data expressed as mean ± SEM test-session latency in the step-down inhibitory avoidance task. In all cases, short-term memory is shown in open columns, and long-term memory is shown in grey columns. Each animal was tested twice, first for short-term memory and then for long-term memory. Rats received immediate post-training bilateral infusions in the dorsal CA1 region of vehicle (VEH) or KT5720 (KT). The infusions were: 0, 22, 45, 90, 135 or 175 min after training. STM testing was at 90, 112, 135, 180, 180 and 180 min after training respectively. At the 0, 90 and 180 min training-infusion intervals, the effect of KT was studied at two different dose levels, 0.1 and 0.5 μg. At all other intervals, it was studied only at the higher dose level, 0.5 μg. Asterisks indicate significant differences from controls at $P < 0.001$ level in *t*-tests. In addition, double asterisks indicate significant differences from the higher dose at $P < 0.02$ level. In groups not marked by asterisks, the training-test latency differences were significant at a $P < 0.001$ level. KT5720 given 0 min post-training inhibited both short- and long-term memory; given 22–90 min after training it inhibited short-term memory, but not long-term memory; given 135 min after training, it had no effect; given 175 min after training, it inhibited long-term memory but not short-term memory.

DISCUSSION

Since William James (1890) proposed that a 'primary' memory system should be in charge of retention while 'secondary', or long-term, memory was being processed, the question of whether STM is a stage towards LTM or a separate process (Gold and McGaugh, 1975) has been crucial to the understanding of the organisation of memory (McGaugh, 1966, 1968). As pointed out elsewhere (Izquierdo *et al.*, 1998a), the answer to this question can only be given by: (a) an experiment showing that LTM can develop in the absence of preceding STM for the same task in the same animal, or (b) an experiment showing that this is impossible. Emptage and Carew (1993) reported that cyproheptadine is able to cancel serotonin-induced facilitation in *Aplysia*, measured at 1.5 h while leaving facilitation measured at 24 h intact; this effectively discriminated the short- from the long-term version of this phenomenon. More recently, we have been able to show that a variety of pharmacological treatments applied into the hippocampus, entorhinal or parietal cortex are able to block STM measured at 1.5 h, while leaving LTM intact or enhanced for the one-trial avoidance task in rats (Izquierdo *et al.*, 1998a–c). The present results add three more experiments to the list: KT5720 given 22, 45 or 90 min post-training. Therefore, the data now support the concept that STM and LTM for this task, as is the case with drug-induced facilitation in *Aplysia* (Emptage and Carew , 1993), rely on separate and parallel – though no doubt linked (Izquierdo *et al.*, 1998a, c) – mechanisms.

In addition, the present findings provide crucial evidence for the independence of STM from LTM at the biochemical level. Clearly, PKA is necessary for the formation of both memory types. However, it is necessary for LTM formation only immediately after training, and again 3 h later (Figure 2a, f), as shown before (Bernabeu *et al.*, 1997; Bevilaqua *et al.*, 1997). PKA is, instead, necessary for STM formation between 0 and 90 min after training; STM, unlike LTM, requires the continued activity of the enzyme over the first 90 min. It may be noted that the findings obtained with infusions given at 135 and 175 min rule out drug influences on retrieval or performance; PKA activity is clearly not necessary for the test session performance of either STM or LTM. PKA also appears not to be involved in either STM or LTM at 135 min after training.

LTM, therefore, depends on PKA activity in CA1 at the times in which this activity, and the levels of its substrate, P-CREB, are at a peak: at 0 and 3 h from the end of training (Bernabeu *et al.*, 1997). In the meantime, PKA controls short-term memory. The first peak has a priming influence both on the second peak and on short-term memory regulation: without it, neither occurs. Short-term memory regulation by PKA could well involve substrates other than CREB, such as membrane proteins that may mark or tag recently stimulated synapses (Frey and Morris, 1998). It is clear that the distinction between STM and LTM must occur mainly at the post-receptor level, since the synapses involved in the generation of both memory types must to a large extent be the same, inasmuch as both deal with the same set of sensorimotor events (Izquierdo *et al.*, 1998a, c).

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