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MARIZIA DO AMARAL TOMA

ANÁLISE DOS EFEITOS DE LESÕES HIPOCAMPAIS SOBRE A APRENDIZAGEM E MEMÓRIA ESPACIAL DE POMBOS EM SITUAÇÃO DE ESCOLHA ALIMENTAR.

Tese apresentada ao Instituto de Biologia para obtenção do título de Doutor em Biologia Funcional e Molecular, na área de Fisiologia.

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Vivemos de atos, fatos e momentos.

Os bons momentos

passam rápido,

os maus...

também !!!

Os fatos entram pra história.

Só os atos continuam.

Ivo A Siqueira

DEDICATÓRIA

Às pessoas que amo...

Binho

Meu amor, muitas pessoas passam a vida toda procurando alguém especial e na maioria das vezes não encontram. Eu encontrei meu perfume raro, e por isso sou a pessoa mais feliz desse mundo!

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RESUMO

Esse trabalho analisou o papel do hipocampo na memória de pombos numa situação de escolha espacial de alimento. Foram utilizados pombos distribuídos em três grupos: Lesão Hipocampal, Lesão Sham e Não Lesão. No Experimento 1 houve Treino Pré-lesão, para a localização e escolha do comedouro correto numa câmera retangular. No Experimento 2 a lesão hipocampal ocorreu antes do treino. Os pombos experimentais sofreram lesões hipocampais com ácido ibotênico. Após a cirurgia, houve Teste Pós-Lesão e Reversão. Testes de Estratégias avaliaram: mapeamento espacial, preferência por quadrante e guiamento. As sessões foram filmadas para registro da latência de escolha e dos acertos. Nos Experimentos 3 e 4 foi usada uma arena circular, sendo que no Experimento 4 houve lesão antes do treino e teste de retenção 25 dias após o final do treino. Os dados indicaram um maior aumento da latência de escolha quando a lesão antecedeu o treino. A lesão pré-treino afetou as escolhas corretas nos Exp. 3 e 4, em que se evidenciou uso de mapeamento espacial, mas não nos Experimentos 1 e 2. Um profundo déficit na retenção de longa duração foi observado nos pombos lesados. O Experimentos 5 avaliou os parâmetros morfológicos de AgNOR (síntese de proteínas) no hipocampo de pombos nas duas tarefas espaciais. O tamanho e área relativa de AgNOR variou entre as áreas hipocampais e os hemisférios dependendo da tarefa.

ABSTRACT

Three groups of pigeons were studied: Hippocampal lesion, Sham lesion and No lesion. The first two experiments used a task of localization and choice between two food-cups in a rectangular transparent chamber. Experiments 3 and 4 sessions were run in a circular arena with choice among four food cups. Hippocampal lesion was carried out with ibotenic acid infusions both pre-training (Experiments 1 and 3) or post-training (Experiments 2 and 4). The pre and post-training, reversal and probe test sessions were video-recorded for quantification of the latency of choice and number of correct choices. The hippocampal groups showed significantly longer latencies of choice during post-lesion tests and reversal sessions. The pre-training lesion affected the percent of correct choices only when the task favored spatial mapping strategy (Experiments 3 and 4). A retrieval test carried out 25 days after the end training (Experiment 4) indicated a large *deficit* in the retention of the long-term memory in lesioned pigeons. Finally, Experiments 5 and 6 evaluated the morphologic parameters of AgNOR in the hippocampus of pigeons submitted to the two spatial tasks. The training affected the size and relative area of AgNOR, with task-dependent differential effects both between hippocampal areas or hemispheres. These experiments corroborate previous studies with rodents and suggest functional similarity between the hippocampus of different animal species. The data also points to new evidence related to long-term spatial memory in hippocampal pigeons and to plastic changes subjacent to learning in different spatial tasks.

ÍNDICE

INTRODUÇÃO

I. INTRODUÇÃO

1.1. Aprendizagem e Memória

Um dos mais extraordinários aspectos de um comportamento animal é a sua capacidade em alterar o comportamento em função das interações com o meio ambiente, por meio da aprendizagem; as habilidades resultantes desse processo alcançam as formas mais complexas nos seres humanos. Segundo KANDEL (2001), a aprendizagem e a memória são processos fascinantes porque se referem a aspectos fundamentais da atividade humana: a aquisição de novas idéias por meio da experiência e a retenção destas idéias ao longo do tempo na memória.

A memória é a capacidade que têm o homem e os animais de armazenar informações que podem ser recuperadas e utilizadas posteriormente. Difere da aprendizagem, pois esta constitui o processo de aquisição das informações (LENT, 2001). Segundo XAVIER (1993), a memória relaciona-se com a capacidade de alterar o comportamento em função de experiências anteriores e a sua organização depende de estruturas localizadas em diferentes regiões do sistema nervoso. Como são vários os processos de memória, é possível identificar as distinções dicotômicas entre os tipos de memória no sentido de caracterizá-las conceitual e operacionalmente. Temos, assim, entre outras, as memórias de curta e de longa duração (BADDELEY e WARRINGTON, 1970), a memória de referência e a operacional (HONIG, 1978), a memória de procedimento ou implícita e declarativa ou explícita (COHEN e SQUIRE, 1980; COHEN, 1984), entre outras. Discutiremos aqui as distinções mais relevantes para este trabalho.

A mais antiga de todas as dicotomias distingue entre memória de curta e longa duração. Memória de curta duração refere-se à capacidade de armazenar uma pequena quantidade de informações por período de tempo limitado (BADDELEY e

WARRINGTON, 1970); nesse período, a informação é mantida por repetição no sistema de memória. A memória de longa duração representa a capacidade de armazenar uma grande quantidade de informações por período indefinido de tempo; a atenção do indivíduo pode ser desviada da informação crítica sem prejuízo da memória. Acredita-se que as informações que são repetidas na memória de curta duração poderiam resultar em memórias de longa duração, num processo denominado consolidação da memória. Todavia, alguns pacientes com distúrbios da memória de curto prazo são capazes de formar memórias de longa duração, sugerindo uma independência entre esses sistemas.

Uma outra classe de memória, inicialmente definida por HONIG (1978) e OLTON, et al. (1979) como memória operacional, refere-se a um tipo de memória que codifica o contexto temporal específico da informação, e que pode ser apagada depois de ser utilizada. Para testar a memória operacional é utilizado, normalmente, um procedimento conhecido como labirinto radial de oito braços (OLTON e SAMULESON, 1976) que representa uma das tarefas que torna necessário o uso da memória operacional. Os ratos são colocados numa plataforma central octogonal da qual irradiam oito braços, um de cada lado do octógono, sendo colocado alimento na extremidade de cada braço no início de cada sessão de treino. O desempenho ótimo da tarefa consiste em escolher braços sempre diferentes numa dada sessão, evitando revisitar um braço do qual o alimento já foi obtido. Depois de alguns dias de treino os ratos escolhem em média 7,6 braços diferentes nas primeiras 8 escolhas (OLTON e SAMULESON, 1976). Os autores concluem que a memória operacional é utilizada pelos ratos para manter uma lista atualizada dos braços já visitados naquela sessão. O tempo de manutenção de uma informação na memória operacional depende, portanto,

da relevância dessa informação; a simples passagem do tempo não determina inexoravelmente o decaimento da memória, como parece ocorrer no caso da memória recente.

A memória de referência independe do contexto específico da informação; nela, armazenam-se informações aplicáveis a diversas situações (no caso do labirinto radial de 8 braços, a todas as tentativas) (HONIG, 1978). Assim, seriam informações inativas (ou latentes), até que sejam ativadas pela apresentação de estímulos apropriados, o que corresponde à evocação ou lembrança (HONIG, 1978). As tarefas que envolvem as discriminações simultâneas são normalmente utilizadas para testar a memória de referência.

Esses dois tipos de memória possuem uma diferença crítica. No caso da memória operacional o critério de resposta pode se relacionar a estímulos diferentes em tentativas diferentes, havendo uma pequena quantidade de itens de informações passível de preservação. Já no caso da memória de referência o estímulo crítico é constante nas tentativas diferentes e a quantidade de itens de informação passível de ser preservada é praticamente ilimitada.

A memória de procedimento refere-se à informação sobre as regras e os procedimentos que são aplicáveis a uma variedade de circunstâncias diferentes (COHEN, 1984). Este sistema de memória não permitiria o acesso explícito ao conteúdo de conhecimento, e se expressaria apenas através do desempenho; isto é, através da ativação das estruturas de processamento ou procedimentos envolvidos nas tarefas de aprendizagem. A aquisição de habilidades motoras envolve esse tipo de aprendizagem. Todavia, há aprendizagens não-motoras que envolvem um processamento similar de informações, como é o caso do desenvolvimento de

habilidades perceptuais e de habilidades cognitivas. Por esta razão, o sistema seria melhor denominado como não-declarativo. A aquisição e a retenção desse tipo de informação decorreria da plasticidade inerente às estruturas de processamento que se modificam em cada ocasião em que a informação é processada, o que requer múltiplas tentativas de aprendizagem e permite pouca elaboração sobre o que foi aprendido.

A memória declarativa refere-se, por sua vez, a um sistema de conhecimento em que a informação específica e factual é armazenada de uma forma explicitamente acessível para uso posterior, sendo evocável em função da demanda (COHEN, 1984). Este sistema adquiriria e manteria uma representação dos produtos específicos das operações realizadas pelas estruturas de processamento ativadas durante as tarefas de aprendizagem, envolvendo a criação de novas estruturas de dados para representar explicitamente os resultados das experiências.

1.2. A aprendizagem espacial

A percepção da localização espacial – aprendizagem espacial – faz parte da categoria de aprendizagem relacional. Na realidade a aprendizagem espacial envolve a aquisição do conhecimento sobre as relações entre muitos estímulos. Por exemplo, para nos familiarizarmos com os objetos presentes numa sala deveremos, primeiramente, aprender a reconhecer cada um dos objetos e, também, a localização relativa dos objetos entre si. Como resultado, as nossas percepções desses objetos e de suas posições relativas possibilitam saber também a nossa localização (CARLSON, 2002).

Durante toda a evolução, a orientação no espaço foi, sem dúvida, um grande problema. Com o desenvolvimento dos olhos complexos e de cérebros para

processarem a informação, surgiu a possibilidade dos animais orientarem-se através de marcas terrestres visuais. Além disso, a navegação celeste tornou-se possível, com a capacidade de manter ângulos definidos em relação ao sol. Assim, muitos invertebrados, peixes, anfíbios, répteis, aves e alguns mamíferos tornaram-se capazes de empregar o sol como um compasso para voltar ao lar e para migração. Para a maioria dos animais, contudo, a capacidade de usar um compasso solar é aprendida e vários auxílios de navegação são empregados. Sob muitas circunstâncias, as marcas visuais terrestres têm precedência sobre o sol como auxiliares de navegação (DETHIER e STELLAR, 1988).

A navegação é um processo que capacita um animal a ir de um local para outro no espaço a partir de uma trajetória aprendida. Esse processo comportamental é muito importante para os animais, pois a partir dele os animais podem sair à procura de alimento, abrigo ou parceiros e sempre voltar para casa. Uma trajetória requer uma representação da posição do animal no espaço e a posição dos locais para os quais o animal dirige seus movimentos, ou seja, são necessárias as informações acerca de posições e de posicionamentos. Para o animal voltar para sua casa é necessário que, antes de seu deslocamento, ele analise todos os estímulos do ambiente, as suas distâncias e a relação entre eles.

Para isso ele pode se guiar por pistas distais e ou proximais do ambiente. Na orientação por pistas proximais um objeto é visível, audível ou detectado pelo cheiro e então pode ser aproximado de uma certa distância. Na orientação por pistas distais as pistas podem ser invisíveis, inaudíveis e indetectáveis pelo cheiro. Assim, para um animal se localizar no espaço é necessário aprender sobre os estímulos presentes,

associar essas pistas ao local, estabelecer as representações das posições relativas às pistas do meio ambiente e utilizar tais representações para se orientar no ambiente.

Na tentativa de explicar como o organismo aprende e o cérebro processa essas informações espaciais foram desenvolvidas algumas teorias, dentre elas a teoria do mapa cognitivo (O'KEEFE e NADEL, 1978) que se concentrou nos aspectos espaciais do ambiente como sendo atributos críticos da memória. Segundo essa teoria, as informações seriam processadas em dois sistemas distintos: sistema de *taxon* e de mapeamento cognitivo (O'KEEFE e NADEL, 1978).

Segundo O'KEEFE e NADEL (1978), as estratégias baseadas no sistema de *taxon* foram chamadas pelos autores de estratégias de rotas e podem ser subdivididas em estratégia de guiamento (*guidance*) e de orientação (*orientation*).

O uso de estratégias de guiamento pressupõe que o animal efetue a localização de um alvo ou objetivo pelo uso de informações fornecidas por alguma seqüência de estímulos ou pistas que fazem parte do ambiente imediato ou local (por exemplo, intralabirinto; ou seja, identificar um objeto ou uma pista no ambiente da qual deve aproximar-se ou afastar-se). Por outro lado, quando é usada a estratégia de orientação, - também denominada de orientação corpórea ou de estratégia corpórea egocêntrica -, a localização do alvo é intermediada por informações de estímulos motores, não dependem da distribuição das pistas sensoriais intra ou extra ambiente experimental, e corresponde à aprendizagem e à repetição de uma seqüência de movimentos do corpo.

A consideração de um sistema de mapeamento implica que o organismo utiliza estratégias de lugar com mapeamento espacial cognitivo. Para formar um mapa cognitivo são utilizadas as pistas distais, que podem servir para a construção de um sistema de localização de um ponto por triangulação; para tanto, são necessárias pelo

menos duas pistas distais, permitindo cálculos entre a posição do animal e a das pistas. Para formar um mapa espacial, é fundamental conhecer a relação angular entre as diferentes pistas dentro do ambiente e saber que essa relação se altera com o deslocamento; isto envolve a construção de um mapa alocêntrico do ambiente. Os mapas são formados durante a exploração do ambiente pelos animais. Os mapas podem ser utilizados pelos animais para se localizarem no ambiente, incluindo os locais de recompensas e de punições ou, ainda, para se deslocarem de um local para outro por meio das diferentes rotas disponíveis.

As diferentes estratégias não são completamente exclusivas e podem, na realidade, serem utilizadas simultaneamente para a resolução da tarefa, formando um conjunto complementar. A aprendizagem de lugar tende a predominar quando os estímulos visuais e fora da câmara/ labirinto experimental são abundantes. Contudo, quando o ambiente é homogêneo predomina a aprendizagem de resposta, com a utilização da estratégia de orientação ou de guiamento.

1.3. Substratos Neuroanatômicos da Memória Espacial

A teoria de O'KEEFE e NADEL (1978), desde a sua proposição, conduziu a uma série de experimentos e parece existir concordância com o fato de que o labirinto aquático de Morris, equipamento desenvolvido por MORRIS (1981), é o melhor aparato utilizado para testá-la. O labirinto nada mais é que um tanque com água opaca, dentro do qual se encontra uma plataforma de fuga submersa e invisível, localizada em uma posição fixa em relação às dicas distais presentes na sala de experimento. A cada tentativa de treino, os animais são colocados dentro da piscina a partir de pontos diferentes, e a tarefa consiste em localizar a plataforma de fuga. Assim, de acordo com

a terminologia de O'KEEFE e NADEL (1978), para localizar a plataforma submersa os animais devem estar utilizando uma hipótese de lugar. Quando os ratos com lesão do hipocampo são treinados nessa situação, observa-se que, em comparação aos animais intactos, apresentam prejuízos na aprendizagem dessa tarefa, com aumento no tempo para encontrar a plataforma de fuga (MORRIS *et al.*, 1982).

Outro tipo de situação muito usada para estudar a aprendizagem espacial é o labirinto radial (OLTON e SAMUELSON, 1976).

Segundo EICHENBAUM *et al.* (1994), existem dois sistemas de memória hipocampal. O primeiro sistema hipocampo-dependente tem a capacidade para realizar representações relacionais, garantindo a operação de ambas classes de memórias para o armazenamento das relações entre itens perceptuais distintos, além da evocação e da expressão flexível de memórias em contextos novos. A representação relacional constitui uma memória de "espaço", uma elaborada organização que permite o acesso para a memória via novas rotas e suporta a expressão flexível de memórias via caminhos não exercitados previamente (EICHENBAUM *et al.*, 1992).

Na ausência da representação relacional, a aprendizagem poderia, então, ser adquirida por meio de *representações individuais* envolvendo o aperfeiçoamento de rotinas motoras específicas e adaptações de resposta sensorio-motoras. Nesse caso, haveria a operação de um segundo sistema, hipocampo-independente, com a capacidade para armazenar as representações individuais, envolvendo a aquisição de conceitos relacionados a itens individuais e que são expressos somente após repetições dos eventos aprendidos (EICHENBAUM *et al.*, 1992).

Em contraste aos estudos com roedores, a maioria dos estudos hipocampais em pombos focalizou os efeitos das lesões na navegação, em particular no comportamento de voltar para o viveiro. Tradicionalmente os sistemas navegacionais de pombos, que permitem a orientação para o viveiro, após a liberação, foram conceituados como consistindo de dois mecanismos.

O primeiro mecanismo refere-se ao mapa navegacional, e é usado para permitir a um pombo orientar-se de volta para casa quando liberado de um local não familiar distante. O mecanismo exato do uso de um mapa navegacional ainda não está claro mais envolve uma inter-relação entre o sistema de olfação e sistema de compasso baseado pelo sol ou baseado no geomagnetismo (LUSCHI e DALL'ANTONIA, 1993). O segundo mecanismo, chamado ponto de referência de navegação, é usado quando os pombos estão nas vizinhanças de seu viveiro. Esse sistema permite a localização do viveiro por meio do uso de pontos de referências visuais familiares (BINGMAN e IOALÉ, 1989).

Um modelo mais recente desenvolvido por KAMIL e CHENG (2001), propõe que, num estudo de laboratório, os pássaros representam a localização de uma pista tal como a direção e a distância particular de cada marca disponível. De relevância psicológica potencial, a relação de direção e distância para cada marca é representada independentemente de algumas outras marcas (i.e. em uma maneira não configural).

Desde o desenvolvimento da teoria mapa cognitivo hipocampal (O' KEEFE e NADEL (1978), muitos dos estudos publicados interessaram-se em comparar a hipótese do mapeamento espacial com formulações teóricas rivais. Entretanto tem sido muito difícil obter evidências convincentes para manter uma teoria ou excluir outras.

No homem, embora recentemente tenha se tornado possível à obtenção de imagens funcionais durante a realização de operações mentais e comportamentos específicos, a maior parte dos dados de que dispomos sobre o processamento mnemônico teve origem na prática e no estudo de casos clínicos por neurologistas. Um dos primeiros estudos foi realizado com o paciente HM. Após a remoção da maior parte da formação hipocampal e estruturas associadas no lobo temporal medial (a amígdala e áreas hipocampais e parahipocampais), este paciente apresentou um profundo prejuízo em novas aprendizagens, com o esquecimento de eventos da vida diária tão rapidamente quanto ocorriam (SCOVILLE e MILNER, 1957 *apud* EICHENBAUN *et al.*, 1992). Esse trabalho evidenciou que o hipocampo era crítico para apenas para alguns tipos de memória, já que as capacidades motoras, perceptuais, cognitivas e lingüísticas permaneciam intactas e ele podia se lembrar de memórias remotas adquiridas anos antes da cirurgia; o acesso à memória remota estava intacto. A incapacidade de HM era de estabelecimento ou manutenção de novas memórias em longo prazo (SCOVILLE e MILNER, 1957 *apud* EICHENBAUN *et al.*, 1992). Posteriormente, trabalhos com outros pacientes amnésicos mostraram conclusivamente que só certos aspectos de novas aprendizagens ou de memória a longo-prazo são prejudicados, fundamentando a separação de formas de memória que foram denominadas hipocampo-dependente e hipocampo-independente (SCHACTER, 1987; SHIMAMURA, 1986).

A partir dos trabalhos de SCOVILLE e MILNER (1957), no paciente HM, se iniciou uma via promissora para os estudos das bases anatômicas da aprendizagem e memória. Os estudos realizados até hoje permitiram conhecer, com certa profundidade, as características comportamentais e anatômicas da síndrome amnésica, e ao mesmo

tempo contribuíram significativamente para a formulação do conceito de sistemas de múltiplas memórias, tão importante na neurociência moderna.

O rápido desenvolvimento do estudo da síndrome amnésica só se deve pelo desenvolvimento de um modelo de amnésia em primatas não humanos, usando uma específica e cuidadosa série de testes comportamentais (GAFFAN, 1974; SQUIRE e ZOLA-MORGAN *et al.*, 1988; ZOLA-MORGAN e SQUIRE, 1985). Logo após os primeiros experimentos com os modelos primatas, iniciaram-se os estudos com roedores. Apesar das evidências que mostram que as maiores descobertas sobre a formação hipocampal e memória foram desenvolvidas em humanos e primatas, os principais fundamentos experimentais para a consideração de que alguns tipos de memória são hipocampo-dependentes e outras são hipocampo-independentes, vêm de estudos realizados com modelos de amnésia realizados em roedores (O'KEEFE e NADEL, 1978; OLTON *et al.*, 1979).

Alguns anos após o início das pesquisas com roedores houve um aumento do interesse no entendimento da função da formação hipocampal em várias outras espécies animais, dentre eles os pombos, que são um excelente modelo para estudo da formação hipocampal (BINGMAN *et al.*, 1984; GOOD, 1987). O comportamento de navegação dos pássaros é uma área atrativa de estudos não só por constituir um fenômeno curioso e complexo, mas também porque é um extraordinário modelo que explora como o cérebro é capaz de representar na memória a relação espacial entre os estímulos ambientais, que são usados para criar um mapa.

Atualmente o estudo da aprendizagem e memória consiste em modelos animais de amnésia e de tarefas. Entretanto, apesar de tantos estudos, a natureza específica do

processamento da informação na formação hipocampal ainda permanece muito controversa.

1.4. O hipocampo de mamíferos e aves são comparáveis

O complexo hipocampal de aves consiste de um hipocampo situado medialmente (H) e uma área parahipocampal situada dorsomedialmente (APH) (KARTEN e HODOS, 1967). Do ponto de vista embriológico, o hipocampo de aves emerge da mesma porção do tubo neural a partir da qual o telencéfalo e o hipocampo de mamíferos se desenvolvem (COLOMBO e BROADBENT, 2000).

Existe uma questão que há muito tempo os pesquisadores tentam responder referente à comparabilidade entre as aves e os mamíferos, ou seja, se o complexo hipocampal de aves é comparável anatomicamente ao hipocampo de mamíferos. Dado que existem 300 milhões de anos de evolução independente entre mamíferos e aves, pode não ser surpreendente que exista um número notável de diferenças estruturais entre o hipocampo de aves e mamíferos.

Uma dessas diferenças está na estrutura do hipocampo de aves, que se distingue consideravelmente da estrutura do hipocampo dos mamíferos. Em contraste com a estrutura em três camadas muito bem descrita nos mamíferos, o hipocampo de aves apresenta células que se organizam numa forma em V. Apesar da estrutura diferente, existem propostas que sugerem que a área em V corresponderia ao corno de *Ammon* de mamíferos; a região mais dorsal a essa área em V seria comparável às camadas granulares e hilar do giro denteado (ERICHSEN *et al.*, 1991), enquanto que a região parahipocampal poderia ser comparada ao *subiculum* dos mamíferos (BENOWITZ e KARTEN, 1976). Entretanto, existem poucas evidências que realmente

comprovem que as estruturas do cérebro de aves tais como o *subiculum*, o córtex entorrinal e o córtex perirrinal são similares aos de mamíferos (COLOMBO e BROADBENT, 2000).

Outras comparações estruturais abordam as conexões eferentes e aferentes do hipocampo de aves. No complexo hipocampo-parahipocampo há um feixe de fibras que emerge desta região e passa medialmente ao ventrículo, e entram ipisilateralmente no *septum* onde termina na comissura *pallii.* Na comissura *pallii,* o feixe de fibras é dividido em três vias. Uma via passa através do *septum* ventrolateral para alcançar o *núcleo taeniae*, onde termina. Uma segunda via continua ventralmente até atingir as proximidades do núcleo hipotalâmico lateral. Aí, este feixe de fibras muda de direção e projeta-se posteriormente, continuando a manter uma posição lateral com respeito ao núcleo hipotalâmico lateral (CASINI *et al.*, 1986; KRAINIAK e SIEGEL, 1978).

Os aferentes que chegam no complexo hipocampo-parahipocampo originam-se ipisilaterailmente do *palidum* medial e lateral, hipocampo, parahipocampo, área corticóide dorsolateral, núcleo da banda diagonal, *septum* ventral e *núcleo taeniae.* Também há projeções originárias dos núcleos mamilares laterais, *estratum* celularis interno e núcleo paramediano interno do tálamo, da área ventral de *Tsai*, do núcleo *reticularis pontis oralis,* e do núcleo da rafe (CASINI *et al.*, 1986; KRAYNBIAK e SIEGEL, 1978). Outra região que envia projeções para a região parahipocampal é o cortex piriforme (BINGMAN *et al.*, 1994).

Outras comparações estruturais abordam as conexões eferentes e aferentes do hipocampo de aves. Estas são consideradas similares ao hipocampo de mamíferos porque ambas as regiões em ambos animais recebem projeções do hipocampo contralateral, tálamo, hipotálamo, núcleo da banda diagonal, *locus coeruleus*, e núcleos

da ... O hipocampo de ambas as espécies projetam-se para o núcleo septal, banda ...nal e hipotálamo (KRAYNIAK e SIEGEL, 1978; CASINI *et al.*, 1986).

Além disso, os neurotransmissores e os neuropeptídeos da formação hipocampal de aves e mamíferos são similares. As fibras aferentes que se projetam ao hipocampo de aves com terminações serotoninérgicas, colinérgicas e catecolaminérgicas são similares às encontradas em mamíferos (KREBS *et al.*, 1991). Também foram descritas similaridades entre o hipocampo de mamíferos e aves quanto aos neuropeptídeos, tais como a ocorrência de imunorreatividade para a somatostatina, o neuropepitídeo Y, o peptídeo intestinal vasoativo, as encefalinas e a substância P (ERICHSEN *et al.*, 1991; ROSINHA *et al.*, 2003).

Finalmente, encontramos alguns paralelos na eletrofisiologia. Foi verificado que em pombos - correio existem formas de LTP dependentes de receptores NMDA similares às observadas em mamíferos (SHAPIRO e WIERASZKO, 1996). LTP independente de NMDA foi descrita em pombos não correio (SHAPIRO e WIERASZKO, 1996) e no hipocampo de galinhas (MARGRIE *et al.*, 1998). E, assim, é importante reconhecer que as sinapses no hipocampo de aves, como em mamíferos, mostram plasticidade na forma de LTP (MARGRIE *et al.*, 1998). Por outro lado, o ritmo Theta também foi verificado no hipocampo de pombos (SIEGEL *et al.*, 2000).

