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MODULAÇÃO TEMPORAL DE PROCESSOS DE APRENDIZAGEM. IMPLICAÇÕES PRÁTICAS E TEÓRICAS

Tese apresentada ao Instituto de Biologia da Universidade Estadual de Campinas, para obtenção do título de Doutor em Ciências Biológicas, área de Fisiologia

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RESUMO

Estudos da variação temporal de processos de aprendizagem e memória são relativamente escassos e inconsistentes, fundamentalmente, devido à falta de uma padronização dos métodos utilizados e especificação dos tipos de controles necessários, que permitam isolar componentes temporais endógenos da aprendizagem propiamente dita. Assim, no presente trabalho discutimos as dificuldades deste tipo de estudo e sugerimos estratégias que poderiam ajudar a controlar alguns dos fatores mascaradores. Foram realizados três estudos tentando aplicar algumas destas estratégias. No primeiro, analisamos o efeito da hora do dia na habituação a um contexto novo em camundongos, quantificando o comportamento ambulatório por meio de observação direta. Em condições de claro-escuro um ritmo diurno foi detectado, porém nenhum efeito temporal foi observado em animais submetidos a um fotoperíodo esqueleto, situação em que se evita os efeitos mascaradores de um ciclo claro-escuro. Estes resultados demonstram que o ciclo de iluminação pode alterar significativamente a resposta a um contexto, reafirmando a necessidade de um controle preciso desta condição externa. Em estudos de ritmicidade de aprendizagem, devido à elevada freqüência de amostragem necessária e à necessidade de testes em horários pouco convenientes, a automatização da medida comportamental resulta indispensável. Assim, no segundo estudo foi validado um equipamento computadorizado para a medida do comportamento de imobilização em situações de condicionamento aversivo. As medidas comportamentais obtidas por este método automático mostraram uma elevada correlação com as medidas obtidas por observação direta. Consequentemente, este sistema foi utilizado no terceiro estudo onde se analisou o condicionamento aversivo durante as fases de atividade e de inatividade de camundongos submetidos a um fotoperíodo esqueleto. Uma diferença significativa entre os testes matutinos e os testes vespertinos foi detectada na expressão e extinção do condicionamento aversivo a um contexto, enquanto que nenhum efeito temporal foi observado no condicionamento aversivo a um som. O fato de que estes dois tipos de aprendizagens envolvem estruturas neurais diferentes sugere que o relógio biológico estaria tendo um efeito modulatório nas vias responsáveis pelo condicionamento a um contexto.

ABSTRACT

Studies that analyze a temporal variation in learning and memory processes are relatively scattered and inconsistent, mainly due to the lack of systematic methods and specifications of the necessary controls that would allow the dissection of a temporal component in the learning process per se. Here we analyze the difficulties of this kind of study and suggest a few strategies that could help control some of the masking factors. Three studies were performed applying some of these strategies. In the first study we analyzed habituation to a novel environment in mice by quantifying ambulation in an openfield through direct observation. Mice submitted to a light-dark cycle showed a diurnal rhythm in learning however, no temporal effect was observed in animals submitted to a skeleton photoperiod (two15-minute bright light pulses separated by 12 h of green dim light). Under these conditions the masking effects of a light-dark cycle are avoided. These results demonstrate that the response to a novel environment is strongly affected by the illumination cycle, thus reinforcing the need for precise control and specification of this condition. When analyzing learning at different times of the day automation of behavioral scoring becomes essential due to the need for high frequency in data collection and testing which occurs around the clock. Therefore, a second study was performed where a computer-assisted system for scoring freezing behavior in mice during fear conditioning situations was validated. The computer measures obtained during fear conditioning tests showed high correlations with hand-scored freezing. Consequently, this data collection system was used in a third study where fear conditioning was analyzed during the active and inactive phases of mice submitted to a skeleton photoperiod. A significant difference in the expression and extinction rate of context-dependent fear conditioning was observed between mice trained and tested in the morning versus the evening. In contrast, no diurnal rhythm was detected for tone-cued fear conditioning. The biological clock may have a modulating effect on the hippocampal-dependent pathway which underlies context fear conditioning and no effect on the hippocampal-independent pathway underlying tone-fear conditioning.

SUMÁRIO

Os resultados apresentados foram obtidos em experimentos realizados no Center for Circadian Biology and Medicine, Neurobiology and Physiology Department, Northwestern University, Evanston, Il, USA, com financiamento do CNPq, bolsa SW, processo # 201609/93-2 (PR).

INTRODUÇÃO

A. Ritmos circadianos: conceitos básicos e importância

Uma das características básicas dos organismos animais é a capacidade de modificar o comportamento ao longo das 24 horas. A bem conhecida alternância atividade-repouso constitui a mais evidente flutuação deste tipo. Embora com manifestações menos óbvias, virtualmente todos os sistema fisiológicos, assim como os processos comportamentais complexos de aprendizagem e memória, manifestam este tipo de variação diária. Tais mudanças diárias do organismo estão diretamente correlacionadas às mudanças do ambiente físico resultantes da rotação da Terra no seu eixo (Moore-Ede *et al.*, 1982).

Estes ritmos comportamentais/fisiológicos diários não são apenas respostas aos ritmos ambientais, porém surgem de um sistema de temporização interno (Pittendrigh, 1960). Este sistema de temporização, ou relógio(s) biológico(s), sob condições experimentais constantes, sem nenhuma pista temporal, regula ritmos com períodos de aproximadamente 24 horas, portanto referidos como ritmos "circadianos" (i.e., de aproximadamente um dia). Nessas condições diz-se que o organismo está em "livre-curso" ou seja, manifestando seu próprio período endógeno. Esta característica endógena dos ritmos permite que o organismo possa predizer e preparar-se com antecedência, fisiológica e comportamentalmente, para enfrentar as mudanças do meio ambiente associadas ao dia e à noite (Turek *et al.*, 1995; Marques *et al.*, 1997). Porém, o relógio biológico não é independente das mudanças diárias do ambiente físico. As variações diárias do meio ambiente sincronizam os ritmos endógenos dos organismos, ajustando, diariamente, a fase destes de tal forma que o período endógeno em

condições de sincronização será igual a 24 horas. O exemplo mais claro deste processo de sincronização é o fato da maioria dos animais serem ativos somente durante o período claro (espécies diurnas) ou durante o período escuro (espécies noturnas) e inativos durante a outra fase do dia. Esta sincronização é de importância fundamental para a sobrevivência do organismo, permitindo que "seja feito o certo na hora certa" (Turek, 1994, pag. 43).

É importante mencionar que além de ritmos circadianos, os organismos manifestam outros tipos de ritmos de diferentes freqüências, superimpostos aos ritmos circadianos (Marques *et al.* 1997). Ritmos infradianos são ciclos de baixa freqüência com períodos maiores a 28 horas (i.e., ritmos sazonais de reprodução). Ritmos ultradianos são oscilações de elevada freqüências com períodos menores a 20 horas (i.e., liberação pulsátil de hormônios hipofisiários).

B. Aprendizagem e memória

A aprendizagem é uma mudança adaptativa do comportamento que resulta de uma experiência. A memória é definida como a armazenagem e recuperação destas experiências. A memória é o conjunto de mecanismos pelos quais as informações ou relações adquiridas pela experiência são incorporadas no organismo. Dessa forma ficam disponíveis para a utilização posterior participando de mudanças comportamentais adaptativas. A observação e medida dessas mudanças comportamentais constitui a principal evidência dos processos de memória. Segundo o período de tempo em que tais mudanças perduram, a memória é chamada de curto-prazo (minutos ou horas) ou de longo-prazo (dias ou anos) (Rosenzweig, 1996).

Sob um ponto de vista experimental e com a finalidade de facilitar a análise, a aprendizagem é categorizada em associativa e não-associativa. Aprendizagens associativas são aquelas onde se estabelecem relações estímulo/estímulo ou estímulo/comportamento (condicionamento clássico e operante, respectivamente). Aprendizagens não-associativas são aquelas onde não se estabelecem relações óbvias entre estímulos ou entre estímulos e comportamentos. Entre as aprendizagens não-associativas podemos mencionar a habituação (diminuição progressiva da resposta a estímulos repetidos ou contínuos) e sensitização (aumento da resposta como conseqüência de um estímulo aversivo).

Aprendizagem e memória implicam mudanças em circuitos neuronais específicos. O conhecimento das bases celulares da aprendizagem e memória desenvolveu-se principalmente a partir de postulados iniciais de Donald Hebb, em 1949. A idéia básica é que quando um neurônio é ativado, suas conexões sinápticas tornam-se mais eficientes. Esta eficiência pode dever-se a um aumento na excitabilidade de curta duração (no caso de memória a curto-prazo) ou pode envolver alguma mudança estrutural na sinapse (como no caso de memória de longo-prazo). Numerosos estudos (Rosenzweig, 1996) têm demonstrado a existência de diferentes tipos de alterações sinápticas, que se acredita, representarem as bases celulares da aprendizagem. Entre estes tipos de plasticidades sinápticas está a potenciação a longo-prazo, processo que requer a depolarização coincidente de células pré- e pós-sinápticas resultando num aumento da eficácia da atividade sináptica que persiste no tempo. A potenciação a longo-prazo é o modelo experimental primário na investigação das bases celulares da aprendizagem e memória (Bliss & Collingridge, 1993).

Embora todo o sistema nervoso possua a capacidade de modificar a eficiência sináptica, existem regiões cerebrais que são críticas para a formação de determinados tipos de memórias. Nos mamíferos, podemos citar o hipocampo e as estruturas associadas do lobo temporal-medial, referido conjuntamente como sistema hipocampal (Eichenbaum & Otto, 1992).

C. Bases anatômicas e fisiológicas do sistema circadiano: relação com aprendizagem e memória?

O relógio biológico mestre em mamíferos, que controla e coordena quase todos os ritmos circadianos endógenos, está localizado em dois núcleos bilaterais no hipotálamo anterior, os núcleos supraquiasmáticos (NSQ). Estes núcleos recebem a informação externa necessária para a coordenação dos ritmos endógenos com pistas ambientais externas. O sincronizador (*zeitgeber*, doador de tempo) externo mais potente é o ciclo claro-escuro. A supremacia deste *zeitgeber* é evidenciada pelas conexões neurais entre a retina e os NSQ (trato retino-hipotalámico) como uma das principais e mais óbvias vias aferentes do sistema circadiano (Moore-Ede *et al.* 1982). Além de estímulos luminosos, sinais ambientais que aumentam o estado de exitação, e, consequentemente o nível de atividade do animal, também são capazes de reajustar o relógio biológico. A questão de como esta informação do estado comportamental do organismo chega ao NSQ, e influencia seu funcionamento tem recebido estudo intenso, porém ainda não foi elucidado totalmente (Mrosovsky, 1996). Uma possibilidade é que informação sobre este estado de ativação generalizada chegue ao NSQ através do núcleo geniculado lateral e do núcleo da rafe.

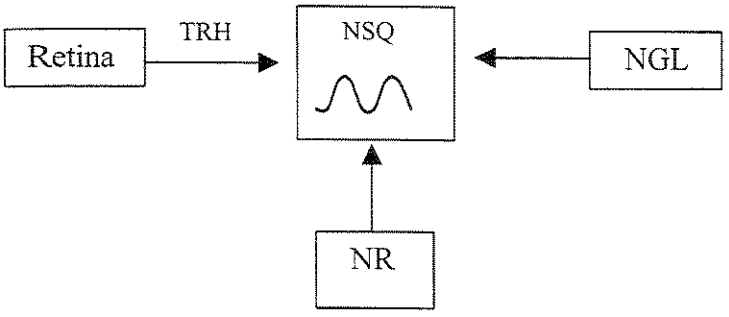

Figura 1. Aferências dos núcleos supraquiasmáticos (NSQ). TRH: trato retino-hipotalámico; NR: núcleo da rafe; NGL: núcleo geniculado lateral.

Os NSQ transmitem a informação temporal endógena (em condições de livre-curso) ou sincronizada (em ambiente rítmico) a todo o organismo através de diversas vias, que garantem que informações rítmicas cheguem a estruturas cerebrais, algumas das quais estão envolvidas direta ou indiretamente com processos de aprendizagem. Os NSQ produzem e liberam ritmicamente neurohormônios no líquido cerebrospinal que poderiam fornecer informação temporal a outras estruturas cerebrais. Por exemplo, um destes hormônios é a vasopressina liberada no líquido cerebroespinal durante a fase inativa em ratos (Jolkkonen *et al.*, 1988). Sabe-se que a vasopressina afeta processos de aprendizagem (Fujiwara *et al.*, 1997), embora ainda não existam evidências de que a vasopressina presente nos ventrículos participe de funções relacionadas com aprendizagem.

Conexões neurais indiretas seguramente têm um papel importante na transmissão de informação temporal dos NSQ para estruturas/sistemas que participam de processos mnemônicos (Watts, 1991). Por exemplo, os núcleos da rafe e os núcleos cerúleos produzem serotonina e noradrenalina, respectivamente, com um padrão rítmico, portanto devem receber

informações dos NSQ. Por sua vez, tanto os núcleos da rafe como os núcleos cerúleos mandam projeções ao hipocampo (Loughlin *et al,* 1986; Gray, 1995). Essas vias, NSQ-NR/NC-Hipocampo poderiam determinar a liberação rítmica de serotonina e noradrenalina no hipocampo, estrutura que tem participação essencial em vários tipos de aprendizagens. Por sua vez, estes dois neurotransmissores afetam mecanismos celulares essenciais para a potenciação a longo prazo, processo que tem sido sugerido como uma das bases fisiológicas da aprendizagem (Rosenzweig, 1996).

Uma outra via de comunicação entre o sistema circadiano e os processos de aprendizagem poderia ser neurohumoral, envolvendo a glândula pineal. Em mamíferos a pineal recebe projeções neurais provenientes dos NSQ e, consequentemente, informação temporal. Esta informação é expressa pela liberação rítmica de melatonina na corrente sanguínea. Tem sido sugerido um papel modulador deste hormônio no desempenho cognitivo em humanos (Dollins *et al.*, 1993). Adicionalmente, a estreita relação anátomo-funcional dos NSQ com o eixo hipotálamo-hipofisário determinaria a secreção rítmica de vários hormônios. Todos os hormônios hipofisiários e hipófise-dependentes manifestam flutuações diárias pronunciadas (Van Cauter & Aschoff, 1989). Estes hormônios rítmicos têm um efeito em diversas estruturas cerebrais e, portanto, podem influenciar temporalmente outros processos, entre os quais a aprendizagem. Por exemplo, é conhecido o fato de que os níveis séricos de corticosterona tem um efeito significativo em processos cognitivos, principalmente através da sua ação no hipocampo. Tem se observado um efeito modulatório da corticosterona em processos como potenciação a longo-prazo, aprendizagem associativa e memória espacial (Lupien & McEwen, 1997). A função hipocampal é modulada por

variações dos níveis de corticosteróides. Por exemplo, corticosteróides regulam a ativação de receptores de glutamato (Joels *et al.* 1996), ou podem regular a excitabilidade hipocampal por meio da regulação de subunidades de receptores GABA-A (Orchinik *et al.* 1994).

Uma outra forma de modulação temporal indireta de processos de aprendizagem poderia ser através do ciclo sono-vigilia. O ciclo sono-vigília é controlado diretamente pelo relógio biológico e, por outro lado, vários outros ritmos circadianos em mamíferos são dependentes do ciclo sono-vigília (Van Cauter *et al.*, 1992). Acredita-se que o sono é importante para a consolidação de memórias, pelo menos para alguns tipos de memórias (Wilson & McNaughton, 1994; Karni *et al.*, 1994). Esta relação sono/memória, poderia outorgar um parâmetro temporal a processos comportamentais que envolvem aprendizagem e cognição. Ou seja, durante o ciclo sono-vigília, as estimulações apresentadas antes da fase de sono seriam, de acordo com essa postulação, melhor lembradas do que aquelas ocorridas no início da fase de vigília.

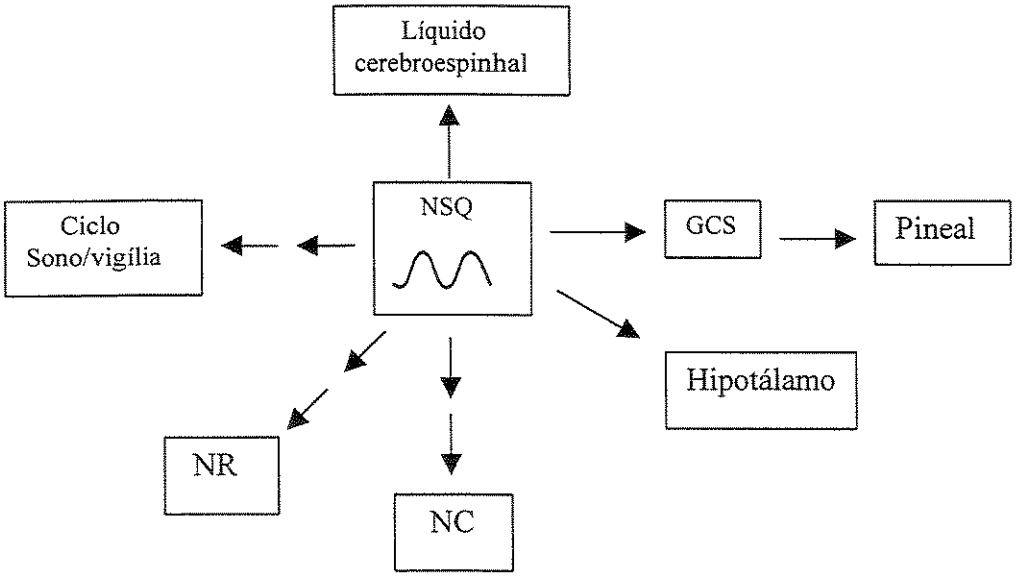

Figura 2. Possíveis vias de transmissão de informação temporal pelos núcleos supraquiasmáticos. NSQ: núcleo supraquiasmático; GCS: gânglio cervical superior; NC: núcleo cerúleos; NR: núcleo da rafe.

Todas estas, e possivelmente outras, vias de transmissão de informação temporal contribuem para a expressão rítmica de diversas variáveis cerebrais que, por sua vez, podem influenciar os processos de aprendizagem. Como exemplos, teríamos a expressão de mRNA de receptores de glucocorticóides e mineralocorticóides (Holmes *et al.*, 1995) e de 5HT2C (Holmes *et al.*, 1997) no hipocampo, assim como também de mRNA de jun B no cortex cerebral e corpo estriado (Menegazzi *et al.*, 1994). Outras variáveis são o nível de proteínas Fos no hipocampo e núcleos caudato e putamen (Kononen *et al.*, 1990), ligação de serotonina a receptores específicos no córtex, hipocampo e tronco cerebral (Wesemann & Weiner, 1990), renovação dos níveis de GABA no córtex cerebral e cerebelo (Kanterewnicz et al., 1993), excitabilidade sináptica no hipocampo (Barnes *et al.*, 1977) e no núcleo geniculado lateral (Hanada & Kawamura, 1984), e inclusive a potenciação a longo-prazo no hipocampo (Dana & Martinez, 1984; Harris & Teyler, 1983). Portanto, não é de se surpreender que o desempenho de animais em processos de aprendizagem manifeste também um componente temporal. De fato, vários estudos tem documentado evidências deste tipo de modulação circadiana, como se discute a seguir.

D. Ritmicidade e aprendizagem

As pesquisas que buscam a relação entre os ritmos biológicos e as variações na aprendizagem são relativamente escassas e inconsistentes. A diversidade dos testes de

aprendizagem e das condições experimentais utilizadas têm aumentado as dificuldades em alcançar uma síntese adequada e definitiva. Apesar disto, tem sido possível detectar variações regulares em vários processos cognitivos, tanto em animais como em humanos.

Em humanos, variáveis psicofisiológicas (tempo de reação, teste de cancelamento de letras, autoavaliação do nível de fadiga ou alerta, etc.) manifestam ritmos circadianos bem definidos. As propriedades desses ritmos são similares àquelas de qualquer ritmo circadiano: persistência em condições constantes e sensibilidade a mudanças de fase do ciclo de atividade-repouso (Lavie, 1980; Leconte 1989; Folkard *et al.*, 1990; Guerin *et al.* 1991). Também foram observadas variações na retenção de informação em função do momento de treino. Mais ainda, a memória a curto e a longo-prazo são afetadas diferentemente pela hora de treino (Folkard *et al.*, 1977; Laive, 1980; Leconte 1989). Por exemplo, a análise do desempenho em responder a um questionário, dado imediatamente depois da leitura de um texto ou uma semana depois da leitura, revelou diferenças entre as duas condições de teste. A memória a curto-prazo era melhor quando a aquisição acontecia no período da manhã, enquanto que a memória a longo-prazo era melhor quando a aquisição tinha sido à tarde (Folkard *et al.*, 1977). Nesca e Kaulack (1994) verificaram que a memorização de uma lista de palavras melhora significativamente quando a aquisição é seguida por um período de sono, observação consistente com a noção de que a consolidação da memória acontece durante a fase do sono (Wilson & McNaughton, 1994; Karni *et al.*, 1994; Koulack, 1997). Porém, a maioria destes dados foram coletados na presença de fatores mascaradores como alimentação, sono, etc. O protocolo de rotina constante permite analizar ritmos endógenos, ao eliminar ou debilitar o efeito mascarador de fatores exógenos. Neste protocolo os indivíduos permanecem

constantemente acordados por 36 a 60 horas em condições comportamentais e ambientais constantes. Johnson *et al.* (1992) e Dijk *et al.* (1992) conseguiram isolar o componente circadiano das variáveis cognitivas estudadas usando este tipo de protocolo. Desta forma eles observaram que memória a curto-prazo (lembrar um texto), o desempenho cognitivo (cálculos completados) e o estado de alerta autoavaliado, manifestaram ritmos circadianos proeminentes.

Em animais experimentais, os estudos tem tido implicações mais amplas. Em ratos, foi observada uma facilitação da aquisição de uma tarefa de esquiva passiva durante a fase inativa e da esquiva ativa durante a fase de atividade (Davies *et al.* 1973). Um fenômeno que foi bastante estudado na década do 70 foi o chamado de "deficiência múltipla de retenção" no condicionamento de esquiva ativa (Holloway & Wansley, 1973a) e de esquiva passiva (Holloway & Wansley, 1973a and b; Wansley & Holloway, 1976), assim como da aprendizagem de tipo apetitivo (Wansley & Holloway 1975; Hunsicker & Mellgren, 1977). A deficiência múltipla de retenção refere-se a uma inabilidade do animal em recuperar a memória de experiências a determinados intervalos treino/teste. Observou-se que intervalos de 12 horas ou múltiplos de 12 horas, entre o treino e o teste, correlacionam-se com melhor desempenho no teste. Como já foi mencionado, a ritmicidade circadiana de numerosas variáveis fisiológicas/comportamentais é controlada pelos NSQ. Lesões dos núcleos supraquiasmáticos eliminam tanto a ritmicidade destas variáveis orgânicas (Turek, 1995) como a deficiência de retenção múltipla na aprendizagem de esquiva passiva (Stephan & Kovacevik, 1978). Mais interessante ainda, foi verificar que, após a lesão dos NSQ, o desempenho de esquiva passiva permaneceu alto em qualquer intervalo treino/teste, ou seja, a

lesão dos núcleos supraquiasmáticos melhorou o desempenho nos intervalos treino/teste que não eram múltiplos de 12 horas. A investigação da deficiência de retenção múltipla não é destacada na literatura atual e, talvez por isso não se tenha uma conclusão definitiva sobre sua origem. Porém podemos dizer que estes dados corroboram a existência de uma estreita relação entre os ritmos fisiológicos/comportamentais com os processos de aprendizagem.

O desempenho de camundongos num labirinto no qual o reforçamento positivo era o retorno à própria gaiola-viveiro, também mostrou um claro efeito da hora do dia. Durante os nove dias de treinamento sucessivo, a aprendizagem melhorou somente durante a fase escura/ativa (Hoffman & Balschun 1992). Em *Octopus vulgaris* a discriminação visual (Bradley & Young, 1975) e no peixe *Serranus scriba*, a aprendizagem alimentar e de esquiva (Kovacevic 1991, Rakic *et al.* 1991) mostraram maior eficiência durante os horários vespertinos, em comparação com os horários matutinos. A aversão à sacarose, induzida por cloreto de lítio (Infurna *et al.*, 1979), e o condicionamento olfatório aversivo (Infurna 1981) flutuam segundo a fase do ciclo claro-escuro. Finalmente, os processos de aprendizagem de tipo não-associativo também são suscetíveis à hora do dia. A habituação de caranguejos a estímulos potencialmente perigosos (Pereyra *et al.*, 1996), assim como a de pombos (Valentinuzzi & Ferrari, 1997) e hamsters (Valentinuzzi, V.S., dados não publicados) a estímulos auditivos, sofre modulação temporal.

Estudos que têm demonstrado uma inequívoca interação do sistema circadiano e os processos de aprendizagem são os de sincronização de ritmos circadianos à disponibilidade alimentar e aqueles referentes à aprendizagem do tipo temporal/espacial (também relacionada com alimento). O papel do alimento como um agente sincronizador é descrita em uma

extensa literatura. Roedores são capazes de discriminar a hora do dia em que vão receber alimento. Se um rato for alimentado uma vez por dia no meio da fase clara, será observado, além da atividade noturna típica nestes animais, um novo surto de atividade que aparece durante a fase clara. Este surto de atividade acontece todos os dias, tipicamente entre 1-3 horas antes do horário de alimentação. Este é um exemplo de um ritmo diário aprendido (Mistlberger, 1990). Por outro lado, a aprendizagem do tipo temporal/espacial foi observada primeiro em abelhas (Saunders, 1982) e depois em aves (Saksida & Wilkie, 1994). Abelhas e pombos expostos a diversos comedouros em diferentes localizações espaciais, aprendem, com uma precisão surpreendente a que hora cada um desses comedouros oferece alimento. Estes dois processos, sincronização alimentar e aprendizagem temporal/espacial, parecem ser essencialmente o mesmo fenômeno. Ambos os processos revelam a capacidade de formar uma memória temporal. Para isto acontecer, as estruturas neurais que atuam na organização do processo de aprendizagem devem interagir com um relógio biológico circadiano que sinalize o tempo transcorrido. A relação destes tipos de aprendizagens com o sistema circadiano é corroborada pela independência deles das condições ambientais. Por exemplo, mesmo com o deslocamento do ciclo ambiental externo ou com a manutenção dos organismos em condições ambientais constantes, tem sido demonstrada a persistência destes fenômenos, corroborando sua característica endógena.

