Faculdade de Medicina

Programa de Pós-Graduação em Medicina: Ciências Médicas

AVALIAÇÃO DO EFEITO DO PADRÃO TEMPORAL SOBRE PARÂMETROS COMPORTAMENTAIS E BIOQUÍMICOS NA RESPOSTA AO ESTRESSE POR RESTRIÇÃO EM RATOS WISTAR

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Orientadora: Prof. Dra. Iraci Lucena da Silva Torres

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"Pensar apenas ou desejar somente, nunca levou ninguém a lugar nenhum. É necessário também ação."

W. Shakespeare.

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SUMÁRIO

LISTA DE FIGURAS

RESUMO

A ritmicidade biológica pode ser entendida como a expressão cíclica de um fenômeno biológico. Marcadores fisiológicos como a melatonina e a corticosterona estão sob a influência dos relógios endógenos e respondem a diferentes variações de intensidades de luz podendo estar envolvidos com alterações de comportamento. Vários relatos têm documentado aumento em suas concentrações na presença de situações estressantes. Esta dissertação teve como objetivo avaliar o efeito do padrão temporal sobre parâmetros comportamentais e bioquímicos na resposta ao estresse por restrição em ratos. Utilizou-se ratos Wistar machos com 70 dias (150-180g), divididos em 4 grupos por *Zeitgebers* (ZT): 0, 6, 12 e 18, subdividindo em: controle, imediatamente, 6 e 24 horas após uma sessão de estresse por restrição. Para a avaliação comportamental utilizou-se os aparatos de campo aberto (CA) e de Labirinto em Cruz Elevado (LCE). Foram analisados os níveis séricos de: corticosterona, melatonina, glicose e das atividades das enzimas NTPDases e 5'nucleotidases. Os dados foram expressos em Média±EPM, e analisados utilizando o teste ANOVA de uma via/SNK, $P<0.05$. As respostas comportamentais avaliadas no LCE mostraram um padrão temporal do número de PHD e NPHD, e no tempo gasto nos braços abertos e fechados. Os dados do CA apresentaram padrão temporal apenas no número de bolos fecais. A exposição ao estresse por restrição foi capaz de romper o padrão rítmico observado nos comportamentos avaliados no LCE e CA. Quanto aos biomarcadores sistêmicos observou-se que imediatamente após estresse há uma perda do padrão temporal dos níveis de corticosterona e 6 h após o estresse há um atraso de fase de ZT0 para ZT18, enquanto que 24 horas após o estresse os níveis retornam ao padrão temporal normal; imediatamente após o estresse houve uma supressão do pico normal de melatonina (ZT18), apresentando o pico pela manhã (ZT0); 6 e 24 horas após o estresse ocorreu uma perda de padrão temporal. Os níveis de glicose tiveram o maior pico em ZT18 similar à melatonina. Observou-se uma inversão do padrão rítmico associado a uma diminuição no ZT18 que permaneceu até 6 horas após nos níveis de glicose, imediatamente após o estresse, sendo que o padrão temporal retornou 24 horas após o estresse. Quanto à atividade enzimática, observou-se que o estresse agudo provoca uma diminuição na atividade das enzimas nucleotidases dependente do período do dia em que o animal é exposto ao estresse, e esse efeito parece persistir por pelo menos 24 horas. Os resultados dessa dissertação demonstram que o horário do dia o qual o experimento é realizado altera parâmetros bioquímicos e comportamentais influenciando os resultados obtidos, o que pode ser um confundidor na interpretação dos resultados.

***Palavras-chave**: Cronobiologia, estresse, comportamento, nucleotidases,soro de ratos.*

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ABSTRACT

The biological rhythm can be understood as an expression of a cyclic biological phenomenon. Physiological markers such as melatonin and corticosterone are under the influence of endogenous clocks and respond to different variations of light intensities may be involved in behavioral changes. Several reports have documented an increase in their concentrations in the presence of stressful situations. This work aimed to evaluate the effect of the temporal pattern of behavioral and biochemical parameters in response to stress by restraint in rats. We used male Wistar rats with 70 days (150-180g) were divided into four groups by Zeitgebers (ZT) 0, 6, 12 and 18, subdivided into: control, immediately, 6 and 24 hours after a session of stress restriction. For the behavioral assessment, we used the open field apparatus (OF) and Plus Maze (PM). We analyzed the serum levels: corticosterone, melatonin, glucose and activity of the enzymes NTPDase and 5'nucleotidase. Data were expressed as Mean + SEM, and analyzed using one-way ANOVA/SNK, $P < 0.05$. Behavioral responses measured in the PM showed a temporal pattern in the number of PHD and NPHD, and time spent in open arms and closed. Data from OF showed only temporal pattern in the number of dung pats. Exposure to restraint stress was able to break the rhythm pattern observed in the behaviors evaluated in the PM and OF. In relation the systemic biomarkers was observed immediately after stress there is a loss of temporal pattern of corticosterone levels and 6 h after stress there is a phase delay of ZT0 to ZT18, whereas 24 hours after stress levels return to standard time normal; immediately after the stress showed a suppression of normal melatonin peak (ZT18), with the peak in the morning (ZT0), 6 and 24 hours after stress was a loss of temporal pattern. Glucose levels had the highest peak at ZT18 similar to melatonin. Observed a reversal of the rhythmic pattern associated with a decrease in ZT18, held until 6 hours after glucose levels immediately after stress, and the temporal pattern returned 24 hours after stress. In enzymatic activity, we observed that acute stress causes a decrease in activity of enzymes dependent nucleotidases time of day at which the animal is exposed to stress, and this effect seems to persist for at least 24 hours. The results of this dissertation show that the time of day which the experiment is performed biochemical and behavioral changes influencing the results, which may be a confounder in the interpretation of results.