Em sumário, dado o número de evidências mostrado acima, parece haver fundamentação favorecendo considerações de que o hipocampo de aves seria similar ao de mamíferos. Essas evidências de similaridades também estimularam estudos com lesões hipocampais em aves na medida que tais estudos permitiriam a ampliação do entendimento da função hipocampal na memória. Deve-se lembrar, contudo, que o conhecimento sobre o hipocampo de aves é escasso, fato este que justifica a

importância de novos estudos morfológicos e funcionais que possam delimitar com maior precisão as semelhanças e diferenças entre aves e mamíferos.

1.5. Lesões experimentais na formação hipocampal e o estudo da memória em pombos

O estudo do efeito de lesões sobre a memória de animais, atualmente é muito utilizado na tentativa de esclarecer a natureza das representações hipocampais da memória. Esses estudos mostram que após lesões hipocampais a aprendizagem de algumas tarefas é prejudicada, enquanto que em outros tipos de tarefas não são prejudicados. Esse tipo de evidência experimental relaciona-se diretamente com a proposição da classificação de tarefas como hipocampo-dependentes ou hipocampo-independentes (EICHENBAUN *et al.*, 1992). Apesar dessas divisões, parece que há diferentes tipos de memórias, relacionadas talvez a diferentes tipos de informação, e que são localizadas em muitos, possivelmente na maioria dos sistemas neurais.

A memória pode ser estocada em diferentes áreas neurais, correspondendo talvez, àquelas áreas responsáveis a formas específicas de informação processada. Dentro desta aceitação não existe um conceito de uma única área de memória. Entretanto, deve-se considerar que existem diferentes áreas no cérebro, cada uma responsável por diferentes formas de informação estocada. O hipocampo, por exemplo, tem papel fundamental na construção e armazenamento de informações espaciais na forma de mapas cognitivos.

Em pássaros, foi analisada uma série de tarefas espaciais e não espaciais que são afetadas por lesões do hipocampo tarefa hipocampo-dependentes. Por exemplo, GOOD (1987), mostrou que os pombos com lesões hipocampais são prejudicados na

reversão, mas não na aquisição, de uma tarefa de discriminação em um labirinto em T envolvendo orientação à esquerda ou à direita. BINGMAN *et al.* (1987, 1988) relataram que pombos-correio com lesões hipocampais tiveram prejuízos na memória de referência espacial referente à orientação em tarefa de voltar para o viveiro.

SHERRY e VACCARINO (1989) também examinaram os efeitos da lesão hipocampal em pássaros estocadores e verificaram um prejuízo de memória para a localização de alimento estocado em um aviário. FREMOUW *et al.* (1997) programaram uma situação de teste que seria considerada comparável ao labirinto aquático de Morris. Usaram uma arena circular seca, na qual os pombos forrageavam livremente e encontravam comida em 8 comedouros. Apesar dos pombos lesados e controles aprenderem a tarefa, os pombos lesados foram mais lentos e tiveram uma menor precisão na escolha dos comedouros. REIS *et al.* (1999) estudaram a memória ao contexto após condicionamento clássico aversivo em pombos e verificaram menor *freezing* ao contexto em animais lesados. WATANABE (1999, 2001) estudou pombos lesados em uma câmara operante com três discos de resposta e verificou prejuízo na aquisição de discriminação espacial (WATANABE, 1999) e um aumento do número de sessões de treino para atingir o critério de aprendizagem (WATANABE, 2001).

Outros estudos analisaram o desempenho em tarefas que não foram afetadas por lesões hipocampais tarefas hipocampo-independentes. REILLY e GOOD (1987) usaram uma tarefa de condicionamento operante de alternação espacial e não observaram prejuízos após a lesão hipocampal. GOOD (1987) não encontrou alterações em pombos lesados que foram treinados na aquisição e na reversão de discriminação de padrões simultâneos em um labirinto em T. WATANABE (1999)

estudou pombos com lesões hipocampais e não encontrou prejuízo em uma tarefa de discriminação de cor em uma câmara operante.

1.6. Papel do hipocampo na aquisição e recuperação de memórias

Uma variedade de tarefas que treina os animais antes e após cirurgia foi desenvolvida ao longo do tempo para melhor entender o funcionamento hipocampal e com isso novas descobertas têm surgido. Dentre essas descobertas alguns pesquisadores verificaram que se os animais aprendem uma tarefa antes do hipocampo ser removido, eles apresentam um prejuízo temporário. Dentre os trabalhos que relatam um prejuízo no tempo para completar a tarefa aprendida ou para voltar para casa podemos citar os de BINGMAN *et al.* (1987; 1988); FREMOUW *et al.* (1997); WATANABE (2001); COLOMBO *et al.* (2001); AMARAL-TOMA e FERRARI (*in press*).

Essa recuperação das informações aprendidas pode ser explicada pelo modelo de consolidação de memória de ALVAREZ e SQUIRE (1994). Considerando o hipocampo de mamíferos, eles postularam que o hipocampo e estruturas relacionadas ao lobo temporal medial servem como um arquivo temporário de memória e que o neocórtex seria um arquivo permanente da memória de longa duração. De acordo com ALVAREZ e SQUIRE (1994), a consolidação da informação ocorreria gradualmente como um resultado dos processos intrínsecos do neocórtex que requerem a participação ativa do hipocampo e estruturas relacionadas ao lobo temporal medial.

Quando o hipocampo é removido antes da aquisição os resultados são controversos. Alguns pesquisadores verificaram que os animais são incapazes de aprender. Dentre eles, STRASSER *et al.* (1998), em um estudo de campo verificou que os pombos lesados antes do treino são incapazes de aprender e de formar uma

representação de marcas espaciais. WATANABE (1999) também relatou um prejuízo na aquisição de discriminação espacial operante. Apesar desses dados outras pesquisas mostram que os pombos que passaram por um supertreino são capazes de aprender a tarefa mesmo na ausência do hipocampo, sugerindo o uso de outro tipo de estratégia que não as utilizadas com o hipocampo intacto. Dentre esses estudos encontramos os de COLOMBO *et al.* (2001), que mostraram que os pombos com lesão do hipocampo mostraram prejuízo numa situação de automodelagem em uma câmara operante, tanto no número de respostas quanto no treinamento necessário para atingir o critério. AMARAL-TOMA e FERRARI (*in press*) também verificaram resultados semelhantes em uma câmara com localização espacial e escolha simples de alimento, onde os pombos são capazes de aprender, mas com um tempo de latência de escolha maior que os controles.

Apesar dos dados mostrarem que os animais conseguem aprender uma tarefa sem um hipocampo funcional, pouco é estudado sobre quanto tempo essa informação permanece estocada, já que, (como visto acima) é necessário um hipocampo intacto para ocorrer à consolidação. RAMOS (2000), contudo, preocupou-se com esse problema. Ele desenvolveu um experimento com ratos em uma tarefa espacial em labirinto radial de quatro braços para verificar o que acontecia quando os animais, que haviam aprendido, foram retestados 24 dias após a realização do treino. Os resultados mostraram que os ratos com lesão hipocampal apresentaram um profundo déficit quando testados 24 dias após atingirem o critério de aprendizagem. Esses resultados mostraram que a retenção das informações aprendidas necessita um hipocampo intacto. O tempo de 24 dias foi utilizado porque, num estudo anterior, BONTEMPI *et al.* (1999), mapearam regiões selecionadas do cérebro com o uso de (^{14}C) 2-deoxy-glicose

para medir a atividade metabólica da formação hipocampal de curto prazo após o treino espacial. Verificaram, contudo, que após 25 dias, quando o processo de consolidação, provavelmente, já havia sido concluído, a ativação hipocampal diminuiu.

1.7. A seletividade das lesões experimentais

Outra preocupação que surgiu nas últimas décadas foi com o tipo de lesão e sua eficácia no estudo da memória. O uso de neurotoxinas para destruir células hipocampais aumentou muito. Este aumento se deu porque as lesões convencionais tais como a aspiração, as eletrolíticas e as de radiofreqüência, causam danos em áreas adjacentes e em estruturas distantes, danificando os axônios que passam na área lesada e também a vascularização do tecido. Com as neurotoxinas, este problema é minimizado (JARRARD, 1989).

Dentre os aminoácidos excitatórios mais potentes e mais utilizados em pesquisas de lesões hipocampais encontramos o ácido ibotênico (IBO). O IBO é um aminoácido heterocíclico excitatório, análogo ao ácido glutâmico (KIZER *et al.*, 1978; BÜRES *et al.*, 1983).

O IBO foi originalmente isolado da *Amanita muscaria*, um tipo de cogumelo (JOHNSTON *et al.*, 1968). Essa neurotoxina parece interagir com o receptor aminoácido excitatório pós-sináptico (NMDA), despolarizando por um período prolongado todos os neurônios que apresentam este receptor (NADLER, 1979), resultando em um desequilíbrio iônico (SHINOZAKI e KONISHI, 1970), decorrente de um grande influxo de cálcio e sódio na célula e um aumento extracelular de potássio, conseqüentemente levando à morte celular (EVANS, 1981). Apresentam também um

dramático efeito farmacológico: em concentrações tóxicas destroem os corpos celulares (KIZER, 1978).

Um dos principais motivos para a grande utilização do IBO nas pesquisas neurológicas é que a infusão local de IBO resulta em áreas mais restritas de degeneração (SCHAWARCZ, 1979) e os efeitos fisiológicos anormais são de menor gravidade (ALDINIO *et al.*, 1981).

JARRARD (1989) foi o primeiro pesquisador a usar o acido ibotênico (IBO) para destruir seletivamente as células do hipocampo de ratos. O modelo envolveu múltiplas injeções locais com pequenas quantidades de IBO guiadas por um aparelho esterotáxico. Esta pesquisa também mostrou que o IBO não interrompia os axônios.

Dada a complexidade da neuroanatomia as técnicas de lesões convencionais podem ser variáveis e produzirem mudanças comportamentais difíceis de serem interpretadas. Por exemplo, lesões eletrolíticas e aspiração têm sido usadas para lesar o hipocampo em muitos estudos, mas estas técnicas interrompem as fibras extrahipocampais de passagem no *alveus* e fímbria que se projetam em ambas direções, causam dano extenso para a vascularização, e geralmente causam dano direto ao *subiculum* em mamíferos. No caso de lesões do córtex entorrinal, muitos pesquisadores usaram lesões eletrolíticas ou por radiofreqüência, resultando em um dano que incluem o *subiculum* e o giro denteado (JARRARD, 1991).

Pelas razões apresentadas acima o uso do IBO nas lesões hipocampais permite uma lesão seletiva dos componentes da formação hipocampal ou do próprio hipocampo.

1.8. As análises moleculares auxiliam o estudo da memória

As técnicas celulares ou moleculares que auxiliam o entendimento das funções mnemônicas atualmente estão sendo muito utilizadas para desvendar os processos cognitivos relacionados com a aprendizagem e a memória. Esses processos podem ser prontamente relacionados com análises celulares ou moleculares (KANDEL, 2001).

Diversos trabalhos relatam que a aprendizagem pode levar o sistema nervoso central a mudanças significantes nos processos celulares. Modificações na síntese de proteína (NOGUÉS *et al.*, 1996; COLOMBO *et al.*, 1997; LUO, *et al.*, 2001), alterações bioquímicas (ROBERSON e SWEATT, 2001), aumento da expressão dos genes de expressão precoce tais como c-*fos* (HERRERA e ROBERTSON, 1996) e zif-268 (BRITO, 2002), mudanças na expressão ou ativação de receptores (LUSCHER e FRERKING, 2001; NEWCOMER e KRYSTAL, 2001), alterações da eficácia sináptica (LAROCHE, 2000; HE *et al.*, 2002; GUZOWSKI, 2002), modificações no tamanho das espinhas dendríticas (YUSTE e BONHOEFFER, 2001) e no tamanho dos núcleos e dos corpos neuronais (QÜ *et al.*, 1994; GARCIA-MORENO, 2000; VARGAS *et al.*, 2000) são alguns dos diversos relatos científicos que comprovam o envolvimento do processo de aprendizagem propiciando a memória do sistema nervoso central.

Todas essas mudanças complexas podem atuar na consolidação do processo de aprendizagem e memória. A síntese de proteína é decisiva nos processos de consolidação da memória em longo prazo. O treinamento dos animais é uma tarefa específica que leva a um aumento das taxas da síntese de proteínas no cérebro e aumento das quantidades da expressão do RNA e, consequentemente, a consolidação da memória (KANDEL, 2001).

A aprendizagem resulta em mudanças significantes no cérebro, por exemplo, no tamanho dos núcleos e dos corpos neuronais (QÜ *et al.*, 1994; GARCIA-MORENO, 2000; VARGAS *et al.*, 2000). Essas alterações estruturais podem refletir o processo de consolidação da aprendizagem e da memória. A relação entre a consolidação das informações aprendidas e o aumento da síntese de proteínas no neurônio é um fenômeno presente em todos os vertebrados (ROSENZWEIG, 1996). Por exemplo, GARCIA-MORENO *et al.* (2000), verificaram um maior aumento da atividade biossintética na região dorsal do hipocampo de ratos após o treino. Outro estudo, que analisou a capacidade de aprendizagem em ratos novos e velhos em uma tarefa de esquiva condicionada, mostrou um aumento na síntese de RNA ribossômico no hipocampo de ratos que aprenderam a tarefa (QÜ *et al.*, 1994). Em pássaros, foi também verificado o aumento da síntese de RNA e proteínas no cérebro após a estampagem (BATESON *et al.*, 1972) ou após o treino de escolha passiva (BULLOCK *et al.*, 1992). FREEMAN *et al.* (1995) analisaram a esquiva passiva em galinhas e verificaram amnésia após o uso de inibidores de síntese de proteínas administrados 0,5 h a 4-5 h após o treino. Também foram verificados vários resultados interessantes em peixes, tais como um aumento da síntese de RNA neuronal após a aprendizagem (ROTHER *et al.*, 1995; SHASHOUA, 1976), prejuízo na consolidação da memória após a utilização de inibidores da síntese de proteínas em uma tarefa de escolha ativa (SCHMIDT *et al.*, 1995) e aumento da região organizadora nuclear argirofílica após a aprendizagem espacial (VARGAS *et al.*, 2000).

A maioria da síntese de RNA ribossômico (rRNA) ocorre na região organizadora nucleolar (NOR) do núcleo da célula. Essa região é uma região ativa do núcleo, onde ocorre a maior parte da síntese de RNA ribossômico. Essas regiões (NORs) são

compostas de ácido desoxiribonucleíco ribosomal e proteínas, algumas das quais são argirofílicas (TRÖSTER *et al.*, 1985). Essas regiões NORs podem ser demonstradas pelo método de coloração AgNOR (PLOTON *et al.*, 1986), que coram argirófilos NOR associados a proteínas (BUYS e OSINGA, 1980; MÉHES *et al.*, 1993). Como também ocorre um aumento da AgNOR durante o aumento da síntese de proteínas, o número e a área do AgNOR refletem a atividade de transcrição gênica de rRNA (CROCKER e NAR, 1987; MORTON *et al.*, 1983). Então, a atividade celular relacionada à atividade transcricional pode ser avaliada pela medida do número e área de AgNOR (GONZÁLEZ-PARDO *et al.*, 1994; LOUIS *et al.*, 1992; MORTON *et al.*, 1983). Esta técnica histoquímica e morfométrica foram aplicadas com sucesso no estudo da atividade transcricional em células cancerosas (TOMOBE *et al.*, 2001; BUCHINSKA e POLISHCHUCK, 2001; HEBER *et al.*, 2002), células em desenvolvimento (DÁMASO, *et al.*, 1988; GONZÁLEZ-PARDO *et al.*, 1994) e dimorfismo sexual (GONZÁLES-GONZÁLES *et al.*, 1996; GONZÁLES-PARDO *et al.*, 1994), e pouco utilizada nos estudos de atividade transcricional em relação aos processos de aprendizagem e memória (VARGAS *et al.*, 2000; GARCIA-MORENO *et al.*, 2000; QÜ *et al.*, 1994).

OBJETIVOS

II. OBJETIVOS

O comportamento de navegação dos pássaros é atrativo como uma área de estudo não só por causa do interesse estético para mentes curiosas, mas também porque fornece um extraordinário modelo para explorar como o cérebro é capaz de representar na memória a relação espacial entre os estímulos ambientais, que são usados para criar um mapa. Utilizando esse modelo, este trabalho buscou investigar a aprendizagem espacial, no sentido de contribuir para uma maior compreensão do envolvimento do hipocampo de pombos em diferentes tarefas espaciais. Para isso foram utilizados diferentes procedimentos para a análise da aprendizagem espacial, associado a técnicas de lesão hipocampo e de análises moleculares. Os objetivos específicos foram os seguintes:

Experimento 1. Propôs-se analisar em uma tarefa espacial simples (1) os efeitos da lesão hipocampal na recuperação das informações espaciais já aprendidas e consolidadas antes da lesão; (2) os efeitos da lesão hipocampal na capacidade de estabelecer novas relações espaciais, após a reversão da localização dos comedouros; e (3) as estratégias utilizadas para o desempenho eficiente na situação.

Experimento 2: Pretendeu analisar em uma tarefa espacial simples (1) os efeitos da lesão hipocampal na aquisição de informações espaciais; (2) na capacidade de estabelecer novas relações espaciais, após a reversão da localização dos comedouros; e (3) as estratégias utilizadas para o desempenho eficiente na situação.

Os objetivos que orientaram o desenvolvimento dos Experimentos 1 e 2 são discutidos em função dos resultados apresentados na forma do **ARTIGO 1,** " Effects of

hippocampal lesions in a food location task in pigeons", que compõe o presente trabalho.

Experimento 3. Procurou analisar em uma tarefa espacial complexa (1) as relações espaciais envolvidas no comportamento de escolha alimentar; (2) os efeitos da lesão hipocampal na recuperação das informações espaciais já aprendidas e consolidadas antes da lesão; e (3) a utilização de estratégia de mapeamento cognitivo para o desempenho eficiente na situação.

Os objetivos que orientaram o desenvolvimento do Experimento 3 serão discutidos em função dos resultados apresentados na forma do **ARTIGO 2:** "Effect of ibotenic acid hippocampal lesion on spatial choice in pigeons", que faz parte deste trabalho.

Experimento 4. Analisou em uma tarefa espacial complexa (1) os efeitos da lesão hipocampal na aquisição de informações espaciais; (2) a utilização da estratégia de mapeamento para o desempenho eficiente na situação e (3) a retenção a longo prazo das relações aprendidas durante o treino.

Os objetivos que orientaram o desenvolvimento do Experimento 4 serão discutidos em função dos resultados apresentados na forma do **ARTIGO 3:** "Impaired long-term retention of spatial memory in pigeons with hippocampal lesion", relatado neste trabalho.

Experimento 5. Investigou as relações entre a aprendizagem espacial e os parâmetros de AgNOR no hipocampo de pombos, para determinar se a tarefa de aprendizagem espacial aumenta a síntese de proteína nas áreas hipocampais e parahipocampais nas duas tarefas utilizadas nos experimentos anteriores.

Os objetivos que orientaram o desenvolvimento do Experimento 5 serão discutidos em função dos resultados apresentados na forma do **ARTIGO 4:** "Hippocampal AgNOR increase after learning spatial in pigeons: Evidences of lateralization", apresentado neste trabalho.

Artigo 1: Effects of hippocampal lesions in a food location task in pigeons

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Abstract

This study investigated the role of the hippocampus in pigeons learning of a food-related choice task. The effects of lesions induced by ibotenic acid were analyzed in two experiments. Experiment 1 investigated the effects of hipocampal damage on postoperative memory retrieval and in reversal learning. Experiment 2 investigated the effects of hippocampal lesions on the acquisition and reversal of learning. In both experiments probe tests were used to assess the behavioral strategies underlying the choice. In Experiment 1 hippocampal lesions impaired the preoperative learned performance in terms of choice latency but not choice accuracy. Experiment 2 data showed that, in postoperative learning sessions, latency and well as choice accuracy were impaired by hippocampal damage. The probe tests, in which a curtain was placed around the chamber, revealed behavioral patterns of a non-mapping strategy. This was true in both experiments and groups (experimental and controls). Immediatly after training, during the probe tests of both experiments, in which food cups were omitted, the three groups spent more time in the target quadrant. However, immediatly after the reversal condition, neither hippocampal damaged nor control pigeons showed a preference for the target quadrant. This may be interpreted as evidence for a hippocampal role in stimulus location learning involving non-mapping strategies.

1. Introduction

The hippocampus and related structures, referred to as hippocampal formation, have been identified as playing a critical role in the neural regulation of memory processes, particularly those involved in spatial behavior. Most of the research about hippocampal functions reports lesion-induced impairments in learned behaviors of humans [7,31,45,51], monkeys [32,41], mice [8], rats [1,15,19,34,38,45,47], birds [10,15,17,40,48,49] and fishes [30].

A growing number of recent studies have emphasized the functional, morphological and anatomical similarities between avian and mammal hippocampus. As is the case for mammals, the avian hippocampus has been related to learning and memory processes [9,12]. The hippocampal complex of pigeons consists of a medially situated hippocampus (Hp), which extends around the posterior poles of the hemispheres, and the dorsomedially situated area parahippocampalis (APH) [22]. Its homology with the mammalian hippocampus complex has been recognized based on criteria of hodology [6,23,25,26], embryologic development [21,28], neurochemical organization [14,27,29], neural connections [6], and neurotransmitters [6,27].

The specific nature of the information processing carried out by the hippocampal formation remains highly controversial. The hippocampus has been pointed out as critical for numerous learning tasks involving place learning and a variety of spatial problems requiring map solution [8,10,16,17,34,42,43,47,48,49]. However, issues related to non-spatial memory impairments after damage to the hippocampus continue to be the subject of considerable debate. In non-spatial tasks the hippocampal formation is required for some tasks such as acquisition of conditional object-choice discriminations in monkeys [41], Y-maze version of a visual conditional discrimination

[35], reversal discrimination [42,44,50], and contextual fear conditioning [3]. These results may indeed indicate that the functional role of the hippocampal formation is not restricted to just one kind of information or learning/memory task.

The present experiment addresses questions related to the role of the hippocampus in pigeons exposed to learning of a food-related choice task. The effects of lesions in the hippocampus were analyzed in two experiments. Experiment 1 investigated the effects of hipocampal damage on behavior during postoperative memory retrieval sessions and in reversal learning. Experiment 2 investigated the effects of hippocampus lesioning over the acquisition of the food choice task. In both experiments after each training condition, probe trials (tests) were used to assess the behavioral strategies underlying choice. These tests could also support arguments concerning the function of the hippocampus in spatial mapping and non-mapping spatial tasks. The lesion induced by intra-hippocampal infusions of ibotenic acid (IBO) was used for a more precise analysis of hippocampal involvement in learning and memory deficits [2,8,18,20,34,40].

2. Experiment 1

The purpose of this experiment was to analyze (1) the spatial relationships involved in the behavior of food choice; (2) the effects of hipocampal lesion in the recovery of spatial information already learned and consolidated before the lesion; and (3) hippocampal lesion effects on the capacity of establishing new spatial relationships, after reversing the food cups location.

2.1. Material and methods

2.1.1. Subjects

Twenty-four adult male pigeons (*Columba livia*) weighing about 325 grams were used. The birds were housed in individual home-cages on a 12:12 h light-dark cycle (lights on at 6:00 a.m.). The experiments were carried out during the light phase of the cycle (between 1:00 p.m. to 4:00 p.m.). Temperature was maintained at about 25° C. The birds were randomly divided in three groups: hippocampal lesion (HL, *n*=8), sham lesion (SL, *n*=9), and no-lesion (NL, *n*=7). During the experiment, the animals were food-deprived to 85% of their *ad libitum* weights. The experimental protocol was approved by the Ethics Committee for Animal Experimentation of the Biology Institute–UNICAMP, Brasil (219-1).

2.1.2. Apparatus

All testing was conducted in an experimental chamber (Fig. 1) consisting of a rectangular box made of transparent plexiglas (50W x 50H x 115L, cm); two identical compartments (20x20x33 cm), externally located at each end of the box, served as initial boxes. Both of them contained a sliding door for access to the experimental chamber. The food cups were two semicircular glass cups (5.0 cm diameter at top, 3.5 cm diameter at bottom, and 5.0 cm high) covered with aluminum paper, located 30 cm apart each other in one of the two corners of one wall at the end of the box: one food cup contained food covered with sand and the other had only plain sand. The wall containing the food cups was always the one located opposite to the starting box in accordance with the experimental phase. Throughout the training the positive food cup had a fixed position at left relative to the starting box. The experimental chamber was located in the middle of the test room (2.11W x 3.10L x 2.77H, m), surrounded by four

white walls, each one with distinctive features like sockets, light switches, one unidirectional mirror, and two entrance doors. In addition, in each wall there was one of four distinctive pictures - a red circle, a blue square, a green triangle or a yellow star – used as landmarks. A GCP-165CR Panasonic videocamera was mounted directly above the experimental chamber. The camera used to record the sessions was connected to a video-TV Panasonic (AG 1960) system located in an adjacent room.

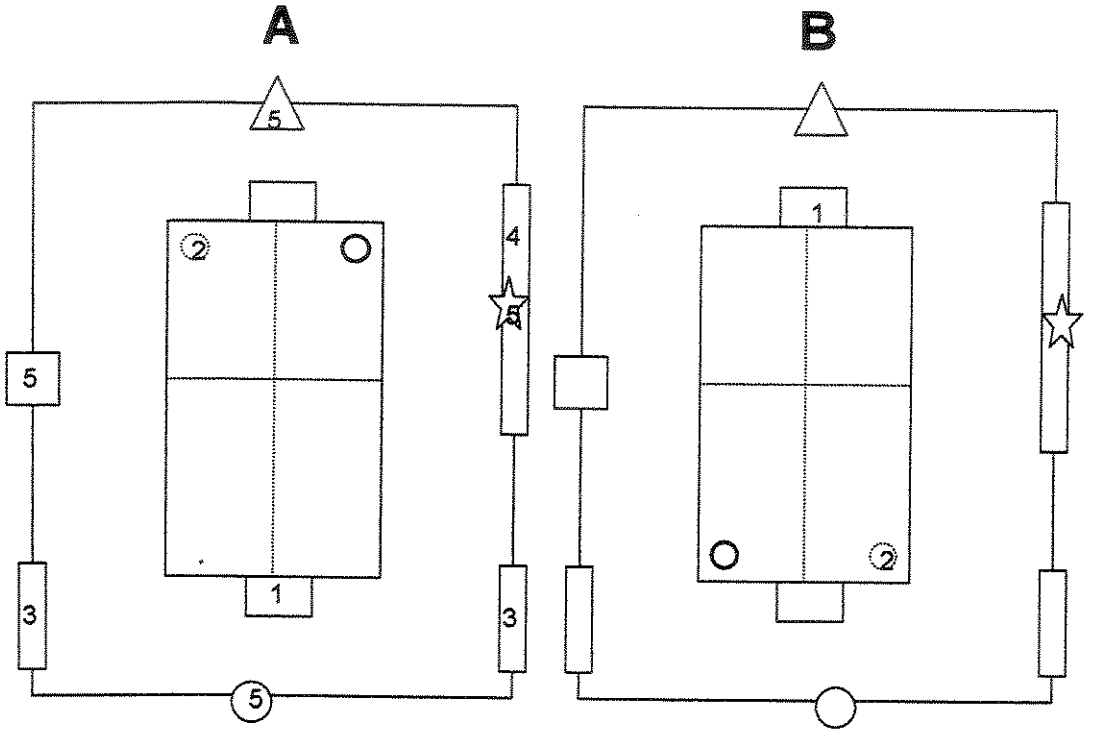

Fig. 1. Schematic representations of the experimental chamber and the experimental room. A: Location of the food cups during the Preoperative and postoperative training sessions; B: Location of the food cups during the reversal sessions. 1: initial boxes; 2: correct food cup; 3: entrance doors in the experimental room; 4: unidirectional mirror; 5: distinctive pictures used as distal cues.