Finalmente, o sistema circadiano pode ser modulado por processos de aprendizagem. Como mencionado anteriormente, o ciclo claro-escuro é a pista temporal dominante no processo de sincronização de ritmos endógenos ao meio externo. A luz que incide diariamente nas retinas de mamíferos alcança os NSQ por meio do trato retinohipotalámico e,

assim, desencadeia uma série de processos celulares. Estes processos vão reajustar diariamente o relógio biológico, permitindo a sua sincronização ao ciclo externo. Foi observado que pistas não-fóticas pareadas adequadamente com as pistas fóticas podem, por meio de aprendizagem associativa, imitar os efeitos de reajuste que se acreditava serem exclusivos da luz. Amir & Stewart (1996) demonstraram, em ratos, que uma corrente de ar previamente pareada com um pulso de luz num condicionamento pavloviano, era capaz de desencadear os processos celulares, fisiológicos e comportamentais típicos de reajuste dependente da fase produzido pela luz. Ou seja, a transcrição do fator Fos nos NSQ e mudanças de fase dos ritmos de temperatura e atividade locomotora. Nós tentamos replicar este experimento em camundongos analisando só a resposta comportamental, porém não conseguimos observar efeito de condicionamento entre uma corrente de ar e o pulso de luz. Diferenças metodológicas referentes a espécie (camundongos em vez de ratos), alojamento (conjunto em vez de individual) e manipulação experimental podem ser responsáveis pela diferença dos resultados (Valentinuzzi, V.S., dados não publicados). Adicionalmente, Amir e Stewart (1998) mostraram que este tipo de condicionamento não só outorga a estímulos não-fóticos a habilidade de ativar mecanismos que medeiam a expressão fótica nos NSQ, mas também reduz a eficiência da própria luz em ativar estes mecanismos, reafirmando que processos de aprendizagem podem efetivamente modular o funcionamento do sistema circadiano.

Na mesma linha, Golombek *et al.* (1994) mostraram que a glândula pineal ou as vias que a inervam, podiam "aprender" a responder, ou seja, a liberar melatonina quando um estímulo não-específico (disponibilidade de água), que tinha sido pareado com o estímulo

ambiental sincronizador normal (i.e., início da fase escura no momento da transição claro-escuro do ciclo diário) era apresentado sozinho. Os resultados destes experimentos de Amir & Stewart e de Golombek *et al.* sugerem que estruturas que fazem parte do sistema circadiano e/ou as vias aferentes possuem a capacidade de convergir e associar estímulos, alterando, consequentemente, a resposta do sistema a estímulos externos. Este tipo de plasticidade caracteriza processos de aprendizagem.

Em resumo, baseado no que foi dito até agora, aparentemente existe uma importante relação entre o sistema circadiano e os processos de aprendizagem. O sistema circadiano é capaz de modular temporalmente estruturas cerebrais responsáveis por processos de aprendizagem, e, adicionalmente, as estruturas que fazem parte do sistema circadiano possuem a plasticidade necessária para "aprender" novas respostas. Porém, o estudo interativo de ritmos biológicos e processos de aprendizagem está apenas no seu começo. Uma série de fatores devem ser considerados e controlados para alcançar conclusões definitivas. Nas seguintes seções tentaremos abordar alguns destes pontos.

E. Avaliação de ritmos circadianos: importância em estudos da organização temporal da aprendizagem

Para estudos do sistema circadiano é necessário o registro contínuo de algum ritmo circadiano do organismo. A fase da oscilação desse ritmo circadiano com relação ao sincronizador ambiental (i.e., ciclo claro-escuro), e o período em condições de livre-curso são duas propriedades dos ritmos circadianos que refletem com elevada precisão o estado do relógio biológico (Turek, 1995). Em roedores, o ritmo de atividade locomotora é a oscilação

mais freqüentemente utilizada tanto pela facilidade de medida, quanto pela regularidade de cada ciclo de atividade-repouso. A precisão com que o ritmo de atividade locomotora reflete o funcionamento do relógio é corroborada pela estreita correlação entre este ritmo e parâmetros fisiológicos/bioquímicos dos NSQ (Kornhauser *et al.*, 1990). Em outras palavras, por meio da análise do ritmo de atividade locomotora é possível inferir características do funcionamento do relógio biológico. Assim, esta medida comportamental é muito utilizada para determinar a hora interna própria do animal, a chamada hora circadiana (fase de um ritmo circadiano em situação de livre-curso).

Para a análise temporal da aprendizagem é essencial o conhecimento da fase circadiana do relógio biológico, seja em condições de iluminação constante ou em condições de claro-escuro. Em condições constantes, na ausência de pistas temporais externas, a única referência temporal é a hora circadiana do animal. Além disto, devido à ausência do potente sincronizador claro-escuro, sempre existe a possibilidade de que as condições do teste de aprendizagem determinem atrasos ou adiantamentos de fase, o que implicaria que os testes seguintes aconteceriam em fases diferentes às desejadas. Todo evento imprevisível determina respostas de ativação ou alerta do animal e, consequentemente, um aumento no nível de atividade. Está bem determinado que estas mudanças no estado comportamental têm um efeito modulatório no relógio biológico, determinando alterações de fase e/ou período, segundo o momento do ciclo em que esse evento acontece (Mrosovsky 1996).

Em condições de claro-escuro também é necessário o registro de atividade locomotora. Embora seja esperado que os ritmos endógenos estejam sincronizados ao ciclo

claro-escuro, ou seja, que a hora circadiana seja igual à hora do *zeitgeber*, é preciso uma confirmação desta sincronização.

F. Dificuldades no estudo de ritmicidade em processos de aprendizagem e algumas sugestões para contorná-las

Baseados na literatura discutida acima, podemos dizer que o sistema de temporização circadiana, de alguma forma afeta processos de aprendizagem e memória. Porém, os estudos existentes mostram resultados muitas vezes conflitivos quanto à caracterização da relação ritmos circadianos/aprendizagem. Isto pode ser explicado pelo fato de que numerosas variáveis, externas e internas, podem interferir em processos de aprendizagem o que dificulta o controle das condições experimentais. Tentaremos aqui espicificar algumas destas variáveis:

1. O ciclo claro-escuro ao qual os animais experimentais são normalmente submetidos, pode interferir nos resultados obtidos em estudos da análise de ritmicidade em aprendizagens. Se as pistas visuais são necessárias para a realização da tarefa, a diferente visibilidade na fase clara e na fase escura pode ter um marcado efeito no desempenho. Portanto, o controle desta variável externa (ciclo claro-escuro) é essencial neste tipo de análise. Cabe destacar que todos os trabalhos existentes, com exceção de uns poucos em humanos (Johnson *et al.*,1992; Dijk *et al.*, 1992), foram feitos em condições de claro-escuro.

Uma solução para este tipo de problema seria manter os animais em condições de iluminação constante (claro constante ou escuro constante). Porém, devido à existência de uma variabilidade intra-específica no período endógeno, cada animal entraria em livre-curso

com seu próprio período resultando, aos poucos, na dessincronização entre os animais do grupo. Simultaneamente, o horário endógeno de cada animal, aos poucos, ficaria fora de fase com o horário do experimentador. Tudo isto levaria a uma distribuição aleatória dos testes de aprendizagem ao longo das 24 horas dificultando a vida do experimentador. Adicionalmente, o uso de escuro constante ou claro constante acarreta outros problemas. Em escuro constante teríamos o problema de uma diminuída capacidade visual durante os testes de aprendizagem. Já em condições de claro constante surge o problema do efeito aversivo da luz no comportamento de animais noturnos (Marques & Waterhouse, 1994). É sabido que a luz intensa pode inibir a atividade de roedores e, assim, poderia mascarar o resultado de um teste de aprendizagem.

O fotoperíodo esqueleto oferece um procedimento adequado para lidar com estes problemas. Este tipo de esquema de iluminação representa um modelo de arrastamento discreto ou não-paramétrico onde um pulso de luz de poucos minutos, apresentado aproximadamente a cada 12 horas, é suficiente para o arrastamento dos ritmos endógenos (Pittendrigh, 1965). Para isto os pulsos periódicos devem cair numa fase da curva de resposta de fase que provoque o deslocamento de fase diário necessário para a sincronização ao fotoperíodo esqueleto. As condições de iluminação no período entre os pulsos de luz é fundamental; tipicamente, é utilizado escuro total. Porém, como mencionado previamente, no caso de testes de aprendizagem é de interesse que os animais recebam informações visuais sobre estímulos ambientais, as quais serão utilizadas na identificação/memória do contexto em que ocorreu a aprendizagem. Ao mesmo tempo, é também necessário evitar o efeito inibitório da luz intensa. Neste sentido, luz de baixa intensidade entre os pulsos de luz de

elevada intensidade, pode representar uma boa solução segundo a espécie utilizada. Por exemplo, no caso de camundongos foi demonstrado que a luz verde é um comprimento de onda que permite uma adequada percepção visual (Balkema & Pinto, 1982), e, se a intensidade é mantida baixa (~1-2lux), é possível evitar o efeito inibitório.

2. O ritmo circadiano de atividade-repouso representa outro mascarador potencial em testes de aprendizagem. Um exemplo representativo é o caso da aprendizagem de esquiva na qual o desempenho em cada fase do ciclo claro-escuro pode depender do tipo de resposta exigida. No que se refere a esquiva ativa, o desempenho é melhor durante a fase ativa de ratos, enquanto que o desempenho de esquiva passiva é melhor durante a fase inativa do animal. Isto sugere que o ritmo circadiano de atividade poderia facilitar ou dificultar o desempenho segundo o tipo de resposta exigida no teste. Este possível efeito mascarador do ritmo de atividade fica difícil de controlar, considerando-se que todos os organismos apresentam níveis incondicionados/basais dos diferentes comportamentos que caracterizam os respectivos repertórios. Todo processo de aprendizagem em animais experimentais envolve quantificação por meio do aumento ou diminuição na freqüência de ocorrência de determinados comportamentos. Se, por exemplo, na análise de condicionamento aversivo em diferentes horários for detectado que o nível de imobilização é maior durante a fase ativa e menor durante a fase inativa, poderíamos dizer, com certa segurança, que o nível basal de atividade não estaria mascarando o desempenho. Se pelo contrário, um resultado oposto é observado, ou seja, maior imobilização durante a fase inativa e menos imobilização durante a fase ativa, alguma outra manipulação seria necessária para tentar dissociar o ritmo de atividade da possível ritmicidade do comportamento aprendido. Horlington (1970) tentou fazer isto por

meio de métodos farmacológicos. A resposta de sobressalto a estímulos sonoros em ratos manifesta um ritmo circadiano. Aparentemente, o circuito neural que organiza este reflexo é modulado pelo sistema circadiano de tal forma que a intensidade de sobressalto é maior durante a fase ativa do animal e menor durante a fase inativa (Chabot & Taylor, 1992; Frankland & Ralph, 1995). Horlington (1970) administrou uma droga que aumentava o nível de atividade durante a fase inativa e outra que diminuía a atividade durante a fase ativa. Apesar destas mudanças no nível de atividade basal o ritmo de sobressalto à estimulação acústica permaneceu inalterado, sugerindo uma independência entre este e o ritmo de atividade locomotora.

Mencionamos anteriormente a necessidade da medida contínua do ritmo de atividade locomotora com o fim de confirmar a hora interna do animal. Segundo o mencionado no parágrafo anterior, uma outra finalidade desta medida contínua seria correlacionar o nível de atividade basal com o nível da resposta comportamental exigida pelo teste de aprendizagem.

3. Um outro problema a considerar que é da área de aprendizagem em geral, porém aplicável ao tipo de estudo que está sendo discutido aqui, é a ocorrência simultânea de diferentes categorias de aprendizagens. Assim, quando se procura um ritmo num determinado tipo de aprendizagem é importante tentar, ao mesmo tempo, isolar outros tipos de aprendizagens presentes e analisar também nestas um possível componente temporal. Por exemplo, em qualquer aprendizagem associativa, além do processo de condicionamento, sempre acontecerá um processo de habituação à caixa experimental. Para isolar este componente de habituação seria necessário um grupo de animais controle só exposto à caixa experimental no qual se

analise a reatividade ou exploração ao contexto durante o teste. Este componente não-associativo deve ser considerado na análise do componente associativo.

4. Devemos considerar que os processos de aprendizagem são influenciados diretamente ou indiretamente por múltiplas variáveis comportamentais, fisiológicas e bioquímicas. Muitas destas variáveis manifestam ritmos circadianos. Ao mesmo tempo, podem manifestar ritmos ultradianos e/ou infradianos superpostos aos ritmos circadianos. Isto poderia contribuir para as diferenças observadas entre os diversos estudos. A análise dos padrões ultradianos e infradianos fica difícil, porém as variações circadianas de alguns destes processos fisiológicos podem ser controlados mais facilmente. Dentre esses processos podemos destacar: **(a)** Sensibilidade a estímulos envolvidos no processo de aprendizagem: pode acontecer que o componente temporal observado num determinado tipo de aprendizagem seja, na realidade, devido a uma ritmicidade na sensibilidade aos estímulos envolvidos e não ao processo de aprendizagem propriamente dito. Neste sentido é necessário sempre fazer experimentos paralelos onde se avalie a sensibilidade aos estímulos em diferentes horários. Por exemplo a sensibilidade a estímulos auditivos pode ser analisada por meio da regulação da resposta de sobressalto acústica através de pré-pulsos (Chabot & Taylor, 1992). Ou seja, um estímulo auditivo apresentado antes do estímulo que desencadeia um sobressalto, diminuirá a resposta de sobressalto proporcionalmente à intensidade do pré-pulso. Assim, é considerado que a magnitude de diminuição do sobressalto reflete com precisão o limiar auditivo ao pré-pulso. A sensibilidade nocioceptiva a um choque em diferentes horários do dia pode ser avaliada pela freqüência e intensidade da resposta comportamental imediata ao choque (i.e., vocalização, pulo, corrida); **(b)** A motivação para executar ou deixar de executar um

determinado comportamento também pode manifestar ritmicidade. Por exemplo, se o reforçamento é o alimento, um ritmo na motivação para se alimentar, que seguramente depende de ritmos de variáveis tais como níveis hormonais e atividade de enzimas digestivas e metabólicas, pode afetar o desempenho no teste de aprendizagem. No caso de processos de aprendizagem que implicam estímulos aversivos (i.e., choque) adiciona-se um componente emocional aversivo à análise temporal. Neste casos pode-se perguntar se existiria maior tendência a sentir medo durante determinados horários ou se a capacidade de formação da memória emocional é que manifestaria variação temporal. O labirinto em cruz elevado permite avaliar o nível de ansiedade em roedores e, assim, as possíveis variações desse estado emocional em diferentes horários. Quanto maior o tempo de permanência ou maior o número de escolha/entradas nos braços fechados do labirinto, considera-se que a ansiedade é maior. Em hamsters foi detectado um ritmo circadiano neste tipo de comportamento no labirinto em cruz (Yannielli *et al.*, 1996); **(c)** O nível de atenção tem um papel crítico em processos de aprendizagem. Uma forma de avaliar e, assim, poder determinar a existência de um componente temporal no nível de atenção, é pela resposta de imobilidade comportamental à uma repentina diminuição de um barulho de fundo. Quanto maior o grau de imobilização, maior o nível de atenção (Buwalda *et al.*, 1995); **(d)** A análise de aprendizagens que envolvem o uso de substâncias exógenas ao organismo (ex., aversão a sacarose induzida por cloreto de lítio) é mais complicada devido a ritmos metabólicos que podem alterar tanto a preferência por sacarose como a sensibilidade às conseqüências da administração do LiCl (Infurna *et al.*, 1979).

5. Pode ser que ritmos biológicos afetem ou não os processos de aprendizagem segundo a fase (aquisição, memória a curto-prazo, consolidação, memória a longo-prazo) do processo que está sendo analisado. Embora, isto não seja necessariamente uma dificuldade, é importante que seja considerado no momento da interpretação dos dados. Em alguns casos, observou-se que as variações diurnas em aprendizagem acontecem só durante o processo de consolidação de uma memória. Em polvos, a discriminação visual mostra variação diurna só nos primeiros dias de treino (Bradley & Young, 1975). Em ratos, o déficit de retenção múltipla começa a desaparecer na medida em que a resposta fica treinada (Holloway & Wansley, 1973b). Estas observações coincidem com a idéia de que o processo de consolidação corresponde a uma fase em que as memórias são lábeis e susceptíveis às alterações do meio interno ou externo (Eichenbaum *et al.*, 1992). Na mesma linha de raciocínio, ritmos endógenos e/ou ambientais poderiam influenciar a expressão destas memórias ainda no processo de consolidação. No entanto, quando a memória está totalmente consolidada existiria maior estabilidade desta. Contrariamente a estes exemplos, Hoffmann & Balschum (1992) observaram em camundongos testados num labirinto, o aparecimento de uma variação diurna somente depois do quarto dia de treino. Os animais foram aprendendo treino após treino, somente durante a fase escura, enquanto que aqueles testados durante a fase clara mantiveram o nível inicial de desempenho. Outro exemplo de um efeito temporal diferencial em diferentes fases do aprendizagem é em humanos onde, como mencionado anteriormente, a memória a curto- e a longo-prazo são afetadas diferentemente pelo horário do dia (Folkard *et al.*, 1977). Estes autores observaram que a memória a curto-prazo era melhor de manhã entanto que a memória a longo-prazo era melhor à tarde.

G. Tipos de aprendizagens analisadas no presente trabalho

Primeiramente, foi analisada uma aprendizagem de tipo não-associativa (habituação a um ambiente novo), utilizando um campo aberto e monitorando o comportamento por observação direta. Após os primeiros experimentos, o método de registro comportamental foi automatizado. Nestas condições, a análise da organização temporal da aprendizagem foi estendida a processos associativos, condicionamento aversivo a um contexto e a um estímulo discreto. Simultaneamente, foi analisado o processo de habituação à caixa de condicionamento (habituação a um contexto novo, porém, um contexto de características diferentes à de um campo aberto).

. Habituação a um ambiente novo (teste de campo aberto ou *openfield*)

O teste de campo aberto permite uma avaliação do estado de ativação e do estado emocional eliciado por um ambiento novo. O nível de ativação é quantificado através de comportamentos exploratórios tais como farejar, locomoção e levantar (Cerbone & Sadile, 1994), enquanto que o estado emocional (medo, ansiedade) é normalmente quantificado pelo nível dos comportamentos de imobilização, fuga ativa e defecação (Gentsch *et al.*, 1981). A primeira exposição a um campo aberto vai desencadear um determinado nível de um ou de vários destes comportamentos; a exposição contínua ou repetida a esse contexto vai tornando o mesmo familiar, resultando numa diminuição gradual da(s) resposta(s). Este processo de habituação é uma forma básica de aprendizagem que permite que o animal ignore estímulos sem nenhum valor biológico (Thompson & Spencer, 1966; Cerbone & Sadile, 1994).

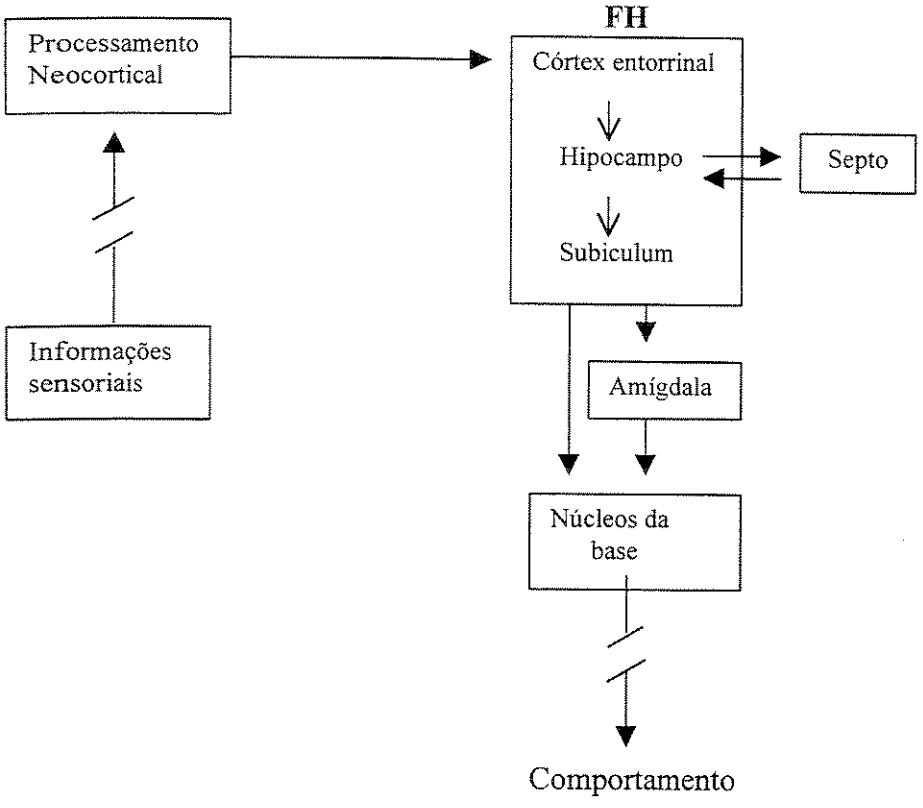

Figura 3. Algumas das estruturas e vias que participam na reação e habituação a um contexto novo. FH: formação hipocampal.

A formação hipocampal tem uma participação importante no padrão de comportamento de um animal num contexto novo. O'Keefe e Nadel (Em: Crusio *et al.*, 1989) sugeriram que o hipocampo forma uma representação interna das propriedades do meio ambiente do animal. Ou seja, estabelece relações entre diferentes estímulos (tanto espaciais como não espaciais) apresentados simultaneamente (formação de um mapa cognitivo do contexto). Portanto, quando o animal é apresentado a um ambiente novo, o hipocampo atuaria

como um comparador, detectando a novidade contextual (estabelece relações entre conjunto de estímulos apresentados sequencialmente, *temporal tagging*), e iniciando, por meio de conexões com sistemas motores, comportamentos exploratórios, permitindo assim, a coleta de informações sobre o meio (Eichenbaum *et al.*, 1992; Gray, 1995). O papel do hipocampo na regulação do comportamento exploratório é reforçado pelo fato de que foram detectadas correlações entre variações estruturais do hipocampo e respostas comportamentais num campo aberto ao comparar-se diferentes linhagens de camundongos (Lipp *et al.*, 1987; Crusio *et al.*, 1989; Roullet & Lassalle, 1990; Cerbone & Sadile, 1994). A amígdala e a área septal também têm um papel importante na geração do componente emocional da reação ao contexto assim como no processo de habituação desta resposta (Da Cunha *et al.*, 1992).

Condicionamento clássico aversivo

No condicionamento clássico aversivo, um estímulo neutro, tal como um som ou o próprio contexto experimental, é pareado com um estímulo aversivo incondicionado, tal como um choque elétrico. O som ou o contexto, em virtude da sua relação (de contiguidade e de contingência) com o estímulo incondicionado, adquire propriedades aversivas condicionadas (estímulo condicionado) e passa a controlar respostas normalmente eliciadas pelo estímulo aversivo (Kim & Fanselow, 1992; Phillips & LeDoux, 1992; Phillips & LeDoux, 1994). Durante o processo de extinção da resposta adquirida, ou seja, durante apresentação repetida apenas do estímulo condicionado, o animal aprende que esse estímulo já não sinaliza o estímulo aversivo e, portanto diminui a freqüência/magnitude da resposta

condicionada. Este processo de extinção é, aparentemente, controlado por substratos neurais diferentes daqueles que controlam a aquisição da resposta condicionada (LeDoux, 1994).

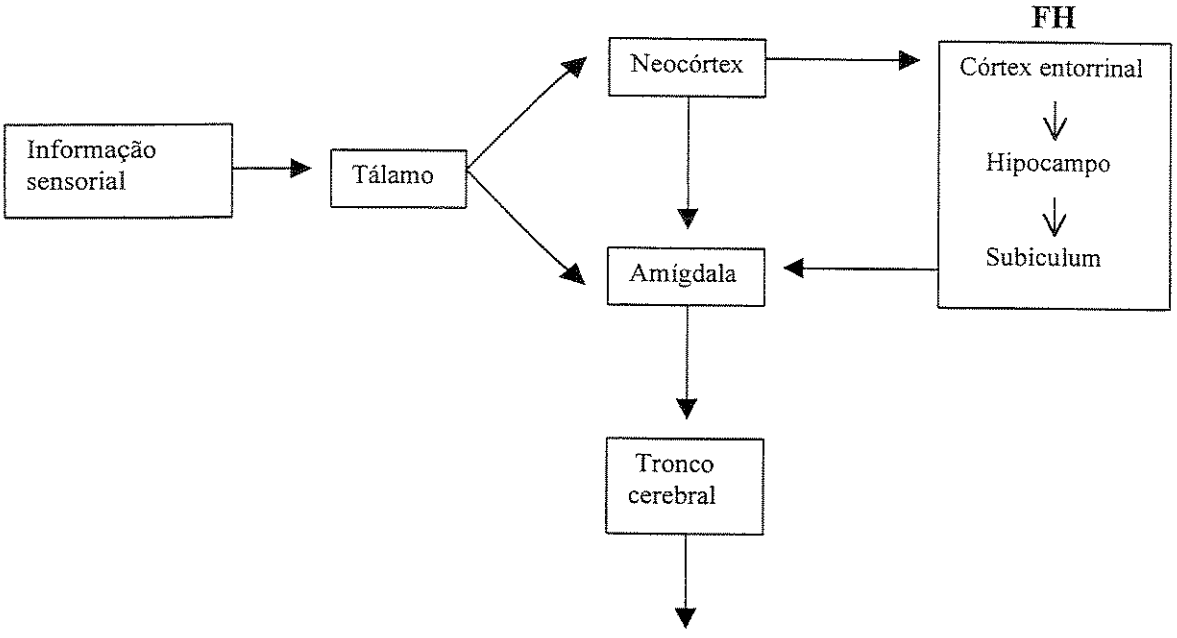

Figura 4. Algumas das estruturas e vias que participam no condicionamento aversivo. FH: formação hipocampal.

Em processos de condicionamento aversivo, os estímulos condicionados podem ser discretos e simples (som) ou complexos e multisensoriais (contexto). Embora o tipo de medida comportamental seja idêntica, as bases neurais destes dois tipos de processos são diferentes. A amígdala está envolvida na formação das associações entre estímulos não-condicionados aversivos e uma variedade de estímulos condicionados, como também no

subsequente controle da resposta aversiva (Maren & Fanselow, 1996). Porém, diferentes vias neurais medeiam a análise das propriedades dos estímulos condicionados segundo as características destes. Vias talâmicas e/ou corticais transmitem a informação sobre estímulos discretos para a amígdala (Kim & Fanselow, 1992; Paylor *et al.*, 1994; Phillips & LeDoux, 1992; Phillips & LeDoux, 1994). Já estímulos mais complexos, como as pistas espaciais de um contexto, requerem o hipocampo e as projeções do *subiculum* até a amígdala. Como mencionado anteriormente, o processamento neural no hippocampo é fundamental para o estabelecimento de relações entre estímulos apresentados simultaneamente (representação configuracional) e estímulos apresentados seqüencialmente (função de comparador) (Eichenbaum *et al.*, 1992; Gray, 1995). Ambos os processos são subjacentes ao processo de condicionamento a um contexto.