Keywords: Chronobiology, stress, behavior, nucleotidases, serum of rats.

LISTA DE ABREVIATURAS

ACTH = Hormônio Adrenocorticotrófico

ADP = adenosina 5’ difosfato

AMP = adenosina 5’ monofosfato

ATP = adenosina 5’ trifosfato

ATP = adenosina trifosfato

CA= Campo-Aberto

CRH = Hormônio Liberador de Corticotrofina

(E-)NPP = (Ecto-)nucleotídeo pirofosfato/fosfodiesterase

(E-)NTPDase = (Ecto-)nucleosídeo trifosfato difosfohidrolase

EPM = *Pluz Maze*

GCs = Glicocorticóides

HHA = Hipotálamo Hipófise Adrenal

HIOMT = hidroxi-indol-O-metil-transferase

IGL = folheto intergeniculado

LCE = Labirinto em Cruz Elevado

NAT = N-acetiltransferase

NSQ = Núcleo Supraquiasmático

PKA = proteína quinase A

OF = *Open-Field*

SN = sistema nervoso

SNC = sistema nervoso central

I. INTRODUÇÃO

INTRODUÇÃO

A cronobiologia é a ciência que investiga e quantifica o mecanismo temporal biológico, descrevendo as manifestações rítmicas da vida. Sabe-se que os chamados ritmos biológicos apresentam frequências que variam de 1 milisegundo a anos e são geneticamente determinados, evolutivamente conservados e endogenamente gerados (Cardoso, 2009). Oscilações periódicas que variam de 20 a 28 horas são chamadas de ritmos circadianos, sendo influenciados por dicas do ambiente externo (principalmente do ciclo claro-escuro) e do ambiente interno (relacionadas à fisiologia do organismo) (Hanifin & Brainard, 2007) e sua influência em processos biológicos tem chamado a atenção de pesquisadores de diversas áreas do conhecimento (Ichikawa, 2001).

Os ritmos circadianos têm origem endógena e sincronizam com o ciclo claro-escuro (Morin, 1994). A geração endógena desse ritmo é provavelmente causada pelo padrão sequencial de reações orgânicas em nível celular e molecular, possibilitando que o metabolismo orgânico ocorra em fases e sempre em uma mesma frequência, e assim, funcionando como um marcador de tempo (*timer*) (Cipolla-Neto *et al*., 1988). A temperatura corporal e as taxas metabólicas possuem importantes padrões circadianos, objetivando manter a homeostase quanto ao processamento de alimentos e energia, por meio da regulação da expressão e/ou atividade de enzimas envolvidas no metabolismo de lipídeos, aminoácidos, glicogênio e glicose (Mortola, 2007). Adicionalmente, muitos hormônios envolvidos no metabolismo exibem oscilação circadiana, como a insulina, o glucagon, a adiponectina, o cortisol (corticosterona em ratos), a melatonina, a leptina e a grelina, (Froy, 2007).

Nos mamíferos, os ritmos são controlados por um sistema hierárquico constituído de um grupo de estruturas, incluindo o núcleo supraquiasmático (NSQ), o folheto

intergeniculado (IGL) e a glândula pineal (Bob & Fedor-Freybergh, 2008). A glândula pineal exerce importante papel na produção da melatonina, um marcador de ritmo biológico.

A luz exerce um papel crucial na complexidade do ritmo circadiano. Especificidades geográficas da luminosidade podem ser relevantes no processo de adaptação, fortalecendo ou enfraquecendo o impacto de componentes da rede circadiana que podem influenciar as diferentes respostas orgânicas incluindo a resposta ao estresse (Moser *et al*., 2006).

Selye, em 1936, demonstrou que qualquer situação de estresse (negativas ou positivas que se refiram a situações de forte emoção) ativa o eixo Hipotálamo-Hipófise-Adrenal (HHA) com consequente aumento do cortisol (corticosterona em ratos). A ativação do eixo HHA resulta no aumento da secreção de hormônio liberador de corticotrofina (CRH) pelo hipotálamo, de hormônio adrenocorticotrófico (ACTH) pela hipófise anterior e de glicocorticóides pelo córtex da adrenal (De Kloet *et al*., 1993). Em humanos, a secreção noturna de ACTH e de cortisol ocorre de modo pulsátil, alcançando seu nível mais baixo na primeira metade da noite, aumentando rapidamente ao aproximar-se o despertar, quando sua secreção é máxima (entre as 6 e as 10 horas da manhã). No rato, a secreção de corticosterona ocorre no turno inverso, com picos no início da noite (Perreau-Lenz *et al*., 2003). Marcadores fisiológicos como melatonina e a corticosterona estão sob a influência de relógios endógenos e respondem a diferentes variações de intensidades de luz podendo estar envolvidos com alterações bioquímicas e comportamentais (Goldbeter, *et al*., 2010).