2.1.3. Procedure

2.1.3.1. Preoperative training

Birds were trained to choose the positive food cup– that is, the one containing food and sand - in five-trial daily sessions, along four consecutive days. The pigeons were transferred from the home-cage and gently put inside the starting box where they remained during 1 min. Each trial began as soon as the pigeon entered in the experimental chamber after the entrance door was open and ended 30 sec after the choice of one of the two food cups or after 10 min, which ever came first. During the four training sessions the food cups remained in the same location throughout the five trials. The bird's task was to locate the correct food cup that contained food bellow the sand [57]. A choice response was recorded when the pigeon approached and pecked one of the two food cups; the correct choice response was defined as the choice of the positive food cup. The latency of the choice response - the time between the opening of the entrance door and pecking one of the two food cups - was measured with a digital chronometer. The pigeons' behavior was video-recorded during the sessions in order to afford reliability control of the data.

2.1.3.2. Probe tests

Each one of the following tests were carried out in one experimental session, with a between-session interval of 24 h.

Test 1

The experimental session was run as usual, except that it had only three trials. This test was carried out after the two initial trials. In the third trial the chamber was surrounded with a white curtain that rendered the extra chamber environment visually uniform and prevented access to the wall landmarks. The objective of this test was to determine whether the pigeons used the constellation of distal landmarks to localize the positive food cup. Impairments in the choice behavior could support arguments favoring the use of a spatial strategy.

Test 2

This test was also carried out after the two initial trials run according to the usual procedure in the experimental session. A new food cup, with a different shape and color, was introduced into the location used for the positive food cup. The chamber was surrounded by a white curtain, which prevented the use of distal landmarks. Pigeon behavior was videotaped in each trial in order to assess a possible non-mapping strategy. In a positive case, the correct choice should be related to the location and not to the characteristics of the new food cup.

Test 3

This test was carried out after two initial trials. During the third trial, the two food cups were removed from the chamber and the pigeons' behavior was tape-recorded for 3 min. The analysis considered the four locations in the chamber defined by imaginary intersection lines on the longitudinal and lateral axes. The time spent in each quadrant was measured. The objective was to determine how much time the pigeons required,

during the omission of the food cups, to respond to the quadrant representing the positive food cup location in comparison to the others.

2.1.3.3. Surgery

Pigeons were anesthetized with Ketamine and Xilazine (0.1 mg/kg, 1:1, i.m.) and placed in a stereotaxic apparatus (David Kopf, model 1204) with the Revzin adaptor for pigeons. The lesion was carried out by bilateral ibotenic acid (IBO) infusions (1 mg/0.1 ml in 0.1 M PBS, pH 7.4) with a 10-μl Hamilton syringe connected to a cannula (0.40 mm) attached to the stereotaxic tower. The following Karten & Hodos [22] coordinates were used: anteroposterior (AP), 4.0, 5.0, 7.0 mm; vertical (V) 1.5 mm; lateral (L), 1.0 mm lateral to the midline. IBO infusions lasted 1 min and the cannula was maintained in the same position for an additional 3 min. Sham-operated birds were submitted to anesthesia, fixation in the stereotaxic apparatus, scalp incision, and skull exposure and perforation procedures, but not to any substance infusion. After surgery, the pigeons were kept in their home cages.

2.1.3.4. Postoperative test

Following a three-day recovery period, the birds were submitted to the postoperative test. General procedures were identical to those used in the preoperative training. The objective was to analyze the effect of hippocampal lesion on the retrieval of the previously consolidated information.

2.1.3.5. Probe tests

The general procedures of tests 1, 2 and 3 were identical to those used in the preoperative training.

2.1.3.6. Reversal training

Following the postoperative probe tests, the locations of the food cups in the experimental chamber were reversed, with the food cups placed at the opposite extremes of the chamber. The positive food cup location was maintained at left relative to the starting box. All the other extra-chamber spatial characteristics of the experimental situation remained unchanged. The aim of this procedure was to analyze the capacity of the pigeons to establish new relationships between the stimuli present in the experimental choice situation.

2.1.4. Histology

At the completion of the behavioral testing the pigeons were deeply anesthetized with Ketamine and Xilazine (0.1 mg/kg, 1:1, i.m.) and transcardially perfused with 0.9% saline solution followed by 10% formaldehyde solution. The brains were removed and maintained in the 10% formaldehyde solution for at least 1 week. Subsequently, the brains were prepared for histology, embedded in paraffin and sectioned. The frontal slices (7 μm) were double stained for neuronal bodies and myelinated fibers by the technique of Klüver-Barrera [24]. The extent of the lesion was evaluated histologically using the pigeon brain atlas [22].

2.1.5. Statistical analysis

Before any comparison of the medians, all dependent variables were submitted to a test of homogeneity of variances and to a normality test. Because most median latency values failed to satisfy either one or both of these criteria, and because data transformation was mostly unsuccessful, the statistical analysis for latency was based on nonparametric tests. Between-group differences were determined by the Kruskal-Wallis test and within-group analyses by the Friedman test for between session comparisons. *Post-hoc* multiple comparisons analysis were carried out with the Dunn test. Variations of the correct choice responses of tests 1 and 2 were determined by the X^2 test.

Two-way ANOVA with group and quadrants as factors, followed by post-hoc multiple comparisons by the Fisher's test, was used for analysis of variation of time spent in the quadrants in the probe Test 3 and Preoperative training.

2.2.Results

2.2.1. Histological analysis

Hippocampal lesions were reconstructed in schematic drawings as illustrated in Fig.2. Cellular disorganization in the hippocampus of the HL group was more evident in the dorsal region (Fig. 4-left) than in the ventral region (Fig. 3-left). Cell disorganization and decreased cell number was evident in the dorsal (Fig. 4 A-C) and ventral (Fig. 3 A-C) regions compared to control animals.

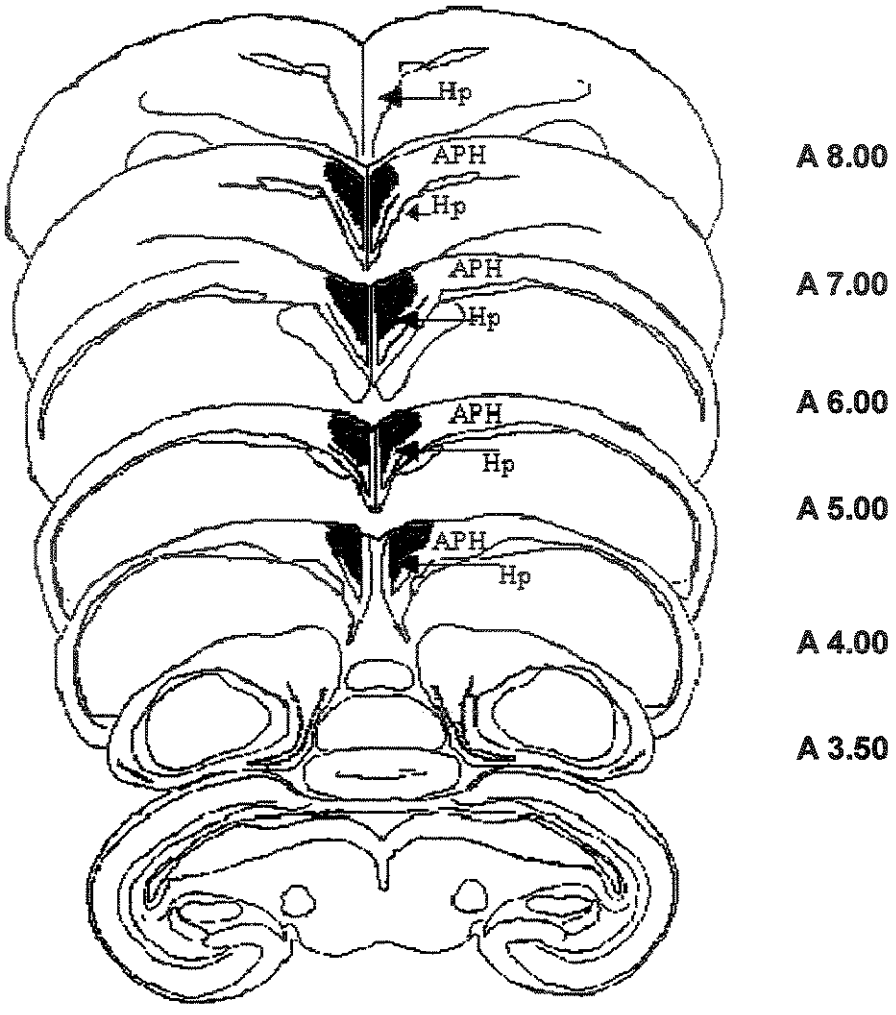

Fig. 2. Schematic representations of the hippocampus of pigeons reconstructed according to frontal sections from the atlas of Karten & Hodos (1967). Gray areas represent the extent of the lesions induced by ibotenic acid in the HL pigeons. APH= area parahippocampalis; Hp= hippocampus.

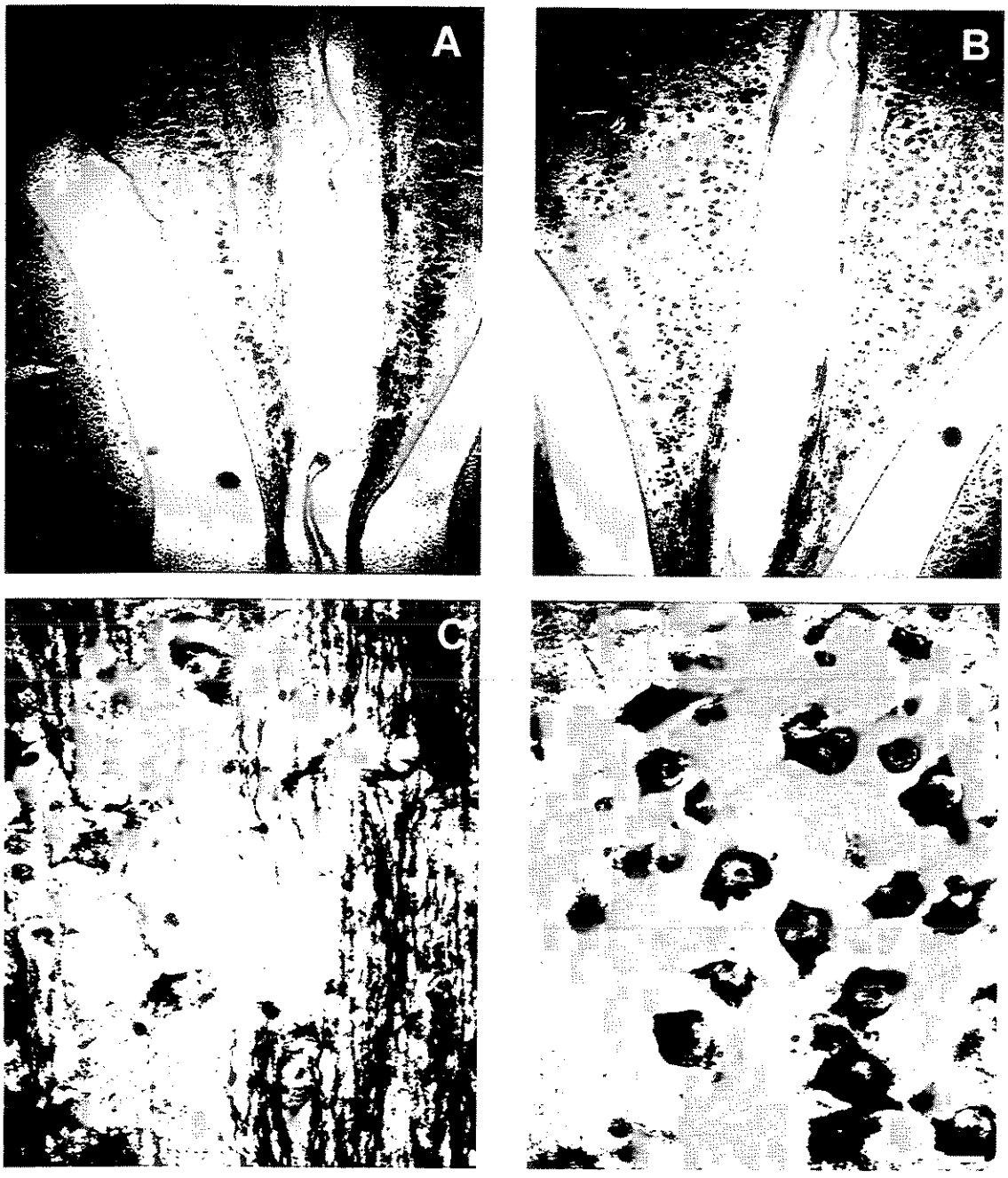

Fig. 3. Photomicrographs showing the ventral region of the hippocampus in frontal sections (10μm) of the pigeon's brain. Scarce neuronal cells can be observed in the brain sections of one HL pigeon (A) as compared to the brain sections of one control pigeon (B). Scale Bars: (C) e (D), 100 μm. Data processing used the Image Pro-Plus software.

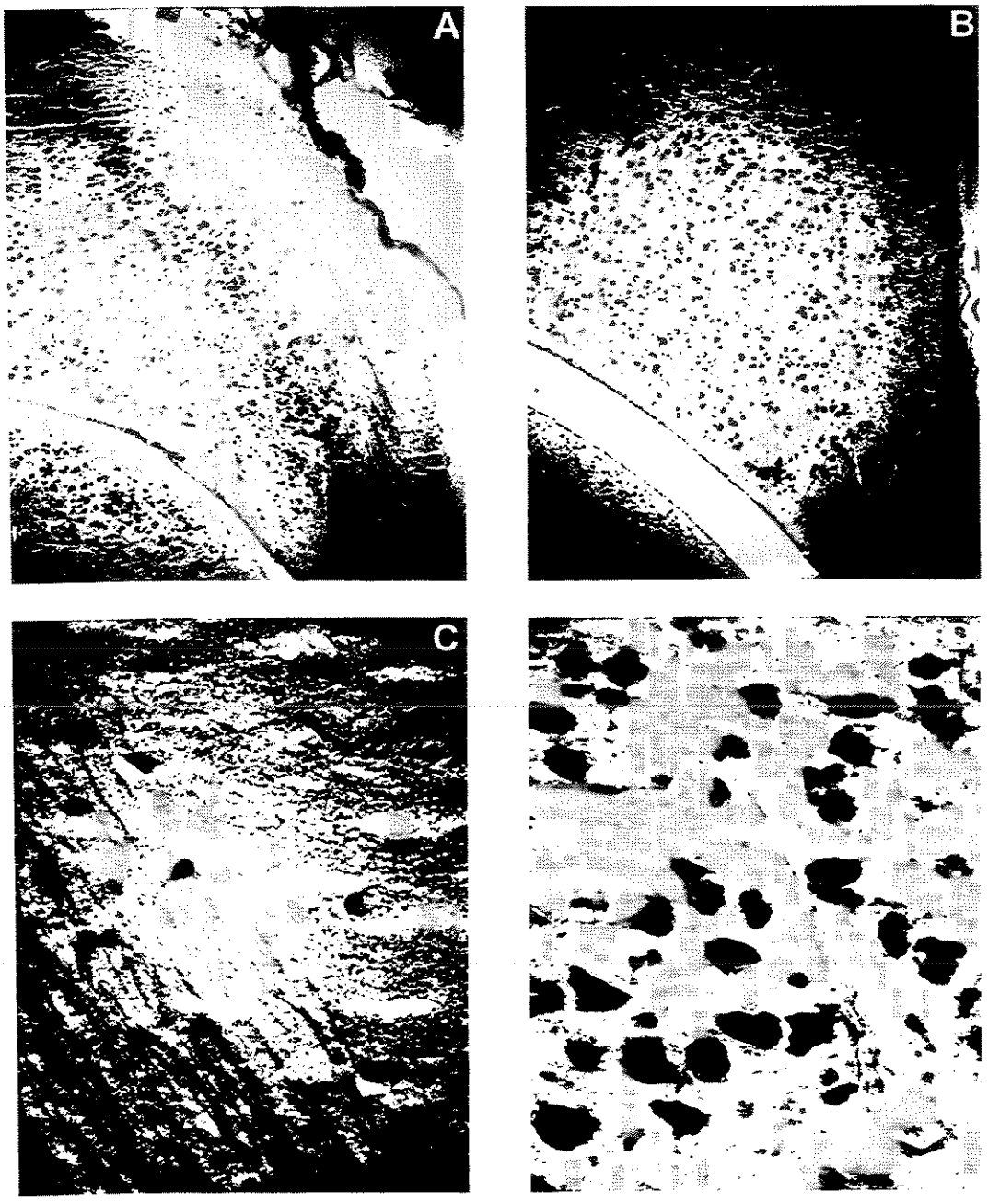

Fig. 4. Photomicrographs showing the dorsal region of hippocampus in frontal sections (10μm) of the pigeon's brain. Scarce neuronal cells can be observed in the brain sections of one HL pigeon (A) as compared to the brain sections of one control pigeon (B). Scale bars: (C) e (D), 100μm. Data processing used the Image Pro-Plus software.

2.2.2. Behavioral results

The acquisition of choice response by pigeons in the preoperative condition is represented in Fig. 5 by the curves of percentual latency values relative to the first training session. The latency values in session 1 were taken as 100% and the values plotted in session 2, 3 and 4 were calculate as percent of session 1. The relative decreases in latency along the training were indicate an effect of session that was very close to significance ($F_{3,21}$=2.73, P<0,051). No between group differences were detected (P>0.05).

Fig. 6 (left) compares the fourth preoperative training session, considered as baseline, with the postoperative test sessions. In this fourth preoperative session no group differences were detected in the median latency values more in the percentage of correct choices (P>0.05). The differences in latency between experimental and control groups, observed in all the four postoperative sessions, were revealed in a significant group effect (P<0.0001). The comparison between the fourth preoperative session and the postoperative sessions (Fig. 6, left) indicated a significant latency increase in the first postoperative session only for the HL group (P<0.0001). In contrast to HL, both control groups showed latency decreases from session to session. The percent of correct choices, in the three groups, was higher than 91.0%.

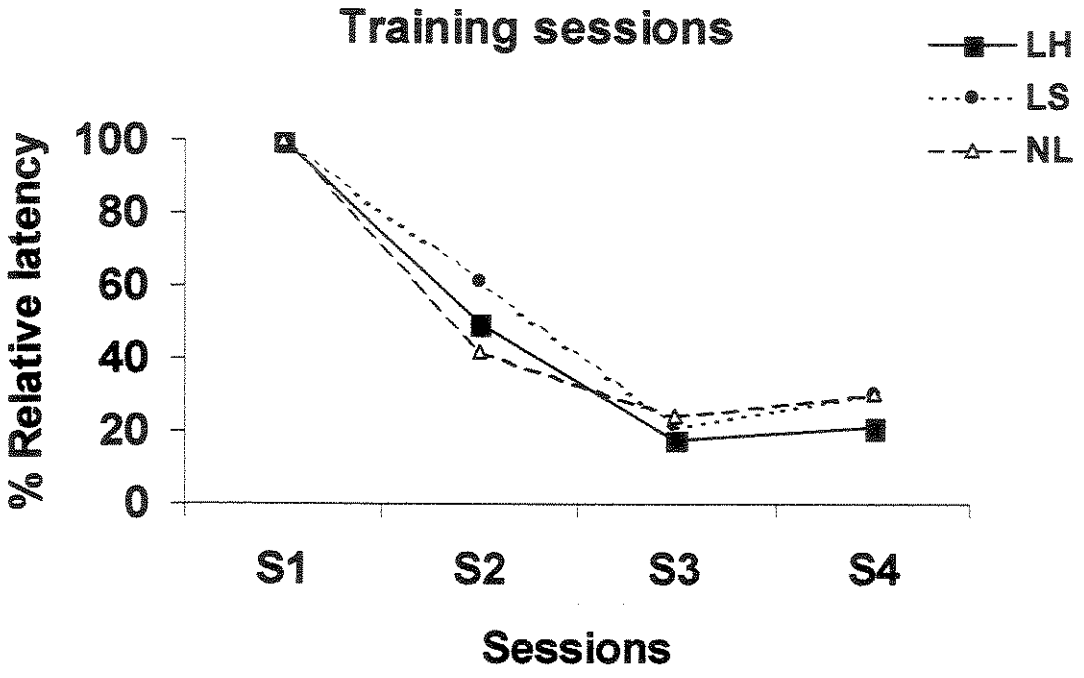

Fig. 5. Percentual latency values relative to the first training session. The latency values in session 1 were taken as 100% and the values plotted in session 2, 3 and 4 were calculed as percent of session in the training sessions of the preoperative condition for the pigeons with hippocampal lesion (HL, *n*=8), sham lesion (SL, *n*=9) and no-lesion (NL, *n*= 7).

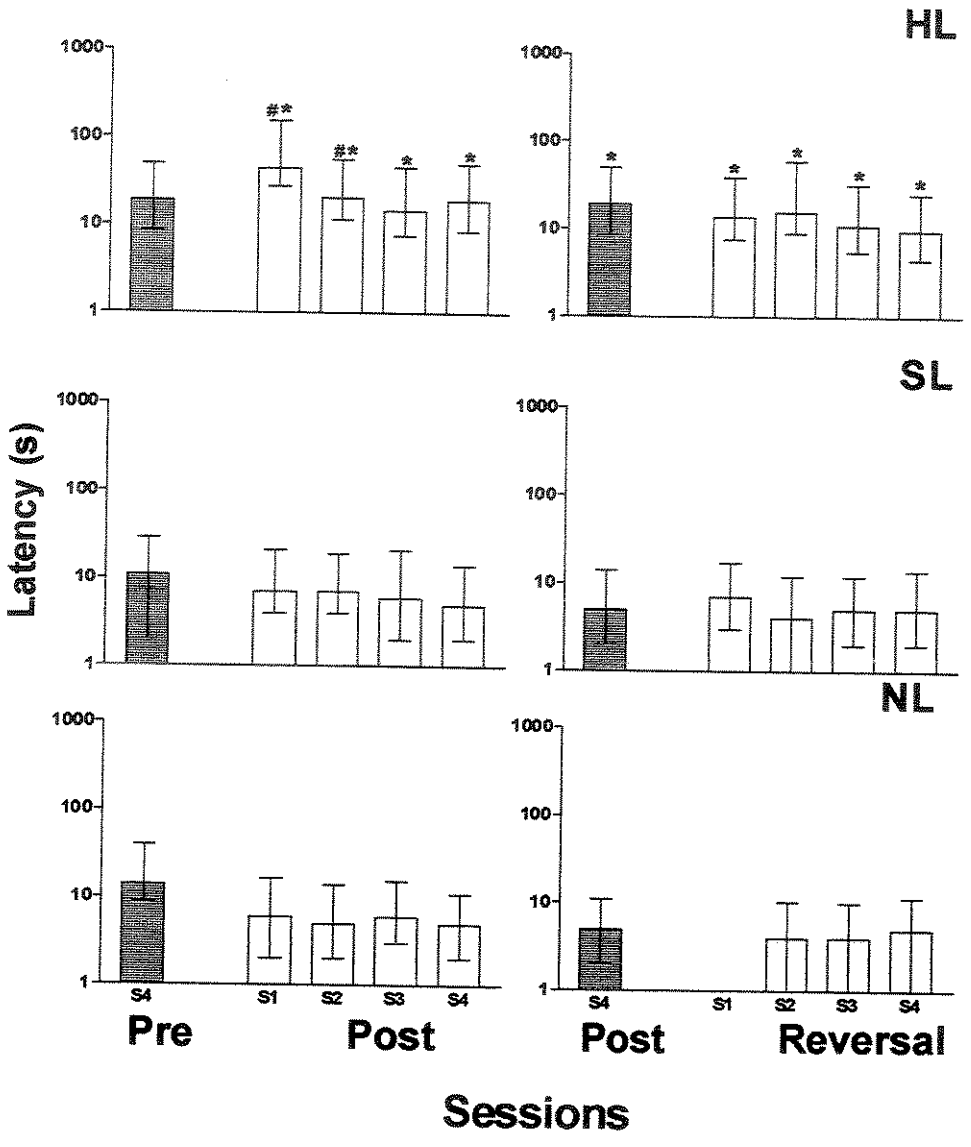

Fig. 6. (left) Median values of the latency of choice (seconds) in the last session of the preoperative condition and in the postoperative sessions. (right) Median values of the latency of choice (s) in the last postoperative session and in the reversal learning sessions. Top: hippocampal-lesion (HL, n=8). Middle: sham lesion (SL, n=9) and bottom: no-lesion (NL, n= 7). * Significantly different (p<0.05) from the controls; # Significantly different (p<0.05) from 4^{TH} preoperative session.

2.2.3. Reversal

A significant group effect was also present during the reversal condition, which was related to the HL's higher latency values during the four reversal sessions as compared to controls ($P<0.001$; Fig. 6–right). Comparisons between the last postoperative session and the median latency of the reversal sessions revealed no intra-group effect of session ($P>0.05$). Percentage of correct choices analysis did not reveal any statistical difference ($P>0.05$).

2.2.4. Probe tests

Test 1

No between groups differences in performance were noticed when the curtain blocked visual access to distal landmarks, both during preoperative, postoperative or reversal conditions. The analysis of the median latencies in the test trial (3rd trial) relative to the second trial in the session (latency after the curtain minus latency prior to the curtain-Fig. 7-left) showed no significant differences between groups or between conditions ($P>0.05$). The X^2 analysis also revealed no significant differences in the number of correct choices ($P>0.05$).