O condicionamento clássico aversivo é um processo de aprendizagem que permite o estudo da relação entre memórias e emoções (memória emocional), em particular de um determinado tipo de emoção, o medo. A resposta de medo inclui numerosos componentes vegetativos (i.e., aumento da pressão arterial, freqüência cardíaca, liberação de hormônios adrenais) e comportamentais (i.e., imobilização, defecação, piloereção).

Automatização do registro comportamental. O comportamento de imobilização é a resposta de medo mais comumente registrada, sendo utilizada como um índice da formação de memória emocional. O comportamento de imobilização é geralmente quantificado por observação direta, registrando se o animal está imóvel ou ativo a cada intervalo de tempo, normalmente 5 a 10 segundos (Phillips & Le Doux, 1992). Este método de observação direta é árduo e lento. A análise do condicionamento aversivo sob um ponto de vista temporal

requer o teste de vários animais, muitas vezes em condições desfavoráveis para o registro visual (baixa intensidade de luz, em horários de reduzido estado de alerta do observador). Neste sentido a possibilidade de automatização apresenta-se como altamente vantajosa.

Considera-se que a resposta de imobilização pode ser quantificada indiretamente como a medida oposta à atividade do animal. Se o nível de atividade é registrado por meio da interrupção de sensores infravermelhos, a imobilização poderia ser quantificada como a latência entre a interrupção desses sensores infravermelhos. Embora seja claro que esta medida traz implícito o comportamento de imobilização, o nível geral de atividade (ou melhor, de inatividade) também estará implícito. Isto exige a necessidade de análise simultânea de animais controles, expostos só ao contexto, para poder distinguir o nível incondicionado de atividade do nível de atividade condicionado.

Uma vez controlado este nível incondicionado, ainda podemos dizer que a latência entre a ativação dos sensores não explicita as características do comportamento dadas por movimentos lentos e cautelosos que identificam um comportamento defensivo, definido por Blanchard e Blanchard (1988), como exploração cautelosa. Com base em observações destes autores, considera-se que há uma relação entre o nível de atividade e diferentes graus de medo. Estudando comportamentos defensivos gerados por predadores em ratos, estes autores introduziram o conceito de níveis de estados defensivos: exploração cautelosa (perigo incerto), imobilização/fuga (perigo identificado porém longe da distância crítica) e luta/fuga (quando o perigo está muito perto ou em contato com o animal).

Os primeiros dois níveis são claramente observados em condições de condicionamento aversivo. Quando o animal é colocado pela primeira vez na caixa de

condicionamento (ambiente novo) é observado um elevado nível de atividade que poderia ser interpretado como ausência de medo. O primeiro nível de comportamento defensivo, exploração cautelosa, acontece quando o perigo é incerto, por exemplo quando a fonte de perigo, embora não esteja presente nesse momento, já esteve presente anteriormente no mesmo contexto. Nestas condições são observados movimentos lentos e cautelosos, ou seja, uma diminuição do nível geral de atividade. Quando a fonte de perigo é identificada, o animal tentará fugir imediatamente ou, se a fuga for impossível, acontecerá uma inibição geral da atividade, ou seja imobilização (Graef, 1994). Como nesse caso a fuga é impossibilitada, existe uma relação mais simples e direta entre nível de atividade e nível de apreensão, em outras palavras quanto mais inativo maior o medo. Isto eliminaria possíveis respostas "falso negativas" em que medo é induzido, porém não é registrado devido ao nível de atividade provocado pelo comportamento de fuga. Assim, acreditamos que o nível de atividade quantificado pela interrupção de sensores infravermelhos pode refletir com precisão adequada o estado emocional numa situação de condicionamento aversivo.

OBJETIVO GERAL

O conjunto de experimentos realizados foram orientados pelo interesse em examinar a existência de uma modulação circadiana em diferentes tipos de aprendizagens em camundongos, tentando isolar/controlar possíveis mascaradores dos supostos ritmos mnemónicos .

OBJETIVOS ESPECÍFICOS

1. Identificar um efeito temporal num processo de aprendizagem não-associativa, habituação a um campo aberto, tentando confirmar a independência da flutuação deste processo do ciclo externo claro-escuro e do ritmo endógeno de atividade locomotora.

2. Padronizar uma medida automatizada da resposta de imobilização (*freezing*) em situações de condicionamento aversivo, com o interesse de facilitar e aumentar a eficiência e objetividade deste tipo de teste em estudos circadianos.

3. Identificar um efeito temporal em processos de aprendizagem associativa, especificada pelo condicionamento clássico aversivo utilizando métodos de registro automatizados, procurando confirmar a independência entre este processo e potenciais mascaradores externos (ciclo claro-escuro) e do próprio organismo (ritmo de atividade locomotora, variação temporal da habituação a caixa de condicionamento, sensibilidade ao choque).

LOCOMOTOR RESPONSE TO AN OPENFIELD DURING C57BL/6J ACTIVE AND INACTIVE PHASES: DIFFERENCES DEPENDENT ON CONDITIONS OF ILLUMINATION

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Running head: Diurnal variation of openfield behavior

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Abstract. C57BL/6J mice maintained in a 12:12 light:dark cycle were tested in an openfield at six different times of day. A diurnal rhythm of ambulation in the openfield was observed with greater levels of activity exhibited by those groups tested at night. Long-term and short-term habituation of the response, as well as defecation scores, were also affected by phase of the light/dark cycle. A second experiment was performed where other groups of animals were entrained to a skeleton photoperiod (two15-minute bright light pulses separated by 12 h of green dim light). These groups were tested during subjective night or subjective day either 2 or 14 h after the onset of the active period, as measured by wheel-running behavior. No effect of circadian phase on ambulation or habituation of this response to the openfield was observed in these animals. The results show that spatial novelty is equally arousing regardless of circadian phase and that the conditions of illumination can dramatically alter the response to a novel environment.

Key words: Habituation; Circadian rhythm; Diurnal variation; Locomotor activity; Learning; spatial novelty.

Introduction

Behavioral arousal or activation as well as the emotionality elicited by a novel environment is frequently analyzed by the openfield procedure. Behavioral arousal can be quantified by exploratory behaviors such as sniffing, walking and rearing (Cerbone and Sadile, 1994), while emotionality (fear, anxiety, etc) is usually quantified by measures of freezing behavior, active escape or defecation (Gentsch *et al.*, 1981).

The magnitude and frequency of any particular behavior elicited by a novel environment may be a function of the interaction of diverse factors. For example, stimulation of removal from a familiar environment and the transfer procedures prior to exposure can influence the response to a novel context. Many aspects of the novelty such as isolation, low levels of pheromones and ultrasounds, differences in levels of illumination or temperature gradient and the physical characteristics of the environment may each have a different impact on openfield behavior (Walsh and Cummins, 1976). In addition, any prior experience in the test situation also has a direct effect on the behavioral response; continuous or repeated exposure to initially novel stimuli results in the gradual alleviation of these responses. This process of habituation to novelty is a basic form of learning that allows animals to ignore stimuli that are of little or no biological significance (Thompson and Spencer, 1966; Cerbone and Sadile, 1994). Among other independent variables that may control behavior in an openfield are the direct effect of the light-dark cycle and the circadian phase of the endogenous rest-activity rhythm at which testing occurs.

Few studies have analyzed time of day effects on activity in an openfield in animals submitted to a light-dark cycle. Hostetter (1966) found increased activity during the dark phase of DBA/1J mice compared to the light phase but no phase effect in C57BL/6 mice. Connolly and Lynch (1981) observed a diurnal rhythm in some strains of mice but not in others. However these authors tested the same animals at different times therefore, long-term habituation may have posed a confounding factor in the interpretation of the results. In rats, Gentsh *et al.* (1982b) and

Vassaut *et al.* (1982) found a clear diurnal variation in locomotion in an openfield while Theander *et al.* (1997) found no effect. On the other hand, Kräuchi *et al.* (1983) observed the same level of reactivity during light and dark phases, for the first ten minutes that rats were exposed to a novel environment. These authors also observed that the rate of habituation in a 2h session was slower in the dark than in the light phase. Other openfield studies neglect to specify time of testing.

These conflicting results are likely due to the very different conditions and methods used. To our knowledge, the potential confounding effect of circadian phase of endogenous activity on the assessment of openfield behavior has not been considered at all. The present study was designed to assess the role of the circadian clock on the diurnal rhythm of openfield behavior. Specifically, we sought to 1) Support or refute the existence of a diurnal rhythm in locomotor reactivity to novelty and/or in the habituation of this behavior, and 2) Separate the influence of internal circadian drive from the strong external stimuli presented by the light-dark cycle.

To eliminate the direct effect of the differences in illumination, we worked with animals in a skeleton photoperiod (Pittendrigh, 1965) where a single 15-min pulse of white light presented every 12h was capable of synchronizing the rest-activity cycle. Thus, mice entrained to the skeleton photoperiod were tested under the same conditions of illumination but at different phases of the circadian cycle.

Methods

Subjects. Male C57BL/6J mice purchased from Jackson Laboratories (Bar Harbor, Maine) were housed in the animal facility under a 12h light:12h dark cycle (LD 12:12; lights on at 06:00 central standard time).The temperature was maintained constant at 23 ± 2 °C. Food (Teklad) and water were available *ad libitum.*

Apparatus. Animals were tested in a water-proof painted wooden open-field box (66x56cm) with its floor divided into nine equal squares, bordered by walls 30cm high. In Experiment I the

openfield was illuminated by normal fluorescent room light (about 150 lux) during the day and by three infrared lights during the night (complete darkness to the naked eye). During the night animal handling was aided with an infrared viewer (Find-R-Scope, FJW Optical Industries). In Experiment II the field was illuminated by dim green light (0.5-1.0 lux at the floor level) during both testing times. The dim green light was provided by three night-lights (Limelite, Austin Instruments, TX) evenly distributed on the openfield walls. Green light was chosen to assure that the mice could assess the visual cues in the chamber (Balkema and Pinto, 1982). The sessions were filmed using an 8mm video camera (Sony) during fluorescent lighting and an infrared camera (RCA) during infrared and green light sessions. The camera was suspended in a vertical position over the center of the openfield.

Openfield procedure. Starting three days before the beginning of the experiment, animals were handled for 2-3 minutes every day at random times to reduce the arousal associated with handling. Each session began by placing the mouse in the same corner of the openfield box. The animal was left undisturbed for 30min. At the end of the session, the animals were returned to their home cage, all fecal boli were counted, and the floor of the openfield was cleaned with a 1 % acetic acid solution. Twenty-four hours later each animal was submitted to an identical 30-minute session in the openfield.

Locomotor activity in the openfield was quantified as the number of crossings from one square to another during each 3-minute bin. A crossing was scored if at least the animal's ears had crossed the plane separating one of the nine regions in the openfield. Three independent observers later scored the videos (Experiment I). The intra- and inter-observer reliability was calculated. Each observer scored six different sessions three times, each on a separate day. There was no significant difference in the number of crossings detected either within or between observers (ANOVA, $p>0.05$). Scoring in Experiment II was performed by only one of those three observers.

Experiment I

The purpose of this experiment was to analyze the effect of time-of-day on openfield behavior in animals submitted to a LD cycle. Mice were purchased at 2-3 months of age and were group housed three per cage in the conditions described above. Subjects were randomly assigned to one of six groups, each tested at a different time of day. Three groups were tested during the light phase corresponding to the inactive phase of mice: 08:00 (n=5), 12:00 (n=5) and 16:00 (n=5). The other three groups were tested during the dark phase which corresponds to the active phase of mice: 20:00 (n=7), 24:00 (n=6) and 04:00h (n=7). Twenty-four hours before the initiation of the sessions, the animals were transported from the animal facility to an experimental room maintained on the same LD cycle. Openfield testing was carried out as described above.

Experiment II

The purpose of this experiment was to analyze the effect of the circadian rhythm of locomotor activity on openfield behavior. For this, any direct effects of the LD cycle were eliminated. Mice were purchased at 2 months of age. Upon arrival, the animals were group housed (5 per cage) in the animal facility for a week and held under the conditions described above. The animals were transported to the experimental room where they were placed in individual acrylic cages (15x32cm) equipped with running- wheels (11cm in diameter). Cages were placed in light-proof, ventilated wooden chambers (44cm high x180cm long x 53cm deep) with an LD cycle identical to that of the animal facility (LD 12h:12h, light provided by 40W fluorescent bulb; 200 lux). After three days, the 12h dark phase was replaced by 12h of dim green light (1 lux at the level of the cage lids); this condition continued for two cycles. Immediately thereafter, a skeleton photoperiod of two 15-min bright 40W fluorescent light pulses separated by 11.5 and 12h of green dim light was established (i.e., 0.25 h white: 11.5 h green: 0.25 h white: 12 h green) and continued for the duration of the experiment (Pittendrigh,

1965; see Figure 1). The dim green light was provided by 6 night-lights distributed along the rear wall of each lightproof chamber. As every periodic environmental cycle capable of entraining endogenous rhythms, the skeleton photoperiod described here is referred to as a *zeitgeber* (time-giver; Aschoff, 1960). In accordance with the conventions in the chronobiology literature, the evening light pulse (signalling dusk) was considered *zeitgeber* time 12 (ZT12) and the morning light pulse (signalling dawn), *zeitgeber* time 24 (ZT24). If synchronization to the skeleton photoperiod is adequate, activity onset should occur close to ZT12. This ZT was used as reference for the time of testing in each group. This skeleton photoperiod procedure allowed testing of the openfield response under identical conditions at different circadian times, to reveal the endogenous component of openfield responsivity separately from the exogenous component related to the light or dark conditions during the test.

Twenty-four mice were randomly divided in two groups (n=12 per group). The day group was tested 2-3 h after the beginning of the morning light pulse, at *zeitgeber* time 02 (ZT 02) or 14-15h after activity onset. The night group was trained 2-3 hours after the end of the evening light pulse (ZT 14) or 2-3h after activity onset. Openfield testing was carried out as described above.

Activity recording. Throughout the experiment wheel-running activity was continuously recorded with the Chronobiology Kit (Stanford Software Systems, Stanford, CA). Each turn of the wheel activated a microswitch that was registered as one pulse of activity. The resulting activity data was displayed as actograms, which were used to confirm entrainment to the skeleton photoperiod. Only those animals exhibiting a circadian period of 24h, indicating entrainment to the skeleton photoperiod were allowed to proceed in the experiment. Data from animals whose activity onset varied 20 minutes or more on three successive days were excluded. The data from one mouse in the subjective night group and two mice in the subjective day group were excluded by these criteria.

Statistical analysis. Data were analyzed as the number of crossings in 3-min bins of each session. Within-session decrements in activity, defined here as short-term habituation, are expressed as the mean number of crossings for each 3-minute bin. Inter-session decrements in activity, defined here as long-term habituation, are expressed as mean number of crossing per session. For every group of mice the level of crossings reached by 12 minutes into the session remained the same for the rest of the 30-minute session, therefore, the last 18 minutes were excluded from the analysis. A three-way ANOVA with one grouping variable (time of day) and two repeated measures (session 1 and 2, bins 1 through 4) was performed on each experiment as a whole. A two-way ANOVA with one grouping variable (time of day) and one repeated measure (sessions 1 and 2) was performed on the defecation score data. A post-hoc Scheffe's test was used for multiple comparisons.

RESULTS

Experiment I

During exposure to a full light-dark cycle, a diurnal rhythm of activity in the openfield was detected. No differences between the three groups tested during the light phase (08:00, 12:00 and 16:00h) were observed. Also, no differences between the three groups tested during the dark phase (20:00, 24:00 and 04:00h) were detected. Therefore, the data from all mice tested during the light phase or dark phase were averaged.

The activity level in response to an openfield was analyzed as mean crossings for each 3-min bin within the first 12 minutes of each session (Figure 2) and as the mean number of crossings for the first 12 minutes of each session (Figure 3). A diurnal rhythm in the response to an openfield was observed. The frequency of crossings in the openfield was lower during the light phase compared to the dark phase. The significance of phase on the response was confirmed by ANOVA ($F(1,33)=24.60$; $p< 0.001$).

We also analyzed the pattern of change of activity in the openfield within a given session. Phase of the light-dark cycle significantly affected short-term habituation. A significant

(Scheffe's test, $p<0.05$) decrease from one bin to another was observed only during the dark phase, both during the first and second sessions. During the light phase no within-session decrease in crossings was detected. Accordingly, the ANOVA revealed a significant main effect of bin ($F(3,99)=14.41$; $p< 0.001$) and a significant time-of-day by bin interaction ($F(3,99)=13.25$; $p< 0.001$). Thus, acquisition of short-term habituation depended on the time of day.

The pattern of change in frequency of crossings from one session to another can be better visualized in Figure 3. Phase of the light-dark cycle significantly affected long-term habituation. A significant (Scheffe's test, $p<0.05$) decrease in reactivity from one session to another was observed only during the light phase. During the dark phase the levels of crossings in the first and second sessions did not differ significantly. The ANOVA revealed a significant main effect of session ($F(1,33)=19.40$; $p< 0.001$) and a significant time by session interaction ($F(1,33)=4.94$; $p<0.05$). Thus, acquisition of long-term habituation depended on the time of day.

The defecation scores (left side of Figure 7) showed higher values during the light phase relative to the dark phase in both session 1 and session 2. The analysis of variance revealed a significant time of day effect ($F(1,30)=4.48$; $p<0.05$), but no session ($F(1,30)=2.58$; $p=0.1$) or time of day by session interaction ($F(1,30)=0.08$; $p=0.8$). Thus, a simple diurnal rhythm in defecation was detected.

Experiment II

Stable entrainment to the skeleton photoperiod was confirmed by the activity records. Figures 4A and B show two representative actograms of the wheel-running activity of two well entrained animals, one mouse tested in the openfield at ZT02 and another tested at ZT14. During the first six days a clear activity rhythm synchronized to the LD cycle was observed. Wheel-running would start every day at almost precisely the same time (at lights off) revealing perfect entrainment to the external LD cycle. The typical pattern of wheel-running behavior in mice is seen in figure 4 where one big bout of activity occurs at the beginning of the dark phase followed

by one or more short bouts in the early morning. For the rest of the light phase, more or less between 12:00 and 18:00, animals do not turn the wheel more than once or twice despite constant access to it. At the start of the skeleton photoperiod (second arrow), a phase delay of about an hour is observed in Figure 4.A. This most likely results from this individual's particular response to discrete pulses of light as assessed by its Phase Response Curve (Daan and Pittendrigh, 1976). Despite this initial phase delay in activity onset, the activity rhythm remains entrained (activity onset starts every day almost precisely at the same time yielding a period of 24h). The individual depicted in Figure 4.B entrains at nearly the exact same phase whether in a full LD cycle or in the skeleton photoperiod. Individual variation in the exact phase of entrainment is expected, the important point is that each mouse's wheel-running begins at a characteristic time day after day.

The activity level in response to an openfield was analyzed as mean crossings for each 3-min bin within the first 12 minutes of each session (Figure 5) and as the mean number of crossings for the first 12 minutes of each session (Figure 6). No diurnal rhythm in the response to an openfield was observed under these conditions. Despite actograms demonstrating robust diurnal rhythms in wheel-running activity under these experimental conditions, the frequency of crossings in the openfield was similar during both active and inactive phases of the rest-activity cycle. Accordingly, analysis of variance revealed no significant effect of *zeitgeber* time ($F(1,19)=3.17$; $p=0.09$).

In Figure 5 the pattern of change of ambulation in the openfield within a given session can be analyzed. Within-session decreases in crossings were observed, however, the phase at which testing occurred had no affect on short-term habituation. Contrary to what was observed in Experiment I, the animals tested during their inactive phase show short-term habituation to the openfield. Habituation also occurred during the active phase. Thus, ANOVA revealed a significant main effect of bin ($F(3,57)=17.63$; $p<0.001$) however, no phase by bin interaction ($F(3,57)=0.66$; $p=0.58$).

The pattern of change in frequency of crossings from one session to another are better visualized in Figure 6. A decrease in the number of crossings from one session to another was

observed at both phases, thus, we found no evidence of an effect of circadian phase on long-term habituation in this experiment. Contrary to the results from Experiment I, the animals tested during their active phase do show long-term habituation to the openfield. The ANOVA revealed a significant main effect of session ($F(1,19)=30.08$; $p<0.001$), but no phase by session interaction ($F(1,19)=3.07$; $p=0.096$).

It is interesting to compare visually the absolute number of crossings in the openfield between Experiment I and Experiment II. Compared to Experiment I, mice used in Experiment II appear to be less reactive especially during the active phase. This change in reactivity to the openfield illuminated with dim green light account for the loss of diurnal rhythmicity in Experiment II and to the difference in acquisition of short- and long-term habituation between the two experiments.

The defecation scores in Experiment II (right side of Figure 7), showed higher values during the inactive phase relative to the active phase. Thus, a diurnal rhythm in defecation was detected in this experiment as well as in Experiment I. A significant (Scheffe's test, $p<0.05$) increase in defecation from the first to the second session is observed only during the inactive phase. The ANOVA revealed significant phase ($F(1,16)=34.28$; $p<0.001$), session ($F(1,16)=4.66$; $p=0.046$) and phase by session ($F(1,16)=4.66$; $p=0.046$) effects. The high level of defecation which occurred during the inactive phase of session 2 accounts for both the main effect of session and the interaction between circadian phase and session. Nothing unusual was observed during session 2 to explain this response.

DISCUSSION

The results presented here demonstrate that the arousing nature of the openfield and the habituation process to this novel environment are strongly dependent on the conditions of illumination but not on endogenous circadian rhythmicity. Mice tested during the dark phase are more aroused by the test environment than those tested during the light phase as evidenced by

significantly increased ambulation during the dark. In contrast, the endogenous circadian rhythm of rest and activity has no visible effect on the locomotor response in an openfield. Thus, mice tested under identical illumination conditions during their normal active or rest periods, showed no diurnal variation of the locomotor response to an openfield stimulus.

In Experiment I the change in ambulation in the openfield both within a given session and the difference in ambulation between the two sessions was also affected by phase of the light-dark cycle. For mice tested in complete darkness, the number of crossings decreases within the session demonstrating short-term habituation. In contrast, mice tested during the day failed to exhibit short-term habituation. It is always possible that the animals would show habituation during the day if the session were continued longer, however, 30 minutes has been demonstrated to be more than enough time by others (Walsh and Cummins, 1976; Roullet and Lasalle, 1990).

Reactivity to the environment was lower in the second sessions of the groups tested during the light phase, suggesting the occurrence of long-term habituation. In contrast, the groups tested during the dark phase do not exhibit long-term habituation even though the same mice demonstrated short-term habituation within both first and second sessions. Other studies have also shown opposite temporal effects on short- and long-term memory (Leconte, 1989). These observations are consistent with evidence showing that short- and long-term memory storage appear to be independent processes (Izquierdo *et al.* 1989). However, we can not exclude the possibility that the lack of a visible long-term habituation during the night sessions is due to a ceiling effect. In other words, perhaps the mice were exploring the openfield as rapidly as possible during the first session and their level of arousal may have been greater than that reflected by their maximal rate of ambulation. Therefore, we may have only an apparent lack of long-term habituation in Experiment I.

Several factors could determine these time-of-day differences, including circadian rhythmicity, aversion to bright light, and altered sensory perception in the dark. Because mice exhibit a robust circadian rhythm of rest and activity, the frequency of crossings maybe higher during the dark phase simply because this is their normal active period. Similarly, ambulation

may be lower during the light phase because it corresponds to the mouse's normal rest period. The aversive effect of light on mouse behavior is another potential determinant for the effects observed in the first experiment. Bright light may inhibit activity in nocturnal rodents (Marques and Waterhouse, 1994). Therefore a low activity level during the light phase could result. On the other hand there may be a facilitation or disinhibition of activity during the dark phase. Finally, sensory perception of animals tested in complete darkness is undoubtedly different from the perception of animals tested during the day. Visual cues during the dark become irrelevant or less important while the significance of olfactory, proprioceptive and auditory cues may be increased. Thus, the animal's perception of the environment could have an effect on the behavior in the open field. The higher exploration during the dark could be a way to compensate for the lower visual acuity in total darkness. The absence of long-term habituation could also be due to lower efficiency in collecting the necessary information for the context to become familiar.

Experiment II was designed to control for the factors of circadian time, the aversive nature of light for the nocturnal mouse, and for differences in sensory perception durin sting. We were able to control for any direct effect of the light-dark cycle by keeping the animals in dim green light where entrainment was maintained by a skeleton photoperiod. Mice were tested at two circadian phases under the same conditions of illumination while wheel-running records were used to confirm the persistence of robust rest-activity cycles.

In these animals, we found no time-of-day effect on the arousal level produced by the novel environment or on the acquisition of short- or long-term habituation. This suggests that the differences observed in Experiment I were mainly due to the light-dark cycle. Thus, we conclude that activity levels in the openfield were not affected by circadian phase. In other words, the behavioral arousal produced by spatial novelty is independent of the behavioral arousal that leads to spontaneous basal locomotor activity.

Because the mean level of openfield activity in dim light (Experiment II) and in bright light (Experiment I) were very similar, we would argue that bright light inhibition of activity is unlikely to have contributed to the light/dark differences observed in Experiment I. The high

frequency of crossings during the dark phase which was primarily responsible for the light-dark difference detected in Experiment I, is not observed during the active phase of the group maintained in the skeleton photoperiod. Presumably, when in dim green light the animals were able to collect information more efficiently than mice tested in complete darkness. As a result, less exploration was necessary. Moreover, this increased efficiency in collecting information may mean that the openfield rapidly becomes less novel leading to habituation. Therefore we conclude that the difference in visibility during the light and dark phase directly affects openfield behavior.

It is important to mention that in Experiment II the animals were individually housed in cages with running wheels for 2-3 weeks before the tests, as opposed to group housing (3 per cage) of the LD animals. The effects of isolation on openfield behavior are well known (Gentsch *et al.*, 1981; Gentsch *et al*, 1982a and 1982b). However, we do not believe this was a significant factor in the present experiment. Isolation is known to significantly increase activity in an openfield as well as to retard habituation, while we observed the opposite effects in the mice housed singly with running-wheels. Permanent access to running-wheels may decrease an isolation effect, just as such access appears to mitigate the effect of other stressors (Solberg *et al.*, In Press).

Defecation scores are thought to reflect the emotionality or fearfulness experienced by the animal in openfield studies (Walsh and Cummins 1976; Crusio *et al.*, 1989). This relationship is supported by rat data from Gentsh *et al.* (1981) that showed a positive correlation between post-openfield corticosterone levels and defecation scores. In the present study, animals tested during their inactive phase, either in bright light (Experiment I) or green light (Experiment II), showed significantly higher defecation scores with respect to their counterparts tested during the active phase, either in the dark (Experiment I) or in green light (Experiment II). We could interpret this as higher emotional reactions in animals pulled out of their nests and placed in a novel openfield arena in the middle of their inactive period, compared to the reaction of animals tested during their active period. In other words, if defecation scores accurately reflect emotionality,

fearfulness of the openfield experience is more pronounced during the inactive phase. However, these data should be interpreted cautiously. Mice show a feeding rhythm in which eating is concentrated mainly during the active phase. This alone could lead to a time of day effect in defecation. Consequently, it is possible that the defecation response produced by aversive situations could show a phase effect due to intestinal contents and not due to a different sympathetic activation. We know of no prior studies on a defecation rhythm in unstressed C57BL/6J mice for comparison with the present data.