O sistema purinérgico, composto por nucleotídeos e nucleosídeos extracelulares como o ATP, ADP, AMP e adenosina, tem pronunciado efeito em uma variedade de processos biológicos, entre eles o desenvolvimento, o sistema cardiovascular, a inflamação, o sistema imune (Robson *et al*., 2006) e a neurotransmissão (Agteresch *et al*., 1999; Cunha, 2001). A sinalização purinérgica parece também estar envolvida com remodelamento tecidual seguida de eventos isquêmicos, trauma ou desordens neurodegenerativas (Burnstock, 2002).

Adenosina é particularmente usada como mensageiro transcelular para sinalizar desequilíbrio metabólico. Vários relatos têm documentado aumento em sua concentração na presença de alterações metabólicas estressantes (Latini & Pedata, 2001).

É importante salientar que a cronobiologia é uma área que se encontra em franco desenvolvimento, e que estudos experimentais devem ser estimulados, pois seus resultados podem contribuir para experimentos futuros, pois o período do dia em que o experimento é realizado pode influenciar diretamente os resultados experimentais, criando, assim, vieses nas pesquisas.

II. REVISÃO DA LITERATURA

REVISÃO DA LITERATURA

2.1. CRONOBIOLOGIA

A partir da verificação de que alguns fenômenos biológicos ocorrem de forma cíclica e regular durante as fases do dia, do ano e do desenvolvimento dos seres vivos, surgiu a cronobiologia (Martínez-Carpio & Corominas, 2004). Esta ciência estuda as características temporais da matéria viva em todos os seus níveis de organização dos ritmos circadianos. O entendimento das relações temporais dos fenômenos fisiológicos com o meio ambiente é fundamental para a compreensão dos mecanismos adaptativos dos seres vivos. Deste modo, na maioria das espécies, podemos observar ritmos que se caracterizam como estados funcionais que variam periodicamente no tempo. Os ritmos circadianos variam em torno de 24h e podem ser apresentados como eventos bioquímicos, fisiológicos ou comportamentais, importantes para sobrevivência (Reppert & Weaver, 2002).

O sistema temporizador determina mudanças fisiológicas no curso do dia, que se expressam por meio de variáveis como: pressão arterial, atividade do sistema imune, coagulação sanguüínea, bem como funções gastrointestinais, renais e endócrinas (Goldbeter, *et al*., 2010). Os ritmos temporizados encontram-se sincronizados não somente com o meio externo como também internamente (Moser et al., 2006). Um exemplo disso é a ritmicidade circadiana da secreção do cortisol que apresenta seu pico plasmático matinal e o nadir (menor nível) ao deitar (Fernandes *et al*., 2009). A regulação desse ritmo depende do relógio biológico, mas também pode ser afetado por estímulos ambientais (sincronização externa) (Moser *et al*., 2006).

A glândula pineal é parte integrante desse sistema de temporização endógeno produzindo o hormônio melatonina durante a noite. Esse hormônio participa da regulação de

funções fisiológicas, principalmente na transmissão de informações relativas ao ciclo claro/escuro para a organização da ritmicidade sazonal e circadiana dos diversos eventos fisiológicos e comportamentais (Arendt, 1998), como o controle do ciclo sono-vigília, a função imunológica, a neutralização de radicais livres, o controle de crescimento tumoral, entre outros (Pandi-Perumal *et al*., 2006). Nesse sistema a informação fótica captada pelas células granulares da retina, através das células envolvidas no processo de percepção do claro e do escuro que expressam melanopsina. Essa informação projeta-se via o NSQ hipotalâmico para os neurônios simpáticos do núcleo intermediolateral do segmento superior da medula espinhal torácica. Desse núcleo, as fibras entram no tronco simpático, fazendo sinapse com células nervosas do gânglio cervical superior que inervam diretamente a pineal. Esse circuito elétrico é desligado quando há incidência de luz pela ausência de potenciais de ação disparados a partir do NSQ. Na vigência do escuro, essa via entra em ação, e os neurotransmissores, noradrenalina e ATP, são liberados, atuando respectivamente via receptores noradrenérgicos (alfa e beta) e purinérgico P2Y1 localizados nos pinealócitos, desencadeando a ativação da via biossintética da melatonina (Markus *et al*, 2003; Ferreira & Markus, 2001). A melatonina, em humanos e em ratos, apresenta um ritmo circadiano, atingindo seus mais altos níveis plasmáticos durante a noite (entre 2 e 3 da manhã) (Nowak *et al*., 1998).

O ritmo e a concentração plasmática ou salivar de melatonina é o melhor marcador fisiológico do tempo do relógio biológico interno disponível, permitindo uma monitoração mais prolongada dos ritmos biológicos. Segundo Josephine Arendt (2005), a análise do ritmo da melatonina é mais efetiva que a medida do ritmo de temperatura (que pode ser mascarada pela atividade física) ou que a medida isolada de cortisol/corticosterona (que pode ser afetado pelo estresse e alimentação).