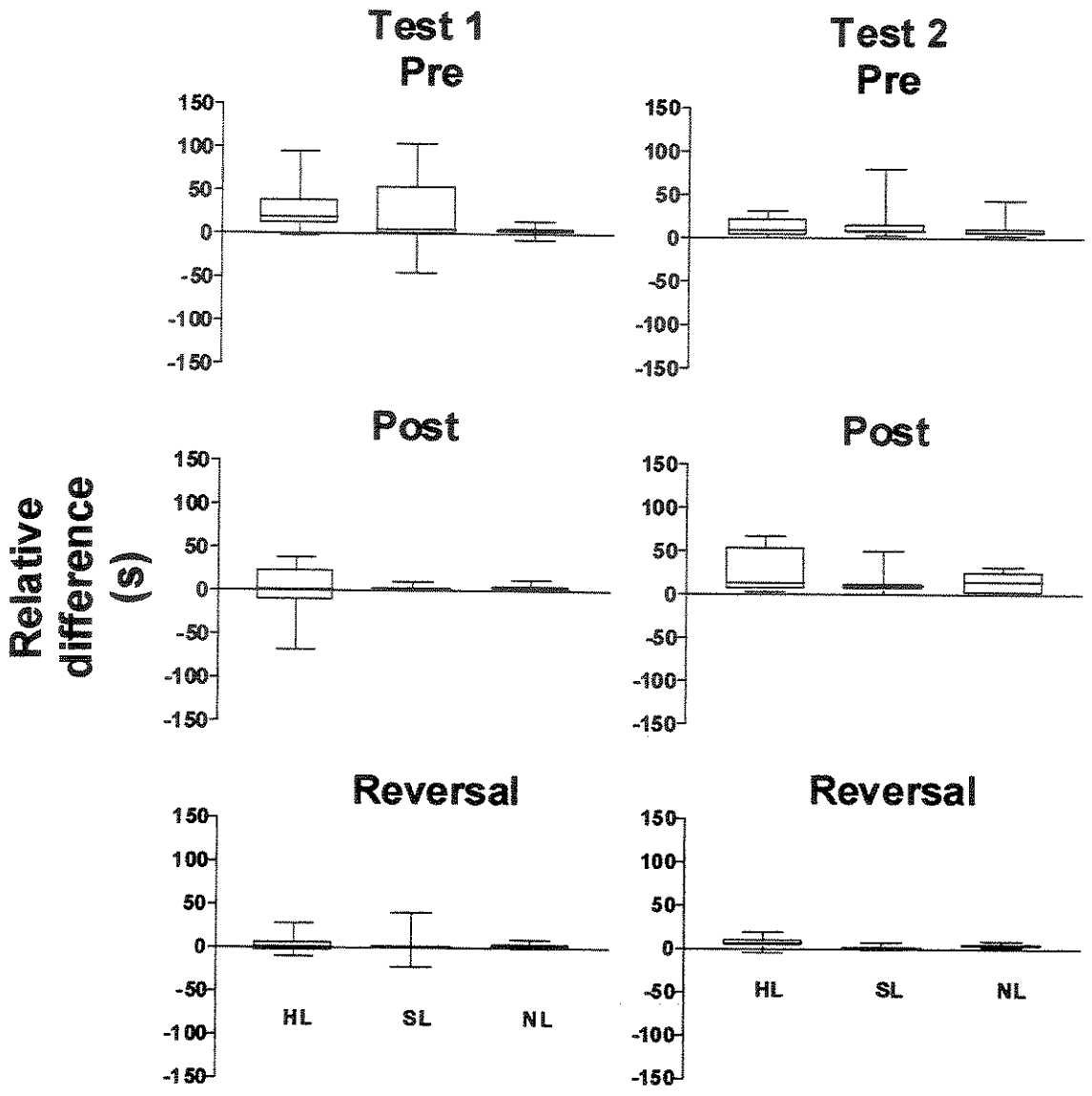

Fig. 7. (left) Differences between the latencies in trial 3 and trial 1 (difference= T1 – T3) during probe test 1. (right) Differences between the latencies in trial 3 and trial 1 (difference= T1 – T3) during probe test 2 (new food cup + curtain). hippocampal-lesion (HL, *n*=8), sham-lesion (SL, *n*=9) and no-lesion (NL, *n*=7).

Test 2

The data from the probe test with the new food cup and the white curtain, Test 2 (Fig. 7, right), are presented as the difference between the median latency of the second trial and the test trial (with the new food cup and white curtain; Diff=t3-t2). No significant between groups differences were observed both relative to the latency nor to the percentage of correct choices ($P>0.05$).

Test 3

A significant increase in the time spent in the in the target quadrant, compared to the other quadrants, was observed for the three groups, both in the Preoperative (top; $F_{3,54}= 40.21$, $P<0.0001$), and Postoperative test (middle; $F_{3,54}= 35.07$, $P<0.0001$) situations. In contrast, after Reversal training (bottom), there was no significant difference ($F_{3,54}=0.96$, $P>0.05$) in this parameter (Fig. 8).

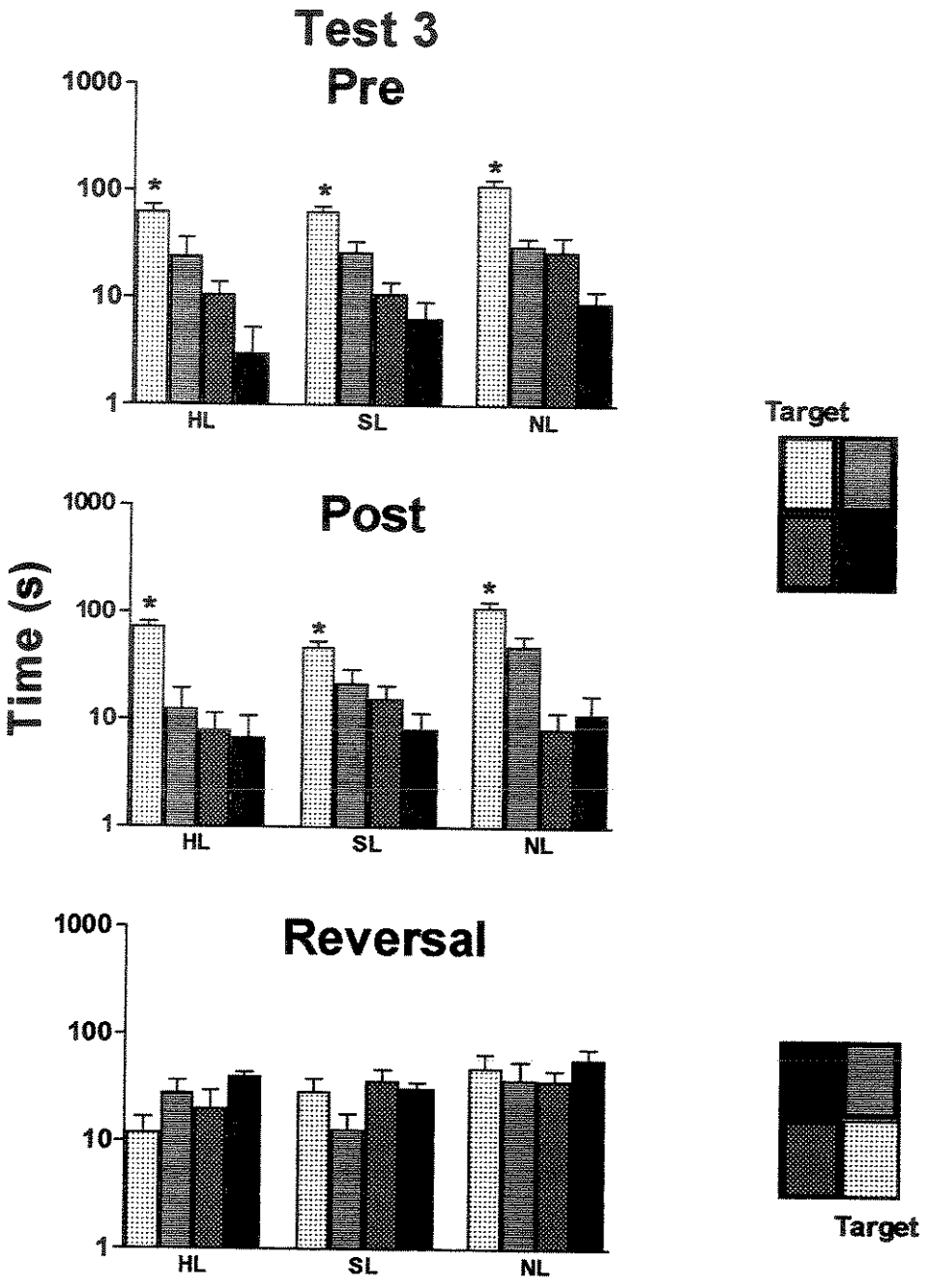

Fig. 8. Overall mean values of the time spend (±S.E.M.) by hippocampal-lesion (HL, *n*=8), sham-lesion (SL, *n*=9) and no-lesion groups (NL, *n*=7). During this time the food cups were removed. (A) preoperative condition, (B) postoperative condition and (C) reversal condition. * Significantly different ($p<0.05$) between the target quadrant and from other quadrants.

3.Experiment 2

In Experiment 1 the data indicated a transitory increase of the choice latency response during the first postoperative session and significant increases in latency during the four reversal sessions of the lesioned animals compared to the controls. These results may be indicating that the information learned before the lesion was already stored in other brain areas [39]. In this ways, Experiment 2 was designed to investigate the effects of hippocampal lesions on the acquisition of new information as well as on the reversal of a food location choice task.

3.1. Materials and methods

3.1.1. Subjects

Twenty-seven adult, male, pigeons (*Columba livia*), weighting around 300 grams were used. The birds were randomly distributed in three groups: hippocampal lesion (HL, n=10); sham lesion (SL, n=10); no-lesion (NL, n=7).

3.1.2. Apparatus and procedure

The apparatus and general procedures were the same as in Experiment 1. The only difference was that the training procedures occurred three days after hippocampal lesioning. In other word there was no preoperative condition.

3.1.3. Statistical analysis

All data were analyzed parametrically using analysis of variance (ANOVA) and *post hoc* analysis with the Tukey-Kramer method for multiples comparisons. Variations of the correct choice responses of tests 1 and 2 were determined by the X^2 test.

3.2. Results

3.2.1. Histology

The analysis of the brains of the experimental and sham lesioned birds confirmed the lesion location as described for Experiment 1.

3.2.2. Behavioral results

3.2.3. Postoperative training and reversal

Fig. 9 (left) represents the median latency of the first four training sessions (left) and the reversal training sessions (right). The lesioned birds learned the task but had significantly higher latency values (lesion effect; $F_{2,24}$=7.48, P<0.005) relative to control groups. While the control groups had an abrupt decrease in latency in the second session, the HL birds showed an increase. This yielded a significant session effect ($F_{3,72}$=3.12, P<0.05) and a significant lesion x session interaction ($F_{6,72}$=3.17, P<0.01). Throughout the reversal condition (right) the HL group maintained significantly higher latencies in comparison to the control groups (group effect; $F_{2,24}$=7.29, P<0.005).

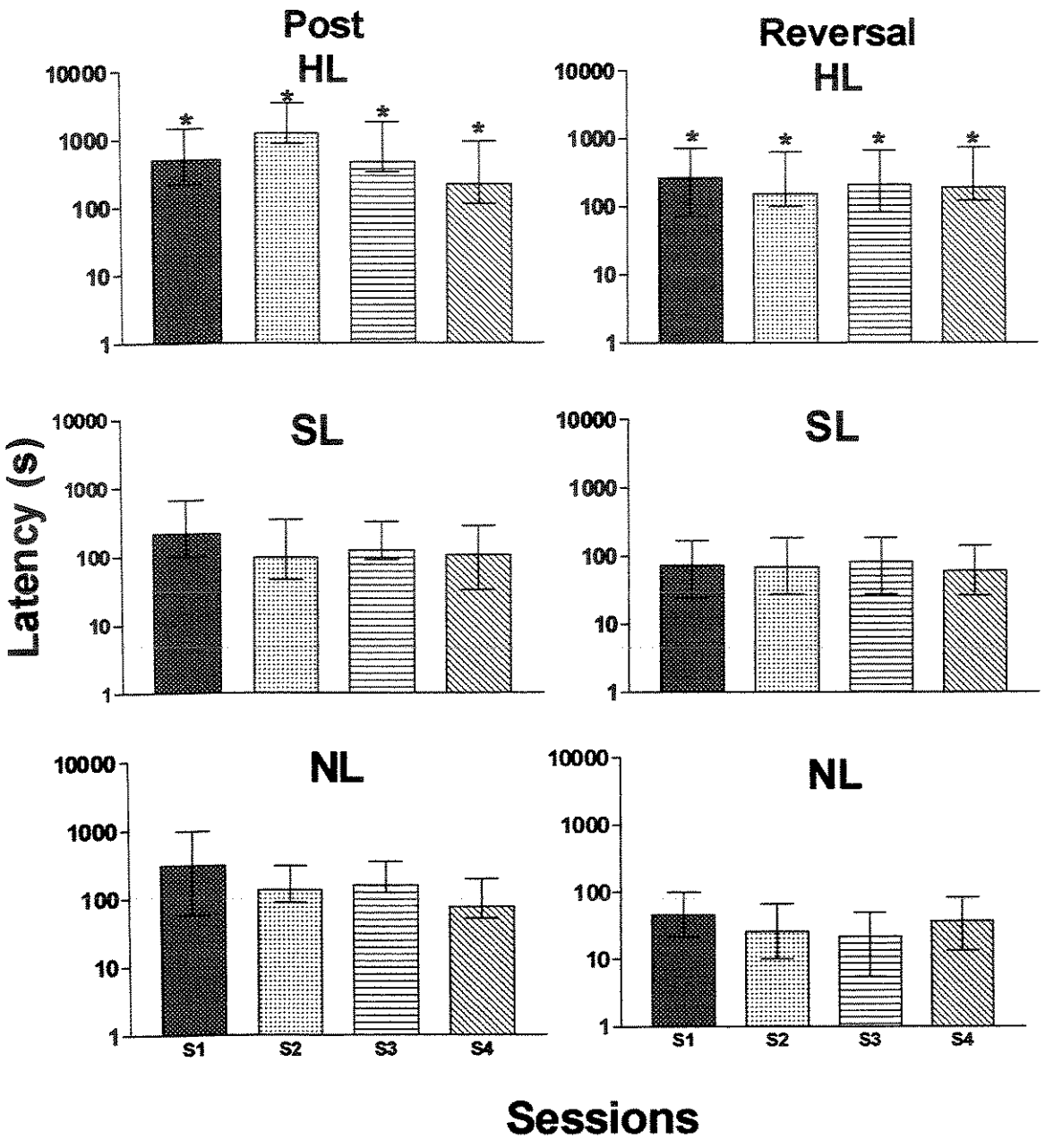

Fig. 9. Mean (±S.E.M.) of the latency of choice (s) in the four sessions Postoperative (left) and reversal (right). Top: hippocampal-lesion (HL, n=10). Middle: sham lesion (SL, n=10) and bottom: no-lesion (NL, n= 7). * Significantly different (p<0.05) from the controls.

Fig. 10 represents the mean percent of correct choices during the 4 training (top) and reversal (bottom) sessions. The HL birds showed less accuracy in their choice as indicated by the percent of correct responses and significant statistical differences compared the control groups ($F_{2,24}$=6.49, P<0.005; Top). There were also significant variations across the sessions in the postoperative condition (session effect; $F_{3,72}$=2.96, P<0.05). No between-group significant differences were detected in the accuracy data of the reversal condition (bottom). There was a significant effect of session probably due to fact that all three groups, showed decreased performance during the first reversal session ($F_{3,72}$=9.93, P<0.0001).

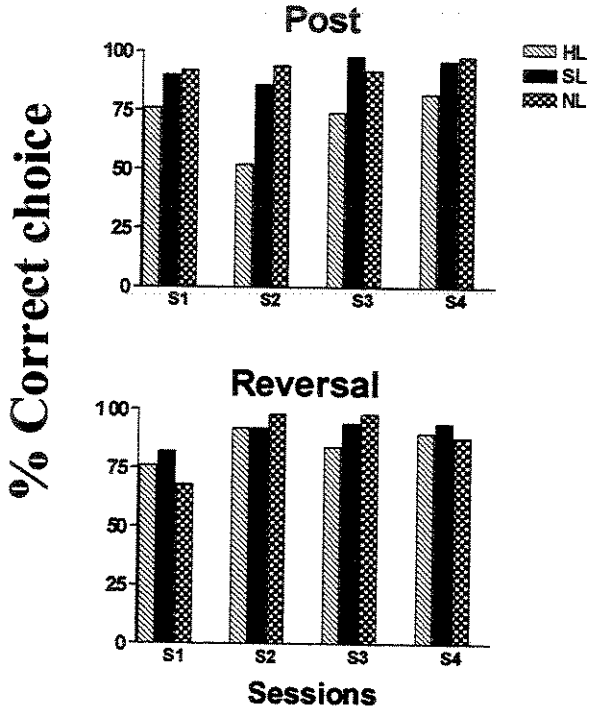

Fig 10. Percentage of correct choices of the positive food cup in the four sessions Postoperative (top) and Reversal (bottom) of the hippocampal-lesion (HL, n=10), sham lesion (SL, n=10) and no-lesion (NL, n= 7) groups. * Significantly different (p<0.05) from the controls.

3.2.4. Probe tests

Test 1 and 2

Fig. 11 (left panel) shows the relative difference between the median latencies in the test trial (3rd trial) and the second trial in the probe test 1, with the curtain around the chamber, and in the probe test 2, with the curtain and the new food cup (right). During the probe test 1 the performance of the pigeons showed no significant differences between groups ($F_{2,24}$=0.65, P>0.5) nor between the conditions of the postoperative and reversion ($F_{2,24}$=0.11; P>0.5). In contrast, during the probe test 2 following training the HL group showed increased latency but shorter latency after reversal training. ANOVA indicated a main effect of lesion ($F_{2,24}$=4.82, P<0.05), of condition ($F_{1,24}$=9.21, P<0.01) and interaction of lesion x condition ($F_{2,24}$=13.94, P<0.0001).

Fig. 12 presents the mean percent of correct choices of food cup location during probe test 1 (left) and probe test 2 (right). Statistical analysis indicated no significant differences both in the probe tests following training or reversal learning.

Test 3

Fig. 13 represents the absolute time spent in each quadrant of the experimental chamber during probe test 3, with no food cup present in the chamber, following the training (top) and the reversal (bottom) conditions. When the test occurred immediately after training all three groups spent more time in the target quadrant, where the food cup was located previously ($F_{3,69}$=8.52, P<0.0001). After the reversal condition (Fig.12-bottom) the absolute time spent in each quadrant was statistically equivalent in all quadrants ($F_{3,69}$=1.36, P>0.05).

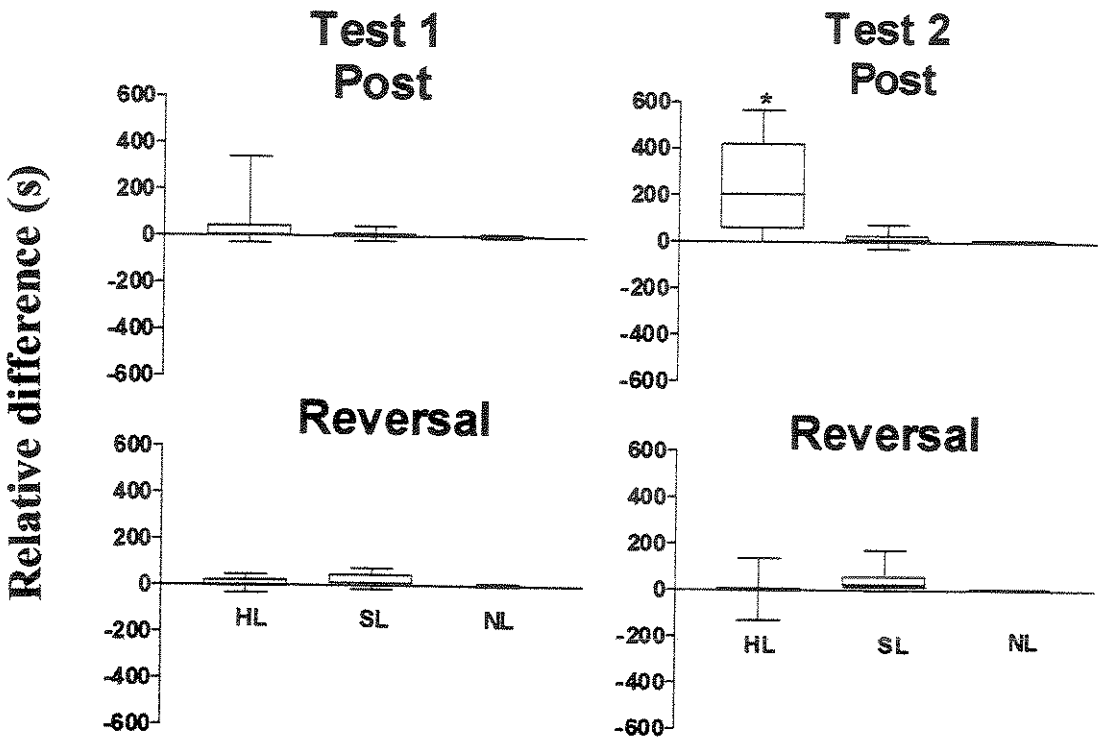

Fig. 11. (left) Differences between the latencies in trial 3 and trial 1 (difference= T1 – T3) during probe test 1(right) in the conditions training (top) and reversal (bottom). Differences between the latencies in Trial 3 and Trial 1 (difference= T1 – T3) during probe Test 2 (new food cup + curtain) during the postoperative (top) and reversal (bottom) conditions: hippocampal-lesion (HL, n=10), sham-lesion (SL, n=10) and no-lesion (NL, n=7). * Significantly different (p<0.05) from the controls.

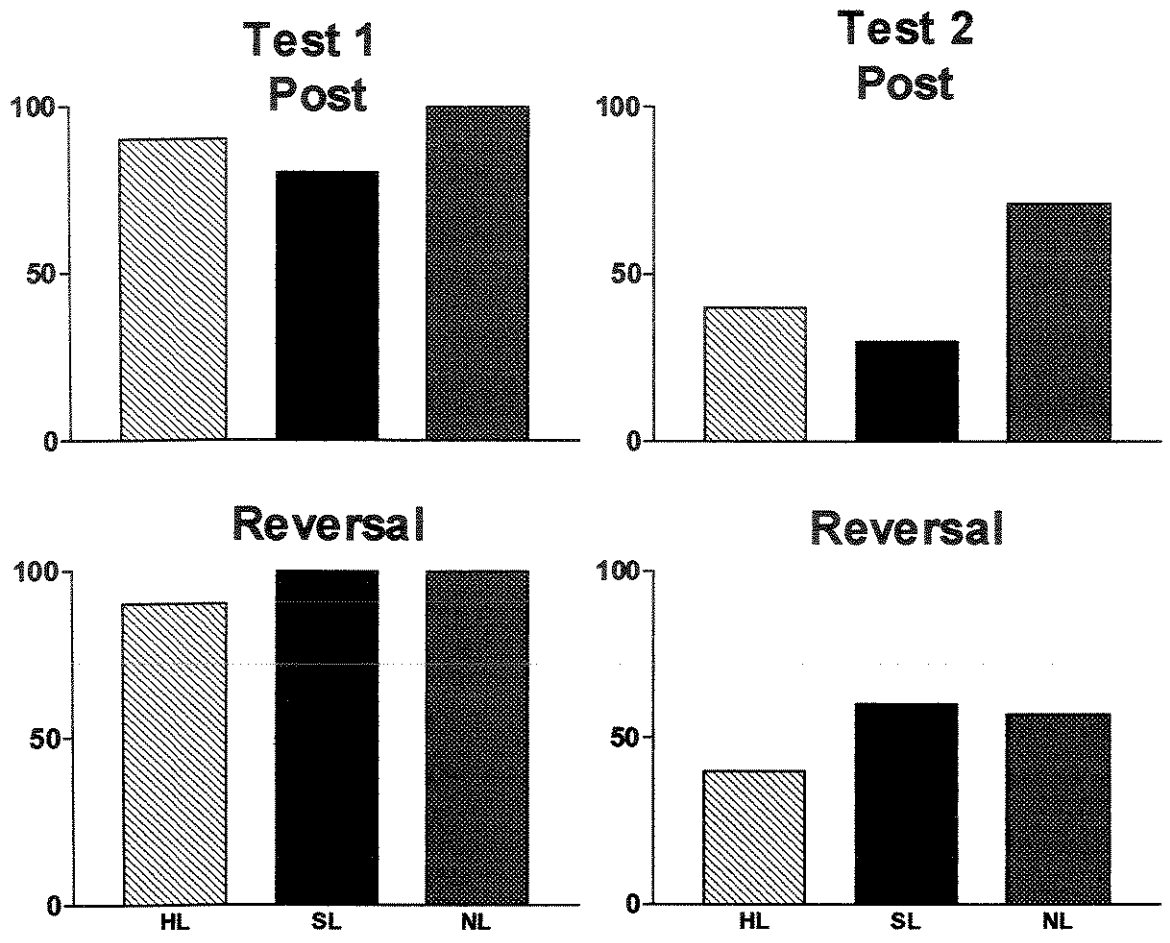

Fig. 12. Percentage of the correct choices of the positive food cup in the Trial 3 during probe test 1(left) and probe test 2 (right) in the training (top) and reversal (bottom) conditions: hippocampal-lesion (HL, *n*=10), sham-lesion (SL, *n*=10) and no-lesion (NL, *n*=7).

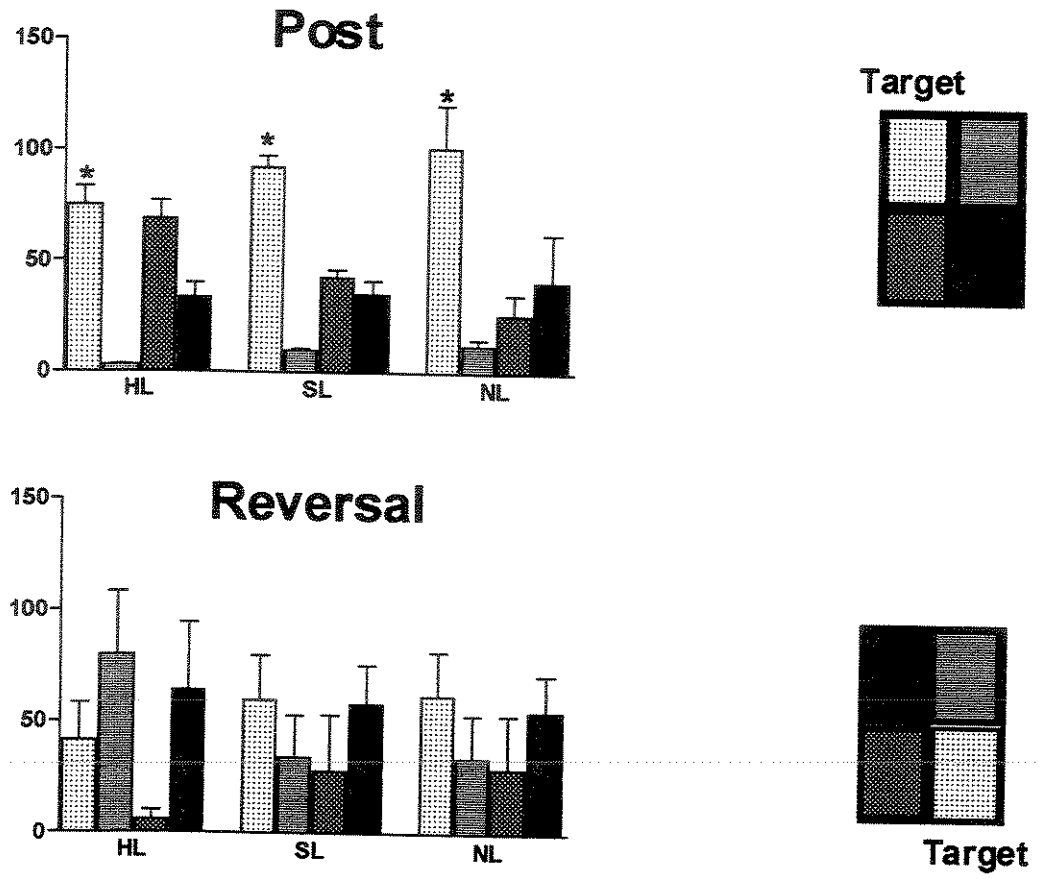

Fig. 13. Overall mean values of the time spend (±S.E.M.) by hippocampal-lesion (HL, n=10), sham-lesion (SL, n=10) and no-lesion groups (NL, n=7). During this time the food cups were removed from the chamber. (A) postoperative training and (B) reversal condition. * Significantly different (p<0.05) between the target quadrant and from other quadrants.