We predicted that defecation would decrease from one session to another as a result of what has been called "emotional habituation" (Papa *et al.*, 1993). However, we did not find any evidence for this phenomenon under light-dark conditions. While in the skeleton photoperiod, an increase in the second session among mice tested during their inactive period was observed. These data are not fully understood at present. Although we did observe decrease behavioral arousal with experience in the openfield we did not observe decreases in this measure of emotionality.

In conclusion, these data demonstrate that results of openfield studies are strongly affected by the illumination cycle and thus reinforce the need for precise control and specification of this condition. In addition, contrary to intuitive expectations, the circadian rhythm of activity has no effect on the behavioral locomotor reactivity produced by a novel environment.

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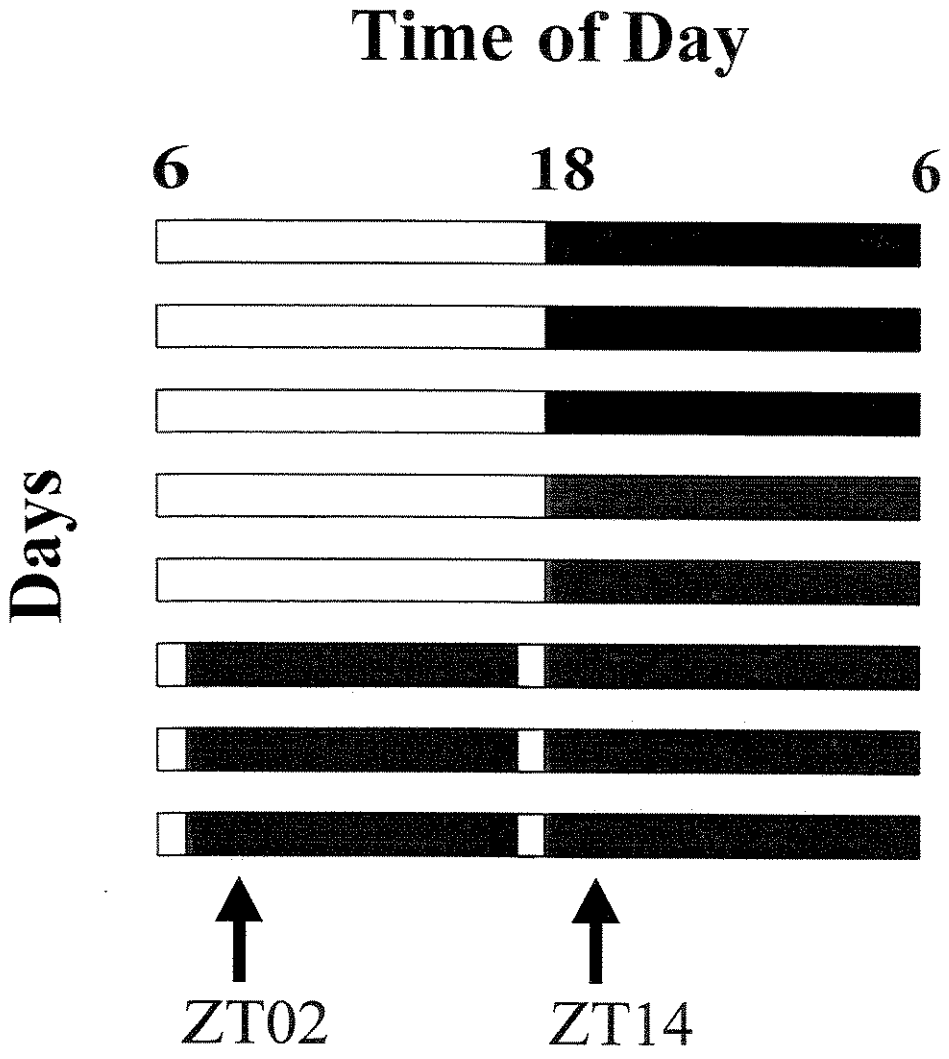

Figure 1. Schematic representation of the light-dark schedule and the transference to the skeleton photoperiod of Experiment II. Time is plotted across the horizontal axis (each bar represents 24h) and successive days are plotted beneath one another. White areas indicate lights-on, black areas indicate lights-off and grey areas represent dim green light. The arrows indicate the times of testing for both groups.

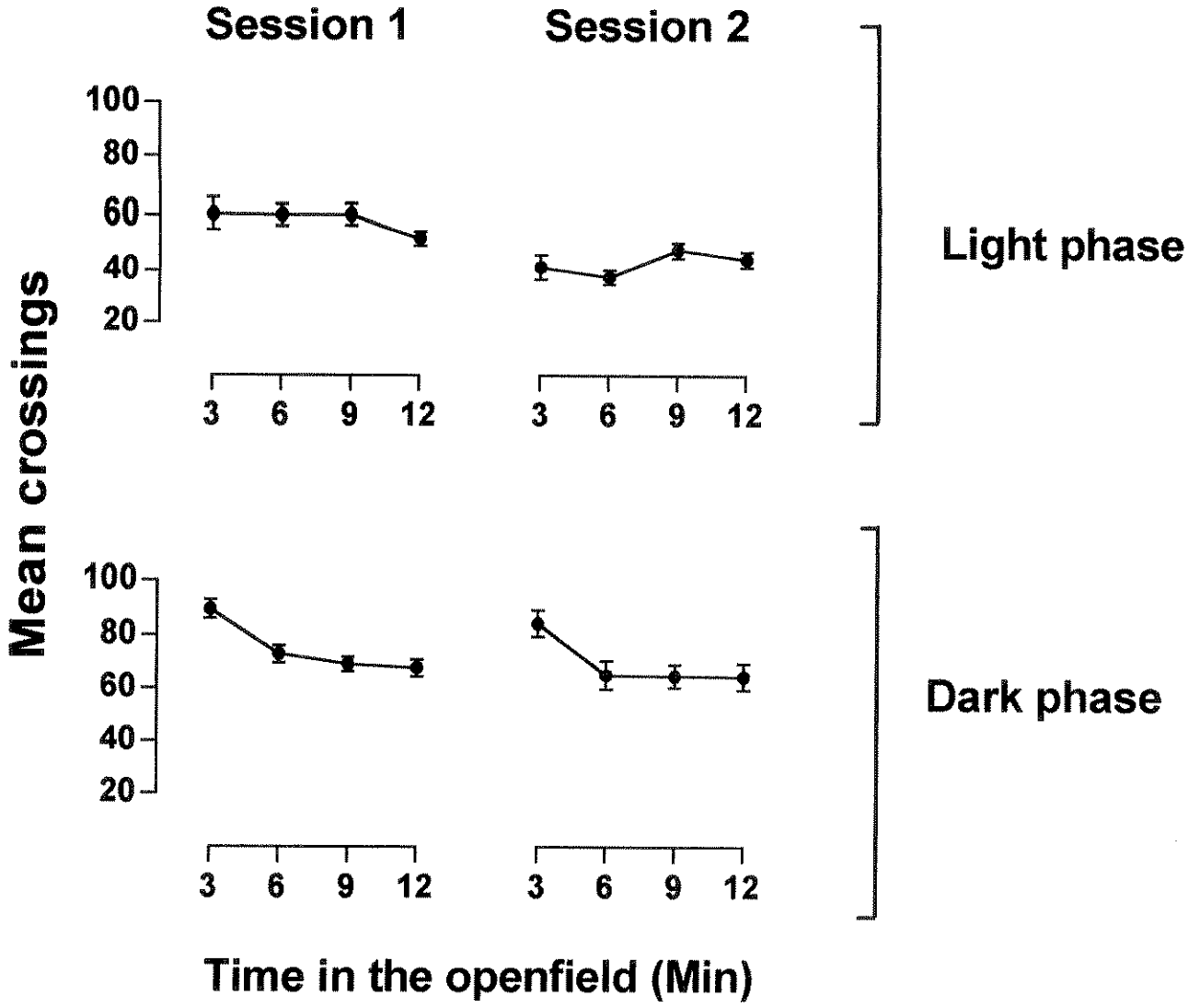

Figure 2. Mean ± SEM number of crossings in an openfield for every 3 minute bin, of the animals tested during the light phase (upper panels) and dark phase (lower panels) during the first (left panels) and second (right panels) testing sessions.

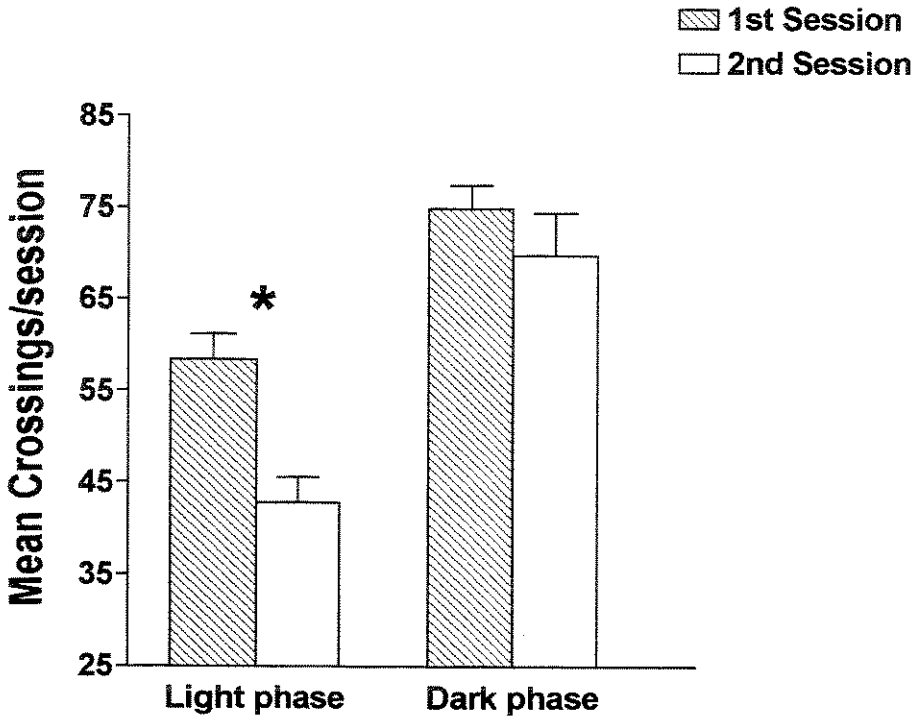

Figure 3. Mean ± SEM number of crossings per session of the first and second sessions of the groups tested during the light and dark phases.

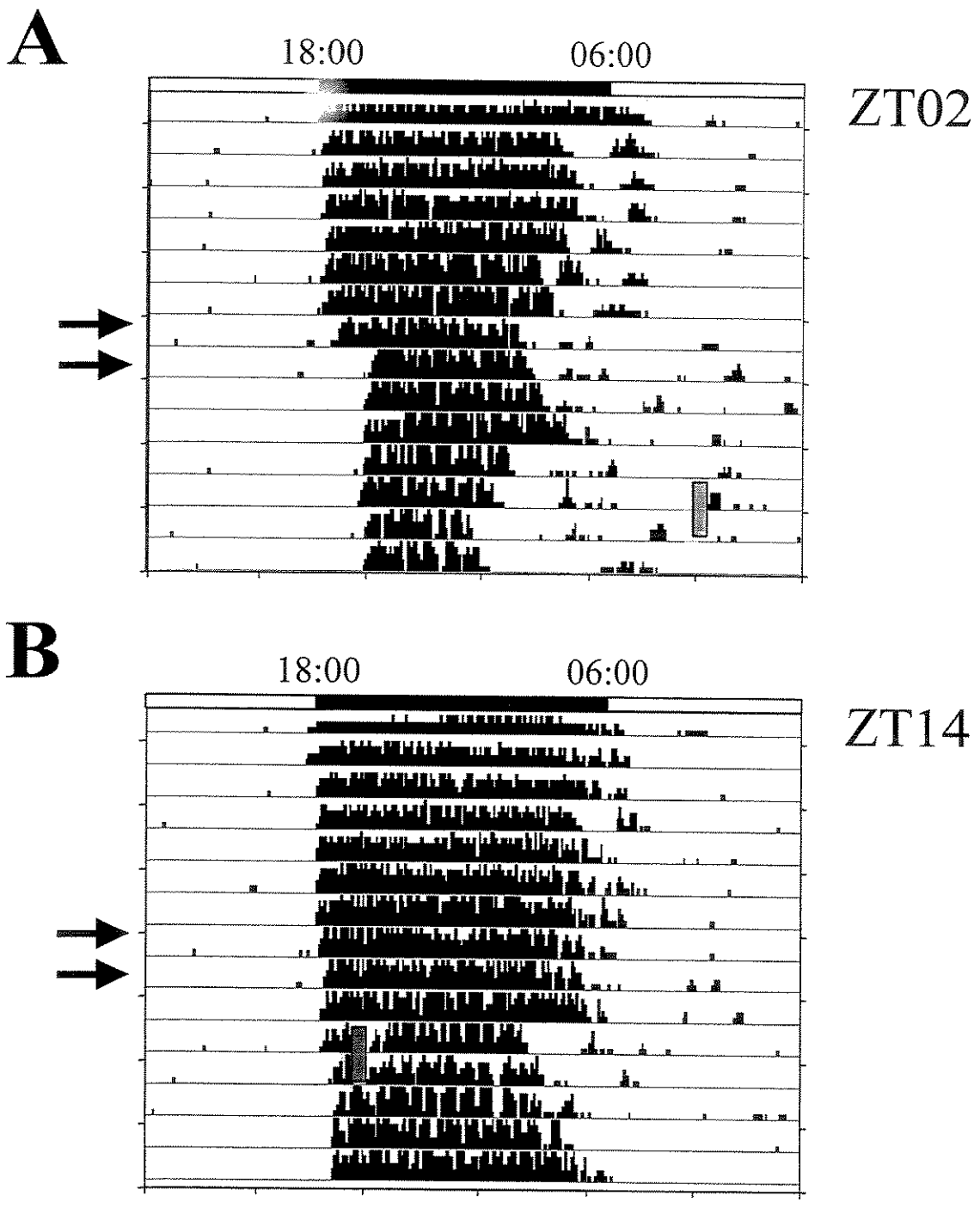

Figure 4. A. Representative actogram of an animal submitted to the openfield test at ZT02. **B.** Representative actogram of an animal submitted to the openfield test at ZT014. Time is plotted across the horizontal axis (24h per line) and successive days are plotted beneath one another. The bar on the top indicates the LD cycle during the first six days. The first arrow indicates when the dark phase was replaced by green dim light and the second one indicates when the skeleton photoperiod started. The vertical grey bars indicate when, during the activity/rest cycle, openfield testing occured.

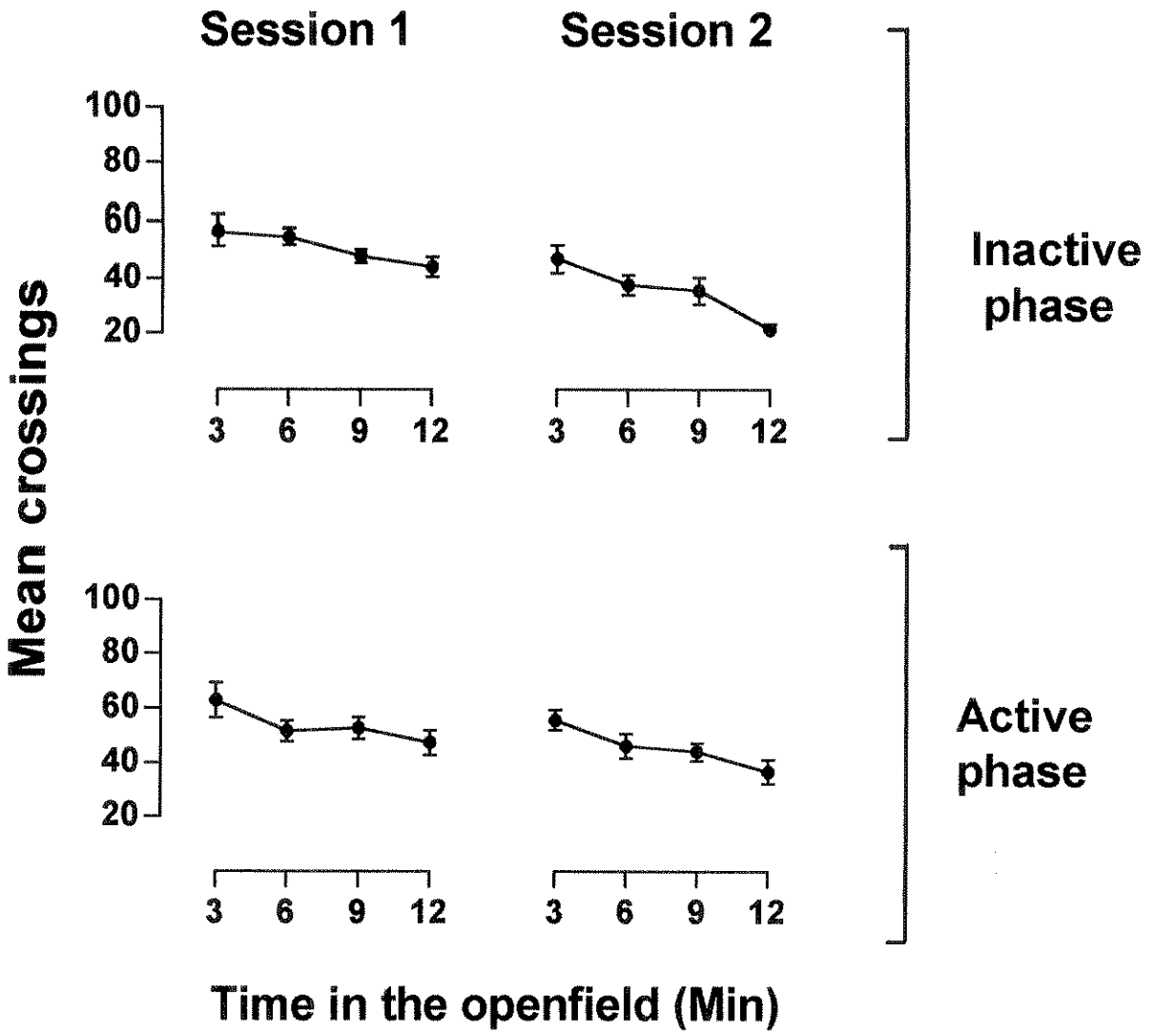

Figure 5. Mean ± SEM number of crossings in an openfield for every 3 minute bin, of the animals tested during the inactive phase (upper panels) and active phase (lower panels) during the first (left panels) and second (right panels) testing sessions.

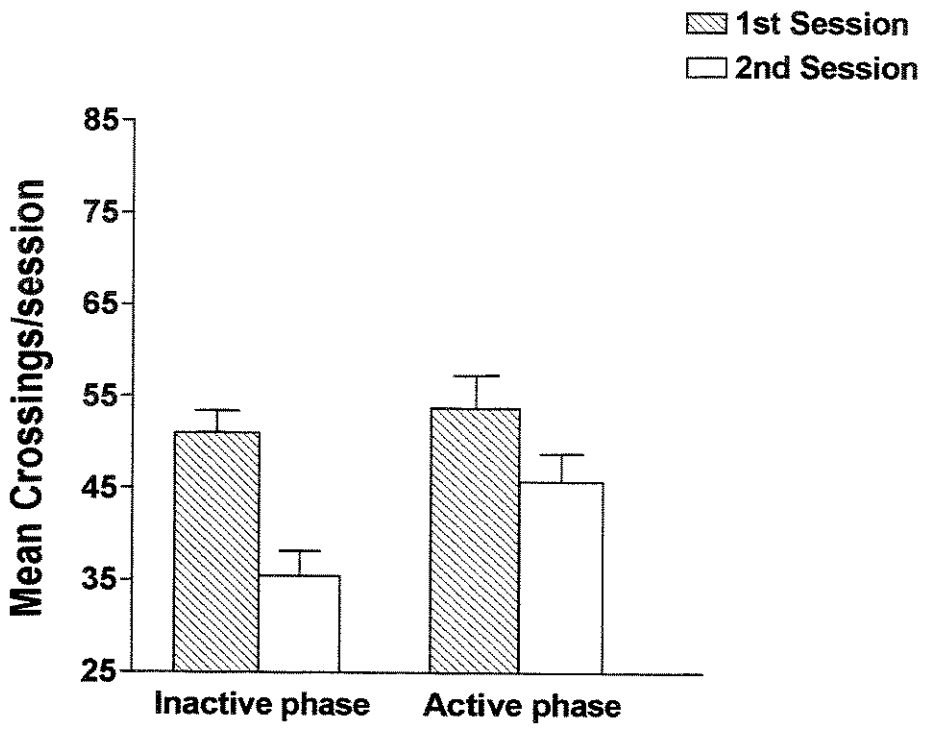

Figure 6. Mean ± SEM number of crossings per session for the first and second sessions of the groups tested during the inactive and active phases.

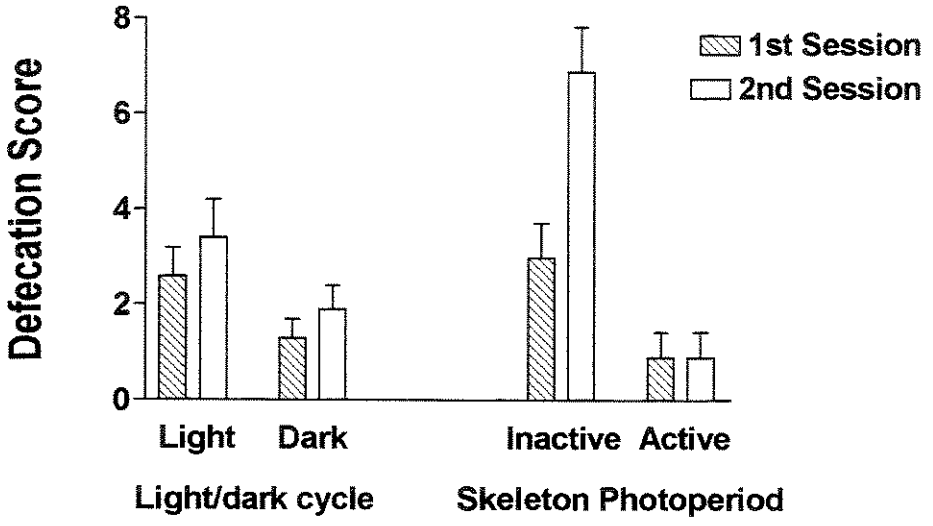

Figure 7. Mean ± SEM of fecal boli produced per session. Animals submitted to a light/dark cycle on left and those submitted to a skeleton photoperiod on right.

AUTOMATED MEASUREMENT OF MOUSE FREEZING BEHAVIOR AND ITS USE FOR QUANTITATIVE TRAIT LOCUS ANALYSIS OF CONTEXTUAL FEAR CONDITIONING IN (BALB/cJ X C57BL/6J)F_2 MICE.

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Running head: Automated and QTL analysis of freezing

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ABSTRACT

The most commonly measured mouse behavior in fear conditioning tests is freezing. A technical limitation, particularly for genetic studies, is the method of direct observation used for quantifying this response, with the potential for bias or inconsistencies. We report the use of a computerized method based on latency between photobeam interruption measures as a reliable scoring criterion in mice. The different computer measures obtained during contextual fear conditioning tests showed high correlations with hand-scored freezing; r values ranged from 0.87 to 0.94. Previously reported strain differences between C57BL/6J and DBA/2J in context-dependent fear conditioning were also detected by the computer-based system. In addition, the use of computer-scored freezing of 199 (BALB/cJ x C57BL/6J)F_2 mice enabled us to detect a suggestive gender-dependent chromosomal locus for contextual fear conditioning on distal chromosome 8 by QTL analysis. Automation of freeze scoring would significantly increase efficiency and reliability of this learning and memory test.

Key words: fear conditioning, freezing, photobeam interruptions, Freeze Monitor, Quantitative trait loci.

INTRODUCTION

Emotional responses such as fear are rapidly acquired through classical conditioning. Fear responses are elicited by previously neutral conditioned stimuli, such as a distinctive chamber (context) or auditory cue after the conditioned stimulus has been paired with an aversive unconditioned stimulus, such as footshock. In mice, freezing is the common and easily measured response used as an index of fear conditioning (Blanchard and Blanchard, 1988; Graef 1994). Fanselow (1990) and Paylor *et al.* (1994) define freezing as the absence of any movement except for respiratory-related movements. Freezing behavior is measured by direct observation, scoring an animal as either freezing or active per interval of time, usually every 5 to 10 sec (Fanselow 1990; Paylor *et al.* 1994) or measuring freezing duration with a stopwatch (Phillips and Le Doux 1992).

One of the central goals of cognitive science is to elucidate the molecular mechanisms of learning and memory. Genetic analysis, when combined with the powerful tools of molecular biology, promis to address this need. Fear conditioning is a good candidate for genetic analysis since it appears subject to complex genetic regulation. Behavioral analyses of targeted mutants have demonstrated roles for specific genes such as *αCaMKII*, *PKCγ*, *SynII*, *mGluR1*, or *CREB* in contextual fear conditioning (Abeliovich *et al.*; 1993, Aiba *et al.*, 1994; Chen *et al.*, 1994; Silva *et al.*, 1996; Bourtchuladze *et al.*, 1994). Inbred strains of mice differ in both contextual and cued fear conditioning, and analyses of recombinant inbred strains as well as segregating generations (intercrosses or backcrosses) have indicated both significant heritability and polygenic control for these traits (Paylor *et al.*, 1994; Owen *et al.*, 1997a and b; Wehner *et al.*, 1997). Recently, quantitative trait loci (QTL) analyses have identified multiple candidate gene loci for contextual fear conditioning (Owen *et al.*, 1997a; Wehner *et al.*, 1997; Caldarone *et al.*, 1997). The loci identified could be limited by the number of parental strains involved, and more loci affecting this behavior could be identified as more strain combinations are examined.

A variety of approaches may be applied to the examination of the genetic regulation of complex behaviors such as fear conditioning and the identification of candidate genes involved in learning and memory processes (Takahashi *et al.*, 1994). Forward genetic methods such as QTL analyses or mutagenesis screens all necessitate quantifying the behavior of large numbers of animals, which could extend over a period of months. Observer based measures are slow and labor intensive. In addition, they are open to subjective bias and the assessment may vary both over time and between observers. The automation of learning and memory tests such as fear conditioning could speed and enhance the reliability, consistency, and practicality of such tests.

Here we report the results of three experiments. The first two experiments were intended to test the validity of a computer system based on the measure of latency between photobeam interruption in the detection and quantification of freezing behavior, while the third experiment used computer-based freezing measures for QTL analysis of contextual fear conditioning.