O cortisol é o hormônio resultante da ativação do eixo HHA, o que inicialmente promove liberação de hormônio liberador de CRH pelo hipotálamo, seguida de liberação de ACTH pela hipófise anterior e de glicocorticóides (GCs) pelo córtex da adrenal (de Kloet *et al*., 1993). O ACTH ativa a conversão do colesterol em pregnenolona, aumentando a produção e a liberação de GCs pelo córtex da adrenal. Esses últimos hormônios atuam em todo organismo para mediar modificações nos processos metabólico, imune e inflamatório requeridos para adaptação e preparação do organismo para responder a uma situação de estresse, incluindo mudanças na forma de obtenção de energia e no metabolismo (Cullinan *et al*., 1996; Herman *et al*., 1995). Em humanos, a secreção noturna de ACTH e de cortisol se faz de modo pulsátil, alcançando seu nível mais baixo na primeira metade da noite, aumentando rapidamente ao se aproximar o despertar, quando sua secreção é máxima (entre as 6 e as 10 horas da manhã) (Scheer, Van Doornen, Buijs, 1999). No rato, a secreção de corticosterona ocorre no turno inverso, com picos no início da noite (Perreau-Lenz *et al*., 2003).

Os ritmos de atividade e repouso, social, de temperatura corporal e de níveis hormonais (melatonina e cortisol) são exemplos de ritmos biológicos no organismo que podem ser medidos. Segundo Kaplan *et al*. (1997), quando um indivíduo se encontra em um estado saudável, todos os seus ritmos têm uma relação natural, estando sincronizados. Quando o sistema é perturbado (situações de estresse), certos ritmos biológicos são desordenados (ritmos do cortisol ou melatonina), considerados dessincronizados. Nesse caso, um determinado ritmo pode ter um avanço de fase anormal, começando mais cedo do que o habitual, ou um atraso de fase, começando mais tarde que o habitual. Sob condições experimentais, é possível observar um avanço ou um atraso de fase causado por um determinado estímulo (luz) quando apresentado em diferentes momentos de um ciclo, como o de sono/vigília.

Os ritmos circadianos podem ser regulados por uma variedade de indicadores externos, como pulsos de temperatura ambiental, refeições e horário de dormir e acordar (Gronfier *et al*., 2007). Porém, o ritmo claro/escuro é uma variável fundamental na sincronização (ou dessincronização) (Gronfier *et al*., 2007). Esse estímulo regulador é denominado na Cronobiologia de *Zeitgeber*, que, provindo do alemão, significa "aquele que regula o tempo" (*time giver*). Na ausência de estímulos exógenos (chama-se um indivíduo em livre curso), o período dos ritmos circadianos humanos é um pouco maior que um dia, entre 24 e 25 horas (Kaplan, Sadock e Grebb, 1997). Os ritmos endógenos não garantem ao organismo sua adaptação ao meio ambiente, que pode ocorrer por meio do fenômeno de arrastamento, garantindo que a expressão de cada atividade ou função aconteça quando as condições ambientais estão favoráveis. A causa primária do arrastamento, ou seja, o estabelecimento de uma relação de fase estável entre dois ritmos é também o princípio que fundamenta a ordem temporal interna do organismo. Esta garante a coordenação dos processos orgânicos por meio da sincronização de diferentes osciladores. A ruptura da ordem temporal interna denomina-se dessincronização (Kern, 1996).

A instabilidade da ritmicidade circadiana, com repetida dessincronização/ ressincronização, pode enfraquecer os mecanismos homeostáticos. Um exemplo desse fenômeno é a exposição a situações de estresse cada vez mais comum na organização da sociedade atual. A consequência desse enfraquecimento possivelmente esteja implicada na gênese ou aceleração do processo saúde-doença (Kern, 1996).

2.2. RELAÇÃO MELATONINA E EIXO SIMPATO-ADRENAL

As variações rítmicas do relógio central (NSQ) chegam a todo o organismo, principalmente por meio de um sinal humoral. O tráfego de potenciais de ação pelo terminal

simpático que se origina no gânglio cervical superior ocorre na fase de escuro. Os neurotransmissores noradrenalina e ATP controlam a produção de N-acetiltransferase (NAT), que é uma enzima-chave na produção de melatonina. A glândula pineal é capaz de captar o aminoácido 5-hidroxitriptofano e transformá-lo em serotonina (5-hidroxitriptamina), e sua concentração durante o dia é alta. Na fase de escuro, há síntese da enzima NAT, que metaboliza a serotonina em N-acetilserotonina, parte deste produto lipossolúvel sendo lançada na circulação, mas parte sendo metabolizada pela enzima hidroxi-indol-O-metil-transferase (HIOMT) em melatonina. A melatonina é uma molécula lipossolúvel, e também é lançada prontamente na circulação (Markus, *et al*, 2007).

Dados da literatura mostram que a produção de melatonina está alterada de forma consistente após um estresse físico, tanto em roedores (Lopes *et al*., 1997 e 2001) quanto em humanos (Pontes *et al*., 2006). Em camundongos, foi observado que, em condições de inflamação crônica, a produção noturna de melatonina pela pineal depende de uma ação conjunta da atividade simpática, que está aumentada no escuro, e dos níveis de corticosterona circulante, aumentados pelo processo inflamatório. Em humanos, foi demonstrada uma correlação negativa entre TNF-α e melatonina (Pontes *et al*., 2006, 2007).