4.Discussion

The set of findings in the present studies showed, that hippocampal lesions impaired the preoperative learned performance in terms of the latency of choice response did not affect accuracy of choice. Second, in the case of postoperative learning, both latency as well as accuracy of choice response was impaired by hippocampal damage.

The effectiveness of the preoperative training for the choice response was confirmed since all the three groups showed between session decreases characteristic of learning curves. As is characteristic of learning situations with free behaving animals we had individual differences that were reduced through training. This is a clear indication that the experimental contingences effectively resulted in lower behavioral variability and in equivalent performance along the preoperative training. Simultaneously to a decline in latency along the sessions, there was an increase in the accuracy reaching values higher than 80% correct choices in the fourth training session. These results agree with those from the literature considering the acceptable performance levels [17,48]. The postoperative increase in latency seen in Experiment 1 may be interpreted as an effect of the lesion

For this reason we used the data of the fourth training session as baseline for comparison with the postoperative training. When we compared the last preoperative training session with the first postoperative session, there was a major increase of latency in the first postoperative session for the lesioned animals. Along the sessions, such increase was attenuated although the latency of experimental animals remained higher than the controls. In addition, during the reversal condition the latency of operated animals was also higher than the controls. Both during the postoperative

training as well as during reversal lesioned birds never reached the performance level of controls. These data agree with previous studies in which hippocampus lesioned pigeons showed increased latency to find food in similar food tasks [16], and decreased performance in spatial discrimination tasks as well as [49] in homing tasks [17,33].

The fact that the lesion impaired latency but did not affect accuracy is particularly interesting. At a first glance it might be suggestive of a differential function of the hippocampus in behavioral systems controlling motor and the accuracy characteristics of the response. A faster or slower locomotion could be due to motor impairments resulting from the lesion. However, we do not believe this was the case since the animals appeared to have normal locomotion pattern and body posture. Moreover, the demonstration that hippocampal birds behaved like the controls when access to contextual cues were avoided implies that the behavioral deficits were not due to altered motility. This observation agrees with those already reported for hippocampal rats in the water maze [37, 46]. Lesioned animals would walk into the chamber as soon as the door was open; then they could remain still or explore the area before walking directly toward the feeder. As indicated by the choice accuracy data, they went to the correct feeder more than 80% of the times. This leads us to consider that the extra time computed in their latency was actualles used by the animals to explore environmental cues that probably guided their behavior.

The data from Experiment 2 indicated that hippocampal lesioned animals were capable of learning the task although showing deficits both in the latency and accuracy of response as compared to the other groups. These data are in agreement with those found in homing studies in pigeons reporting that hippocampal lesions impaired landmark navigation and increased time to return to the loft [46]. They also agree with

studies in rats that showed increased training to learn a spatial task in rats [33] and increased latency in the water maze in rats [33, 37] after hippocampal damage.

One point worth considering is that with pre-training hippocampal lesions a double impairment in performance was observed; an increased latency as well a decreased accuracy. Post-training lesion in Experiment 1 did not produce choice accuracy deficits, although latency values were increased. This finding may suggesting that in the post-operative condition of Experiment 1 the memories about the task were already stored outside the hippocampus. Several studies have postulated that the hippocampus has a temporary role in memory storage and other structures, such as the neocortex in rodents [4,5], play a permanent storage role. Although these facts are not well clarified in pigeons, it is tempting to suggest that the deficits observed in Experiment 2 may indicate that damaged hippocampus and the structures to which it is related are not sufficient for accurate learning of the task.

The probe tests with hidden distal cues of the extra-chamber environment done in both experiments, revealed behavioral patterns characteristic of a non-mapping strategy, both for experimental and control pigeons. During these tests the animals had no possibility of using distant spatial cues however they were not impaired either in their latency nor in their accuracy, suggesting the use of other types of information. On the other hand, in Experiment 2 when the curtain was around the chamber and a different food cup was presented, lesioned birds showed decreased performance expressed as increased latencies. This suggests that animals are using the stimuli provided by the food cups consequently characterizing the use of a non-mapping strategy.

In order to better understand the behavioral strategies used by pigeons, we carried out another probe test. Probe test 3 consisted of food cups omission. The results

were interesting and raised some questions. First, in the two experiments the three groups spent more time in the training quadrant when the test occurred after the preoperative or the postoperative training sessions. That is, even with the omission of internal cues the animal went to the training quadrant. Since in the previous probe test there was no evidence of a mapping strategy, in this situation the animals could be just using cues or information already learned which are given by the sequence of movements of the body, such as, turning toward the food O'Keffe & Nadel [36]. Second, when this test followed the reversal condition, neither hippocampal damaged nor control pigeons showed a preference for the correct choice quadrant, as they did after training and in the postoperative test. These data suggest that the relationships with the environment established previously by the animals may have been disrupted after reversal training. When exposed to a new environment or after it has been changed, the animal has to obtain and memorize new information. This is necessary to establish as many relationships as possible among the environmental events, which may participate in the elaboration and modification of the general knowledge about the world [13].

We may thus suppose that when the internal cues of the experimental chamber provided by the food cup were missing the pigeons had to link the information of this new situation to the previously consolidated environmental relationships characteristic of the training sessions and of the reversal conditions. Actually, the reversal situation was peculiar both for submitting the animals to a situation of stimulus omission in the case the food cups and because these stimuli had different possibilities of environmental arrangements. Since this was observed both for the experimental and control pigeons, it seems that the difficulty does not depend on hippocampus integrity. Possibly, a longer duration of this test would identify some differences among groups.

Taken together, the findings of Experiment 2 present a more robust effect of hippocampal lesion on both latency and accuracy of choice. This fact may be related to the function of the hippocampus as a transitory storage system of acquired information. In Experiment 1 the birds already had preoperative consolidated memories and the information may have been stored elsewhere at the moment of the lesion. However, in the Experiment 2, the hippocampal dysfunction observed when it was lesioned before learning may indicate an important integrative role of the hippocampus concerning the spatial information involved in the task. The deficits seen during or after the reversal learning may be related to impairment in the establishment of new relationships among environmental stimuli. The hippocampus may link episodic representations together and compose generalizations among types of information acquired from different experiences, which may be stored as relational or declarative memory. Accordingly, Eichenbaum [13] propose that the hippocampus might be capable of recording sequential and context-specific information and to link then together. However, we still need answers about how the neural circuitry may operate in order to accomplish their complex functions both concerning the mammalian and the avian hippocampus.

In summary, the present study, although using a simple location task that involved orientation and locomotion to a specific site, and choice of one food cup, was efficient in demonstrating an effect of hippocampal lesion on learning. During the different conditions of the two experiments the pigeons started each trial from the same initial box. During the reversal condition the animals were released from a different starting box and the feeders were transferred to the opposite wall, both the proximal events of the environment and the animal location related to the distant events were changed. This probably imposed a need for a new composition of the already known

contextual events. In this situation, pigeons with hippocampal damage showed significantly higher latencies in comparison to the controls, and no improvement along the sessions. Our data may be related to the issues raised by Eichenbaum *et al.* [11] who studied rats in a water maze task and allowed them to repeatedly start from the same starting position. With this procedure they eliminated the demand of constructing a contextual map. In this condition, animals with hippocampal damage identified the escape site as readily as the controls. Nevertheless, the rats with hippocampal damage were unable to locate the platform when they were released from other points.

Finally, it is worth saying that the hippocampus should not be analyzed as an independent system, but as a structure extensively connected to other cerebral systems. The deficits caused by lesioning the hippocampus may be properly interpreted as evidence of the intricate functional network where the hippocampus operates. The data of the present study may be regarded as indicating that damage localized in the hippocampus alters the activity of such network and impairs learned performance even in a simple task as the one used here.

Acknowledgements

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Artigo 2

Artigo 2: Effect of ibotenic acid hippocampal lesion on spatial choice in pigeons

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ABSTRACT:

The present experiment was aimed at the investigation of the hippocampal lesions effects on the behavior of pigeons in a spatial learning task requiring mapping solution. Pigeons were trained before and after the ibotenic acid lesioning in a food choice location in a circular arena that had one food cup in each quadrant. In each trial of the sessions the pigeon was released from a different starting point. Preoperative and postoperative probe tests without the food cups in the arena were carried out at the end of training conditions. The data showed that damage to the hippocampus resulted in increased latency of choice in the first sessions of postoperative condition. The higher latency values observed for the hippocampal pigeons decreased with training but never reached the scores of control animals during the postoperative sessions. The pigeons were impaired in the correct choice of the food cup, although this damage was transitory. The probe test confirmed that the lesioned pigeons were incapable to retrieve the correct quadrant location when the food cups were omitted from the arena. These results Indicate that localized damage in the hippocampus impairs a learned performance that uses spatial mapping and contribute for comparatives analyses of the role of hippocampus in birds and mammals.

1. Introduction

The hippocampus and related structures have come to be characterized as playing an important role in the neural organization of certain mnemonic processes (Eichenbaum et al., 1994; O'Keefe and Nadel, 1978). These processes include spatial representations that guide navigation when environmental landmarks are represented in a map-like or relational navigational map (Eichenbaum et al., 1990).

Accordingly, the lesion in the hippocampal formation impairs spatial cognition in mammalians in a wide variety of experimental tasks including place learning (Morris et al., 1982; Compton et al., 1997; Hannesson and Skelton, 1998; Pouzet et al., 2002), context learning (McDonald et al., 2002), relational learning (Rudy and Sutherland, 1989), discrimination learning (Rothblat et al., 1993; Arns et al., 1999; Ridley et al., 2001).

However, in many cases, the resultant post lesioning impairment is not absolute, since accurate spatial performance eventually emerges with extend training. For example, Hannesson and Skelton (1998) investigating spatial performance of rats in the Morris water maze verified that the performance of the lesioned rats recovered near to control levels with further training, when training was increased to 12 days. Pouzet et al. (2002) reported that rats with dorsal hippocampal lesions learned to escape to a fixed location of the platform in a water maze when training was extended to 16 days as compared to training during 8 days.

Recent growing interest in the understanding of the role of the hippocampus in spatial memory in different animals has been extended to the avian hippocampus. Indeed, the pigeon's hippocampus plays an important role for spatial memory both

under semi natural and natural conditions (Bingman et al., 1988; Sherry and Vaccarino, 1989; Bingman et al., 1990; Gagliardo et al., 1999; Strasser et al., 1998; Bingman and Able, 2002). However few experiments on the avian hippocampus have been accomplished in the laboratory as compared to the investigations of rodent's hippocampus. These experiments approached issues related to the acquisition and reversal of a left-right position, T-maze discrimination (Good, 1987), open-field spatial task (Colombo et al., 1997), three colored keys conditional discrimination in operant chamber (Watanabe, 2002), place learning in a circular arena (Fremow et al., 1997), conditional discrimination of landmarks in a room (White et al., 2002). In a previous study, Amaral-Toma and Ferrari (in press) find out that damage in the hippocampus of pigeons impairs learned performance with increase in latency and decrease in correct choice even in a simple no-mapping task.

The present experiment was aimed at the investigation of how ibotenic acid hippocampal lesions might affect the behavior of pigeons in a spatial learning task requiring mapping solution.

The dry version of the water maze as used by Fremow et al. (1997) was changed to provide four similar food cups, one in each quadrant, and just one correct location of the food.

2. Materials and methods

2.1. Subjects

The subjects were 25 adult pigeons (*Columba livia*), with 320 g mean weight. The animals were housed in individual home-cages, under a 12:12 h light: dark cycle with

lights on at 6:00 a.m. The pigeons were fed with a mixture of seeds, corn and sand. During the experimental period the birds had restrict amount of food in order to maintain their experimental body (85% of the *ad libitum* body weight). The birds were randomly attributed to three groups: hippocampal lesion (HL, *n*=8), sham lesion (SL, *n*=8), and no lesion (NL, *n*=7). The experimental protocol has been approved by the Ethics Committee for Animal Experimentation of the Instituto de Biologia–UNICAMP, Brazil (219-1) and were conducted in accordance with the recommendations of the Canadian council on animal care and with the ethical guidelines for investigations of experimental pain in conscious animals (Zimmerman, 1983).

2.2. Apparatus

All testing were conducted in a wood made circular arena (Fig.1) with 1.5 m diameter and 50 cm high, with the floor and the wall painted white. The floor was covered with a rough brown paper that was changed in each session. It was positioned in the center of a room (2.11W x 3.10L x 2.77H, m) that had distal visual stimuli like sockets, light switches, one unidirectional mirror and two entrance doors. In addition, in each wall there was one distinctive picture - a red circle, a blue square, a green triangle or a yellow star –, provided as landmarks

Four points equally spaced along the circumference of the arena were arbitrarily assigned as: N (north), E (east), S (south) and W (west). These points served as the starting positions at which the pigeons were lowered into the chamber. The area of the arena was also conceptually divided into four quadrants (NE, SE, SW and NW) of the equal size. In each quadrant there were one food cup; in three of these quadrants the

food cup contained plain sand and only in the SW quadrant there was a food cup with food covered with sand.

The behavior of the pigeons in the chamber was recorded with a GCP-165CR video camera mounted 2.60 m above the center of the experimental chamber. The camera used to record the sessions was connected to a video-TV Panasonic (AG 1960) system located in an adjacent room.

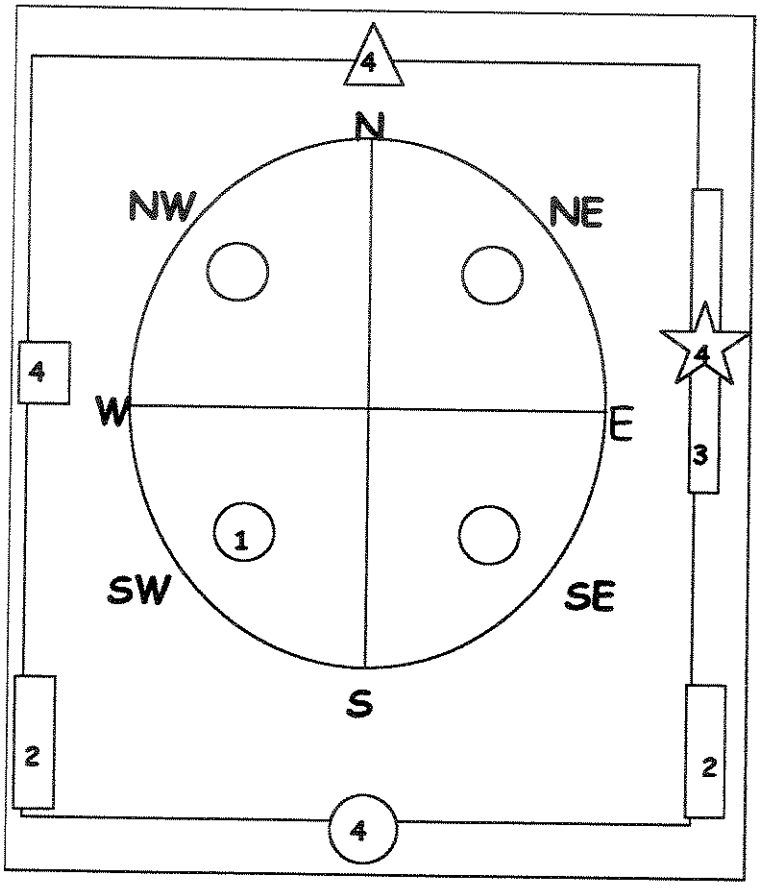

Figure 1. Schematic representations of the experimental arena and the experimental room. 1: correct food cup; 2: entrance doors in the experimental room; 3: unidirectional mirror; 4: distinctive pictures used as distal cues.

2.3. Preoperative training

The preoperative training consisted of the seven days of training. Pigeons were given six trials per day to find the correct food cup. In each trial the light in the room was turned off and the pigeons were placed in the arena with its head facing the wall of the chamber. The lights were then turned on and the experimenter recorded the latency of the choice response and the accuracy correct choice (correct or error) by the pigeons. The session was videotaped for subsequent analysis. The choice response was defined as the first peck in one of the food cups. After the choice response the pigeons were allowed to a 30 s period in the chamber. If no choice occurred during a 10 min period the trial was finished. On each of the six trials of the session, a different starting point (N, E, S, L and W) was used and the location of the correct food cup was fixed in the center of SW quadrant, 18 cm from the arena wall. Before the beginning of a trial, the pigeon rested in a cage located in an adjacent room. At the end of the session, the pigeons were taken back to their home cages.

2.4. Probe test 1

Immediately following the completion of the last trial, the four food cups were removed from the chamber. During the test trial with omission of food cups the pigeon was put in the arena and the behavior was tape-recorded during 3 min. The analysis considered the four locations in the chamber defined by imaginary intersection lines on the longitudinal and lateral axes. The time spent in each quadrant was measured. The objective was to determine how much time the pigeons required, during the omission of the food cups, to respond to the quadrant representing the correct food cup location in comparison to the others.

2.5. Surgery and Histology

Details of the surgical and histological procedures were according to techniques previously described in Amaral-Toma and Ferrari (in Press). Pigeons were anesthetized with Ketamine and Xilazine and placed in a stereotaxic apparatus for pigeons. The lesion was carried out by bilateral ibotenic acid (IBO) infusions following Karten & Hodos (1967). Coordinates were used: anteroposterior (AP), 4.0, 5.0, 7.0 mm; vertical (V) 1.5 mm; lateral (L), 1.0 mm lateral to the midline. Sham-operated birds were submitted to anesthesia, fixation in the stereotaxic apparatus, scalp incision, and skull exposure and perforation procedures, but not to any substance infusion. After surgery, the pigeons were kept in their home cages.

At the completion of the behavioral testing the pigeons were deeply anesthetized and transcardially perfused with 0.9% saline solution followed by 10% formaldehyde solution. Subsequently, the brains were prepared for histology.

2.6. Postoperative training

Following a three days recovery period, the birds were submitted to the postoperative training. General procedures were identical to those used in the preoperative training. The objective was to analyze the effect of the hippocampal lesion on the retrieval of the previously consolidated information about the food location in the arena.

2.7. Probe test 2

The general procedure of the probe test 2 was identical to those used in the probe test 1.

2.8. Statistical analysis

The data were analyzed with two-way ANOVA having group and condition as factors and session as repeated measured. *Post hoc* analysis used the Tukey-Kramer method for multiple comparisons.

3. Results

3.1. Histological analysis

The hippocampus lesions were reconstructed in schematic drawings of the lesion as illustrated in Fig. 2. Cellular disorganization in the hippocampus of the HL group was more evident in the dorsal region than in the ventral region. Altered structural cell organization and decreased number of cells was evident in the dorsal region and ventral compared to control.

3.2. Preoperative training

Fig 3 (top) presents the mean latency of the food cup choice response for the controls (SL and NL) and HL pigeons over seven days of training. Both controls and HL reduced their choice latency as training progressed. ANOVA confirmed a main effect of session ($F_{6,12}$=76.85, P<0,001). There was no effect of group (P> 0.05).

The mean correct choice of the food cup for the controls and HL pigeons over seven days of preoperative training is also seen in Fig 3 (bottom). The three groups showed a learning curve with increased correct choice along the training sessions. The performance of controls and HL pigeons was similar (P>0,05). ANOVA indicated a

significant effect of sessions ($F_{6,12}$ =48.72, P<0,01), related to a higher number of correct choice in the last training sessions.

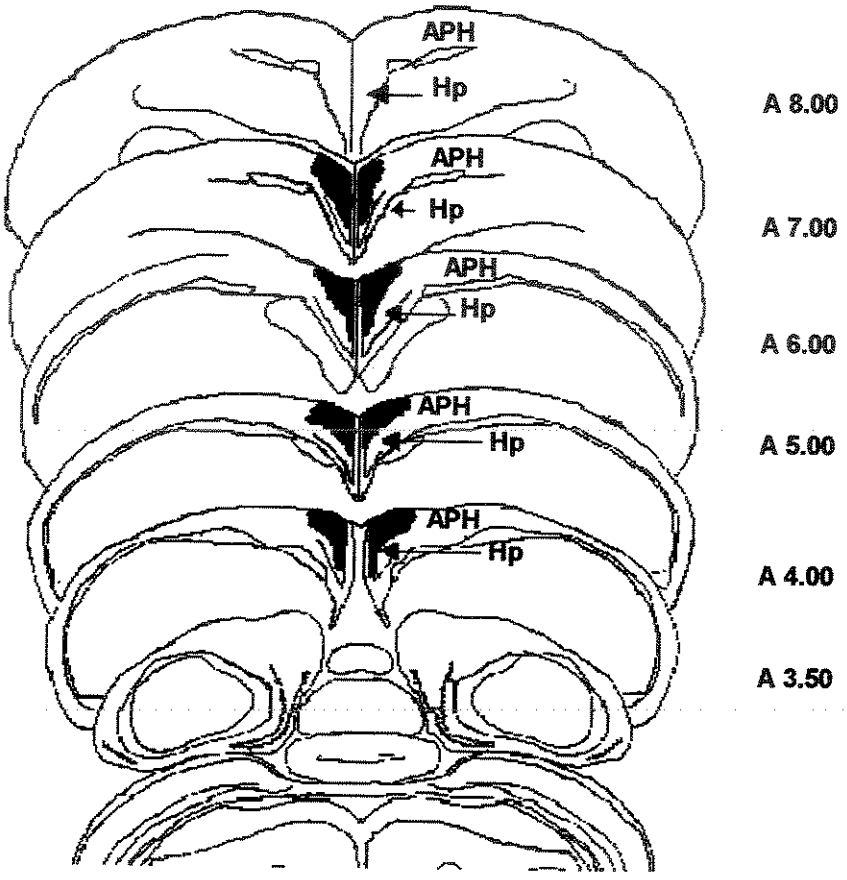

Figure 2. Schematic representations of the hippocampus of pigeons reconstructed according to frontal sections from the atlas of Karten & Hodos (1967). Blacks areas represent the extent of the lesions induced by ibotenic acid in the HL pigeons. APH= area parahippocampalis; Hp= hippocampus.

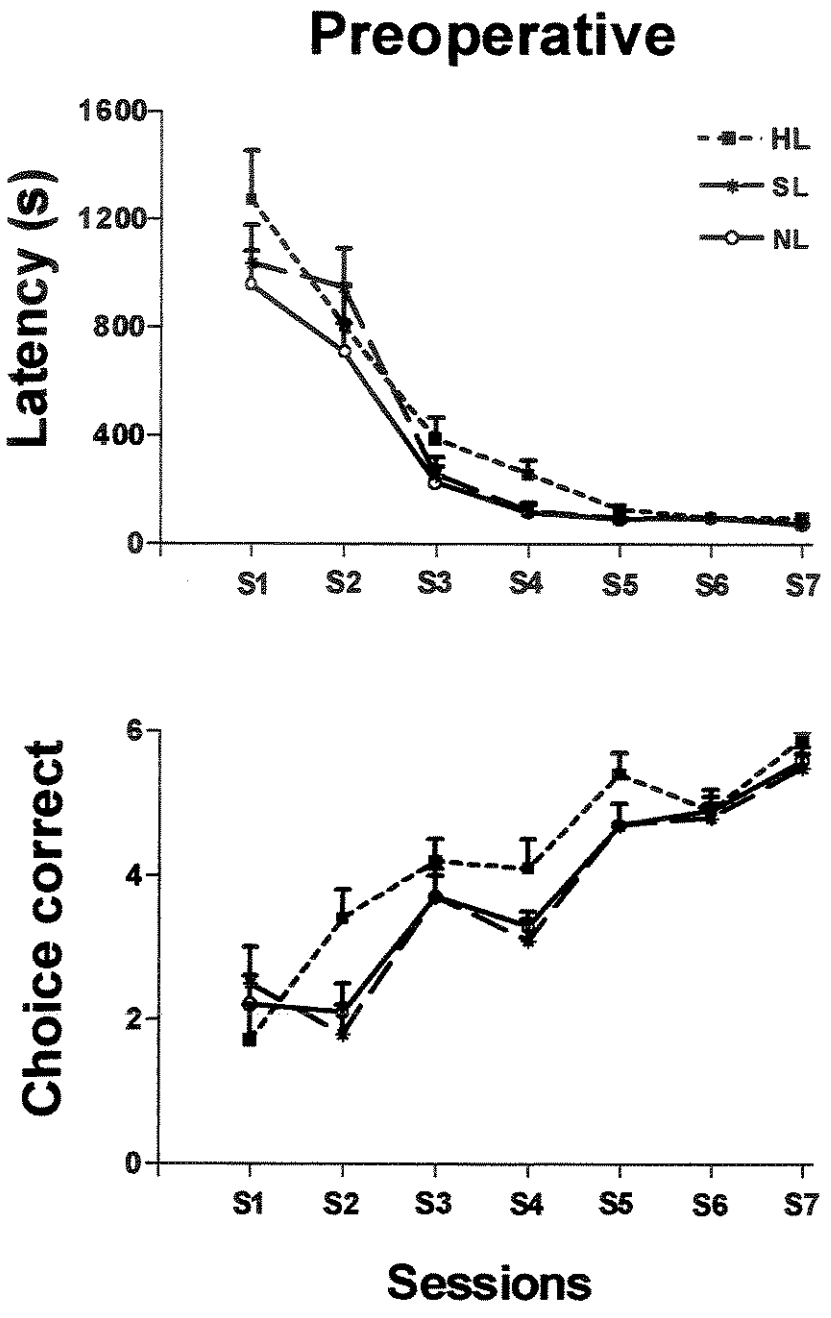

Figure 3. (Top) Mean choice of the food cups latencies (second ± S.E.M.). (bottom) Mean ± S.E.M. of the correct choice in the seven sessions of the preoperative condition, for the hippocampal lesion (HL, *n*=8), sham lesion (SL, *n*=8) and no lesion (NL, *n*= 7) groups.