MATERIALS AND METHODS

Subjects

All animals of the inbred strains and F1 progeny were purchased from the Jackson Laboratory (Bar Harbor, ME). CD1 mice were originally obtained from Harlan, and were bred in the Center for Experimental Animal Resources (CEAR) at Northwestern University. CB6F2 progeny also were bred at Northwestern University. Mice were grouped-housed (6 per cage) in the animal facility, where the light:dark cycle was LD 12:12 (lights on at 05:00h) and the temperature was maintained constant at 23 ± 2 °C. Food (Harlan Teklad) and water were available *ad libitum*. In Experiments I and III mice were handled daily by the experimenter three days before training and on the day of training the cages were moved to the experimental room. In Experiment II, three days prior to training mice were grouped-housed 3 per cage, moved to the experimental room and placed in ventilated, light-tight wooden cabinets where the conditions were the same as in the animal facility. For Experiment I, 12 B6 female mice 2-3 months old

were randomly divided into two groups: controls (n=6, no shock given during training) and experimentals (n=6, three shocks presented during training). For Experiment II, 12 B6, 12 D2, 12 B6D2F1/J and 10 CD1 mice, all 2-3 months old males were used. In Experiments II and III, all animals were in the experimental group (i.e., no unshocked controls were run). For Experiment III, B6, C, and CB6F1/J mice, all 2 months old were used. Six males and six females from each strain were tested. For the QTL analysis, 199 CB6F2 mice, of both sexes, from 2-5 months old, were used.

Apparatus

The Freeze Monitor (San Diego Instruments) consisted of a transparent acrylic conditioning chamber (33 cm high x 25 cm wide x 21 cm deep). A grid floor made of stainless steel rods separated by 0.5 cm was connected to a shock generator (Coulberg). The test chamber was cleaned with 70% ethanol between subjects. A frame (33 x 33 cm) with 16 infrared photobeams (2.5 cm between beams) in the horizontal plane surrounded the chamber. The freeze monitor software (San Diego Instruments) controlled the shock generator and recorded data from the photobeams. In Experiment II, the conditioning chamber and surrounding frame were located inside a sound attenuated enclosure (interior dimensions were 50 cm high x 65 cm wide x 47 cm deep). The inside of the enclosure was covered with gray acoustical foam. A 15W light bulb was centered on the ceiling. Two night-lights (Limelite, Austin Instruments, TX) were placed on the sides of the sound enclosure. A small fan was located on the top of the right wall. A white noise generator ("Sleep Machine" Radio Shack, USA) was used to deliver the auditory-cue (85dB). The speaker was placed on the floor to the left side of the test chamber. Distinct geometric shapes of white paper were also located on the inside walls of the sound chamber.

Testing Procedure

All training and testing occurred during the light phase, between 10:00 and 17:00. In Experiments I and III the training session consisted of placing the animal in the conditioning

chamber for 6 minutes. After three minutes in the enclosure three shocks (1 sec, 0.6 mA) were given at 1-minute intervals. The mice in Experiment I that were assigned to the no shock condition were simply placed in the test chamber for 6 minutes. All mice were tested for acquisition of the conditioned fear response 24h after the training session. The test session consisted in placing each mouse in the enclosure for 6 minutes (Experiment I) or 8 minutes (Experiment III) and scored for freezing using the sampling procedure described below. No shock was delivered during testing.

In Experiment II, the mice were first placed in the test chamber and two minutes later a 30-second auditory cue (white noise) was presented. Immediately after the auditory-cue terminated a 2-second, 0.6 mA footshock was delivered. Mice were removed from the test chamber 30 seconds later and returned to their home cage. Twenty-four hours later, subjects were placed into the same training chamber for 5 minutes and their freezing behavior was scored as described below. One to three hours later, subjects were tested for their freezing to the auditory cue. For the auditory-cue test, the training chamber and sound enclosure were altered by placing a green plastic cover over the grid floor, illuminating the sound chamber with two green lights (0.5-1.0 lux at the level of the rods), covering with white paper the entire inside of the sound chamber, and placing a container with vanilla extract (5ml) inside the sound chamber. The test chamber was also cleaned with 1% acetic acid between subjects. During the auditory-cue test, mice were scored for 6 min; the white noise generator was turned on for the final three minutes of the session. Before the auditory testing a different person transported the animal in a transfer cage with paper towel in the place of bedding while the lights of the experimental room were off.

Response measures

The basic measure of the Freeze Monitor is photobeam interruptions. Total activity was the total number of beam breaks per session. The software translates beam interruption in latency between photobeam interruptions. For this the whole session is divided in 5-sec bins and the latency to break the first three new photobeams in each 5-second interval was recorded. That is,

the latency from the beginning of each 5-sec interval to the first new beam interruption within that interval (termed Latency 1). The latency between the beginning of each 5-sec interval to the second new beam interruption within that interval (Latency 2). And the latency between the beginning of each 5-sec interval to the third new beam interruption within that interval (Latency 3). If a new beam interruption never occurred during the 5-second interval, a score of 5 seconds was recorded. For each animal, we computed the total amount of time to break the first, second, and the third photobeams for the entire session.

In addition, we established four different criteria that were used to approximate the type of freezing scores obtained using the hand-scored procedures. First, we counted the number of five-second intervals in which the animals took more than one second to cross the first new beam (termed 1sec5sec). Second, we counted the number of five-second intervals in which the animal took more than 2 seconds to cross the first new beam (2sec5sec). The same criteria were also applied to every other 5-sec interval. That is, the number of 10-sec intervals in which the animal took more than one second to cross the first new beam of the interval (1sec10sec). And the number of 10-sec intervals in which the animal took more than two seconds to cross the first new beam of the interval (2sec10sec). For each of these measures we computed the percentage of intervals during which the mouse was freezing.

Simultaneously with the computer scoring, the number of freezes was scored through direct observation by a time-sampling procedure. This was done by two different methods in order to compare the computer scoring with different methods of observer scoring. In Experiment I and III, every 5 seconds observation of the animal would start and continued for the whole 5 second unless freezing was observed. If freezing occurred, observation was halted until the beginning of the next 5-sec interval. In Experiment II, every 10 sec the animal was observed for 1 sec and judged as either freezing or active, this judgment being made at the instant that the sample was taken (Paylor *et al.*, 1994; Owen *et al.*, 1997b; Wehner *et al.*, 1997). Freezing was defined as the absence of visible movement, except for the minor movements required by respiration. All other behavior was considered active. One observer scored Experiment I. In

Experiment II each session was scored by at least two observers; some sessions were scored by three observers.

Genotyping of F2 mice

High molecular weight genomic DNA was extracted from tissue samples (either tail biopsies or liver) collected from each of the 199 CB6F2 progeny using a standard proteinase K digestion and phenol-chloroform extraction procedure (Ausubel *et al.*, 1995). Genotyping of all F2 DNAs used simple sequence length polymorphisms (SSLPs; Copeland *et al.*, 1993; Dietrich *et al.*, 1994, 1996), obtained as MapPairs from Research Genetics, Inc. SSLP genotyping methods were slightly modified from those of Dietrich *et al.* (1992), and are described here. PCR reactions were carried out in 10 µl volumes, using ~25 to 40 ng template DNA in 5 µl H_20, 0.25 units of Amplitaq DNA (Perkin Elmer), 200 nM each dNTP, 0.85X GeneAmpPCR buffer II (1X buffer: 50 mM KCl, 10 mM Tris-HCl pH 8.3), 1 µg/µl BSA, and 1.275 mM MgCl. One hundred ten nM of each primer was used, with all of the forward primer aliquot for each reaction labeled with [γ-^{32}P]ATP (specific activity 6000 Ci/mmol, DuPont/NEN), using T4 polynucleotide kinase. PCR reactions were carried out on either a 96- or 192-well PTC-100 thermal cycler (MJ Research). The thermocycling profile was 94°C for 4 min, 27-35 cycles of (94°C for 15 sec, 55°C for 2 min, 72°C for 2 min), followed by a 7 min extension step at 72°. PCR products were separated on 7% denaturing acrylamide sequencing gels and visualized by autoradiography. The genotype, either homozygous B6 or C, or heterozygous (B6/C), was scored for 121 loci throughout the mouse genome.

QTL analysis

A total of 199 CB6F2 mice were genotyped for 121 SSLPs spaced at approximately 20-30 cM intervals throughout the genome. Five different computer-measured freeze scores were used as phenotypic traits for the quantitative trait loci analysis. These were: 1sec5sec, 2sec5sec, 1sec10sec, 2sec10sec, and Latency 3. Genotypic and phenotypic scores were entered into a MS-

Excel worksheet. The order of SSLP markers was based on the MIT/Whitehead Institute map (Dietrich *et al.*, 1996), and for some chromosomal regions where additional markers were analyzed, the most likely order and genetic distances between markers was calculated using the program MAPMAKER (Lander *et al.*, 1987). Genome-wide scans for linkage of freeze scores were done using the program MapManager QT (Manly, 1993) on a Macintosh computer, as well as the program MAPMAKER/QTL 1.1 (Lander and Botstein, 1989; Lander *et al.*, 1987; Paterson *et al.*, 1988) on a Sun computer. LOD [logarithm of the (odds of linkage/odds of no linkage)] scores for candidate regions were calculated for all phenotypic scores using MAPMAKER/QTL. Data were analyzed first using a "free" genetic model (which assumes no phenotypic effect of C alleles), and subsequently using additive, dominant, and recessive models, where dominant or recessive refers to the B6 allele in each case.

Statistical Analyses

For all statistical analyses, values were calculated as percentages (e.g., bouts of 1sec10sec measures/total possible 1sec10sec bouts). In Experiment I t-tests were performed on hand-scores, 1sec5sec and Latency 3 measures of the testing sessions to compare control and experimental groups. Correlation analysis quantified the strength of association between hand-scored freezes and the different computer measures and between the hand-scored freezes of the different observers. In Experiment II one-way analyses of variance with strain as the grouping variable were performed on observer freeze scores and 1sec10sec computer measures for both the context and auditory-cue testing sessions. In addition a Net 1sec10sec was derived by subtracting the freezing level in the first two minutes of the training session (before the US was presented) from the 1sec10sec freezing value recorded during the context test. This measure was also analyzed through a one-way analysis of variance. CD1s were excluded from the ANOVA since they are not an isogenic group. In Experiment III, variances were not equal between strains. Significant differences in variances were assessed by F-test. Comparisons between groups (either strains or

genders) were done by Kruskall-Wallis tests. All statistical analyses were performed using the program NCSS 6.1 (Utah) on a Windows 95 PC.

RESULTS

Experiment I

In order to assess the Freeze Monitor's ability to detect differences in conditioned freezing behavior, two groups (shock and no-shock) of B6 mice were simultaneously scored by an experimenter and by the Freeze Monitor system.

All computer measures were significantly ($p<0.0003$) correlated with hand scoring. Correlation coefficients ranged from 0.87 (Latency 1) to 0.94 (Latency 3) (Table 1).

Figure 1 presents the freezing data during the training and test sessions. The percentage freezing obtained from direct observation (Figure 1A) is shown with two selected computer measures, the 1sec5sec (Figure 1B) and Latency 3 scores (Figure 1C). Even though all computer measures showed high correlations with hand-scores, Latency 3 was chosen to graph because it had the highest r value (0.94). The 1sec5sec measure was chosen because it is dichotomous and is based on a 5-sec interval similar to the hand-scoring procedure used in this experiment. Low levels of freezing were recorded during the pre-shock part of the training session followed by an increase in freezing during shock presentation. These patterns of data were obtained for all computer measures. Both hand-scored and computer-scored freezing showed that there were significantly higher levels of freezing in experimental animals than in controls (p's <0.01).

Experiment II

Because different strains of mice have different behavior patterns and levels of activity, we considered the possibility that the same scoring criterion may not be ideal for every strain. To address this issue, B6, D2, B6D2F1/J, and CD1 mice were tested in context and cued fear-conditioning paradigms. These strains were selected because it is known that B6 mice display

more contextual, but not auditory-cued, conditioned fear than D2 mice (Paylor *et al.*, 1994) and because CD1 mice have been observed to have low levels of freezing using similar procedures (R. Paylor, unpublished observations).

Correlations between hand scored freezing and all the computer measures were highly significant ($p's<0.0001$) with r values ranging from 0.69 (activity) to 0.79 (2sec5sec) (Table 1). Figure 2 depicts the correlation plots between observer-scored freezing and 1sec10sec (Figure 2A). Even though all computer measures had significant correlations with hand-scoring, 1sec10sec was chosen because of its high r value (0.78) and because it is a dichotomous measure based on 10-sec sampling intervals as was the hand scoring method used in this experiment. The correlation graphs of all the computer measures were very similar. As expected, the freezing values of D2 mice (crosses) were on the lower part of the hand-scored and computer-scored freezing scales while those of B6 (black circles) tended to be at the higher part of the scales. CD1s (squares) had low values and F1s tended to have intermediate values. It is important to note that the levels of freezing were higher in all the computer measures, particularly in animals with low observational freezing scores. This was especially apparent for D2 mice and yielded non-zero y-intercepts (Figures 2A).

We considered possible sources of this apparent false positive rate in computer scored freezing, as well as methods to correct for it. The Freeze Monitor system is not able to detect small head movements which would be scored as "active" by an observer, thus this system tends to have higher levels of freezing when testing mice that show low levels of freezing as measured by an observer. Thus an animal that is relatively inactive or moves slowly may be erroneously scored as freezing. This indicates that under certain circumstances when low levels of freezing are recorded using the hand-scored protocol, the computer-derived scores will record more freezing behavior. We have attempted to compensate for this "over-scoring" by generating a Net 1sec10sec measure. We considered the level of freezing in the first two minutes of the training session (before the US was presented) as a measure of the baseline false positive freeze rate for the 1sec10sec computer-derived measure. By subtracting this value from the 1sec10sec freezing

value recorded during the context test, we were able to correct for different baseline scores. This manipulation sets the baseline level of freezing to zero, which may not be true for all animals. However, we did find that the mean level of hand-scored freezing during the pre-US part of the training session was not significantly different from zero for any of the strains tested. Indeed, correlation coefficients were higher for the baseline-corrected ("Net") values (Table 1; Figure 2B).

The 1sec10sec baseline-corrected computer measure was chosen for statistical analysis between strains. The analysis of variance of hand-scored freezes during testing revealed a significant main effect of strain, $F(2, 33)=10.04$, $p<0.001$. Post hoc analysis with the Newman-Keuls' test indicated that B6 animals were significantly ($p<0.05$) different from D2 (Figure 3A). Similarly, when analyzing the baseline-corrected 1sec10sec values during testing, the analysis of variance revealed a significant main effect of strain, $F(2,33)= 3.35$, $p<0.05$. The post hoc analysis indicated that B6 animals were significantly ($p<0.05$) different from D2 (Figure 3B). No significant effect of strain was detected with uncorrected 1sec10sec values.

Correlations between observer scores and computer measures were also analyzed for the auditory-cued fear conditioning testing during CS presentation. Correlation values were highly significant ($p<0.0001$) with r values ranging from 0.62 (1sec10sec) to 0.74 (Latency 2 and Latency 3) (Table 1). Figure 3 C and D shows the freezing levels for each strain during auditory-cued testing as measured by hand scores (Figure 3C) and Net 1sec10sec computer scores (Figure 3D). Consistent with Paylor *et al.* (1994), no difference between D2 and B6 during auditory-cued fear-conditioning was detected either with hand-scored or any of the computer-scored freezing measures ($p's >0.05$).

Experiment III

We undertook a QTL analysis in order to identify candidate chromosomal regions containing loci which influence contextual fear conditioning as an example for the use of the

Freeze Monitor, in a protocol similar to Experiment I. F2 intercross progeny between BALB/cJ and C57BL/6J were used as the segregating generation (N= 199) for the QTL analysis.

In order to determine the most appropriate of the Freeze Monitor measures to use for the QTL analysis, B6, C, and CB6F1 mice were simultaneously scored by observer and the computer. All computer measures obtained in the context testing sessions of parentals and F1 intercross showed significant ($p<0.0001$) correlations with hand-scored measures; r values ranging from 0.81 (1sec10sec) to 0.87 (Latency 2) (Table 1). The correction for baseline activity (Net values) actually resulted in decreased correlation coefficients (Table 1), consequently the net values were considered inappropriate for these strains, and were not used.

Both observer-scored and computer-scored freezing showed unequal variances among the isogenic generations. In general B6 animals had significantly lower variance than the other groups. When analyzing hand-scored measures the F-tests for equal variance showed that only B6 and F1 had unequal variances ($F(10,11)=5.05$, $p<0.01$). Latency 3 measures had unequal variances between B6 and C ($F(10,11)=6.75$, $p<0.005$), B6 and F1 ($F(10,11)=5.70$, $p<0.005$) and between F1 and F2 ($F(11, 222)=1.06$, $p<0.05$). 1sec10 sec measures showed unequal variances between B6 and C ($F(10,11)=9.96$, $p<0.001$) and between B6 and F1 ($F(10,11)=8.98$, $p<0.001$). All groups showed normal distributions as revealed by Kolmogorov-Smirnov normality tests. Transformation of the data in order to be able to use a parametric statistical test was not possible because some groups do show equal variance. The non-parametric Kruskall-Wallis analysis of observer-scored freezes during testing of parentals and F1s revealed a significant main effect of strain ($F(2,32)= 12.53$, $p<0.001$). Post hoc Z tests for pairwise comparisons revealed no difference between parental strains while F1 animals showed significantly more hand-scored freezing than B6 and C. When analyzing 1sec10sec values during testing of parentals, F1s, and F2s, a significant main effect of strain was observed ($F(3,232)= 3.91$, $p<0.001$). F1 animals showed significantly different values than B6, C and F2 while no difference between parental strains was detected. Similarly, Latency 3 analysis during testing also showed a significant main

effect of strain ($F(3,232)= 3.21$, $p<0.05$). F1 animals were significantly different from B6, C and F2 and, again, no difference between parental strains was evident.

The frequency distributions for 1sec10sec and Latency 3 respectively, of B6, C, CB6F1 and CB6F2 mice are shown in figure 4. These two computer measures were graphed because, they yielded the highest LOD scores. The significantly higher level of freezing behavior of the intercross F1 detected by hand scoring was also detected by the Latency 3 and 1sec10sec computer measures (Figure 4), consistent with overdominance for this trait. The mean values of the CB6F2 are intermediate between the CB6F1 and the parental values, also consistent with dominance towards high freezing. A continuous distribution is observed in CB6F2 mice for both measures, suggesting a polygenic mode of inheritance. The variance of the CB6F2 population is not significantly different than the CB6F1. However, the variances of the two parental groups also are unequal; B6 mice have lower variance than C and CB6F1 mice, so comparison of variance of F1 to F2 may not reflect genetic variance.

The results of the linkage analysis are shown in table 2 where the candidate chromosomal regions with LOD scores over 2.0 were selected. A suggestive QTL (LOD scores between 2.8-4.1) was detected in chromosome 9 when using the 2sec10sec measure. Segregation at D9Mit2 accounts for 8.2% of the phenotypic variance in this computer measure. Similar LOD score values for this location were found for the 1sec5sec and the 1sec10sec measures. Another suggestive QTL in distal chromosome 8, linked to D8Mit13 was located when analyzing males alone. The same result arose when using all the computer-generated freeze measures. Both 1sec5sec and 1sec10sec had LOD scores greater than 2.8 with free genetic models. Segregation at D8Mit13 accounts for 12% of the phenotypic variance in 1sec5sec, and 13.4% of the phenotypic variance in 1sec10sec. The position of this QTL is shown as described by MAPMAKER/QTL (Figure 5). A suggestive LOD score for Latency 3 measure linked to D8Mit 25 was also found in males, however no other computer measure at this chromosomal region supported this. The analysis of only females revealed LOD scores higher than 2.0 for all computer measures on chromosome 16 linked to D16Mit27.

DISCUSSION

Taken together these data constitute convincing evidence that the Freeze Monitor is a good tool to study conditioned fear as measured by behavioral inhibition which is reflected by increased latencies to break new beams. This behavioral inhibition, clearly detected by the equipment is highly correlated with the freezing measure that is typically scored by experimenters. We conclude that the Freeze Monitor is capable of obtaining reliable measures of the freezing response during fear-conditioning tests.

In Experiment I, the levels and patterns of freezing were similar when comparing the hand-scored data to those obtained from the computer. High correlation values were obtained between hand scores and all the different computer measures. These results suggest that using various computer-derived measures from the Freeze Monitor system it is possible to obtain reliable conditioned freezing scores in B6 mice.

Different strains of mice have been shown to exhibit significantly different locomotor and exploratory activity (Lhotellier *et al.*, 1993) and reaction to novelty (Rodgers and Cole 1993). There are also strain differences in learning performance (e.g. Upchurch and Wehner, 1988; Yamada *et al.*, 1992; Paylor *et al.*, 1993; Roullet *et al.*, 1993; Paylor *et al.*, 1994; Mori and Makino, 1994; Owen *et al.*, 1997b). As with any behavioral measure, it would be essential to validate the testing or scoring procedure when adapting it for use in a new strain. The purpose of Experiment II was to extend the use of the Freeze Monitor to some other strains. D2 mice and the F1 intercross of this strain with B6 were tested. Again, high correlation values were obtained between hand scores and computer scores. In addition, the previously reported difference between B6 and D2 mice during context testing (Paylor *et al.* 1994) was also detected with computer scores. However, it is important to point out that we obtained lower r values in Experiment II than in Experiment I. Currently, we do not fully understand the nature of these differences but it is likely due to a combination of factors including different strains, different

training procedures, and differences in the hand-scored protocols. Findings from Experiment II also indicate that the Freeze Monitor is apparently less sensitive when mice display low levels of freezing. This is an issue that requires more testing in order to confirm.

In the first part of Experiment III, C, B6 and CB6F1/J were tested for context fear conditioning. CB6F1/J animals showed a significantly higher level of freezing compared to B6 and C, indicative of overdominance. The intermediate mean values of CB6F2 mice also suggest this type of trait. Overdominance is predicted for phenotypes that have strong direct survival or reproductive effects and is invoked as a basis for so-called "hybrid vigor" (Falconer, 1981). Hence, overdominance is not surprising in the case of learning processes. Indeed, there is evidence of overdominance in the hybrids 129B6F1 and FVB129F1 that show better scores than either of the parental strains during context fear conditioning tests and during Morris probe trails (Owen et al., 1997b). The behavioral analysis of the F2 intercross showed that several computer measures were sufficient to detect suggestive QTLs for context fear conditioning on chromosomes 8 and 9. Other studies using hand scoring during context testing have indicated that there are several genetic regions that have strong influence on performance in this paradigm. QTLs on chromosome 1 and 16 were detected in a study of the BxD recombinant inbred strains (Owen *et al.*, 1997a). Wehner *et al.*, (1997) testing an intercross between B6 and D2 animals found that QTLs for context fear conditioning were associated with specific regions in chromosome 1, 2, 3, 10 and 16. Similarly, Calderone *et al.* (1997) identified strong QTLs in the distal and proximal ends of chromosome 1 in a backcross population generated from B6 and C3H/HeJ mice. Using F2 intercross mice from C and B6 progenitors, our results indicate that there are some additional QTLs to be considered. The suggestive QTL on chromosome 8 was only present in males. Even though no gender difference was observed in the mean freezing levels, context learning is a complex test in which the same ability to perform may reflect different strategies and/ or different sensory inputs. C3H animals which become blind as adults show an increase in freezing in response to a context previously paired with a shock (Owen *et al.*, 1997b) despite being visually impaired, suggesting that other, non-visual, cues are being used to

identify the context. In the present experiment, males and females could be relying on different sensory pathways; consequently the genes involved could be different. The suggestive QTL on chromosome 9 was detected when considering males and females together. None of the suggestive values obtained matched the results of the already published data however a different strain combination was used so it can be expected that different loci or sets of genes responsible for this trait would be detected.

It is important to note that the genetic models used here (free, dominant, additive and recessive) do not exactly predict the allelic effects at an overdominant locus. None of these models were designed for this kind of effect, consequently, the actual linkage values may be higher than the values reported here. As might be expected for a trait in which overdominance is present, the LOD score estimates are highest for the "free" genetic model, followed by the dominant genetic model. Additive and recessive genetic models predict lower LOD scores for all freeze measures.

Testing more animals is necessary to confirm these results and further refinement of the interval on chromosomes 8 and 9 will be required to determine the behavioral specificity of the QTL found.

In general, some computer-scored measures more accurately reflect hand-scored freezes than others. The selection of which should be used or the use of baseline-corrected values will depend on the strain used and the method of hand scoring normally used. Other manipulations can be done to try to increase the sensitivity of the Freeze Monitor like adjusting the frequency of measurements, height, position or number of the photobeams to optimize the measure for other strains' behavior patterns. The possibilities are many and the data shown here are a first step towards improving the method.

These observations support the Freeze Monitor as a way to automate the measure of freezing response. As with any automation method, the benefits are obvious: increase in efficiency, elimination of the subjective component that characterizes direct observation and the possibility of testing more animals and in more diverse conditions such as darkness. The recent

increase in the use of genetic techniques for mapping genes related to learning and memory could significantly benefit with the automation of learning tests.

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TABLES

Table 1. Correlation values (r) between observer-scored freezing and each computer-based measure.

	Experiment I	Experiment II			Experiment III	
Measure	Context	Context	Net Context	Cued	Context	Net Context
Latency 1	0.87	0.78	0.79	0.73	0.85	0.81
Latency 2	0.92	0.76	0.75	0.74	0.87	0.80
Latency 3	0.94	0.75	0.76	0.74	0.84	0.75
Activity	-0.93	-0.69	-0.73	-0.69	-0.84	-0.76
1sec5sec	0.91	0.76	0.77	0.69	0.84	0.72
2sec5sec	0.89	0.79	0.80	0.72	0.85	0.79
1sec10sec	0.92	0.78	0.79	0.62	0.81	0.65
2sec10sec	0.88	0.78	0.80	0.66	0.84	0.79

Table 2. Summary of genome-wide QTL analysis reporting LOD scores for linkage of candidate chromosomal regions (LOD scores ≥ 2.0) for loci affecting fear conditioning and percentage of phenotypic variance explained.