Considerando que a glândula pineal é diretamente regulada pelo sistema simpático, é plausível supor que disfunções do sistema autonômico por estresse físico ou psíquico determinem alterações no eixo imune-pineal, expressas como disfunções no ritmo circadiano e na suscetibilidade a processos infecciosos como gripe e herpes e não infecciosos, como depressão. Essas alterações podem ter influência no eixo HHA e, portanto, nos hormônios marcadores de estresse como o cortisol ou corticosterona (De Kloet, 2009).

A via neural retino-hipotalâmica é responsável pela sincronização do relógio biológico ao ciclo claro-escuro ambiental e a via polissináptica, que liga o relógio a pineal, é responsável pela produção de melatonina na fase de escuro. A inervação da pineal é feita pela

via simpática a partir dos gânglios cervicais superiores, e o desencadeamento da via de transdução da proteína quinase A (PKA) é decorrente da estimulação de adrenoceptores β1. A concentração de melatonina plasmática reflete diretamente a produção de melatonina pela glândula pineal (Markus *et al*., 2003 e Simonneaux & Ribelayga, 2003). Noradrenalina e ATP atuam como co-transmissores, mas o aumento de cálcio intracelular desencadeado pela ativação de receptores purinérgicos $P2Y_1$ (Ferreira *et al*., 1994, Ferreira e Markus, 2001) apenas é capaz de potenciar a produção noturna de melatonina, que é desencadeada pela ativação de adrenoceptores β1.

Embora a melatonina possa ser produzida por outros tecidos (mucosa estomacal) ou células (linfócitos, macrófagos e células do colostro), o pico noturno de melatonina é a real expressão da atividade pineal. A produção de melatonina é função da atividade simpática e, consequentemente, da liberação de noradrenalina. Portanto a razão entre a produção noturna e diurna de melatonina é um marcador da atividade simpática. Recentemente foi realizado um grande estudo sobre a concentração de melatonina em indivíduos normais, conduzido durante o período de um ano em várias regiões geográficas. Nesse estudo, Wetterberg *et al*. (1999) observaram que a população poderia ser dividida em dois padrões: aqueles que secretavam altas concentrações desse hormônio, definidos por concentrações anuais maiores que 0,25 nmol/L, e aqueles que secretavam baixas concentrações. Como fatores demográficos como sexo, idade, altura, peso, estação do ano não foram responsáveis por essa diferença, foi sugerido que esse padrão provavelmente refletisse uma variabilidade genética nos níveis de secreção de noradrenalina, já que a produção de melatonina é regulada via um sinal simpático e/ou na atividade das enzimas de síntese de melatonina durante a noite (Bergiannaki *et al*., 1995). Outro estudo realizado com humanos demonstrou que o estresse e o exercício físico podem aumentar as concentrações plasmáticas de melatonina. Assim, nos mamíferos, a pineal é considerada um órgão endócrino com propriedades de transdutor neuroendócrino, porque a

informação fótica, canalizada através de um sinal da via simpática, chega à glândula e regula a secreção de melatonina (Menna-Barreto, 1993).

2.3. ESTRESSE

A partir dos trabalhos de Selye (1936), tem ocorrido um maior entendimento do mecanismo de estresse, sendo este definido como uma resposta estereotipada, não específica, do corpo a mudanças no ambiente externo ou interno. Esta resposta foi chamada de síndrome de adaptação geral e caracterizada por hipertrofia de adrenal, sangramento gastrintestinal e diminuição da função de órgãos do sistema imune (Selye, 1974). O cérebro recebe impulsos produzidos por vários estressores, e envia respostas por meio dos sistemas nervoso, endócrino e imune. A ativação de sistemas envolvidos com estresse leva a mudanças comportamentais e periféricas que buscam manter a homeostase, aumentando a chance de sobrevivência. Durante o estresse, a atenção é aumentada, as frequências cardíaca e respiratória são aceleradas, o catabolismo é aumentado e o fluxo sanguíneo é redirecionado para prover maior perfusão e combustível para o cérebro, coração e músculos (Tsigos & Chrousos, 2002).

Eixos HHA e simpático-adrenal (SA) são ativados por estressores. Como imediata resposta, altera-se a taxa de descarga dos neurônios simpáticos com consequente secreção de catecolaminas no sangue. A resposta simpática leva a aumento de frequências cardíaca e respiratória e de pressão sanguínea, broncodilatação, dilatação de pupilas, transpiração e palidez. No pico da resposta, fontes fisiológicas de energia são mobilizadas. O estágio mais tardio é caracterizado pela ativação do eixo HHA, resultando no aumento da secreção de CRH pelo hipotálamo, de ACTH, pela hipófise anterior e de GCs, pelo córtex da adrenal (de Kloet *et al*., 1993). Estes últimos hormônios atuam em todo organismo para mediar modificações nos processos metabólico, imune, inflamatório requeridos para adaptação e preparação do

organismo para lidar com uma situação estressante, incluindo mudanças na forma de obtenção de energia e no metabolismo (Cullinan *et al*., 1995; Herman *et al*, 1995). Os GCs inibem a liberação e a síntese de ACTH por atuarem no hipocampo, hipotálamo e hipófise, exercendo retroalimentação negativa sobre a liberação de ACTH (Nestler *et al*., 2002) (Figura 1). O cortisol é o principal hormônio glicocorticóide secretado pelo córtex da adrenal humana, enquanto que a corticosterona é o principal corticóide secretado nos ratos (Bhatnagar *et al*., 1997). Segundo Monk *et al*. (1983), o estresse muda ritmicamente a regulação diurna, bem como a regulação cardíaca e as respostas neuroendócrinas, que parecem ser responsáveis pelos níveis mais elevados de doença cardiovascular encontrada em indivíduos cronicamente estressados. Além disso, a função cardiovascular é um mecanismo básico associado à atenção e à memória, que passam a ser afetadas em caso de estresse (Edelstein *et al*., 2008).