3.3. Probe Test 1

Fig. 4 presents the absolute time spent in the four quadrants of the arena, for controls and HL pigeons over 3 min of the test. As can be seen, the three groups showed preference for the target quadrant. ANOVA revealed a significant main effect of quadrant ($F_{3,6}$=12.51, P< 0.01) but no significant effect of group (P>0.05).

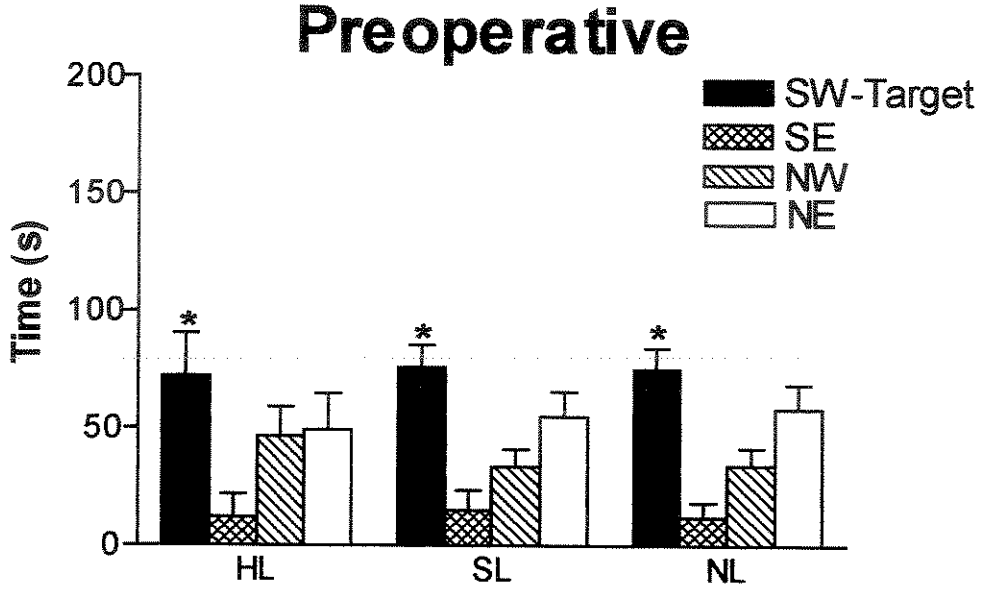

Figure 4. Overall mean values of the time spent (Mean ± S.E.M.) during 3 min of the probe test 1 in the preoperative condition by hippocampal lesion (HL, n=8), sham lesion (SL, n=8) and no lesion groups (NL, n=7). During this time the food cups were removed from arena. * Significantly different (ANOVA: p<0.05) between the target quadrant and the other quadrants.

3.4. Postoperative training

The Fig. 5 (top) provides a direct comparison of the mean latency of the food cup choice response in the last preoperative session (S7) and each one of the seven postoperative sessions, for the controls and HL pigeons. While HL pigeons showed robust increases in the postoperative choice latency values control groups maintained steady and low choice latency values. ANOVA yielded a main effect of group ($F_{2,23}$=16.15, P<0,001), of session ($F_{7,14}$=28.35, P<0,001) and a significant interaction of group x session ($F_{14,161}$=18;95, P<0.001).

Fig. 5 (bottom) presents the mean correct choice of the food cup for controls and HL pigeons in the last preoperative session (S7) and in each one of the seven postoperative sessions. As compared to preoperative training the control groups maintained total accuracy 100% of correct of the choice response while HL birds had a 50% decrease of the correct choice in the first postoperative session. The number of HL correct choice responses increased gradually but did not reach the control values. ANOVA yielded a main effect of group ($F_{2,24}$=25.16, P<0.001), of sessions ($F_{7,24}$=6.30, P<0.001) and a significant interaction of group x session ($F_{2,14}$=3.67, P<0,001) which may be related to the fact that the higher differences among groups occurred in the first and second postoperative sessions.

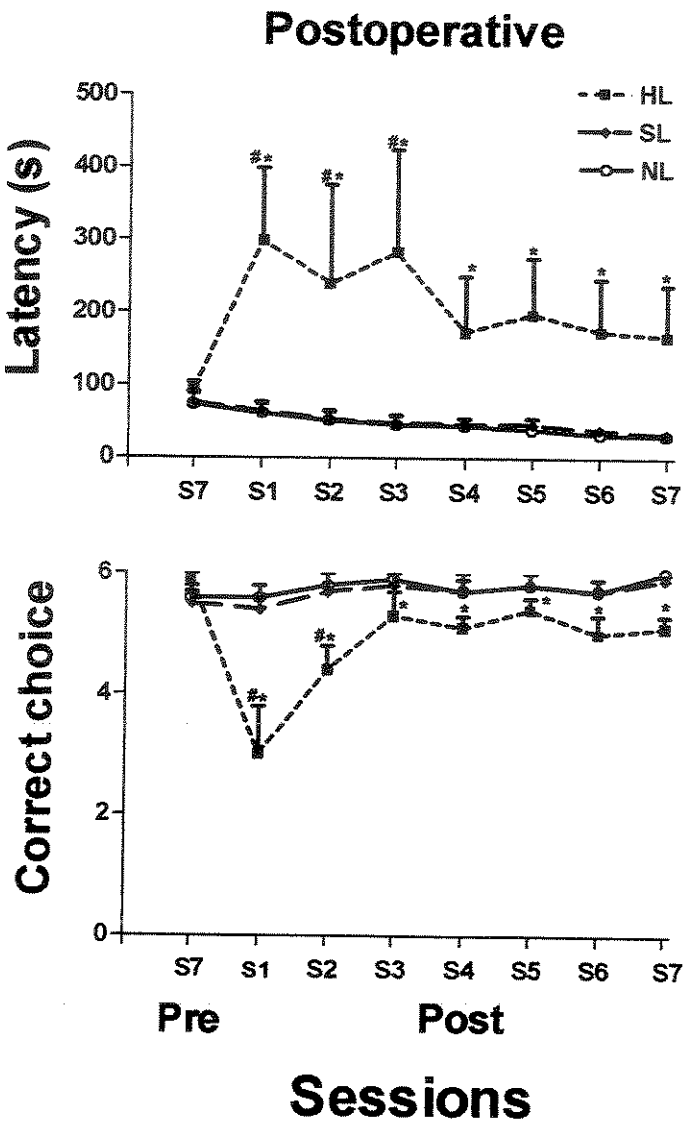

Figure 5. (top) Mean choice of the food cups latencies (second ± S.E.M.). (bottom) Mean ± S.E.M. of the correct choice (second) in the last session of the preoperative condition and in the postoperative sessions. For the groups hippocampal lesion (HL, *n*=8), sham lesion (SL, *n*=8) and no lesion (NL, *n*= 7). * Significantly different (ANOVA: $p<0.05$) from the controls; # Significantly different (ANOVA: $p<0.05$) from 7^{TH} preoperative session.

3.5. Probe test 2

Fig. 6 presents the absolute time spent in the four quadrants of the arena for controls and HL groups. The HL group performed less accurately than controls groups and expressed a low preference for the target quadrant. ANOVA showed an effect of quadrant ($F_{3,6}$=54.41, P<0.001) and a significant interaction of group x quadrant ($F_{3,6}$=21.18, P<0.001).

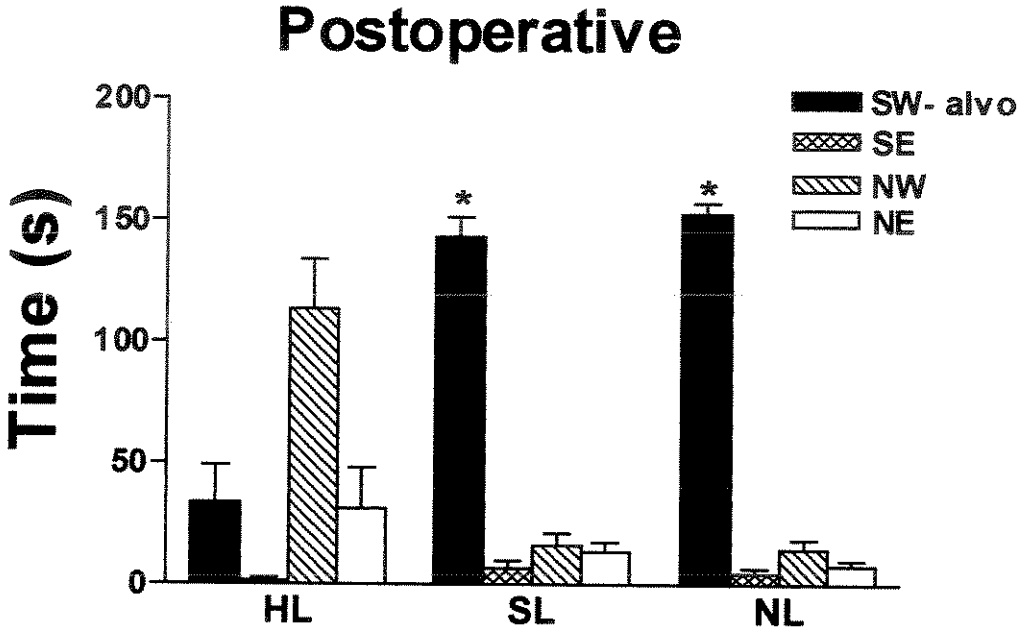

Figure 6. Overall mean values of the time spent (Mean ± S.E.M.) during 3 min of the probe test 2 in the preoperative condition by hippocampal lesion (HL, n=8), sham lesion (SL, n=8) and no lesion groups (NL, n=7). During this time the food cups were removed from arena in the postoperative condition. * Significantly different (ANOVA: p<0.05) between the target quadrant and the other quadrants.

4. Discussion

Three main findings emerged from the current study. First, the data showed that damage to the hippocampus resulted in increased pigeon's choice latency in the first session of postoperative condition. The higher latency values observed for the HL pigeons decreased with training but never reached the scores of control animals during the postoperative sessions. Second, the pigeons were impaired in the correct choice of the food cup, although this damage was transitory. Third, the probe test confirmed that the lesioned pigeons were incapable to retrieve the correct quadrant location when the food cups were omitted from the arena.

Data of the higher latency and decreased accuracy of pigeons with hippocampal lesion may be related to a deficit in the retrieval of information. This deficit is apparently related to a difficulty in recovering the information about the spatial location of the food cups, which may suggest a possible explanation for spending more time than the controls to solve the task during the training trials.

The data referred to increases in spatial choice latency agree with several studies that reported higher latency in hippocampal animals as compared to controls performing in a spatial task. Fremouw et al. (1997) reported that hippocampal lesioned pigeons, operating in a dry version of the Morris water maze, had longer mean latency to find the hidden food well as compared to control group and the mean latency decreased only after 9 or 10 sessions. Bingman et al. (1987) and Gagliardo et al. (1999) studying the homeward orientation based on familiar-site information for navigation observed that lesioned birds were less effective in their time ability to return home. In a study with rats in a Morris water maze, Pouzet et al. (2002) verified that hippocampal lesioned rats showed longer latency to find the hidden platform than the control group. Is interesting to

note that Amaral-Toma and Ferrari (in press) as well as showed that the damage in the hippocampus of the pigeons impairs learned performance with increases in latency even in a simple no-mapping task.

The second set of results concerning the number of correct choice of the food cup also corroborates several reports of the literature. An abrupt decrease in correct choices occurred in the first postoperative sessions. Although the accuracy of choice improved as training progressed it never reached control values. In the study of the spatial discrimination task with pigeons Watanabe (2001) showed that the birds with hippocampal damage required more trials to reach the criterion of the emitted 10 successive correct choices or 80 trials were over in the spatial task than the control group. Sherry and Vaccarino (1989) verified that ckickadees with hippocampal aspiration lesion showed deficits in the accuracy in recovering scattered caches that required remembering the spatial locations of the cache sites. Such a set of results show that the hippocampus plays an important role in the retrieval of the spatial information already consolidated, since in all these studies the task was preoperatively learned. Indeed, our results suggest that in the postoperative condition the memories about the task were already outside the hippocampus (Jarrard, 1991; Alvarez and Squire, 1994; Bontempi et al., 1999). However, the delay in retrieval of long – term memory is probably due to the impairment in the interaction between the hippocampus and other neural structures. Since the hippocampus is extensively connected to other cerebral systems, the deficits in accuracy of choice may be interpreted as evidence that alteration in the intricate functional network where the hippocampus operates results in mapping strategy impossibility. The robust decrease in accuracy followed by a relative

improvement may be indicative that the animals come to use new strategies as far as they could not use any mapping strategy.

The results of the probe test are informative about the effect of the lesion in the retrieval of spatial information. The fact that lesioned animals spent less time exploring the target quadrant may be interpreted as evidence that they were unable to remember the previous location of the food. That is, hippocampal birds were impaired in retrieving previously consolidated information about location of the correct food cup. Is worth saying that during the preoperative probe test the three groups showed a preference for the target quadrant expressed as a significantly longer time of exploration. This result may be regarded as indicative of the use of spatial mapping by the animals with intact hippocampus.

So, the results of the present study with pigeons corroborate previous findings in studies of the literature with mammals tested in a Morris water maze task. Moser et al. (1993) reported that in rats with dorsal electrolytic lesions of the hippocampus the amount of time in the correct quadrant during the probe test was directly related to the extension of the hippocampal lesion. Devan et al. (1996) studying rat with Fornix/fimbria lesion (FF) in the water maze verified that when the platform was removed, the lesioned rats distributed evenly the time within all the four quadrants.

Taken together the data of the present study extend previous results indicating that damage localized in the hippocampus impairs learned performance that uses spatial mapping and contribute for comparatives analyses of the role of hippocampus in birds and mammals. Is worth saying, that although the knowledge about the hippocampal role in spatial mapping in mammals has been favored by the extensive studies of the rodents' behavior in the Morris water maze, the study of avian

hippocampus does not have an experimental situation comparable with the water maze. In this sense, the present results provide a task that has proved to be useful for the discussion of the memory processes and the hippocampal functional role.

5. Acknowledgments

We to thank Walber Toma for assistance with the pigeons, Marco Aurélio Ribeiro de Paula and Norivaldo Celestino for excellent technical assistance in histological preparations, and Francesco Langone for generous use of their microscopy and computer for imaging capture.

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Artigo 3: Impaired in the long-term retention of spatial memory in pigeons with hippocampal lesion

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1. Introduction

The Hippocampus was proposed as a neural region directly responsible for place learning, a process related to forming and storing neural maps in rodents (O'keefe & Nadel, 1978) and pigeons (Bingman & Able, 2002).

Rats and mice with hippocampal lesion have been found to be impaired on a variety of tasks, notably spatial navigation (MacDonald & White, 1994; Compton *et al.*, 1997; Hannesson & Skelton, 1998; Hollup *et al.*, 2001; Bruin *et al.*, 2001; Pouzet *et al.*, 2002; Ramos, 2002). The hippocampal lesions impaired the acquisition of spatial tasks in pigeons (Bingman, *et al.*, 1988; Strasser *et al.*, 1998; Ioalè *et al.*, 2000; Amaral-Toma & Ferrari, in press;) and rats (Moser *et al.*, 1993; Devan *et al.*, 1996; Morris *et al.*, 1990) as well as long-term retention of spatial relations (Ramos, 2000).

In the studies in which the task was preoperatively learned the results show that the hippocampus plays an important role in the retrieval of the spatial information already consolidated. At the moment of the postoperative training the memories about the task are already outside the hippocampus and the deficits could probably be due to the impairment in the interaction between the hippocampus and other neural structures (Jarrard, 1991; Alvarez & Squire, 1994; Bontempi *et al.*, 1999). On the other hand, when the task was postoperatively learned the results indicate that the deficits are more pronounced although the hippocampal lesioned animals are still capable of learning. Most of the studies, have only examined the effects the lesion on acquisition but not on the long-term retention of the learned information.

The participation of the hippocampus in the consolidation and retention of the long-term memory was analyzed by Ramos (2000) in a original study that dissociates

acquisition and retention in preoperative spatial learning in hippocampal rats. The main findings indicate that rats with lesions in the hippocampus can learn a place response at the same rate as the control subjects when a special training is used. However, 24 days later, lesioned rats show a profound deficit in the retention of this information.

The aim of the present work was to analyze the effects of the hippocampal lesion in pigeons on acquisition and retention of the spatial learning.

2. Material and methods

2.1. Subjects

The subjects were 21 adult pigeons (*Columba livia*), with 318 g mean weight. They were maintained on a 12:12 hr light/dark cycle with lights on at 6:00 h. They received water and food *ad libitum* in their home cage except during the experimental period when the birds had restrict amount of food in order to maintain their experimental body weight (85% of the *ad libitum* weight). The birds were randomly attributed to three groups: hippocampal lesion (HL, *n*=7), sham lesion (SL, *n*=7), and no-lesion (NL, *n*=7). The experimental protocol has been approved by the Ethics Committee for Animal Experimentation of the Instituto de Biologia–UNICAMP, Brazil (219-1) and was conducted in accordance with the recommendations of the Canadian council on animal care and with the ethical guidelines for investigations of experimental pain in conscious animals (Zimmernan, 1983).

2.2 Surgery

After acclimation to the animal's quarter room and before any training procedure the birds were submitted to neurotoxic hippocampal lesions. Details of the surgical procedures were according to techniques previously described in Amaral-Toma and Ferrari (in Press). The lesion was carried out under deep anesthesia (Ketamine and Xilazine). The coordinates used for bilateral ibotenic acid (IBO) infusions followed Karten & Hodos (1967): anteroposterior (AP), 4.0, 5.0, 7.0; vertical (V) 1.5; lateral (L), 1.0 lateral to the midline. Sham-operated birds were submitted to anesthesia, fixation in the stereotaxic apparatus, scalp incision, and skull exposure and perforation procedures, but not to any substance infusion. After surgery, the pigeons were kept in their home cages.

2.3. Apparatus

All testing were conducted in a wood circular arena (Fig.1) with 1.5 m diameter and 50 cm high, with the floor and the wall painted white. The floor was covered with a rough brown paper that was changed in each session. It was positioned in the center of a room (2.11W x 3.10L x 2.77H, m) that had distal visual stimuli like sockets, light switches, one unidirectional mirror and two entrance doors. In addition, in each wall there was one distinctive picture - a red circle, a blue square, a green triangle or a yellow star –, provided as landmarks

Four points equally spaced along the circumference of the arena were arbitrarily assigned as: N (north), E (east), S (south) and W (west). These points served as the starting positions at which the pigeons were lowered into the chamber. The area of the arena was also conceptually divided into four quadrants (NE, SE, SW and NW) of the equal size. In each quadrant there was one food cup; in three of these quadrants the

food cup contained plain sand and only in the SW quadrant there was a food cup with food covered with sand.

The behavior of the pigeons in the chamber was recorded with a GCP-165CR video camera mounted 2.60 m above the center of the experimental chamber. The camera used to record the sessions was connected to a video-TV Panasonic (AG 1960) system located in an adjacent room.

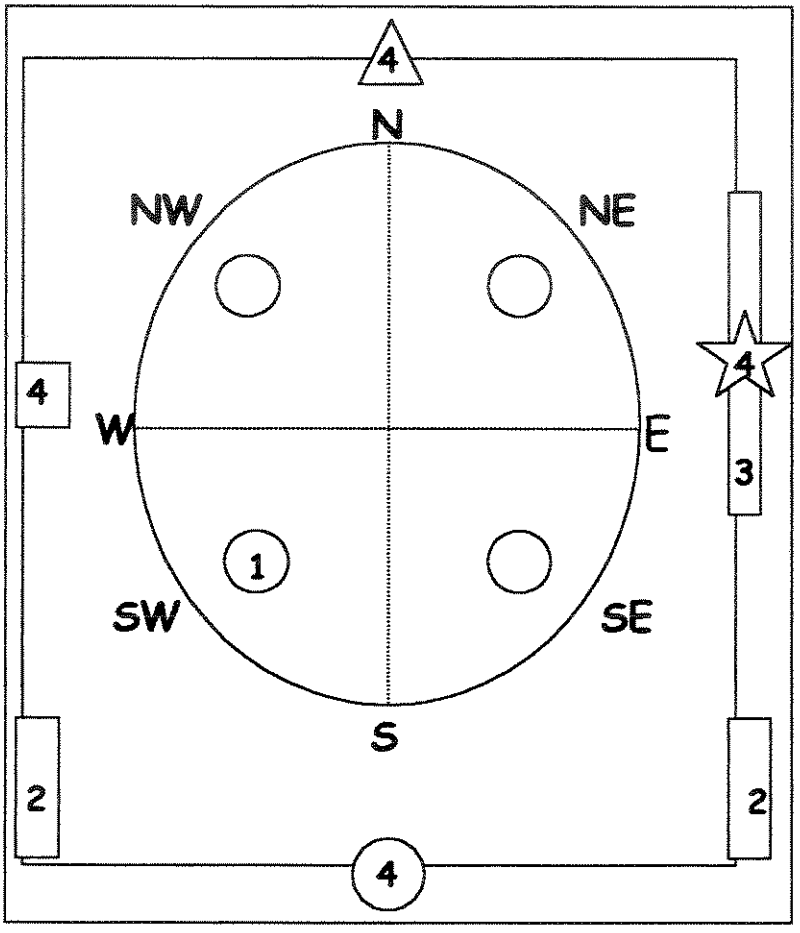

Figure 1. Schematic representations of the experimental arena and the experimental room. 1: correct food cup; 2: entrance doors in the experimental room; 3: unidirectional mirror; 4: distinctive pictures used as distal cues.

2.4.Behavioral Procedures

2.4.1. Training (acquisition)

The training consisted of seven daily sessions, each one with six trials to find the correct food cup. In each trial the light in the room was turned off and the pigeons were placed in the arena with its head facing the wall of the chamber. The lights were then turned on and the experimenter recorded the latency of the choice response and the accuracy correct choice (correct or error) by the pigeons. The session was videotaped for further analysis. The choice response was defined as the first peck in one of the food cups. After the choice response the pigeons were allowed a 30 s period in the chamber. If no choice occurred within a 10 min period the trial was finished. On each of the six trials of the session, a different starting point (N, E, S, L and W) was used and the location of the correct food cup was fixed in the center of SW quadrant, 18 cm from the arena wall. During the intertrial interval (1 min) the pigeon rested in a cage located in an adjacent room. At the end of the session, the pigeons were returned to their home cages.

2.4.2. Probe test

Immediately after the completion of the last trial, the four food cups were removed from the chamber. During the following test trial with omission of the food cups the pigeon was put in the arena and the behavior was tape-recorded during 3 min. The analysis considered the four locations in the chamber defined by imaginary intersection lines on the longitudinal and lateral axes. The time spent in each quadrant was measured. The objective was to determine how much time the pigeons required, during

the omission of the food cups, to respond to the quadrant representing the correct food cup location in comparison to the others.

2.4.3. Retention test

After the completion of training, the subjects were maintained in their respective home cages for 24 days. During this period were not tested in any way. Starting on day 25 the pigeons received retention tests of the spatial task learned during the initial training phase. The procedure used during the retention test was identical to that of last training session (acquisition).

2.5. Histology

At the completion of the behavioral testing the pigeons were deeply anesthetized and transcardially perfused with 0.9% saline solution followed by 10% formaldehyde solution. Subsequently, the brains were prepared for histology according to the Klüver Barrera technique (1953).

2.6. Statistical analysis

The data were analyzed with two-way ANOVA having groups as factor and session as repeated measured. *Post hoc* analysis used the Tukey-Kramer method for multiples comparisons.

3. Results

3.1. Histological analysis

The hippocampus lesions were reconstructed in schematic drawings of the lesion as illustrated in Fig. 2. Cellular disorganization and little cells were show in the hippocampus of the HL group were evident both in the dorsal and ventral regions.

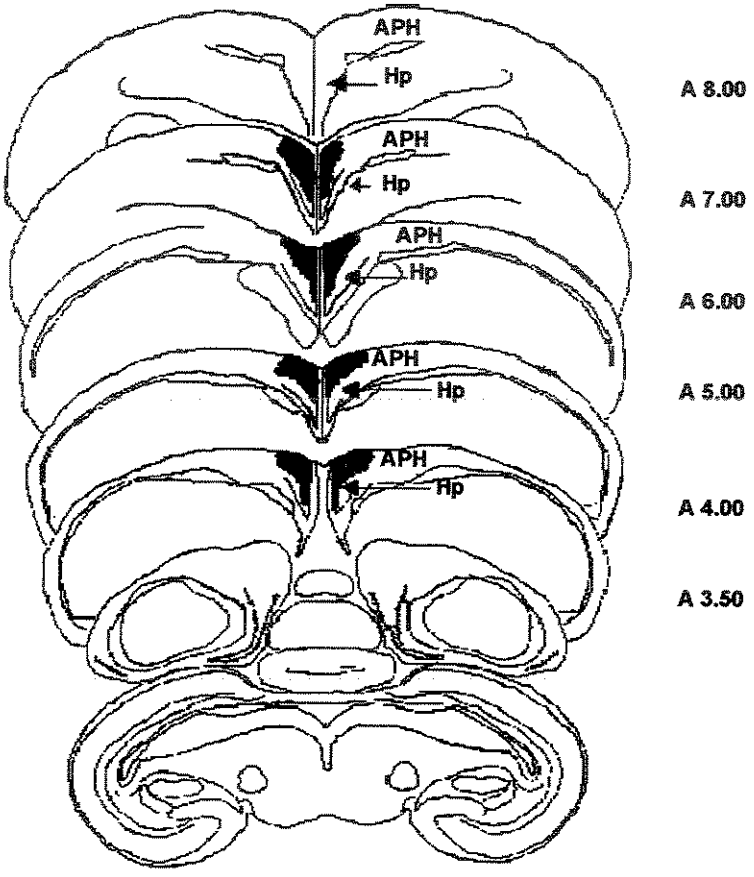

Fig. 2. Schematic representations of the hippocampus of pigeons reconstructed according to frontal sections from the atlas of Karten & Hodos (1967). Black areas represent the extent of the lesions induced by ibotenic acid in the HL pigeons. APH= area parahippocampalis; Hp= hippocampus

3.2. Behavioral

3.2.1. Training (acquisition) and retention

Figure 3 (Top) shows the mean correct choice of the food cup for controls (SL e NL) and HL pigeons along the seven sessions of training and the retention test. The HL pigeons learned task as indicated by correct choices number in the last sessions but had low choice accuracy during the retention test. Controls pigeons showed total accuracy of choice in the 3 last training sessions and maintained high level of correct choice during the retention test. Anova indicated a significant effect of group ($F_{2,18}$ = 15.69; $P < 0.001$), of session ($F_{7,14}$ = 36.01; $P < 0.001$) and the interaction between factors ($F_{14,126}$ = 2.57; $P < 0.005$). The performance of the HL group in the last day of training proved to be significantly higher than in the first day of training (*t*-test, *t*6 = 18.6; $P < 0.001$). In the retention test the performance of HL birds was significantly lower than that of control groups ($F_{2,18}$ = 11.7; $P < 0.005$).