A. All F2 progeny					
Genetic Interval	Trait				
	1sec5sec	2sec5sec	1sec10sec	2sec10sec	Latency 3
D3Mit11-D3Mit17	2.00 8.6%	2.32 10%			2.09 9.3%
D9Mit2-D9Mit4	2.41 6.6%		2.62 7.6%	2.81 8.2%	
D8Mit13-D8Mit14		2.07 4.7%			2.29 5.2
D16Mit27-D16Mit106					
B. Female F2 progeny					
Genetic Interval	Trait				
	1sec5sec	2sec5sec	1sec10sec	2sec10sec	Latency 3
D3Mit11-D3Mit17	2.22 13.9%		2.29 14%	2.16 12%	2.29 14.1%
D3Mit131-D3Mit6		2.01 12.7%			
D9Mit2-D9Mit4					
D8Mit13-D8Mit14					
D16Mit27-D16Mit106	2.21 13.4%	2.66 18%	2.08 11.8	2.3 14.5%	2.25 12.8%
C. Male F2 progeny					
Genetic Interval	Trait				
	1sec5sec	2sec5sec	1sec10sec	2sec10sec	Latency 3
D3Mit11-D3Mit17					
D9Mit2-D9Mit4					
D8Mit13-D8Mit14	2.84 12%	2.63 11.2%	3.2 13.4%	2.25 9.7%	2.55 10.4%
D8Mit25-D8Mit200					2.92 13.8%
D16Mit27-D16Mit106					

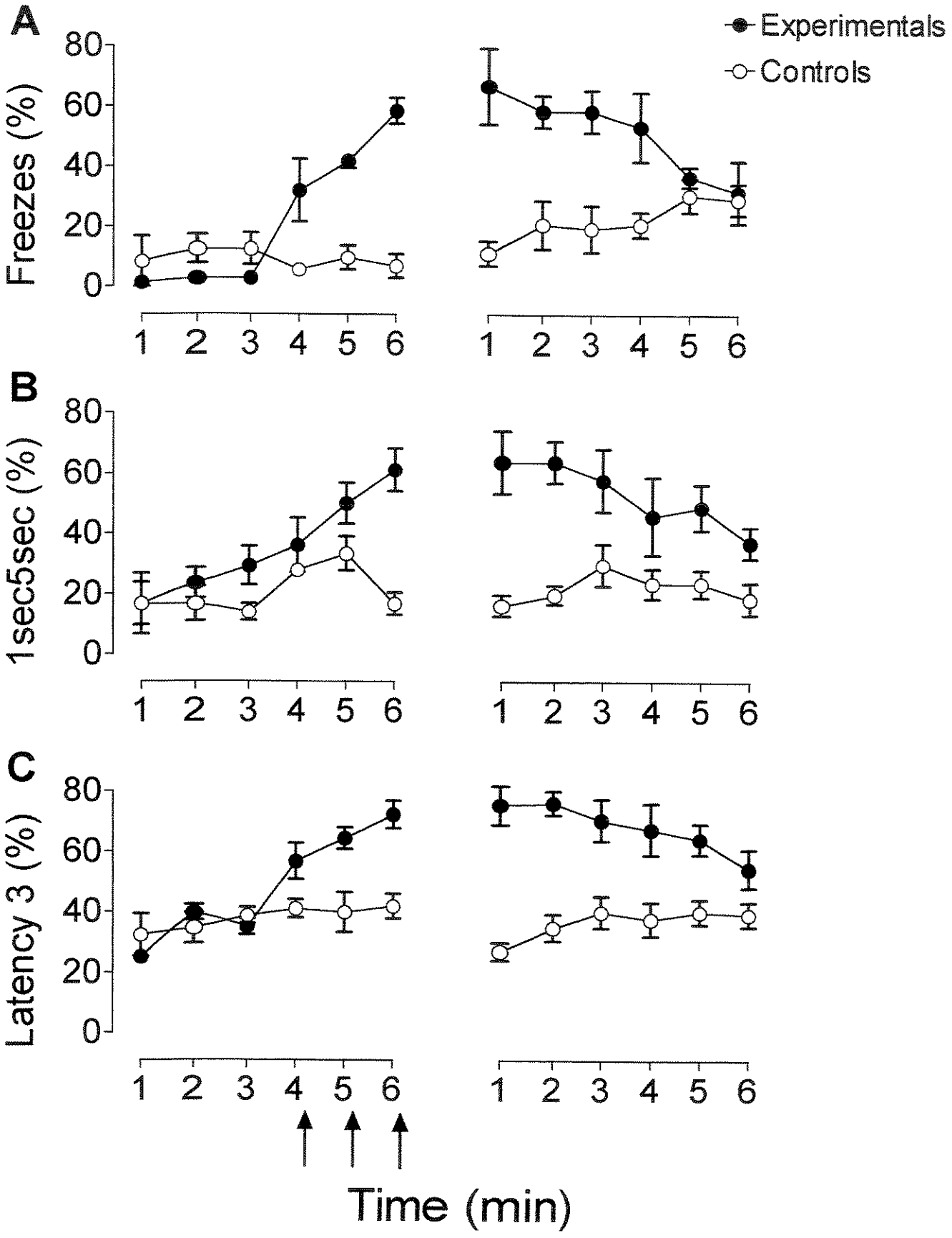

Figure 1. Mean ± SEM number per minute of **A.** % hand-scored freezes, **B.** %1sec5sec computer-scored freezing **C.** % Latency 3 computer-scored freezing, for training (left panels) and testing (right panels) sessions of Experiment I. The control group was not exposed to shocks during training (open circles) while the experimental group was

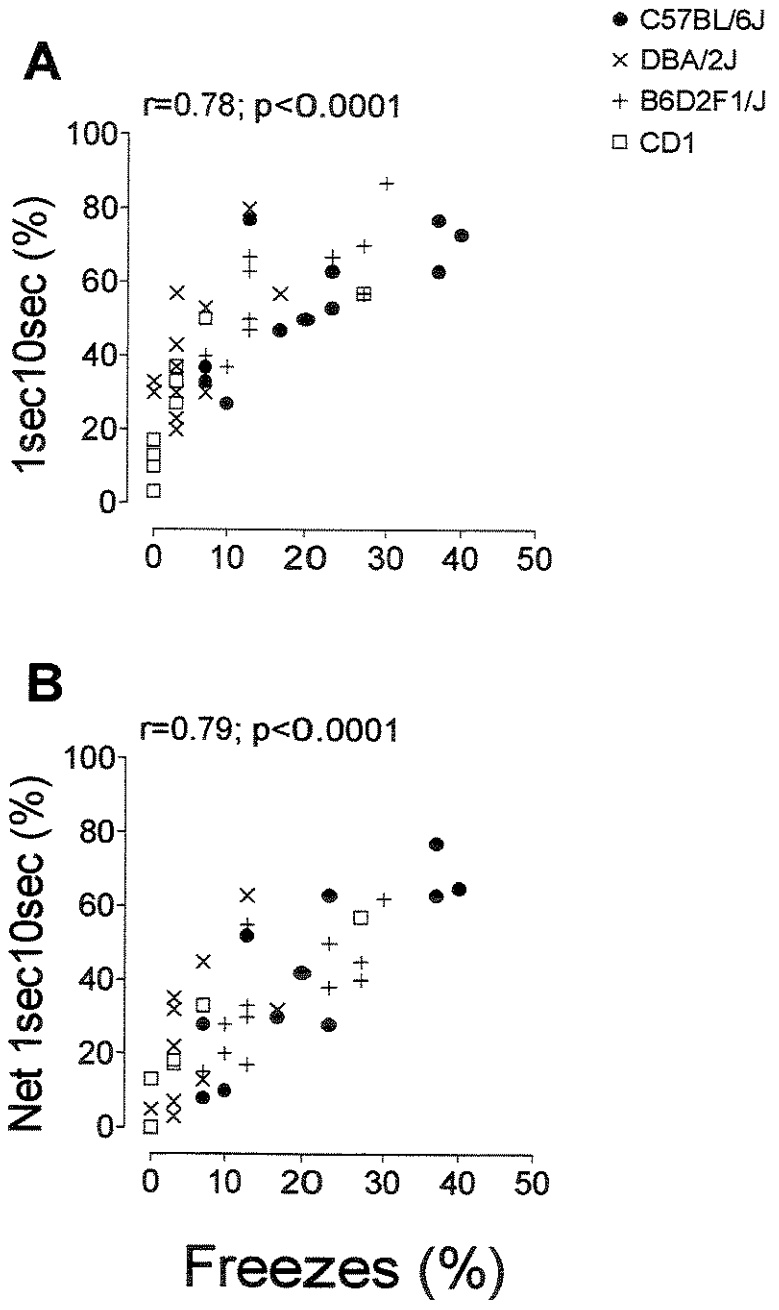

Figure 2. Linear correlation plots between percentage of hand-scored freezes and **A.** %1sec10sec and **B.** % Net 1sec10sec, per testing sessions of Experiment II.

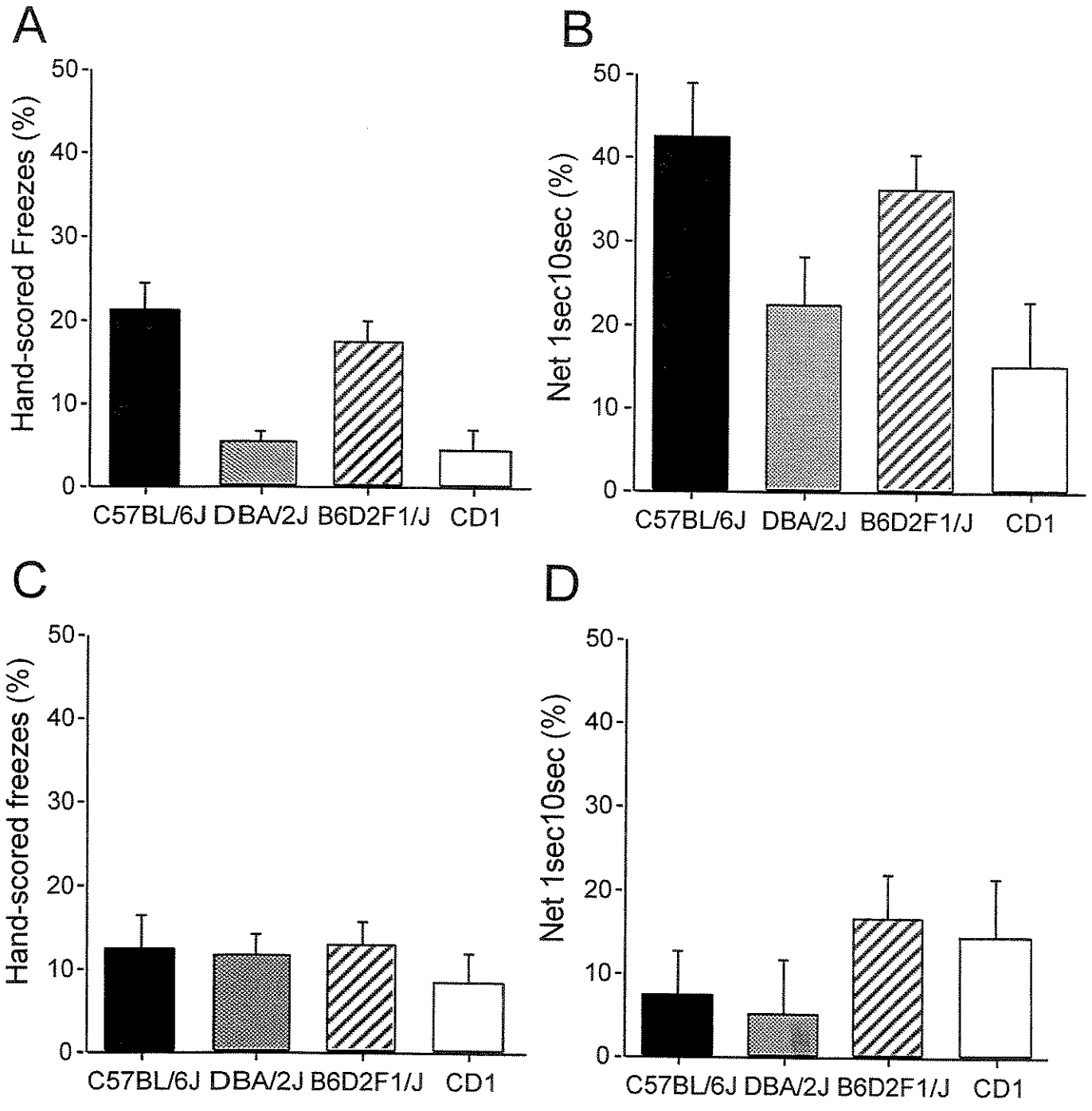

Figure 3. Mean ± SEM of the percentage of **A.** % hand-scored freezes during context testing, **B.** % 1sec10sec baseline-corrected measures during context testing, **C.** % hand-scored freezes during auditory-cued testing, **D.** % 1sec10sec measures during auditory-cued testing, for C57BL/6J, DBA/2J, B6D2F1/J and CD1 strains of Experiment II.

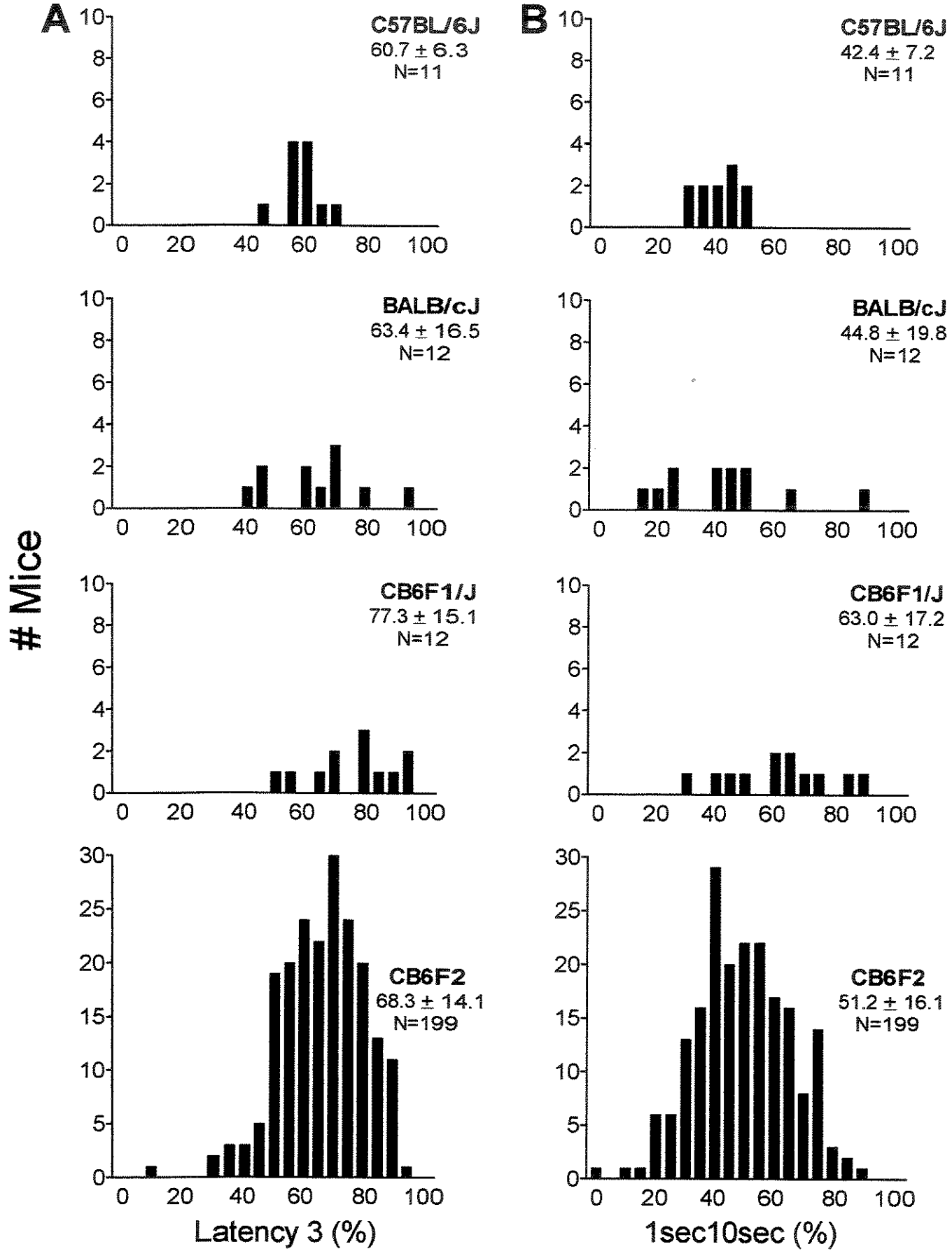

Figure 4. Frequency distributions graphs for **A**. %1sec10sec computer measure and **B.** % Latency 3 computer measure of C57BL/6J and BALB/cJ, CB6F1/J and CB6F2 mice of Experiment III.

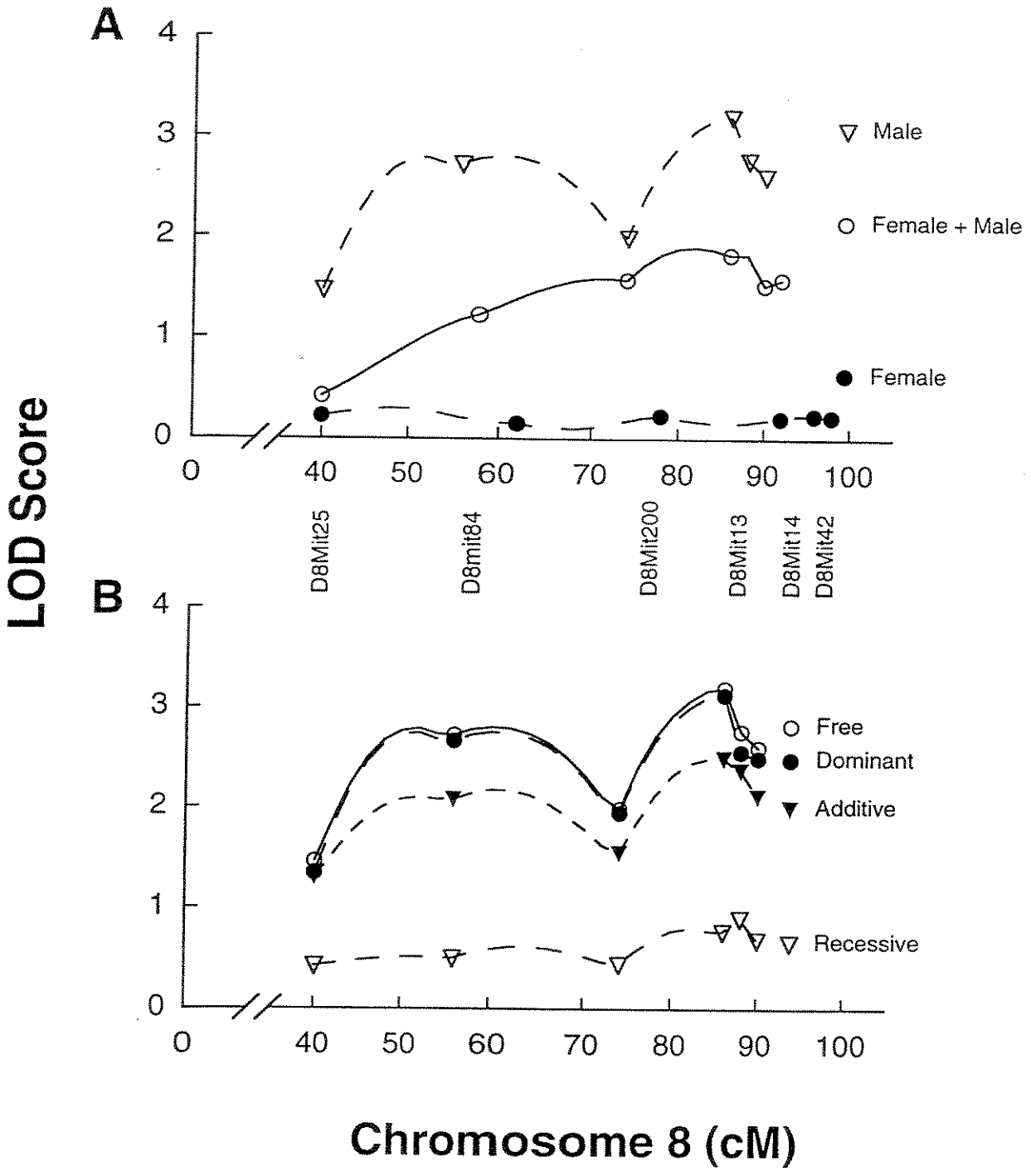

Figure 5. Lod plot on chromosome 8 of contextual learning using 1sec10sec computer measures for **A.** males, females + males and females alone using a free genetic model. **B.** Only males using free, dominant, additive and recessive genetic models. Lod scores are plotted on the vertical axis. Genetic distances along the chromosome (in centimorgans) are plotted on the horizontal axis with markers used for the assessments.

EFFECT OF CIRCADIAN PHASE ON CONTEXT AND TONE FEAR CONDITIONING IN C57BL/6J MICE

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Running head: Phase effect on fear conditioning

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ABSTRACT

This study examined context-dependent and tone-cued fear conditioning during the activity and rest periods of C57BL/6J mice. Wheel running activity was measured continuously as a marker of circadian phase. Two experimental groups kept in a skeleton photoperiod were trained for context and tone fear conditioning at two different time points. Animals were tested every 24 hours for five days to analyze the conditioned response and the rate of extinction. A significant difference between morning and evening groups was observed in the expression and extinction rate of context-dependent fear conditioning but no difference was detected for tone-cued fear conditioning. The evidence of temporal variation of context fear conditioning, but not of tone fear conditioning, two processes based on different neural pathways, suggests that the biological clock may have a modulating effect on the context fear conditioning pathway.

Key words: fear conditioning, C57BL/6J mice, circadian rhythm, skeleton photoperiod, learning, memory.

INTRODUCTION

Rhythmicity has been reported in numerous biochemical, physiological and behavioral variables. These rhythms reflect the combined influences of an endogenous biological clock and a range of exogenous influences (Turek, 1998). Many processes that influence memory display circadian oscillations. Examples include brain gene expression (Holmes et al., 1995; Menegazzi et al., 1994), brain protein levels (Kononen et al., 1990), neurotransmitter binding and synthesis (Wesemann & Weiner, 1990), hormone secretion (Van Cauter & Aschoff, 1989), synaptic excitability (Barnes et al., 1977), and even long-term potentiation (Dana & Martinez, 1984; Harris & Teyler, 1983). In view of this generalized rhythmicity it is not surprising that the efficiency in learning and memory processes also manifest a temporal component. Indeed, several studies have documented evidences of circadian modulation of learning and memory processes.

Non-associative learning seems to have a temporal component. Pigeons submitted to sound stimulation show a lack of habituation in nocturnal sessions as opposed to a clear habituation process during morning sessions (Valentinuzzi & Ferrari, 1997). In the marine fish *Serranus scriba,* avoidance and food-related learning are more effe n the evening than the morning (Kovacevic et al., 1991; Rakic et al., 1991). In rats, acquisi f a passive and active avoidance tasks are facilitated or impaired depending on the time of g (Davies et al., 1973, Hoffman & Balschun, 1992). In addition, multiple retention deficits of active avoidance learning (Holloway & Wansley, 1973a) and passive avoidance learning (Holloway & Wansley, 1973a and b; Wansley & Holloway, 1976), as well as one-trial appetitive learning (Wansley & Holloway, 1975) are well characterized. These retention deficits represent an inability of the animal to retrieve the memory of a learning experience probably as a result of rhythmic changes in some internal state. Consistent with this hypothesis is the observation that lesions of the suprachiasmatic nucleus (the master biological clock in mammals) flatten the multiple retention deficits in active and passive avoidance tasks (Stephan & Kovacevik, 1978). Maze performance

in C57BL/6Ola mice also shows a clear time of day effect (Hoffman & Balschun, 1992). Finally in humans, psychophysiological variables (i.e., reaction time, subjective alertness), short- and long-term memory (remembering a text) and cognitive performance (calculations completed) have also been shown to change as a function of time of day (Leconte, 1989; Guerin et al., 1991; Dijk et al., 1992; Johnson et al., 1992; Koulack 1997).

While very suggestive, these previous studies have failed to establish circadian (as opposed to state-dependent or light-dark dependent) modulation of learning processes. In other words, there are many potential confounding factors when analyzing learning in a circadian context. The different phases of a light-dark (LD) cycle can have a confounding effect due to different visibility during the dark vs the light phase. This can result in different abilities to learn the context. In addition, bright light decreases or masks nocturnal rodents' locomotor activity (Marques & Waterhouse, 1994), thereby interfering with the behavioral manifestation of learning. The locomotor activity rhythm can also have a masking effect on learning processes, depending on the behavioral response being monitored as a measure of learning. For example, active avoidance learning is reportedly enhanced during rodents' active period while passive avoidance is enhanced during the rest period (Davies et al., 1973; Hoffman & Balschun, 1992). In addition, circadian modulation of simple aspects of a task such as baseline values of the behavior measured, reactivity to the novelty of the experimental chamber and subsequent habituation, and sensitivity to cues involved in the learning task should be considered.

We sought to examine whether circadian modulation of different types of learning occurs in mice by controlling confound features such as lighting and independently measuring those factors which could not be controlled (activity, habituation, response to unconditioned stimulus). The acquisition of context and tone-dependent fear conditioning and the extinction of the fear response as well as habituation to the experimental chamber were determined at two different circadian phases. To by-pass any masking effect of a LD cycle we worked with animals in a skeleton photoperiod (Pittendrigh 1965) where a single 15-minute pulse of white light presented every 12 h was capable of synchronizing the rest-activity cycles.

MATERIALS AND METHODS

Animals. Male C57BL/6J mice were purchased from the Jackson Laboratory (Bar Harbor, Maine) at 6 weeks of age. Upon arrival, the animals were group housed (5 per cage) in the animal facility at Northwestern University for 2-3 weeks under a 12h light:12h dark cycle (LD 12:12; lights on at 05:00 central standard time). The temperature was maintained constant at 21 ± 2 °C. Food (Teklad) and water were available *ad libitum*.

The animals were transported to the experimental room where they were placed in individual cages (15x32cm) with running-wheels (11cm in diameter). Cages were placed in lightproof wooden chambers (44cm high x180cm long x 53cm deep) where, for the first three days, the conditions were the same as in the animal facility (LD 12:12, light provided by 40W "cool white" fluorescent bright (300 lux) light). After this interval, the 12h dark phase was replaced by 12h of dim (1 lux at the level of the cage lids) green light; this condition continued for two cycles. Immediately thereafter, a skeleton photoperiod of two 15-min bright white 40W fluorescent light pulses separated by 11.5 and 12h of green dim light was established (i.e., 0.25 white:11.5 green:0.25 white:12 green) and continued for the duration of the experiment (Figure 1A). As is every periodic environmental cycle capable of entraining endogenous rhythms, the skeleton photoperiod is referred to as a *zeitgeber* (time-giver; Aschoff, 1960). The evening light pulse was considered *zeitgeber* time 12 (ZT12), and the morning light pulse *zeitgeber* time 24 (ZT24). If synchronization to the skeleton photoperiod is adequate, activity onset should occur close to ZT12. This ZT was used as reference to determine when training and testing would occur in each group. The dim green light was provided by 6 night-lights (Limelite, Austin Instruments, TX) evenly distributed along the rear wall of each lightproof chamber. Green light was chosen to assure the mice could assess the visual cues in the chamber, since this should be an effective wavelength for the mouse (Balkema & Pinto, 1982).

Forty mice were randomly divided into two groups (n=20 per group). The "day" group was trained 2h after the beginning of the morning light pulse, at *zeitgeber* time 2 (ZT 02). The "night" group was trained two hours after the end of the evening light pulse (ZT 14). Each of these groups was subdivided in control and experimental groups (n=10 per group).