O aumento de adrenalina e de cortisol são respostas endócrinas ao estresse, e os níveis dessas substâncias no plasma, urina ou saliva são indicados como uma forma de mensurar o nível de estresse (Selye, 1974 e Mcclelland *et al*., 1982).

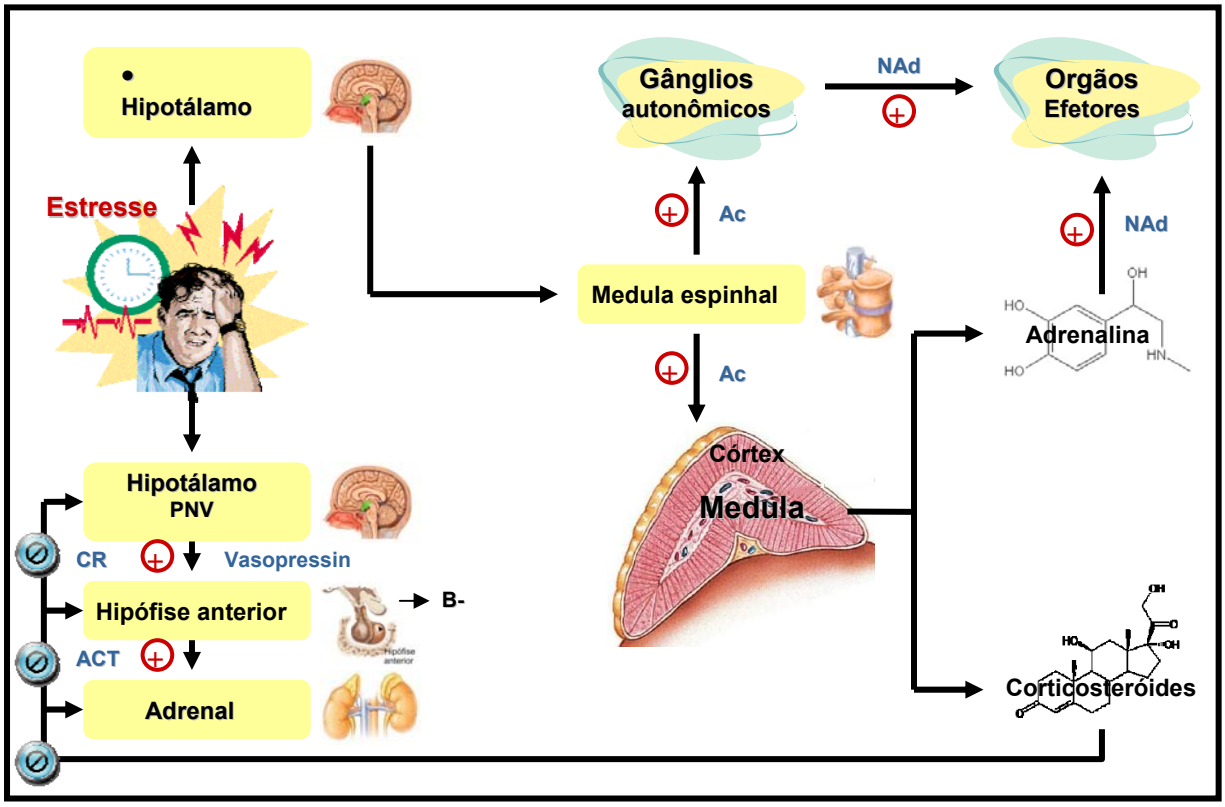

Figura 1: Representação esquemática da ativação de eixos hipotálamo-hipófise-adrenal (HHA) e simpático-adrenal (SA) pelo estresse. PVN= núcleo para-ventricular hipotalâmico.

2.4. RESPOSTA COMPORTAMENTAL

O comportamento animal é resultado de uma interação entre fatores biológicos e ambientais. Os fatores biológicos inatos, pré-programados geneticamente, resultam de processos de seleção natural relacionados com a adaptação da espécie no meio. A experiência individual dos organismos com os fatores ambientais resulta em aprendizagem e envolve processos de aquisição de conhecimento acerca do mundo (Melo *et al.*, 2003).

Habituação é uma das formas de aprendizagem que se caracteriza por uma diminuição de respostas quando um estímulo é continuamente apresentado ou repetido com alta frequência. (Groves & Thompson, 1970, Thompson & Spencer, 1966). A redução frente a

um estímulo, sem significativo funcional, tem valor adaptativo para o organismo, uma vez que continuar respondendo a ele implicaria dispêndio energético desnecessário.