Fig. 3 (botton) shows the mean latency of the food cup choice response for the controls and LH pigeons in the seventh session of training and in the retention test.

The latency of the HL group was longer in the first sessions and in the retention test as compared to the controls. ANOVA yielded a main effect of groups ($F_{2,18}$ = 5.75; $P < 0,01$) and effect of session ($F_{7,14}$ = 32.83; $P < 0,001$). The performance of the HL group in the last day of training proved to be significantly than in the first day of training (*t*-test, *t*6 =4.919; $P < 0.001$).

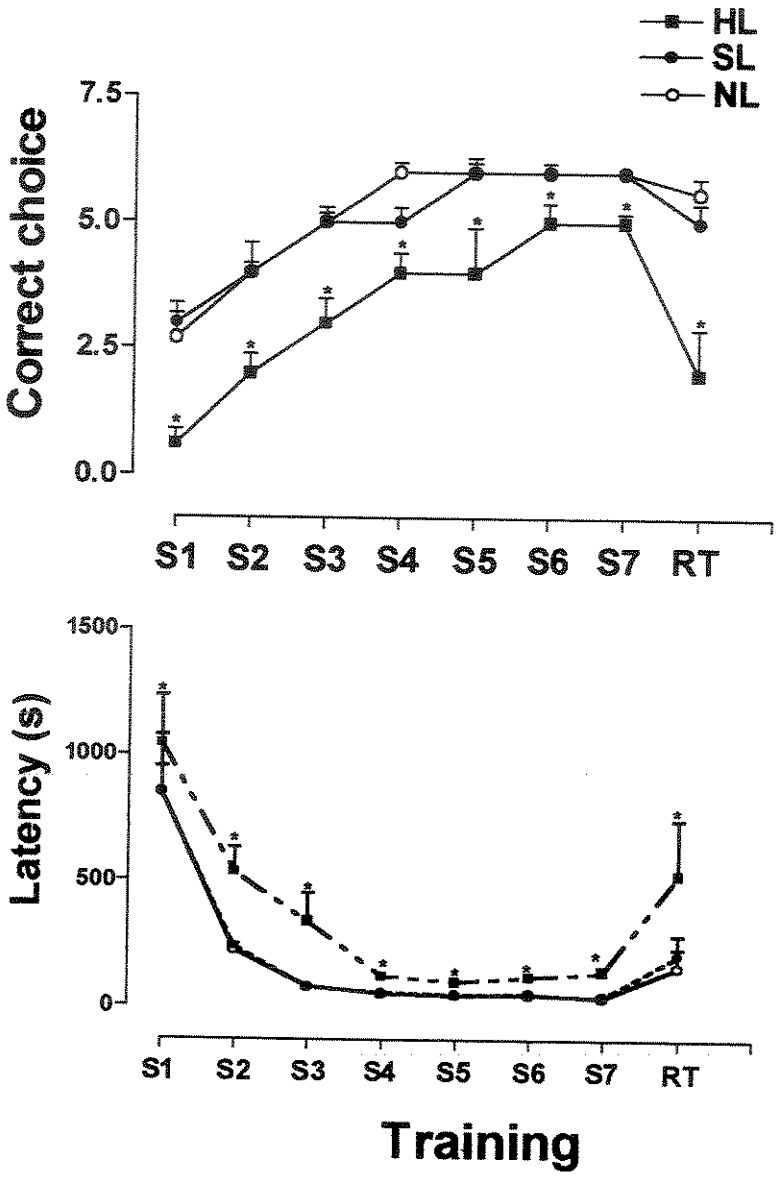

Fig. 3. (Top) Mean ± S.E.M. of the correct choice. (bottom) Mean choice of the food cups latencies (second ± S.E.M.). in the seven sessions of the training condition, and one session of the retention test (RT) for the groups' hippocampal-lesion (HL, n=7), sham lesion (SL, n=7) and no-lesion (NL, n= 7). * Significantly different (p<0.05) from the controls.

3.2.2. Probe test

Fig. 4 presents the absolute time spent in the 4 quadrants of the arena for the controls and HL group. The HL group performed less accurately than controls groups and expressed a low preference for the target quadrant. ANOVA showed an effect of quadrant ($F_{3,6} = 33.87$; $P < 0.001$) and a significant interaction of group x quadrant ($F_{6,54} = 27.16$; $P < 0.001$).

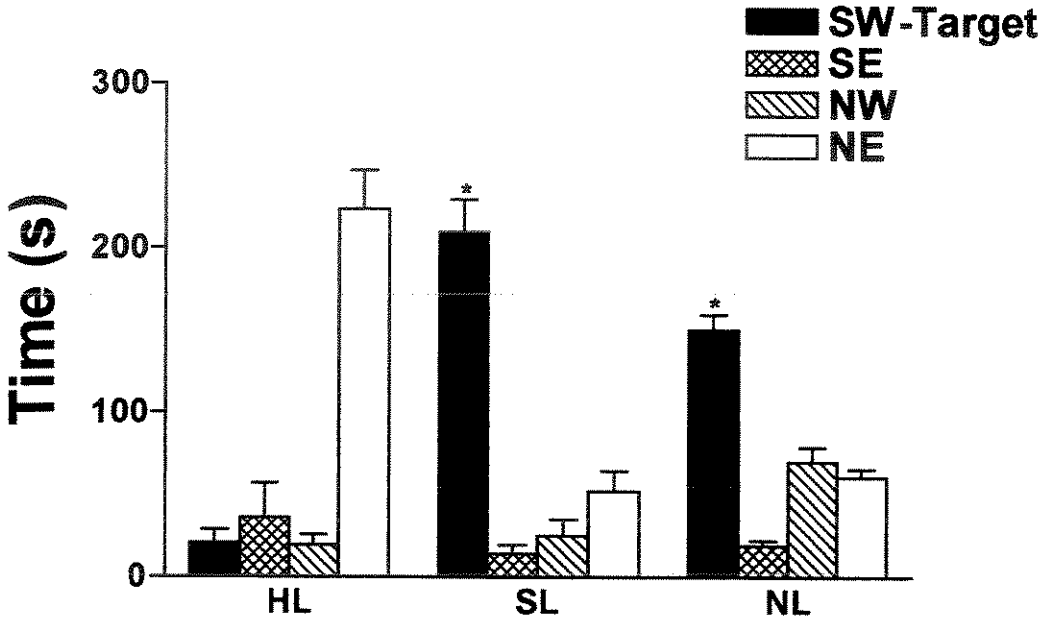

Fig. 4. Overall mean values of the time spent (Mean ± S.E.M.) during 3 min of the probe test 1 in the preoperative condition by hippocampal lesion (HL, n=7), sham lesion (SL, n=7) and no lesion groups (NL, n=7). During this time the food cups were removed from arena. * Significantly different (p<0.05) between the target quadrant and the other quadrants.

4. Discussion

The present results show that hippocampal lesion impaired the acquisition of the food cup choice response in a circular arena. Both the latency as well as the accuracy of choice were affected by hippocampus lesion. However the HL pigeons were sensitive to the training contingences as far as they learned the task, although never reached the same accuracy level as the controls. A profound deficit in the retention of the learned information was observed in HL pigeons 25 days later as compared to the control animals.

Data of the longer latency and decreased accuracy of the pigeons with hippocampal lesion during the sessions of training may be related to a deficit in the acquisition and retention in the spatial learning.

These results agree with previous studies with rodents that suggested that with training, the hippocampal lesioned animals reached an asymptotic level of performance. However, they were incapable of further improvements to control the level of efficiency, indicating impairment in the acquisition of the information. In a study with rats in the Morris water maze Pouzet *et al.* (2002), verified that hippocampal lesioned rats showed longer latency to find the hidden platform than the control group, but improved their to escape learning with extended training.

The results of the probe test complement the observations concerning latency and correct choices deficits as effect of the lesion in the acquisition of learning. These results indicate that the lesioned animals spent less time in the correct quadrant. This may be interpreted as evidence that the animals were unable to remember the location of the food, which may be related to a deficit in retrieval of the spatial location of the

target quadrant. Similar results may be found in previous studies of the literature with rodents. Moser *et al*, (1993) reported that rats with ventral electrolytic lesions to more than 30% of the hippocampus were associated with poorer performance, with a distribution of time equivalent in the four quadrants. Devan *et al*, (1996) studying rat's Fornix/fimbria lesion (FF) in the water maze task verified that in the probe test with a platform removed the rats with FF lesions distributed the time within evenly in all the four quadrants.

Another interesting finding of our data refers to the retention deficit in HL pigeons 25 days after training. The robust difference between hippocampal lesioned birds and controls may be related to a failure in the consolidation process in the long-term retention of spatial memory. Ramos (2000) investigated if lesioning made before training lead to a deficit in the long–term retention of the learned spatial information and showed that after 24 days later of training lesioned rats manifest a profound deficit in the retention of the spatial information. In this sense the present data corroborate these previous observations concerning hippocampal rats.

One important issue arise from these results. First, Bomtempi *et al*. (1999) examined in mice the changes in functional metabolic activity of the hippocampus formation at the 25 days retention interval. After 25 days of learning occurred an increase in the metabolic activity in the frontal, anterior cingulated and temporal cortices, indicating greater recruitment of these regions in the retrieval of remote as compared to recently acquired information. This finding indicates that the reduction in hippocampal metabolic activation supports the idea that the hippocampal formation has a transitory role in memory storage. Studies of inactivation of the hippocampus suggest that the

neocortical areas perform the storage of LTM whereas the hippocampus is necessary for acquisition and short-term storage (McClelland *et al.*, 1995). According Parron *et al.* (2000), the parietal cortex is part of the functional network that mediates the initial acquisition of a task but not part of the network that mediates the updating of memory. In contrast, both networks include the hippocampus. According to this view, memory consolidation would involve a transitory interaction between the hippocampus and related structures of the cortex, as this interaction are compromised, this to be possible impeding the long-term retention of the information.

In conclusion, this data suggest that memory processes are not implemented within a single brain system but within a large network of structures. This functional organization accounts for time-dependent processes, such as memory consolidation (Nadel & Bohbot, 2001), i.e., the transformation of a labile memory trace (dependent on the hippocampal system) into a stable memory trace (dependent on extra-hippocampal structures).

Future studies will be necessary to determine the neocortical structures responsible for acquisition of the short-term memory when the hippocampus no is functional and the neocortical areas perform the storage of LTM.

5. Acknowledgment

We to thank Walber Toma for assistance with the pigeons, Marco Aurélio Ribeiro de Paula and Norivaldo Celestino for excellent technical assistance in histological preparations, and Francesco Langone for generous use of their microscopy and computer for imaging capture.

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Artigo 4

Title: Hippocampal AgNOR increase after learning spatial in pigeons: Evidences of lateralization.

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Abstract

Spatial learning and memory related morphological changes in the argyrophilic nucleolar organizer region (AgNOR) of hippocampus and parahippocampus neurons in pigeon were quantitatively evaluated by means of AgNOR neurohistochemical stain. The AgNORs and nuclei of nerve cells of two hippocampal region and one parahippocampal region of pigeons trained in a simple spatial task or one complex spatial task or no training (Control group) were morphometrically evaluated. Results show the absolute and relative area of AgNOR in Ventral-Anterior, Dorsal-Anterior and Parahipocampal-Anterior areas in the two hemispheres increased significantly in the group simple spatial learning but not in control group. The absolute and relative area of AgNOR in VP, DM in the left hemisphere increased significantly in the complex spatial learning group but not in control group. As the size of AgNORs in the nerve cell nuclei reflect the level of transcriptive activity, these morphological changes could be revealing increased protein synthesis in pigeons hippocampus neurons related with learning and memory.

Keywords: AgNOR, Hippocampus, Spatial learning, pigeons, long-term memory, lateralization

Introduction

The importance that hippocampus has on learning and memory is well known. Most of the research about hippocampus functions reports lesion-induced impairments in learned behaviors of humans (Cave & Squire,1991; Zola-Morgan *et al.*, 1986 Smith & Milner, 1981; Milner, 1972), monkeys (Mishkin, 1978; Ridley *et al.*, 2001), rodents (Aggleton *et al.*, 1986; Hampson *et al.*, 1999; Mumby *et al.*, 1999; Ramos, 1998; Pouzet *et al.*, 2002; Morris *et al.*, 1990), birds (Amaral-Toma & Ferrari, 2003; Colombo *et al.*, 2001; Fremouw *et al.*, 1997, Good, 1987, Reis *et al.*, 1999; Watanabe, 2002; Strasser *et al.*, 1998; Bingman *et al.*, 1988; Ioalé *et al.*, 2000), fishes (López *et al.*, 2000) and reptiles (Rodriguez *et al.*, 2002) in spatial and no spatial memory tasks. It is well established that processes of consolidation of long-term memory require protein synthesis activity (Fride *et al.*, 1989). Indeed, several works show that learning promotes an increase in the protein synthesis in the brain (Greenough *et al.*, 1990). On the other hand, studies using protein synthesis inhibitors provide evidence of impairments in the recall of learned information in chicks trained in a passive avoidance learning task after be use the protein synthesis inhibition, showed no recall when tested after 6 or 24 later in the absence of the drug (Bradley & Galal, 1987; Freman *et al.*, 1995).

The protein synthesis activity can be indirectly measured by analyzing the cell nucleolus. The size and configuration of the nucleolus reflect the level of cellular transcriptional activity and in turn this activity is related to the cellular protein synthesis rate, which is regulated by the cytoplasmatic demand of the rRNA necessary for protein synthesis (Lafarga *et al.*, 1991). The nucleolar organizer region (NORs) is the active region of the nucleolus, in which most of the ribossomal RNA synthesis occurs. The

NORs are structures of central importance in the trasncription of nucleic acid to protein (Alberts *et al.*, 1989). These regions have been shown loops of ribossomal DNA which transcribe to ribossomal RNA (rRNA) and thus ultimately to ribossomes and thence to protein (Derenzini, *et al.*, 1992). NORs can be demonstrated by a simple argyrophilic staining technique, the AG (argyrophilic)-NOR method (Ploton *et al.*, 1986), which stains NOR-associated proteins because they bind silver ions selectively (Buys & Osinga, 1980; Méhes *et al.*, 1993). As the AgNOR is enlarged during increased protein synthesis, the cellular activity related to transcriptional activity can be evaluated by measuring the AgNOR number and area (Louis *et al.*, 1992; Gonzáles-Pardo *et al.*, 1994.).

Silver staining of the nucleolar organizer regions (AgNOR) has proved to be a valuable tool for studies on cell proliferation (Alberts *et al.*, 1989), cytogenetics (Alberts *et al.*, 1989), discrimination of benign and malignant cancer cell (Derenzine & Ploton, 1991; Underwood & Giri, 1988), sexually dimorphic cells (Gonzáles-Gonzáles *et al.*, 1996; Gonzáles-Gonzáles *et al.*, 1997) and neuronal activity in learning and memory (Qü *et al.*, 1994, Vargas *et al.*, 2000; Garcia-Moreno *et al.*, 2000).

This work was designed to assess the relationship between learning and the morphometrical parameters of the AgNOR in the pigeons' hippocampus. Our aim was to determine whether learning of the spatial task increase neuronal synthetic activity in the hippocampal and parahipocampal areas. Specifically, we investigated differences in morphometrical parameters of the AgNor in two spatial tasks differing in complexity and in the behavioral strategies underlying them. For this purpose, we evaluated the neuronal synthetic activity in the two hemispheres of hippocampal, parahipocampal and control areas of the pigeons'brain.

Materials and methods

Subjects

Twenty-four adult male pigeons (*Columba livia*) weighing about 325 grams were used. The birds were housed in individual home-cages on a 12:12 h light-dark cycle (lights on at 6:00 a.m.). The experiments were carried out during the light phase of the cycle (between 6:00 a.m. to 18:00 p.m.). Temperature was maintained at about 25° C. The birds were randomly divided in three groups: complex spatial learning (CSL, n=8), simple spatial learning (SSL, *n*=8) and controls (C, *n*=8). During the experiment, the animals were food-deprived to 85% of their *ad libitum* weights. The experimental protocol had the approval of the Ethics Committee for Animal Experimentation of the Biology Institute–UNICAMP, Brazil (219-1).

Apparatus

Simple spatial learning group (SSL)

The simple spatial learning training were conducted in an experimental chamber (Fig 1 A) consisting of a rectangular box made of transparent plexiglas (50W x 50H x 115L, cm); two identical compartments (20x20x33 cm), externally located at each end of the box, served as initial boxes. Both of them contained a sliding door for access to the experimental chamber. The food cups were two semicircular glass cups, in one of the two corners of one wall at the end of the box: one food cup contained food covered with sand and the other had only plain sand. The wall containing the food cups was always the one located opposite to the starting box. A videocamera was mounted directly above (2 m) the center of experimental chamber, to record the sessions.

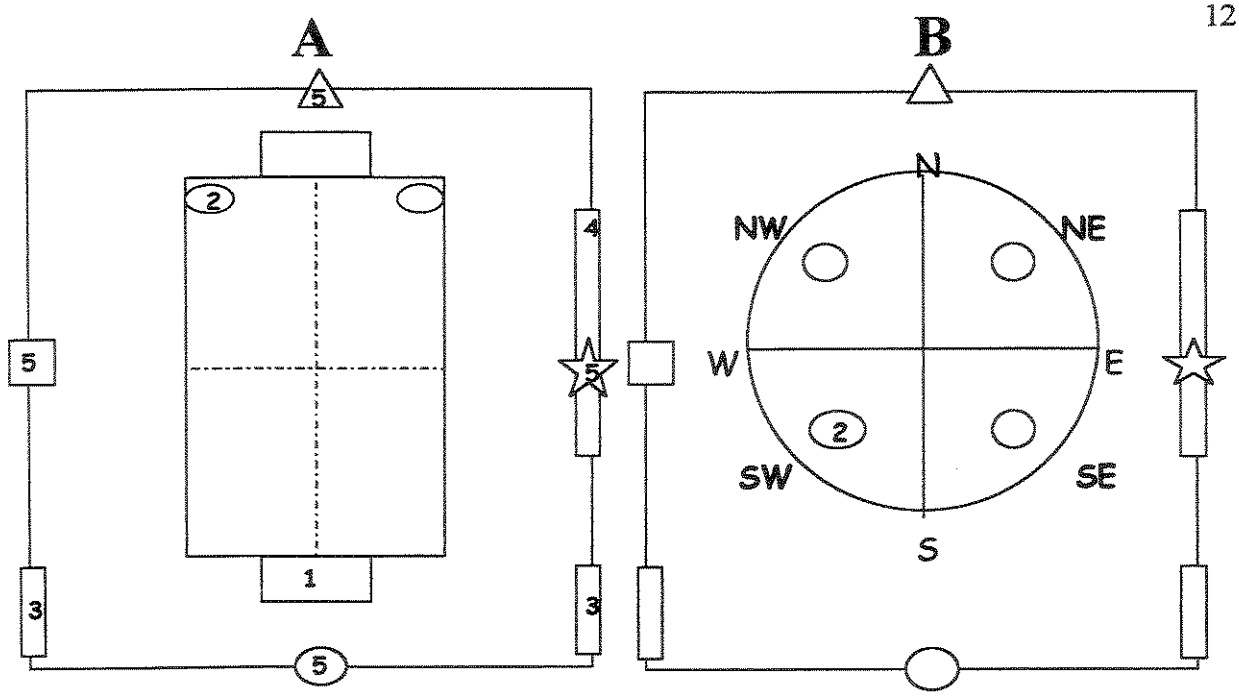

Fig. 1. Schematic representations of the experimental apparatus and the experimental room. A: Location of the food cups in the chamber of the task spatial learning; B: Location of the food cups in the arena of the task of spatial mapping learning. 1: initial boxes; 2: correct food cup; 3: entrance doors in the experimental room; 4: unidirectional mirror; 5: distinctive pictures used as distal cues.

Complex spatial learning group (CSL)

All testing were conducted in a wood circular arena (Fig 1 B) with 1.5 m diameter and 50 cm high, with the floor and the wall painted white. The floor was covered with a rough brown paper that was changed in each session. It was positioned in the center of a room (2.11W x 3.10L x 2.77H, m) that had distal visual stimuli like sockets, light switches, one unidirectional mirror and two entrance doors. In addition, in each wall there was one distinctive picture - a red circle, a blue square, a green triangle or a yellow star –, provided as landmarks

Four points equally spaced along the circumference of the arena were arbitrarily assigned as: N (north), E (east), S (south) and W (west). These points served as the starting positions at which the pigeons were lowered into the chamber. The area of the arena was also conceptually divided into four quadrants (NE, SE, SW and NW) of the equal size. In each quadrant there were one food cup; in three of these quadrants the food cup contained plain sand and only in the SW quadrant there was a food cup with food covered with sand.

The behavior of the pigeons in the chamber was recorded with a video camera mounted 2.60 m above the center of the experimental chamber. The camera used to record the sessions was connected to a video-TV Panasonic (AG 1960) system located in an adjacent room.

Training

Simple spatial learning group (SSL)

Birds were trained to choose the correct food cup that is, the one containing food and sand - in five-trial daily sessions, along four consecutive days. The pigeons were transferred from the home-cage and put inside the starting box where they remained during 1 min. Each trial began as soon as the pigeon entered in the experimental chamber after the entrance door was open and ended 30 sec after the choice of one of the two food cups or after 10 min, which ever came first. During the four training sessions the food cups remained in the same location throughout the five trials. The bird's task was to locate the correct food cup that contained food bellow the sand. A choice response was recorded when the pigeon approached and pecked one of the two food cups; the correct choice response was defined as the choice of the correct food

cup. The latency of the choice response - the time between the opening of the entrance door and pecking one of the two food cups was measured with a digital chronometer.

Complex spatial learning group (CSL)

The training consisted of the seven days. Pigeons were given six trials per day to find the correct food cup. In each trial the light in the room was turned off and the pigeons were placed in the arena with its head facing the wall of the chamber. The lights were then turned on and the experimenter recorded the latency of the choice response and the accuracy correct choice (correct or error) by the pigeons. The session was videotaped for subsequent analysis. The choice response was defined as the first peck in one of the food cups. After the choice response the pigeons were allowed to a 30 s period in the chamber. If no choice occurred during a 10 min period the trial was finished. On each of the six trials of the session, a different starting point (N, E, S, and W) was used and the location of the correct food cup was fixed in the center of SW quadrant, 18 cm from the arena wall. Before the beginning of a trial, the pigeon rested in a cage located in an adjacent room. At the end of the session, the pigeons were taken back to their home cages.

Control Group (C)

The animals were manipulated during 4 consecutive days. Pigeons were carried until the experimental room, after 1 min reconducted to its home cage.

Perfusion

The day after the conclusion of the training period the pigeons were anesthetized with Ketamine and Xilazine (0.1 mg/kg, 1:1, i.m.) and perfused transcardially with saline

solution 9%, followed by 10% formalin in 0.1 M phosphate buffer (pH 7.4). The telencephalon were cut in series of 10-μm tick sections with a microtome (Leica RM-2025 Germany).

AgNOR Staining and quantification

The staining procedures for AgNOR quantification has been described in detail elsewhere (Vargas *et al*, 2000). One of each three sections was silver stained according to the method of Ploton et al. (1986). Briefly, sections were deparaffinized in xylene, hidrated and incubated at room temperature in the dark for 10 min in freshly prepared solution comprising of 2% gelatin (Sigma, USA) dissolved in 1% aqueous formic acid and of 50% silver nitrate solution dissolved in distilled water. The sections were observed under a light microscope using an oil immersion lens (x 1000 magnification). The silver nucleolar organizer regions were clearly visualized as black or black-brown intranuclear dots (Fig 2 A).

A computer assisted interactive image analyzer and specific software were used to measure cellular dimension-al parameters in each selected image. The following variables were quantified for the analysis: mean number of AgNORs per neuron, mean AgNOR and nuclear areas and relative AgNOR area [(absolute AgNOR area/nucleo area) x 100].

The AgNOR quantification was carried out according to anterior –posterior coordinates including anterior (A 8.0), middle (A 5.0) and posterior (4.0) slice sampling of the hippocampus for both hemispheres. In each hemisphere four areas were

analyzed: ventral hippocampus (VH), dorsal hippocampus (DH), area parahipocampalis (APH) and ectostriatum, as control area (E). (Fig.2 B)

Results

Fig. 3 presents the mean correct choices (A) and the mean latency (B) of the correct food cup choice for the SSL over four training sessions. The number of correct choice (A) increased significantly as training progressed as compared to the first session. This was confirmed by ANOVA as a significant effect of session ($F_{3,24}$=8.08, P<0.005). (B) A significant decrease in latency to find the correct food cup along the experimental sessions indicated improvement in spatial task ($F_{3,24}$=7.98, P<0.005).

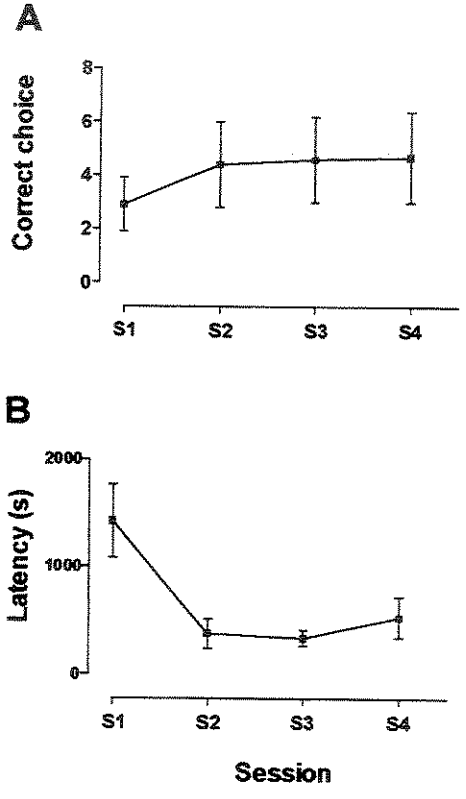

Fig. 3 Mean (± E.P.M.) values of correct choice (A) and latency of choice (B) during the four session in the simple spatial task for the groups SSL (n=8) .

Fig. 4 presents the mean of correct choices (A) and the mean latency (B) of the correct food cup for the CSL over seven training sessions. The pigeons' accuracy of choice (A) improved significantly as training progressed. ANOVA showed a significant effect of session ($F_{6,48}$=11.15, P<0.001). (B) The latency to find the correct food cup decreased significantly as training progressed ($F_{6.48}$=28.75, P<0.001).