Apparatus. The Freeze Monitor (San Diego Instruments) consisted of a transparent acrylic-conditioning chamber 33cm high, 25cm wide and 21cm deep. A grid floor made of stainless steel rods separated by 0.5cm was connected to a shock generator (Coulberg). A frame (33x33cm) with 16 infrared photobeams (2.5cm between beams) in the horizontal plane surrounded the chamber. The conditioning chamber and surrounding frame were located inside a sound attenuated enclosure (interior dimensions were 50cm high x 65cm wide x 47cm deep). The inside of the enclosure was covered by gray acoustical foam. A small fan located on the top of the right wall generated a background noise of 65 dB. A speaker (Mallory, SC628) was placed on the upper part of the back wall. Two night-lights on the ceiling of the chamber generated green dim light (0.5 -1.0 lux at the level of the grid floor). Distinct geometric shapes of white paper were distributed on the inside walls of the sound chamber to increase the number of visual cues.

The Freeze Monitor software (San Diego Instruments) controlled the shock generator and speaker and recorded data from the photobeams. Latency between photobeam interruptions was recorded as the latency to break the third photobeam in each 5-second interval. If a third beam interruption never occurred, a score of 5-sec was recorded for that interval. These latency values were added to obtain the cumulative latency (in seconds) for each minute of each session. Different types of measures based on latency between photobeam interruptions can be obtained with the Freeze Monitor software, each of which have been previously shown to correlate very well with hand-scored freezing (Valentinuzzi et al, 1998). Latency to third beam interruption is presented here, however, statistical analysis of other Freeze Monitor measures indicated the same group differences and treatment effects.

Procedure

Activity recording. Wheel-running activity was recorded continuously throughout the experiment with a 20-channel Esterline-Angus event recorder in order to monitor entrainment to the skeleton photoperiod. The circadian phase at which each animal was trained and tested was confirmed through the resulting activity records. Animals were considered poorly entrained when activity onset occurred more than 2-3 h from the evening light pulse for more than five successive days; data from such animals were excluded from further analysis. These were: three mice from the night-control group, one from the night-experimental group, two from the morning-control group and two from the morning-experiment.

Context fear conditioning. During the training session animals were placed in the conditioning chamber for 6 minutes. At the beginning of minutes four, five and six each animal in the experimental group received a 1-sec shock (0.6mA). Control animals were handled identically but received no shock. Testing for acquisition of the conditioned response was carried out 24h later. The test session consisted of placing each mouse in the enclosure for an 8-min monitoring period. After this first testing session, the animals were submitted to additional tests every 24h for the next four days to analyze extinction of the conditioned response. After each training and testing session the acrylic chamber was cleaned with 70% ethanol.

Tone Fear Conditioning. Twenty-four hours after the last context fear-conditioning test the animals were submitted to a tone fear conditioning paradigm. The training session consisted of placing the animals in the conditioning chamber for 6 minutes. The tone/shock pairing occurred on minutes three, four and five and consisted of a 20-sec tone immediately followed by a 1 sec, 0.6mA shock. Animals were tested at 24h intervals for five days. Between the training and testing sessions the walls of the environmental chamber were covered with white cardboard to alter visual contextual cues and the gridded floor was covered with acrylic to alter tactile cues. Before each session, the conditioning enclosure was cleaned with 1% acetic acid to alter olfactory cues. The 6-min testing session consisted of presenting the 20-sec tone during the

beginning of the third, fourth and fifth minute. Control animals were exposed to the tones during both training and testing sessions, but never received shocks.

Reactivity to shock. To determine if there was an effect of time of day on sensitivity to the shock, the same control animals were submitted to an additional 4-min session at the appropriate time points (5 mice at ZT02 and another 5 at ZT14). Three shocks (1 sec, 0.6 mA) were given at minutes 2, 3 and 4. The behavioral response at the moment of each shock was observed and recorded by two independent observers. Inter-scorer reliability was determined by dividing coincident behaviors judged by the total number of observations. This reliability value between observers was high (0.93). The categories of behaviors recorded were vocalization, jumping and running judged as either occurring or not.

Analysis of data and statistics

Context fear conditioning results were analyzed using the mean latency/minute per session for each animal; i.e. the cumulative latency of the whole session divided by the number of minutes. For tone fear conditioning, the amount of freezing in response to the tone was analyzed as a "net tone latency" for each animal. The net tone latency was the mean latency per minute during tone presentation minus the mean latency before the tone presentation.

In order to analyze the effect of time of day on unconditioned components of the behavior the control groups were first analyzed separately. Two-way analysis of variance with one grouping variable (time) and one repeated measure (training and testing sessions) were used in each fear-conditioning paradigm.

Three-way analysis of variance with two grouping variables (time of day and experimental/control group) and one-repeated measure (testing days 1 through 5) were performed on the whole set of data (i.e., experimentals and controls) for both context fear conditioning and tone fear conditioning.

Additionally, in order to isolate associative components, the differences between each experimental animal's latency (or net tone latency) and the mean latency (or mean net tone

latency) for the appropriate control group (i.e., morning or evening and session number) were calculated. These difference scores for context fear conditioning as well as for tone fear conditioning were subjected to two-way ANOVAs with one grouping variable (time of day) and one repeated measure (session number).

T-tests were used to compare each behavior type between morning and evening groups when analyzing reactivity to shock.

RESULTS

Entrainment to the skeleton photoperiod was confirmed by the activity records. Figures 1B and C show two representative actograms of well-entrained animals, one trained and tested at ZT02 and another trained and tested at ZT14.

Context fear conditioning. The results of the context fear conditioning paradigm are shown in figures 2 and 3 where the mean cumulative latency during training and testing sessions at ZT14 and ZT02 are depicted. The analysis of the control animals is essential to provide a baseline of the unconditioned responses: spontaneous locomotor activity (circadian rhythm of locomotor activity) and exploration of the novel environment, as well as the analysis of habituation to this environment. Control animals tested at ZT14 and ZT02 differ in latency levels (time of day effect $F(1,13)=8.70$; $p<0.05$) reflecting the effect of the circadian rhythm of locomotor activity. As expected, control mice placed in the testing chamber at ZT02 were less active than their counterparts tested at ZT14. Increases in latency from one session to another are also observed (session effect $F(5,65)=17.10$, $p<0.001$), an effect that can be interpreted as habituation to the testing chamber. A significant time of day by session interaction ($F(5,65)=5.36$; $p<0.001$) suggests that this habituation process is modulated by the circadian phase. ZT14 controls showed more moderate increases in latency with values reaching a maximum of 26.0 ± 2.3 sec/min during testing session 3 while ZT02 control animals reached a latency level of 36.9 ± 3.4 in testing session 3.

The analysis of the whole set of data permits a comparison between the experimental and control animals and consequently a comparison between associative and non-associative components of the response. A clear difference between experimental and control animals is observed (condition effect, $F(1,28)= 40.96$; $p<0.001$) revealing that conditioning to the context occurred in experimental animals at both time points and during the different sessions. An effect of time of day on latency levels ($F(1,28)= 9.51$; $p< 0.005$), and between-session change ($F(4,112)= 15.59$; $p<0.001$) is also evident. During training sessions, experimental animals show the same latency levels and patterns at both ZT times. Both time groups show an increased latency during Test 1 and a progressive decrease from session to session. The latency levels and the between-sessions rate of change of the experimental group differ from control animals and show a time of day effect (time by session, $F(4,112)= 3.71$; $p<0.05$; condition by session, $F(4,112)=22.32$; $p< 0.001$; time by condition by session interaction, $F(4,112)=5.25$; $p< 0.001$).

The latency values observed for experimental animals may result from a combination of associative and non-associative processes (fear conditioning as well as habituation to the environment) while control animals represent only the unconditioned behavior. In order to dissect these two classes of processes (habituation and conditioned response) and better visualize the conditioned component, the difference between the latency of the individual experimental animals and the mean value of the control group of the corresponding sessions was obtained (Figure 4). In testing session 1 there was a clear difference in the conditioned response between the ZT02 and ZT14 groups (time of day effect $F(1,15)=8.40$; $p<0.05$). The group tested during the active phase (ZT14) showed a higher latency level (27.3 ± 2.2 sec/min) than the group tested during the inactive phase (ZT02, 15.5 ± 1.9). Both groups showed extinction of the response (session effect, $F(4,60)=50.56$; $p< 0.001$). The rate of decrease in latency differed between groups (two-way interaction time of day by testing session, $F=(4,60)=11.86$; $p<0.001$). The ZT02 group reached an asymptotic level value (7.9 ± 2.6 sec/min) in the second testing session while the ZT14 group did not reach this level (6.9 ± 1.5 sec/min) until the fifth testing session.

Tone fear conditioning. The results from the tone fear-conditioning paradigm are shown in Figures 5 and 6 where they are expressed as mean latency/minute. Statistical analysis was done with the net tone latency in order to isolate the response to the tone. The response to the tone in control animals provides a way to evaluate sensitivity to the tone and subsequent habituation to it. A decrease in latency levels of controls in response to the tone is observed from one session to another ($F(5,65)=4.74$; $p<0.001$). However no time of day difference was evident in either the response to the tone (time effect, $F(1,13)=0.34$; $p=0.571$) or habituation process (time by session interaction, $F(5,65)=1.09$; $p=0.372$).

The analysis of the net tone latency of experimentals and controls permits a comparison between the experimental and control animals. Experimentals differed from the controls (experimental vs control effect, $F(1,28)=67.51$, $p<0.001$) revealing that conditioning to the tone occurred in experimental animals at both time points and during the different sessions. Extinction of the response also occurred at both time points (session effect, $F(4,112)=9.34$, $p<0.001$). A significant effect of time of day on net tone latency levels ($F(1,28)=7.09$, $p<0.05$) was observed revealing the influence of the activity rhythm. However, the lack of significant effects in any of the time of day interactions indicates the absence of a temporal modulation in the expression and extinction processes to a tone (time by condition, $F(1,28)=2.18$, $p=0.15$); time by session, $F(4, 112)=1.46$, $p=0.22$); time by condition by session, $F(4,112)=0.19$, $p=0.08$).

The dissection of the unconditioned (reactivity to the novelty of the tone as well as habituation to it) and conditioned (conditioning to the tone) components was also intended here. The difference between the net tone latency of the individual experimental animals and the mean value of the control group of the corresponding sessions was obtained. This difference score is depicted in Figure 7 . Although there was a trend for mice trained at ZT14 to exhibit greater latency in response to the tone, the effect of time of day did not reach statistical significance. There were no significant effects of time of day ($F(1,15)=1.51$; $p=0.234$), of session ($F(4,60)=0.15$; $p=0.962$) or of a time of day by session interaction ($F(4,60)=0.64$; $p=0.639$).

Reactivity to the shock. As an indirect measure of the animal's perception of the unconditioned stimulus at both phases we quantified the immediate response to the shock. The type and level of these responses were the same at both time points. The t-tests revealed equal vocalization ($t=0.93$; $p=0.389$), jumping ($t=-0.80$; $p=0.446$) and running ($t=0.006$; $p=0.995$) responses. At both times of day running was the most common response to the shock.

DISCUSSION

The present data show temporal modulation of a hippocampal-dependent learning process. Expression of a context-dependent fear-conditioned response was more pronounced and the rate of extinction was slower among mice tested during their active phase than those tested during their inactive phase. In contrast, cued conditioning, a hippocampal-independent learning process, was not influenced by time of day.

During context fear conditioning sessions the analysis of the control animals enabled us to quantify the unconditioned responses. The significantly different latency levels between morning and evening control groups could be the manifestation of the circadian rhythm of locomotor activity. Animals tested during their active period show lower latency values while those tested during their rest period show higher latency values. The increase in latency in the successive testing sessions can be interpreted as habituation. Rodents placed in a novel environment will characteristically show an initial high level of activity that decreases over time (Cerbone & Sadile, 1992). Isolating this habituation component enabled us to detect a statistically significant effect of time of day in this habituation process.

This temporal effect on the habituation component could be the cause of the observed time of day effect on context fear conditioning. In other words, circadian phase could be modulating the unconditioned components rather than the conditioned component of the behavior. To address this issue the associative learning component was isolated by calculating the difference scores. This procedure was effective in extracting a clear time of day effect on the

conditioned component of the behavior. Thus it appears both associative and non-associative learning processes are affected by time of day.

The locomotor activity rhythm can have a masking effect on learning processes, depending on the behavioral response being monitored as a measure of learning. Considering this, one might hypothesize that measures of a freezing response would be elevated during the inactive period as freezing is measured by inactivity. However, our data show the opposite: animals tested during the active phase express higher latency values (more freezing behavior), ruling out a facilitating effect of the inactive phase on freezing. The finding that the behavioral response during training of context fear conditioning is the same at both times of day and that there is no time of day effect on tone fear conditioning also indicate that the rest-activity rhythm and the context fear conditioning response are independent processes. If the activity rhythm had a masking effect on the freezing response, a time of day effect would have been observed during training of context fear conditioning as well as during training and testing of tone fear conditioning.

We can not rule out the possibility of a temporal variation in perception of contextual visual, tactile and/or olfactory cues and/or in attentional states that could alter sensory input. However, this seems unlikely since the level and pattern of latency during training are the same at both times for both control and experimental animals. We see no evidence of a time of day difference in sensitivity to the shock because the level of vocalization, jumping and running in response to the shock was found to be similar at both times of day.

In contrast with context fear conditioning, cued-fear conditioning is independent of circadian phase. This is clearly observed when analyzing the difference scores of Figure 7 which isolates the conditioned component of the response. Similarly, unconditioned components also seem to be time-independent. Because the groups tested at ZT02 and ZT14 showed the same level of reactivity to the tone when evaluated as net tone latency, there is no indication that sensitivity to the sound stimuli is affected by time of day, at least when measuring latency values. Our results agree with Chabot and Taylor (1992) who showed that sensitivity of rats to

sound stimuli was the same at different times of the day. In addition, the control animals' decrease in the net tone latency from session to session may be indicative of habituation to the tone. No time of day effect in this process was detected.

Even though, when isolating the response to the tone, fear conditioning as well as reactivity and subsequent habituation to the tone do not show a circadian phase effect, components related to reactivity to the chamber do show a difference. For example, for both experimentals and controls, the first three minutes of the training session (before the tone/shock pairing) show different latency levels between ZT14 and ZT02. Another example is the first three minutes of tests 4 and 5 (before tone presentation) where both time groups also show different latency levels (Figures 5 and 6). We believe that these different context-dependent responses are mainly due to the previous experience of these animals: repeated exposure to the experimental chamber (controls) and acquisition and extinction of context fear conditioning (experimentals).

The amygdala is involved in the formation of associations between aversive unconditioned stimuli and a variety of conditioned stimuli types as well as in the subsequent control of a fear response (Maren & Fanselow, 1996). However, different neural pathways mediate the analysis of the stimulus properties depending on the characteristics of the specific stimuli involved in the situation (Kim & Fanselow, 1992; Paylor et al., 1994; Phillips & LeDoux, 1992; Phillips & LeDoux, 1994). For modality-specific conditioned stimuli either thalamic or cortical inputs to the amygdala suffice as transmission routes. For more complex stimuli such as those that involve spatial organization, the hippocampus, as part of its general role in spatial processing and the projection from the subiculum to the amygdala are required (Eichenbaum et al., 1992).

The participation of a complex and integrative structure such as the hippocampus (Eichenbaum et al., 1992) in context-dependent fear conditioning but not in tone-cued fear conditioning may lead to increased susceptibility of the former to external modulation. Indeed, a number of variables influence contextual, but not tone-dependent fear conditioning.

Postconditioning isolation (Rudy, 1996), retention interval (Rudy & Morledge, 1994), stimulus preexposure (Rudy & Morledge, 1994; Rudy, 1996) and age (Rudy, 1993) affect conditioning to a context. This suggests a vulnerability in the consolidation process of the context characteristics. Our data add time of day as another modulatory variable.

The biological clock could be directly or indirectly affecting the hippocampal complex. Indeed, several hippocampal variables manifest rhythmicity. The expression of glucocorticoid and mineralcorticoid receptor mRNA (Holmes et al., 1995), as well as 5-HT2C receptor mRNA expression (Holmes et al., 1997) show diurnal rhythms. This 5-HT2C receptor mRNA rhythm parallels time-dependent variations in 5-HT2C agonist-induced behaviors in openfield test (Holmes et al., 1997). The hippocampus also expresses rhythmicity in Jun-B and c-fos mRNA (Menegazzi et al., 1994) and in Fos protein levels (Kononen et al., 1990). In addition, plasma hormonal rhythms have direct effects on the hippocampus. Plasma corticosterone level, which is itself driven by the circadian clock (Van Cauter & Aschoff, 1989) is known to have a significant effect on cognition mainly by acting on the hippocampus (Lupien & McEwen, 1997). Corticosterone levels affect activation of kainate receptors (Joels et al., 1996) and mRNA levels of GABAA receptor subunits (Orchinik et al., 1994) consequently affecting hippocampus excitability. Indeed circadian rhythms in synaptic excitability (Barnes et al., 1977) and long term potentiation (Harry & Teyler, 1983; Dana & Martinez, 1984) have been detected in rat hippocampus. Rhythmicity in biochemical variable that may underlie the diurnal rhythm in context learning still remain to be investigated.

The adaptive value, if any, of having temporal modulation of context fear conditioning but not of tone fear conditioning, remains unclear. Discrete stimuli may be perceived more as an imminent danger which can appear at any time and at any place. An animal cannot afford to have its ability to associate such stimuli with danger reduced at a particular time of day. On the other hand, learning a context may be more related to foraging, mating and establishing territory. These are all activities that take place during the active phase. A decreased capacity to learn

contextual cues during the inactive phase may not be much of a threat to an animal in a well-known context, its nest.

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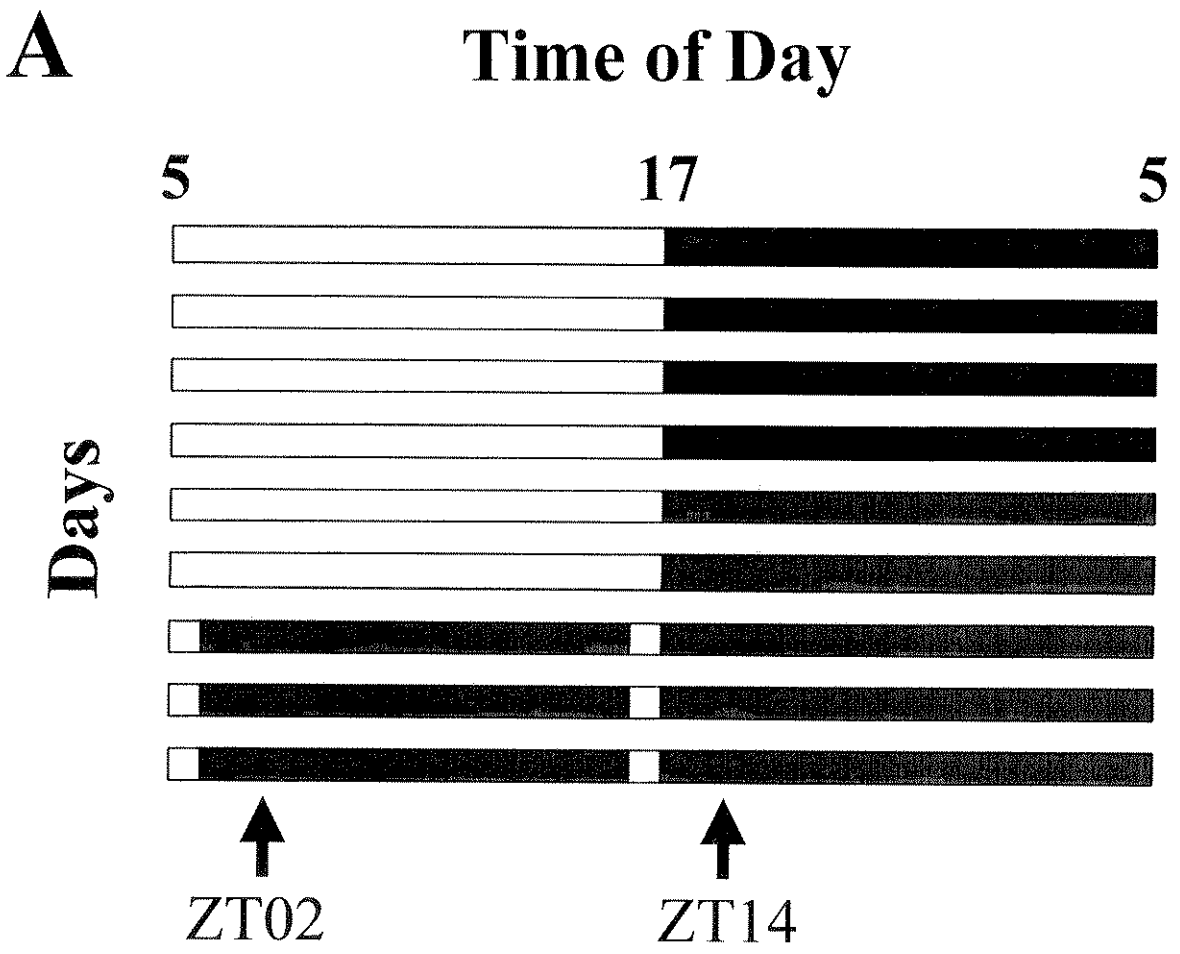

Figure 1. A. Schematic representation of the light-dark schedule and transference to the skeleton photoperiod. Time is plotted across the horizontal axis (each bar represents 24h) and successive days are plotted beneath one another. White areas indicate lights-on, black areas indicate lights-off and grey areas represent dim green light. The arrows indicate the times of testing for the "day" and "night" groups respectively.

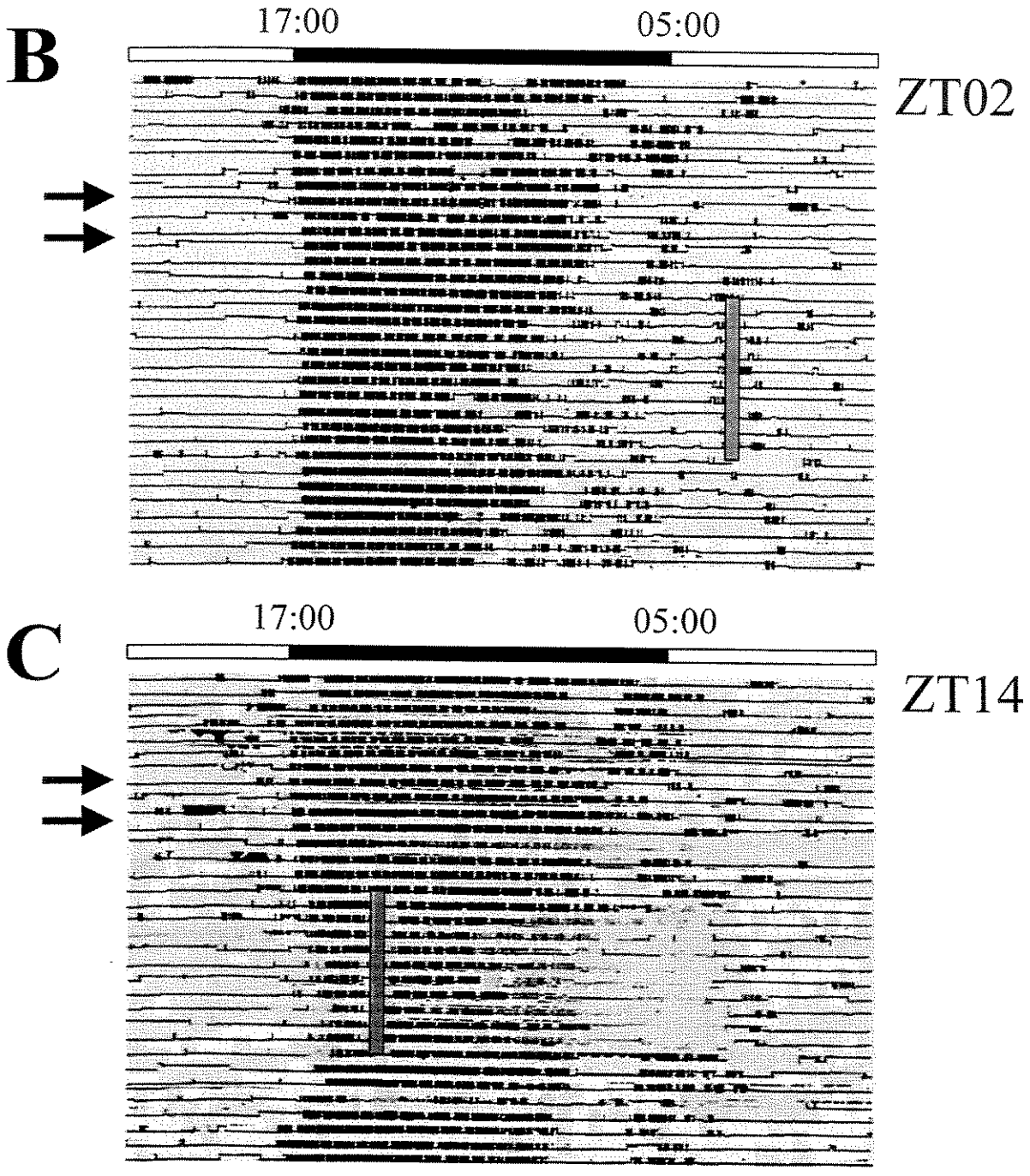

Figure 1. B. Representative actogram of an animal submitted to fear conditioning at ZT02. **C.** Representative actogram of an animal submitted to fear conditioning at ZT014. Time is plotted across the horizontal axis (24h per line) and successive days are plotted beneath one another. The bar on the top indicates the LD cycle during the first week. The first arrow indicates when the dark phase was replaced by green dim light and the second one indicates when the skeleton photoperiod started. The vertical grey bars indicate when, during the activity/rest cycle, training and testing for both context and tone fear conditioning occurred for each group.