A resposta de orientação faz parte do comportamento exploratório, que garante a inspeção, localização, identificação e avaliação precisa de eventos ambientais (Berlyne, 1960, Buzsaki, 1982, Sokolov, 1960). O comportamento exploratório tem componentes vegetativos, reflexos e operantes e se relacionam de maneira direta com a novidade e a intensidade do estímulo.

A exploração também tem sido relacionada com a curiosidade animal (Glickman & Sroges, 1966). Os comportamentos reflexos, relacionados com apresentação de estímulos, que precedem as reações exploratórias caracterizando-se, portanto como comportamentos pré-exploratórios, vêm sendo também estudados.

A avaliação de roedores no aparato de campo aberto é um procedimento muito utilizado, com a finalidade de se observar a atividade locomotora de animais de pequeno porte. Em uma primeira instância, sabe-se que os ratos, assim como os seres humanos, poderão reagir ao ambiente considerado "novo" e apresentar uma resposta aversiva, característica de congelamento (do inglês "*freezing*"), que é um comportamento inerente ao animal, comportamento este muitas vezes utilizado pelos animais como uma forma de diminuir as detecções visuais e auditivas por parte dos predadores. No entanto, em uma segunda instância, ele tende a explorar o ambiente onde se encontra. Este teste consiste na exposição do animal em uma arena com asoalho dividido em quadrados, que avalia tanto o nível de ansiedade, quanto parâmetros de deambulação do animal (Handley e Mithani, 1984).

O labirinto em cruz elevado (LCE) é um equipamento na forma de cruz, elevado 50 cm do chão, com dois braços fechados e dois abertos, opostos entre si (Handley e Mithani, 1984). É outro teste comportamental, com base na aversão natural que roedores apresentam pelos braços abertos do labirinto (Montgomery, 1955), pois, quando eles são forçados a

permanecerem nos braços abertos desse aparato, mostram manifestações comportamentais e fisiológicas de medo, tais como congelamento, defecação e aumento nos níveis de corticosteróides plasmáticos (Pellow *et al*., 1985). Em uma série de estudos, Treit *et al*. (1993) indicaram que o fator de maior contribuição para esta "reação de medo" é a falta das paredes altas dos ramos abertos, que impede a tigmotaxia. A proporção da exploração total nos braços abertos determina uma medida de pouca ansiedade, de tal modo que o aumento nas percentagens de tempo e de entradas nos braços abertos é considerado como indicativo de ação ansiolítica de drogas (Handley e Mithani, 1984; Pellow *et al*., 1985).

Outro fator que pode alterar a resposta comportamental dos animais é o ritmo circadiano, ritmo biológico que persiste mesmo sob condições ambientais constantes (luz, temperatura) com um período de duração de aproximadamente 24 horas (Moser et al., 2006). A manutenção de ritmicidade em um ambiente constante, como a permanência no escuro contínuo, demonstra que o ritmo é gerado de forma endógena ao invés de uma reação ao ambiente externo (Kennaway, 2004). No entanto, esses ritmos são ajustados pelo ambiente, ou seja, apesar de serem mantidos independentes das condições ambientais eles atuam sincronicamente com o ambiente. Esse sistema temporal permite ao organismo antecipar e se preparar para mudanças físicas no ambiente que estão associadas com a noite e o dia. Assim o organismo se adapta tanto comportamental como fisiologicamente para se deparar com desafios associados com essas mudanças resultando em uma sincronização entre o organismo e o ambiente externo (Turek, 1998).

2.5. SISTEMA PURINÉRGICO

Além de seu papel central em metabolismo celular energético, o ATP é um neurotransmissor em sistemas simpático, parassimpático e nervos sensoriais periféricos, assim

como em SNC (Cunha & Ribeiro, 2000). Em sinaptossomas corticais, é co-liberado com acetilcolina e noradrenalina, mas, em geral, é liberado de neurônios não-colinérgicos e não-adrenérgicos (Linden, 1999, Burnstock, 1999). É também co-transmissor de neurônios gabaérgicos (Burnstock, 1999). É liberado em fluido extracelular como resultado de lise celular, permeabilização seletiva de membrana ou exocitose de grânulos secretórios, podendo exercer funções completamente diferentes da função de mediador intracelular.

As ações do ATP extracelular são delimitadas pela sua degradação por enzimas acopladas à membrana plasmática ou encontradas na forma solúvel no meio extracelular (Burnstock, 2007). Assim, por ação dessas enzimas, o ATP é sequencialmente hidrolisado a ADP e AMP (nucleotídeos) o qual é finalmente hidrolisado a adenosina, o nucleosídeo da adenina. Nucleotídeos e nucleosídeos têm pronunciados efeitos em uma variedade de processos biológicos, incluindo neurotransmissão, contração muscular, funções cardíaca e plaquetária, vasodilatação e metabolismo hepático do glicogênio (Agteresch *et al*.,1999).

O ATP e cada um de seus metabólitos têm atividade mediada por receptor específico (Abbracchio *et al*., 2009). Em mamíferos, oito subtipos dos receptores P2Y foram clonados (P2Y1,2,4,6,11,12,13,14) respondendo, em diversos graus, a uma variedade de ligantes endógenos, ATP, ADP, UTP e UDP e estão localizados nos mais diversos tecidos (Burnstock, 2007). Os P2Y1, P2Y12 e P2Y13 são ativados principalmente por nucleotídeos difosfatados; enquanto que os P2Y2, P2Y4 e P2Y6 são ativados por purinas e pirimidinas (Fields & Burnstock, 2006). A adenosina pode exercer sua ação por ativação dos receptores A1, A2A, A2B e A3, subtipos dos receptores P1,todos acoplados à proteína G (metabotrópicos).