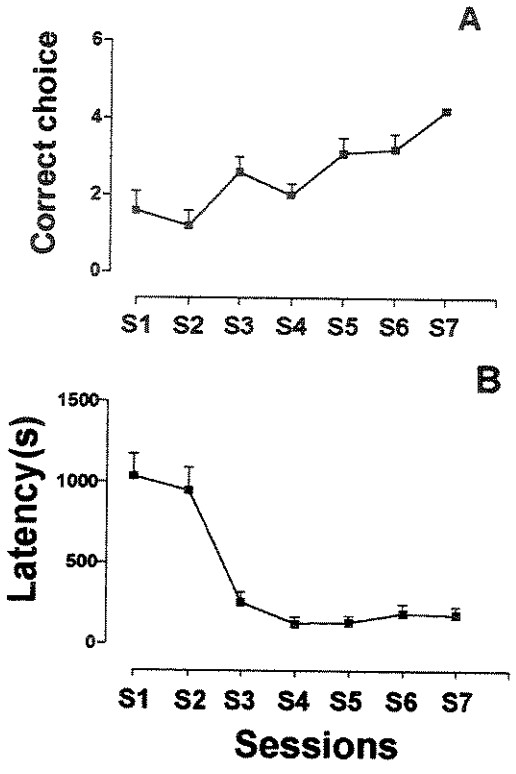

Fig. 4 (A) Mean (± E.P.M.) values of correct choice (A) and latency of choice (B) during the seven sessions of training in the complex spatial task for the group CSL (n=8).

Fig. 5 presents the values of absolute area of the AgNOR for the Ventral-Anterior, Dorsal-Anterior hippocampal areas, and parahippocampal-Anterior regions, in both left (top) and right hemispheres (Bottom), in pigeons trained in the simple spatial learning.

The absolute area of the AgNOR showed significant increases in group the simple spatial learning as compared with control group in the left (VP: Mann-Whitney, U=25.380.500, $P<0.001$; DM: U=21.007.000, $P<0.001$; PA: U=25.639.000, $P<0.001$) and right hemispheres (B) (VA: U=23.374.000, $P<0.001$; DA: U=20.211.500, $P<0.001$; PA: U=26114.500, $P<0.001$).

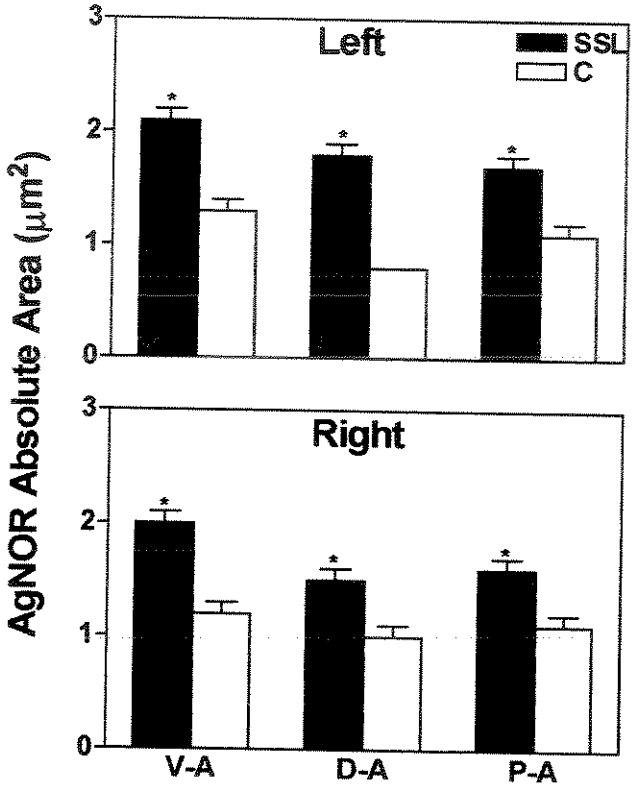

Fig 5. Absolute sizes of AgNORs in the left (Top) and right (Bottom) hemisphere in three formation regions of the hippocampal formation for pigeons of the SSL (n=8) and control (n=8) group. Ventral-Anterior (VA) Dorsal-Anterior (DA) and Parahipocampal-Anterior (PA)). * Significantly different ($p<0.05$) from the control.

Fig. 6 presents the values of relative areas of the AgNOR both in the left (Top) and right (Bottom) hemispheres calculated for the Ventral-Anterior, Dorsal-Anterior hippocampal regions and in the parahipocampal-Anterior region of pigeons trained in the simple spatial learning. AgNOR sizes increased both in the left (VA: Mann-Whitney, U=23.441.500, $P<0.001$; DA: U=22.170.000, $P<0.001$; PA: U=25.242.500, $P<0.001$) and right hemispheres (VA: Mann-Whitney, U=25.271.000, $P<0.001$; DA: U=20.309.500, $P<0.001$; PA: U=28.044.000, $P<0.001$) of trained pigeons as compared to the control.

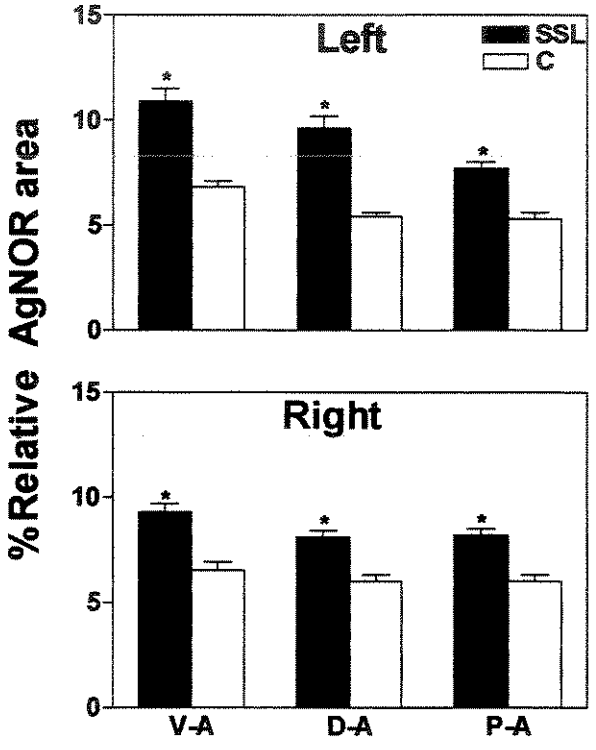

Fig 6. Relative sizes of AgNORs in the left (Top) and right (Bottom) hemisphere in three regions of the hippocampal formation for pigeons of the SSL (n=8) and control (n=8) group. Ventral-Anterior (VA) Dorsal-Anterior (DA) and Parahipocampal-Anterior (PA). * Significantly different ($p<0.05$) from the control.

Fig 7 presents the values of absolute (TOP) and relative (BOTTOM) values of the area of the AgNOR in the left hemisphere in the Ventral-Posterior and Dorsal-Medial hippocampal areas in pigeons trained in the complex spatial learning. A significantly higher increase in the AgNOR was seen in function of the complex spatial learning, as compared to control group, both in absolute area (VP: Mann-Whitney, U=35.187, $P<0.001$; DM: U=16.189.000, $P<0.001$) and in relative area measurements (VP: U=39.667.500, $P<0.001$; DM U=15.444.000, $P<0.001$).

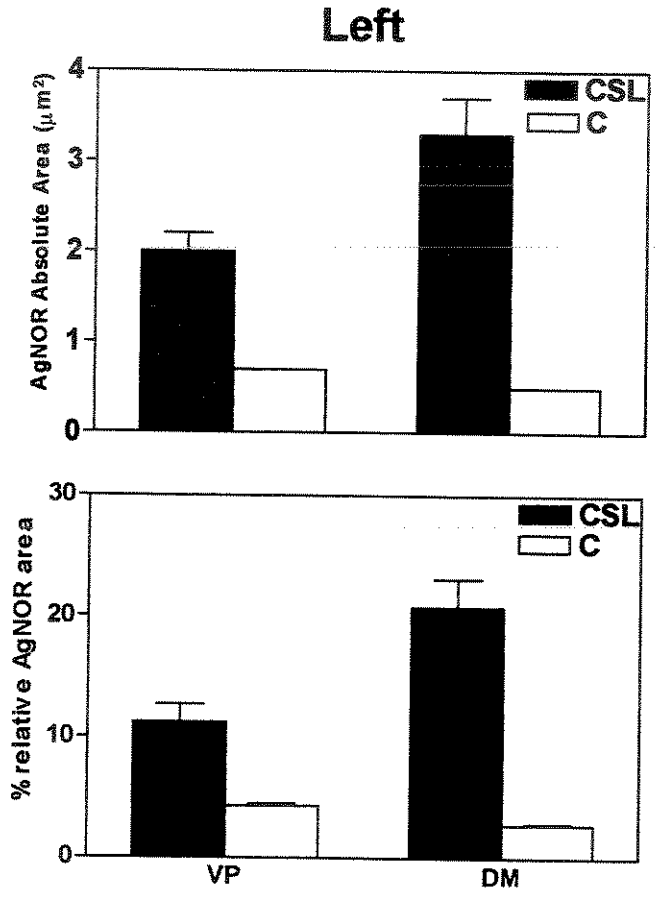

Fig 7. Absolute (Top) and relative (Bottom) sizes of AgNORs areas in the two hippocampus regions in the left hemispheres for pigeons of the CSL (n=8) and control (n=8) group. Ventral-Posterior (VP) and Dorsal Medial (DM) regions. * Significantly different ($p<0.05$) from the controls.

Discussion

Two main findings emerged from the current study. First, the size and the relative area of the AgNOR, in the Ventral-Anterior, Dorsal-Anterior and Parahipocampal-Anterior regions in the hippocampus of pigeons increased significantly after learning a simple spatial task. In the complex spatial task this increase occurred only in the Ventral-Posterior and Dorsal-Medial regions. Second, in the group submitted to training in the complex spatial task only the left hemisphere showed been a significant increase of the size absolute and relative area of the AgNOR.

The differential increases of the absolute size and the relative area of the AgNOR according to different regions of the hippocampus observed in the pigeons trained in the two tasks have a twofold experimental relevance. First, our data show that the AgNOR staining is a good indicator of transcriptional activity underlying learning. AgNORs parameters indicative of increase in the area are related to the increase of protein synthesis and to a high transcriptional activity in normal cells during learning. The AgNORs area/nucleus area ratio attempts to express the activity of the protein synthesis by the existing relationship between both areas. A high value would indicate a greater area of AgNOR in relation to the nucleus and thus a greater protein synthesis. Our data clearly indicate a relationship between this transcriptional activity in the hippocampus and learning extending previous data of the literature in learning and non-learning situations. Moreno *et al.*, (1997) studying NOR activity in hippocampal areas in rats during the postnatal development and ageing find out that the mean AgNOR area and the ratio between AgNOR and nuclear areas per neuronal cells decreased at the older ages. Ploton (1992) also showed that the use the AgNOR area (Nucleus area ratio since

it has been shown to be an efficient indicator of the differences functional in activity between areas hippocampais in rats. Vargas *et al.* (2000) studying the parameters of the absolute and relative area of the AgNOR in the goldfish showed increases in the neurons of telencephalic areas after spatial learning.

Second, the observation that the increases of the size and relative area of the AgNOR was differentiated both relative to the hippocampal areas as well as to the learning situation. During the training of the complex spatial task the AgNOR increases were mostly prominent in some areas of the left hemisphere while in the simple spatial task the increases were evenly observed both in the hippocampal regions and parahippocampal in the two hemispheres. Taken together these data are very interesting and point to the exciting insights concerning a possible regionalization of hipocampal function, both within the hippocampus as well as between hemispheres. The differential activation of the two cerebral hemispheres are well described in the literature. Some studies tested birds under monocular conditions, and showed a number of visual asymmetries, revealing specialization of either hemisphere for different tasks (Güntürkün, 1997; Rogers, 1996; Vallortigara, 2000). More recent studies suggested that the right and left brain hemispheres might control different aspects of spatial information processing (Tommasi & Vallortigara, 2001). Tommasi & Vallortigara (2001) studying chicks in an indoor arena showed that the right avian brain is mainly concerned with relational spatial information, white the left avian brain encodes absolute metric information.

In the experimental situation of complex spatial learning a clear hemispheric difference emerged. The results of the increase of the AgNOR only in the left hemisphere could reflect a specific effect suggestive of lateralization of the brain

processes that participate in fundamental aspects of spatial mapping information processing in pigeons. Ulrich *et al.*, (1999) tested pigeons monocularly on either their left or their right eye for homing performance after they had binocularly learned the homeward route from remote release sites. Birds using their right eye (effectively left hemisphere) showed considerably better homing performance. Gagliardo *et al.* (2001) also demonstrated a left hemisphere advantage, specifically the left hippocampal formation, for navigational map learning in young pigeons. Gagliardo *et al.* (2002) studying pigeons with unilateral lesion showed that the left hippocampal formation plays a dominant role in navigational map learning. Other indication of the left hemispheric superiority during homing in pigeons is showed for Von Fersen & Güntürkün (1990). Their work showed that pigeons have a better long-term memory for visual patterns in the left brain hemisphere. So, it may seem reasonable to consider that the left hemisphere could play a preferential role condition where the navigational map is implicated.

Another interesting finding from the present study is quite revealing. In the experiment of the spatial learning when the pigeons were released from the same start position the increases of the AgNOr were evident in the two hemispheres. There was a similar tendency for both hemispheres to be evenly used for global spatial references in such a simple spatial choice task. Prior *et al.* (2001) tested the effects of monocular occlusion and possible differences between the right and left brain hemispheres in pigeons to find the route to the goal from a start position that was the same during all trials. The search at this location and directedness of the bearings were equally high with both eyes, suggesting that both brain hemispheres have the same competence level for these components of the task. The present study, together with that of Prior *et*

al. (2001) shows evidence that brain processing underlying simple spatial tasks do not show lateralization.

Some previous studies suggested that, in some place-finding tasks, the left hemisphere of young chicks encodes spatial information, but tends to be concerned mostly which absolute (metric) information whereas the right hemisphere tends to be concerned mostly with relational spatial information (Tommasi & Vallortigara, 2001). It is conceivable, therefore, that the hippocampal formation of both hemispheres may participate in familiar landmark navigation near the loft, but perhaps in different ways. For example, it might be that the right hippocampal formation is important for representing landmarks in a relational or map-like fashion whereas the left hippocampal formation uses landmarks more as recognition or guidance cues (Eichenbaun *et al.*, 1994; Gagliardo *et al.*, 1999).

The present study contributes with a set of results indicative of changes in AgNOR parameters as function of learning that corroborates those previous findings from the literature. Moreover, our data extend the evidence concerning hemisphere specialization related to the processing of spatial information. Although both hemispheres contribute to spatial cognition, it seems that the left hemisphere is prominently involved in complex spatial tasks requiring mapping strategies.

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CONCLUSÕES

III. CONCLUSÕES

Os experimentos desenvolvidos para compor essa tese indicaram, no seu conjunto, que o hipocampo de pombos, tal como o de mamíferos participa dos processos de aprendizagem e memória espacial, tanto numa tarefa que requer a estratégia de mapeamento, quanto numa tarefa espacial mais simples que não requer. Os principais resultados dos experimentos podem ser descritos sumariamente como evidências de que (1) danos no hipocampo de pombos causam prejuízos transitórios em tarefas espaciais simples e complexas quando ocorrem após um treino pré-lesão; (2) os prejuízos no desempenho foram mais evidente quando os danos hipocampais ocorreram antes da aquisição da aprendizagem e memória, ou seja, nos animais que sofreram a lesão antes do treino; (3) os pombos treinados na tarefa que requeria mapeamento espacial tiveram um prejuízo na consolidação da memória de longa-duração, avaliada 25 dias após o treinamento pós-lesão; (4) nos dois tipos de tarefas foram evidenciados aumentos na AgNOR, em diferentes regiões hipocampais, indicando um aumento da síntese de proteínas na região hipocampal subjacente à aprendizagem e memorização das relações espaciais. Contudo, os pombos treinados na tarefa que requer o mapeamento espacial apresentaram uma maior síntese de proteínas no hemisfério esquerdo.

Os pontos relacionados a seguir sumarizam as principais implicações dos dados obtidos:

1. Os resultados aqui expostos sugerem que a aprendizagem de ambas tarefas espaciais, de não mapeamento e de mapeamento espacial, envolve a codificação de informações sobre os eventos episódicos e representações sobre a seqüência desses

eventos e associações de eventos a eles relacionados. De acordo com Eichenbaum, (2001), isto implica que o hipocampo pode ser capaz de recordar as informações seqüenciais e específicas do contexto, integrando-as.

Tombol *et al.* (2000) estudando circuitos do hipocampo de pombos, relataram evidências de uma complexa rede que é utilizada nas atividades neuronais relacionadas às relações espaciais envolvidas na orientação e navegação. Qualquer tipo de interferência nessa rede pode desorganizar o circuito prejudicando a recuperação da informação. A questão de como as redes podem operar em seqüência para acompanhar suas funções complexas ainda necessita de respostas, tanto em relação ao hipocampo de aves quanto de mamíferos.

Estudos em ratos envolvendo a inativação hipocampal reversível após a aquisição (Parron *et al.*, 2001) mostram claramente que a organização neural da memória não está restrita ao hipocampo ou à formação hipocampal, mas envolve uma grande rede de estruturas neurais. Como visto em nossos estudos 1 e 3 os pássaros que foram lesados após a aquisição das informações no treino pré-operatório apresentaram prejuízos transitórios, sugerindo que a memória já estaria consolidada e armazenada em outras áreas fora do hipocampo. Então, o efeito transitório de aumento na latência da escolha poderia ser relacionado a um prejuízo relacionado com a recuperação da memória estocada em sistema não hipocampais.

Esses dados aqui apresentados mostram similaridades entre aves e mamíferos em termos dos efeitos da lesão hipocampal. Os mamíferos e os pássaros com danos no hipocampo são prejudicados em tarefas que requerem o processamento e a retenção de informação espacial. Nossos resultados, referentes aos prejuízos na latência de escolha do comedouro correto e ao número de respostas corretas, são diretamente

relacionados com dados verificados em vários experimentos realizados com roedores (Pouzet *et al.*, 2002) mostrando que quanto maior a complexidade da tarefa maior o prejuízo. Resultados citados acima mostram que existem muitas evidências que fundamentam comparações funcionais entre pássaros e mamíferos, já danos hipocampais em diferentes animais resultam em uma mesma classe de prejuízo em tarefas semelhantes.

2. Em nossos experimentos, observou-se que a lesão pós-treino causou um prejuízo apenas na latência para encontrar o comedouro correto, enquanto que a lesão pré-treino causou prejuízos maiores na latência e no número de respostas corretas. Assim, outro ponto a ser destacado é a questão da análise de efeitos da lesão em situações pré e pós-treino. Os presentes dados podem sugerir que nos experimentos em que os animais foram treinados antes da lesão essas informações foram estocadas em outras regiões fora do hipocampo e por isso ocorreu apenas um prejuízo na latência para evocação da informação. Tais observações relacionam-se com vários estudos que postularam que o hipocampo tem uma função temporária na estocagem da memória enquanto que outras estruturas, tais como o neocortex em roedores (Alvarez e Squire, 1994; Bontempi *et al.*, 1999), teriam uma função de estocagem permanente. Apesar desses fatos não estarem muito claros em pombos, pode-se sugerir que nos experimentos em que o treino de aquisição ocorreu após a lesão hipocampal, as estruturas relacionadas com o hipocampo não foram suficientes para a aprendizagem precisa das relações entre o organismo e o ambiente. Desse modo, os dados enfatizam a importância do hipocampo para a aprendizagem e memorização adequadas.

Nos Experimentos 1 e 2 durante a aprendizagem de reversão, quando os animais foram liberados de uma diferente caixa inicial e os comedouros foram transferidos para a parede oposta, ambos os eventos proximais do ambiente e a localização relacionada aos eventos distantes foram mudadas. Isto provavelmente impôs uma necessidade para uma nova composição dos eventos contextuais já conhecidos. Nessa situação, os pombos com lesão hipocampal mostraram aumento na latência se comparados aos controles,indicando a importância da funções hipocampais para o estabelecimento de novas relações entre os eventos ambientais.

3. Os testes de estratégia desenvolvidos nos experimentos 1 e 2 foram importantes na tentativa de desvendar se as estratégias utilizadas pelos pombos eram desempenhadas pelo sistema de taxon ou de mapeamento cognitivo (O'Keefe e Nadel, 1978). O teste de estratégia 1 mostrou que os animais lesados não estavam utilizando o mapeamento cognitivo para localizar o alvo, já que após a colocação da cortina os animais não apresentaram nenhum prejuízo para se localizar. Como a estratégia utilizada não era de mapeamento espacial foram desenvolvidos mais dois testes de estratégia para verificar qual hipótese de táxon os animais estavam seguindo. Como os animais não apresentaram prejuízo no teste de estratégia em que o comedouro era substituído por outro de formato diferente, os dados sugeriram que os animais usaram as estratégias de guiamento e;ou a de orientação

4. Como evidenciado nos experimentos 1, 2 e 5, ocorre o envolvimento do hipocampo em tarefas que não exigem o mapeamento espacial. Entretanto esse prejuízo é mais evidenciado apenas na latência, mostrando que quanto mais complexa

for a tarefa, maiores serão os prejuízos. Contudo, podemos verificar que qualquer alteração na conexão entre o hipocampo e outras estruturas, ocorrerão déficits na aprendizagem de qualquer tarefa espacial. Nesse sentido, os dados apresentados contribuem para o esclarecimento de questões que, nos estudos com mamíferos, são contraditórias, ou seja, no que diz respeito a utilização do hipocampo em tarefas que não requerem o mapeamento espacial.

5. Outros dados interessantes de nossos estudos referem-se aos déficits de retenção após 25 dias do treino, observados nos pombos lesados antes do treino (Experimento 4). Uma diferença robusta entre os pombos lesados e os controles pode estar relacionada a uma falha no processo de consolidação na retenção a longo-prazo da memória espacial. Estudos de inativação do hipocampo sugerem que as áreas neocorticais desempenham a estocagem da memória a longo-prazo enquanto o hipocampo é necessário apenas para a aquisição e estocagem a curto-prazo (McClelland *et al.*, 1995). Concordando com este ponto de vista, a consolidação da memória pode envolver uma interação transitória entre hipocampo e estruturas relacionadas corticais e sub-corticais, e quando essa interação está comprometida é possível que a retenção da informação a longo-prazo seja impedida.

6. Os resultados referentes a marcação de AgNOR nas duas tarefas confirmaram os resultados encontrados experimentos anteriores (Experimento 1, 2, 3 e 4), os quais mostraram o envolvimento do hipocampo em tarefas espaciais simples e complexas. Como evidenciado anteriormente, na tarefa espacial simples ou de não mapeamento

espacial, ocorreram poucos prejuízos nos animais com lesões hipocampais. Nessa tarefa, ocorreu um aumento da síntese de proteínas em todas as regiões hipocampais e parahipocampais, sugerindo que na ausência do hipocampo outras estruturas poderiam estar desempenhando o papel normalmente desempenhado pelo hipocampo.

No entanto, na tarefa de mapeamento espacial ocorreu um aumento da síntese de proteínas foi muito mais robusto nas regiões hipocampais do hemisfério esquerdo. O aumento na expressão da síntese de proteínas verificado nos animais treinados nas duas tarefas indicou que com maior complexidade da tarefa, houve um menor número de regiões marcadas com aumento na síntese de proteínas. Especificamente, apenas o hipocampo mostrou aumento na AgNOR. Portanto, os dados sugerem que como menos regiões processariam essas informações, o dano hipocampal, nesse tipo de tarefa, causou déficits maiores.

No experimento de mapeamento espacial surge,assim, uma clara evidência de lateralização hemisférica. Os resultados do maior aumento da AgNOR no hemisfério esquerdo podem refletir um efeito específico que sugere a lateralização dos processos cerebrais que participam dos aspectos fundamentais do processamento das informações de mapeamento espacial em pombos. Este trabalho, juntamente com o de Gagliardo *et al.* (2002), sugere que o hemisfério esquerdo de pombos pode ter uma preferência funcional nas condições onde o mapeamento espacial é utilizado, tanto em estudos de campo quanto os de laboratório.

7. As condições experimentais programadas nos Experimentos 1 e 2 e nos Experimentos 3 e 4, mostraram-se adequadas para a análise de aprendizagem e

memória espaciais que requerem diferentes tipos de estratégias. Dentre os estudos de aprendizagem espacial com pombos, a maior parte foi realizada em estudos de campo, sendo que poucos experimentos foram realizados em laboratório. Os experimentos existentes estão relacionados com a aquisição e reversão de uma posição esquerda-direita, discriminação no labirinto em T (Good, 1987), tarefa espacial em campo aberto (Colombo et al., 1997), discriminação condicional com três discos coloridos em câmera de condicionamento operante (Watanabe, 2002), aprendizagem de lugar em uma arena circular (Fremouw *et al.*, 1997) e discriminação condicional de marcas em uma sala (White *et al.*, 2002). No presente estudo, pretendeu-se planejar uma situação experimental que garantisse uma condição mais próxima daquela que é a mais utilizada em roedores para o estudo da memória espacial. Procurou-se, então, melhorar a versão seca do labirinto aquático de Morris, desenvolvido por Fremouw *et al.*, (1997). Contudo, é importante salientar que tanto na situação desenvolvida por Fremouw e colaboradores (1997), quanto em no presente estudo, a versão seca do labirinto aquático provê uma situação de desempenho apetitivo, enquanto que o labirinto de Morris constitui uma situação de fuga, portanto aversiva.

8. O padrão de resultados mostrou que a lesão neste estudo causou prejuízos parciais, provavelmente por não ter destruído as fibras de passagem e danificado outras estruturas, além do hipocampo. Talvez por esse motivo os déficits observados nos animais não tenham sido tão profundos como os encontrados na literatura.

Esse prejuízo pode ser considerado o efeito da lesão com ácido ibotênico. Jarrard (1993) mostra efeitos semelhantes em experimentos realizados em ratos com lesões com acido ibotênico (IBO), submetidos a tarefas espaciais. Esses resultados

indicam efeitos muito mais sutis e transitórios do que aqueles encontrados com outros tipos de lesões ainda muito utilizados hoje em dia em situações experimentais semelhantes.

As lesões convencionais (aspiração e eletrolítica) foram e ainda são muito utilizadas na maioria dos experimentos encontrados na literatura, e é sabido que o dano resultante destas técnicas não é limitado ao hipocampo, mas inclui dano nas estruturas extrahipocampais e suas projeções. Este fato leva a concluir que a lesão do hipocampo com ácido ibotênico, sem prejudicar as outras estruturas hipocampais, possa apresentar efeitos muito mais sutis e temporários que a maioria dos experimentos.

9. Finalmente, é necessário lembrar que o hipocampo não pode ser analisado como um sistema independente, mas como uma estrutura muito bem conectada a outros sistemas cerebrais. O déficit causado pela lesão no hipocampo pode ser interpretado como evidência de uma rede funcional complexa onde o hipocampo opera. Os dados do presente estudo podem ser considerados como indicativos de que o dano localizado no hipocampo altera a atividade de tal rede e prejudica a aprendizagem de desempenho em uma tarefa simples como aquela desenvolvida por nós.

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