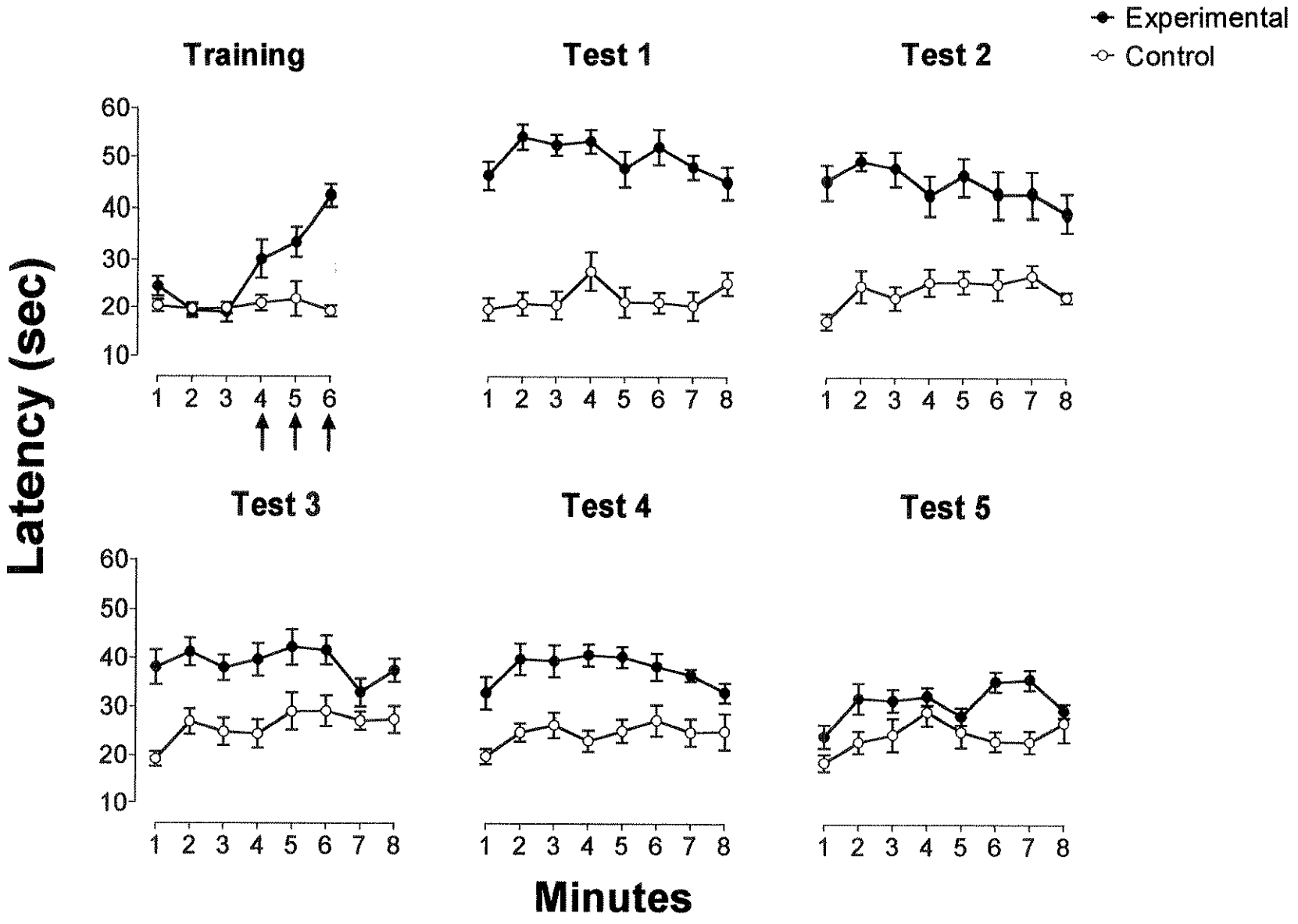

Figure 2. Mean ± SEM cumulative latency for each minute of the training and five testing sessions for the control group (open circles) and the experimental group (black circles) during context fear conditioning of the animals trained and tested two hours after the evening light pulse (ZT14). Vertical Arrows indicate 1-sec, 0.6 mA shocks given to the experimental group.

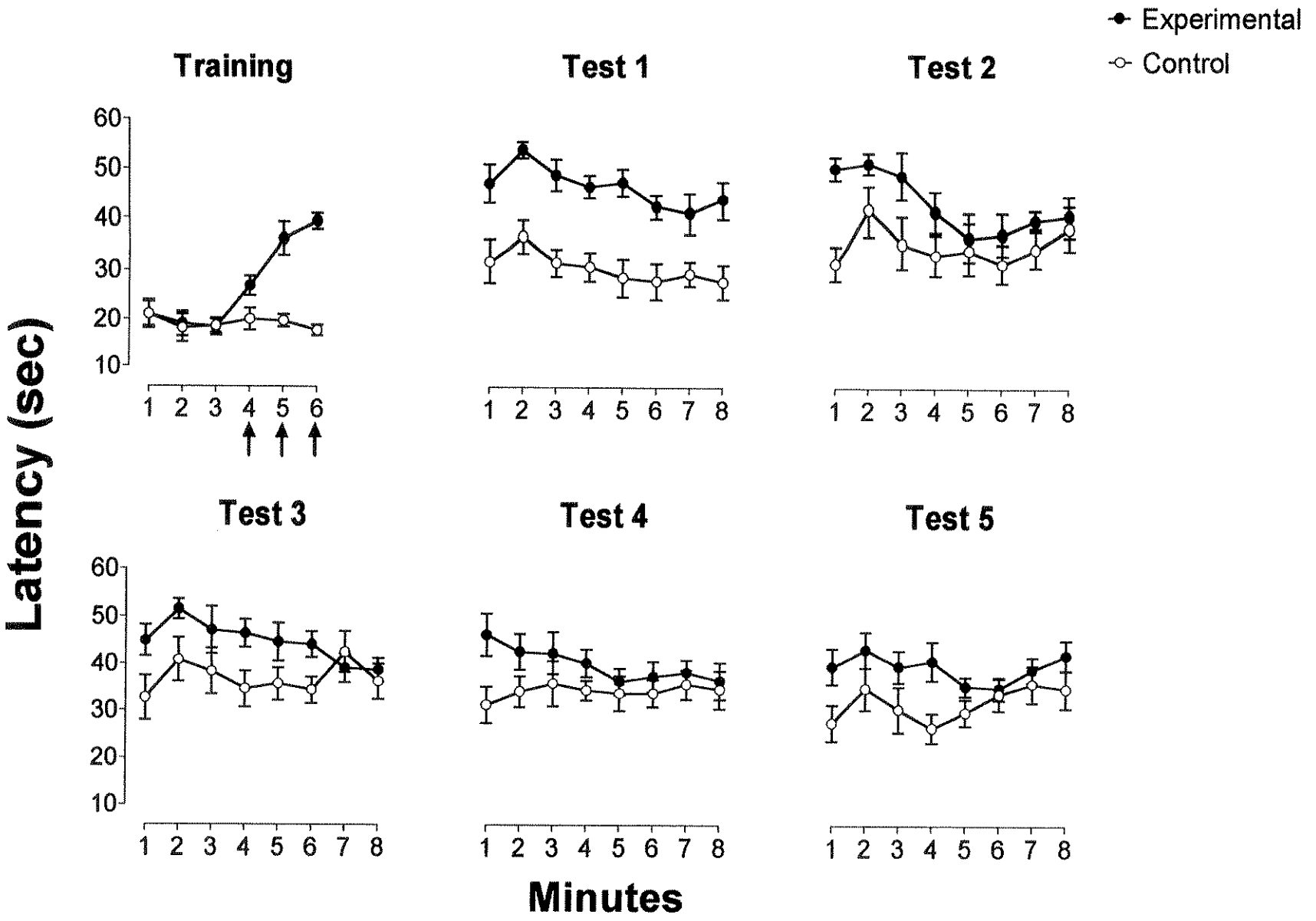

Figure 3. Mean ± SEM cumulative latency for each minute of the training and five testing sessions for the control group (open circles) and the experimental group (black circles) during context fear conditioning of the animals trained and tested two hours after the morning light pulse (ZT02). Vertical arrows indicate 1-sec, 0.6 mA shocks given to the experimental group.

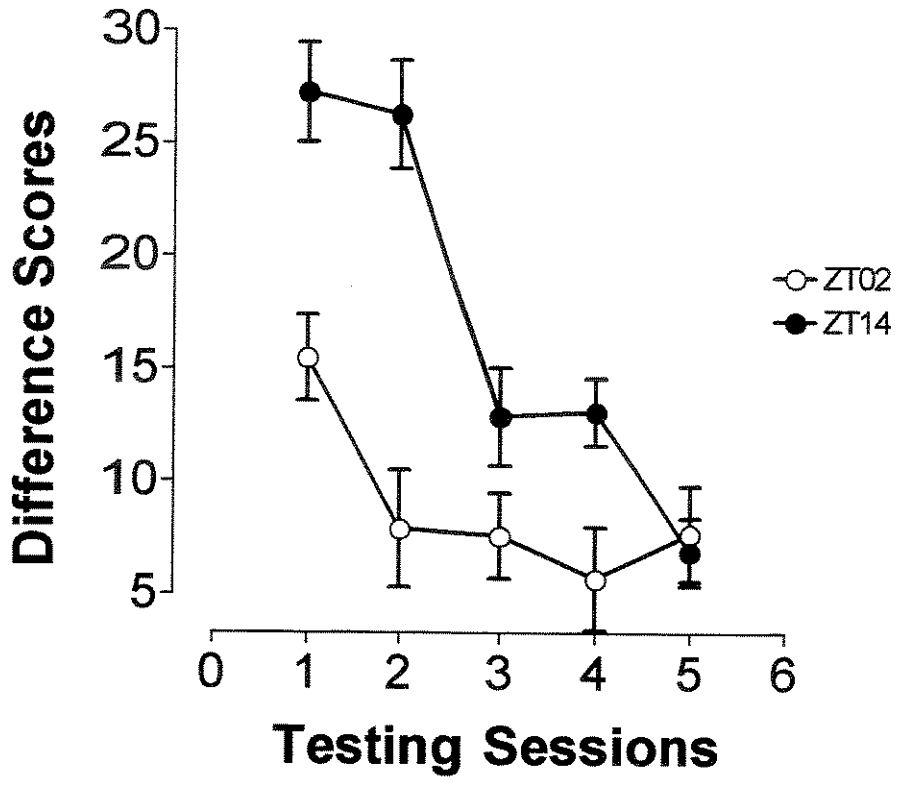

Figure 4. Mean ± SEM latency per minute for each testing session of the experimental animals tested for context fear conditioning at ZT14 (black circles) and ZT02 (open circles) after extracting the respective control values (difference scores, see Methods).

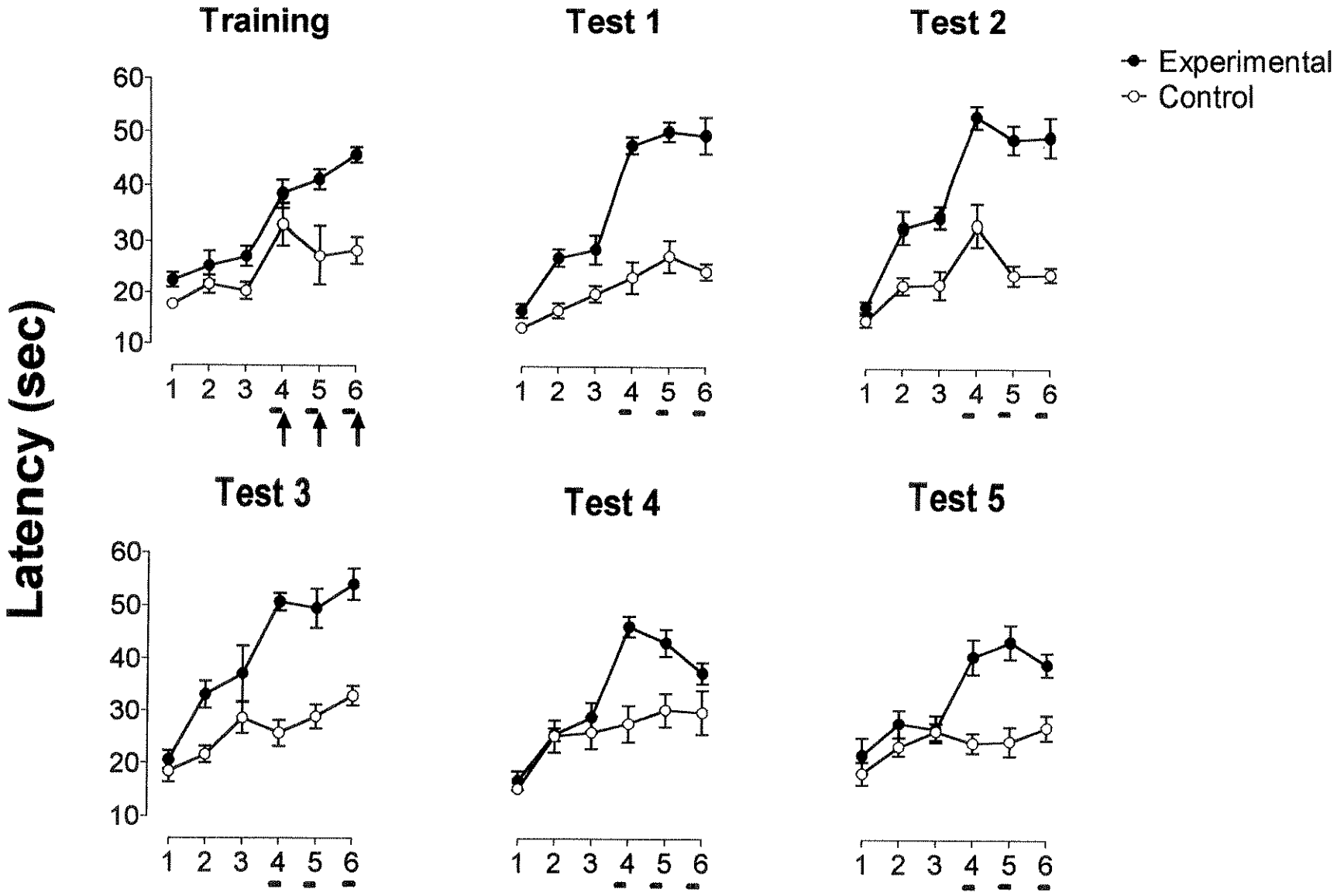

Figure 5. Mean ± SEM cumulative latency for each minute of the training and five testing sessions for the control group (open circles) and the experimental group (black circles) during tone fear conditioning of the animals trained and tested two hours after activity onset (ZT14). Black horizontal bars indicate 20-sec tones presented in every session to experimental and control animals. Vertical Arrows indicate 1-sec, 0.6 mA shocks given to the experimental group.

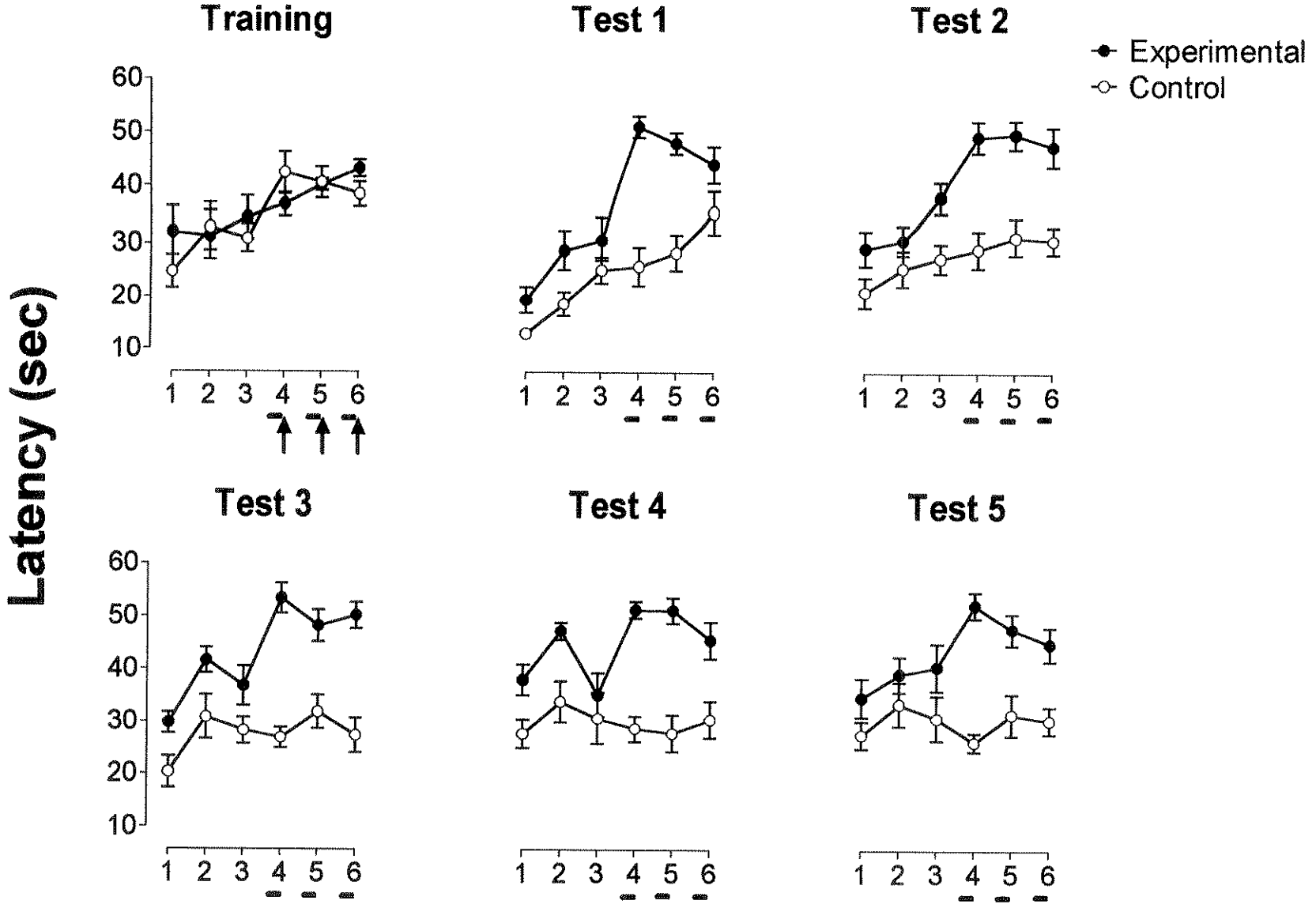

Figure 6. Mean ± SEM cumulative latency for each minute of the training and five testing sessions for the control group (open circles) and the experimental group (black circles) during tone fear conditioning of the animals trained and tested 14 hours after activity onset (ZT02). Black horizontal bars indicate 20-sec tones presented in every session to experimental and control animals. Vertical Arrows indicate 1-sec, 0.6 mA shocks given to the experimental group.

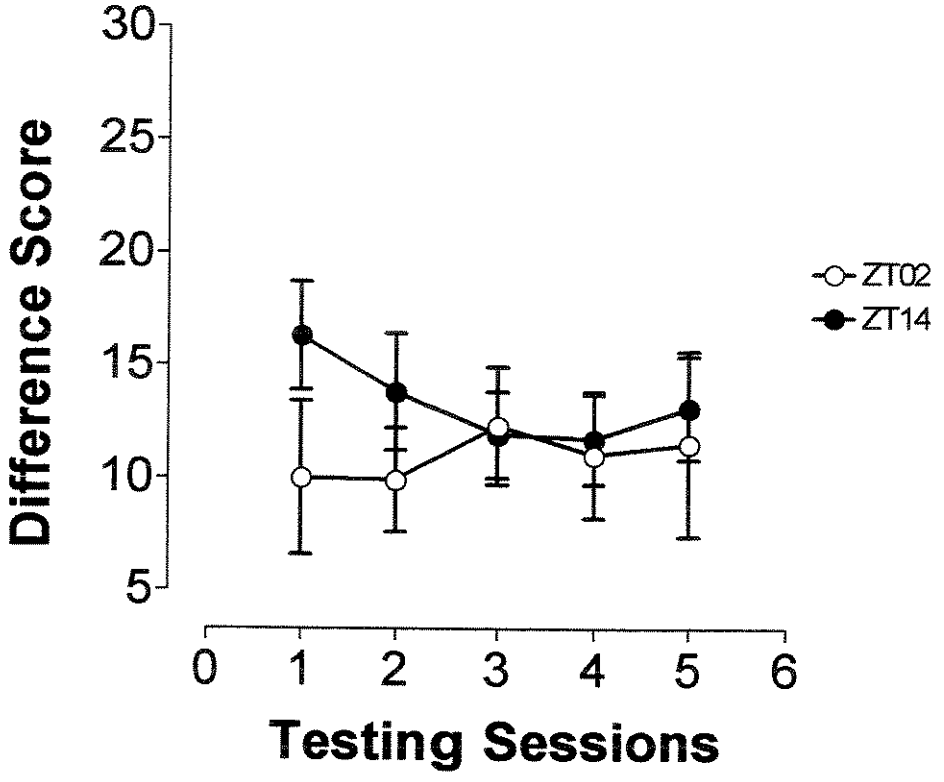

Figure 7. Mean ± SEM latency per minute for each testing session of the experimental animals tested for tone fear conditioning at ZT14 (black circles) and ZT02 (open circles) after extracting the control values (difference scores, see Methods).

CONCLUSÕES

Como discutido anteriormente, na literatura existem evidências de modulação temporal no desenpenho de vários tipos de aprendizagem, porém, em geral, a comprovação de uma ritmicidade na aprendizagem, propriamente dita, fica difícil devido à influência de fatores tanto externos (i.e., ciclo claro-escuro) como internos (i.e., ciclo de atividade locomotora, de sensibilidade a estímulos) que, direta ou indiretamente, influenciam a aprendizagem e a memória. Assim, se faz indispensável controlar estes fatores para que se possa chegar a conclusões definitivas. Este trabalho preocupou-se com o controle de alguns fatores mascaradores e, a partir das possibilidades colocadas analisou-se o componente temporal de dois tipos de aprendizagens.

1. Em condições de claro-escuro, camundongos manifestaram um ritmo diurno da reatividade a um campo aberto, com maiores níveis de locomoção durante o período escuro. A habituação a curto e a longo-prazo desta resposta também foi afetada pela fase do ciclo claro-escuro. A habituação a longo-prazo aconteceu durante a fase clara, enquanto que a habituação a curto-prazo só durante a fase escura.

Por outro lado, em condições de fotoperíodo esqueleto, não foi observado nenhum efeito de fase no comportamento no teste do campo aberto. Os níveis de deambulação, assim como a habituação a curto e a longo-prazo, foram iguais tanto na fase ativa como na fase inativa.

Podemos concluir que a ativação comportamental que a exposição a um campo aberto produz, e a subsequente habituação a este contexto novo, não manifestaram um ritmo circadiano, porém foram fortemente mascarados pelas condições de iluminação.

Em outras palavras, só em condições de claro-escuro foi possível observar uma variação temporal nestes processos, o que sugere que os ritmos observados são apenas respostas às flutuações diárias de iluminação externa.

2. No Experimento I a análise foi feita com medidas comportamentais obtidas por meio de observação direta. No experimento seguinte procurou-se automatizar o registro do comportamento. A avaliação do condicionamento clássico aversivo em camundongos por meio de uma técnica computadorizada, baseada na medida da latência entre a interrupção de sensores infravermelhos, permitiu observar que:

Medidas computadorizadas obtidas durante testes de condicionamento aversivo a um contexto mostraram elevada correlação com as medidas obtidas por observação direta nos mesmos testes em camundongos C57BL/6J.

As bem conhecidas diferenças entre as linhagens de camundongos C57BL/6J e DBA/2J no desempenho de condicionamento aversivo a um contexto identificadas por medidas de observação direta, foram também detectadas por este sistema automatizado.

Como exemplo do uso da técnica, as medidas computadorizadas foram suficientes para detectar, num cruzamento (BALB/cJ x C57BL/6J)F2, um sugestivo *locus* genético para condicionamento a um contexto no cromossoma 8 utilizando a análise genética de QTL.

O conjunto destes resultados permitiu demonstrar a validade desta técnica automatizada na medida do comportamento de *freezing* em situações de condicionamento clássico aversivo. Assim, seguidamente, o método foi utilizado para a análise temporal deste tipo de aprendizagem associativa.

3. O processo de condicionamento aversivo a um contexto variou segundo a fase do ciclo de atividade-repouso em que os treinos e testes aconteceram. A aquisição e a extinção da resposta condicionada, foram maiores durante a fase ativa do animal em relação à fase inativa. Contrariamente, o processo de condicionamento som-choque não foi afetado pela hora do dia em que os testes foram realizados.

A evidência de uma variação temporal no condicionamento aversivo a um contexto, porém não do condicionamento aversivo a um som, processos baseados em diferentes vias neurais, levanta questões relacionadas com as interações entre o sistema circadiano e os substratos neurais desses processos. Sugerimos que o relógio biológico tenha um efeito no hipocampo, estrutura essencial no condicionamento a um contexto.

Adicionalmente, no mesmo experimento, o processo de habituação à caixa de condicionamento foi analisada. Este processo, avaliado pelo aumento de latência para interrupção de sensores de uma sessão a outra (ou seja, a diminuição de atividade entre as sessões), mostrou um componente temporal. Este resultado coincide com a hipótese de modulação temporal da função hipocampal, já que se acredita que esta estrutura também tem um papel no processo de habituação a um contexto novo.

Estas observações sobre habituação numa caixa de condicionamento contradizem os resultados obtidos anteriormente sobre habituação num campo aberto. Por isso, acreditamos ser de interesse a comparação e verificação das diferenças entre estes dois conjuntos de dados. Primeiro, as condições físicas dos contextos utilizados em ambos experimentos são completamente diferentes com respeito às dimensões e tipo de material de construção. As características do contexto podem ser correlacionadas com diferentes graus de medo, estresse, curiosidade, estado de alerta e portanto, diferentes manifestações

comportamentais (Walsh & Cummins, 1976). Tais evidências podem estar relacionadas com nossos dados. A caixa de condicionamento utilizada, além de ser coberta, era um ambiente pequeno (525cm^2), similar ao tamanho da gaiola-viveiro (480cm^2). Por outro lado, o campo aberto caracterizou-se por uma dimensão significativamente maior (3696 cm^2) em relação à gaiola-viveiro, além de ser um espaço aberto. Estas duas características são de natureza aversiva para camundongos que, de acordo com o repertório natural da espécie, procuram espaços pequenos e protegidos. Segundo, o método de quantificação do comportamento utilizado em cada caso foi totalmente diferente. Na caixa de condicionamento a medida era automatizada utilizando sensores infravermelhos distanciados a 2.5 cm, enquanto que no campo aberto, a medida foi manual e a distância entre as linhas utilizadas para quantificar os cruzamentos foi de 20 cm. Em outras palavras, provavelmente a sensibilidade do método utilizado no campo aberto (8 vezes menos sensível que no método automatizado), não foi suficiente para detectar um efeito temporal no processo de habituação ao contexto.

Desta maneira acredita-se que a contribuição do presente trabalho foi no sentido de separar a modulação temporal originada de fatores externos (i.e., ciclo claro/escuro) e internos (i.e., ciclo de atividade locomotora) de uma possível modulação mnemônica propiamente dita. Demonstrou-se a forte interferência que o ciclo claro-escuro pode ter na expressão da habituação a um campo aberto. Além disso sugere-se que o condicionamento aversivo expressa um componente temporal embora a presença deste componente depende do tipo de estímulo condicionado utilizado, e que a habituação à caixa de condicionamento também varia temporalmente.

Em seu conjunto, os dados experimentais e as análises desenvolvidas no presente trabalho revestem-se de importância principalmente por estimularem a investigação da organização temporal de processos de aprendizagem, área que somente agora parece começar a receber atenção sistemática de cronobiologistas. Além disso, as questões levantadas apontam para o interesse na busca das interações entre o relógio biológico e sistemas neurais envolvidos na organização, modulação e controle de processos de aprendizagem e memória.

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