Nucleotídeos extracelulares podem ser hidrolisados por uma variedade de enzimas que estão localizadas na superfície ou estão solúveis no meio intersticial ou nos fluidos biológicos (Zimmermann, 2000). Além disto, nucleotidases solúveis, que hidrolisam sequencialmente o ATP até adenosina, também são liberadas de nervos simpáticos (Todorov

et al.,1997). Trabalhos têm demonstrado que membros de várias famílias de ectonucleotidases podem contribuir para a hidrólise de nucleotídeos. Nucleosídeos 5'tri- e difosfatos (NTP e NDP) podem ser hidrolisados por membros das famílias das E-NTPDases (ectonucleosídeo trifosfato difosfo-hidrolases), E-NPPs (ectonucleosídeo pirofosfatase/fosfodiesterases) e fosfatases alcalinas (Zimmermann, 2000). Estas ectonucleotidases, junto com 5´-nucleotidase, controlam a disponibilidade de ligantes (ATP, ADP, AMP e adenosina) para receptores de nucleotídeo e nucleosídeo, e, consequentemente, a duração e a extensão da ativação do receptor (Chen & Guidotti, 2001). Portanto, a cascata de ectonucleotidases é uma via enzimática com dupla função de remover o sinal (ATP) e gerar um segundo (adenosina). Pode ter função na regulação efetiva de vários processos, uma vez que tem considerável plasticidade em diferentes situações patogênicas. Essas enzimas podem também ter uma função de proteção pela manutenção dos níveis extracelulares de ATP/ADP e adenosina dentro de níveis fisiológicos (Agteresch *et al*., 1999). Além disso, a atividade ecto-enzimática das E-NTPDases pode regular uma variedade de estados fisiológicos, incluindo função cardíaca, secreção hormonal, respostas imunes, neurotransmissão e agregação plaquetária, todos pela modulação dos níveis circulantes de nucleotídeos no sangue (Mulero *et al*., 1999).

As E-NTPDases são representadas por 8 membros. As NTPDases 1, 2, 3 e 8 são proteínas transmembrana, localizadas na superfície celular, com o sítio catalítico voltado para o meio extracelular. As NTPDases 4, 5, 6 e 7 estão localizadas intracelularmente, ancoradas nas membranas de organelas intracelulares, com o sítio catalítico voltado para o lúmen das mesmas (Zimmermann, 2006). Sua atividade catalítica máxima requer a presença dos cátions divalentes Ca^{2+} e Mg^{2+}, sendo inativas na ausência destes íons (Kukulski *et al*., 2004). Estas enzimas hidrolisam os nucleotídeos com diferentes velocidades. A E-NTPDase 1, (apirase, CD39), hidrolisa ATP e ADP igualmente bem, enquanto que a E-NTPDase 2 (CD39L1) prefere os nucleotídeos trifosfatados, em proporção de 30:1 (ATP/ADP). Por essa razão.

também é chamada de Ecto-ATPase (Zimmermann, 2000). As E-NTPDase 3 e 8 preferem o ATP ao ADP, em proporções de 3:1 e 2:1, respectivamente. A E-NTPDase 4 prefere UDP e está ancorada à membrana do aparelho de Golgi. As E-NTPDases 5 e 6 têm preferência pelos nucleotídeos difosfatados e possuem um único domínio transmembrana próximo ao N-terminal. A forma 5 está ancorada ao retículo endoplasmático, e a 6, ancorada à membrana do aparelho de Golgi. Além disso, estas duas enzimas podem sofrer clivagem proteolítica e serem secretadas em uma forma solúvel (Lavoie *et al*., 2004). A E-NTPDase 7 localiza-se ancorada em vesículas intracelulares, e tem preferência pelos nucleotídeos trifosfatados.

Entre as nucleotidases solúveis descritas em soro, está a E-NTPDase-5 (ou CD39-L4), que é secretada de células de mamíferos. Sua presença em macrófagos indica possível ocorrência também em sangue. Tem papel na modulação dos níveis de ADP circulante (Mulero *et al*., 1999); a E-NTPDase-6 (ou CD39-L2) está associada ao complexo de Golgi e, em menor grau, à membrana plasmática, somando-se a 5'-nucleotideo fosfodiesterase (PDEase), enzima que também hidrolisa nucleotídeo e pode estar presente no soro, que é capaz de hidrolisar tanto ADP quanto ATP (Sakura *et al*., 1998).

Os nucleotídeos monofosfatados, como AMP são hidrolisados pela 5'-nucleotidase, formando adenosina (Zimmermann, 1992). Sete 5'-nucleotidases foram isoladas e caracterizadas, cinco enzimas localizadas no citosol, uma na matriz mitocondrial, e uma ancorada à parte externa da membrana, a ecto-5'-nucleotidase/CD37, que é expressa em diferentes tecidos, sendo abundante no cólon, rim, cérebro, fígado, coração e pulmões (Yegutkin, 2008).

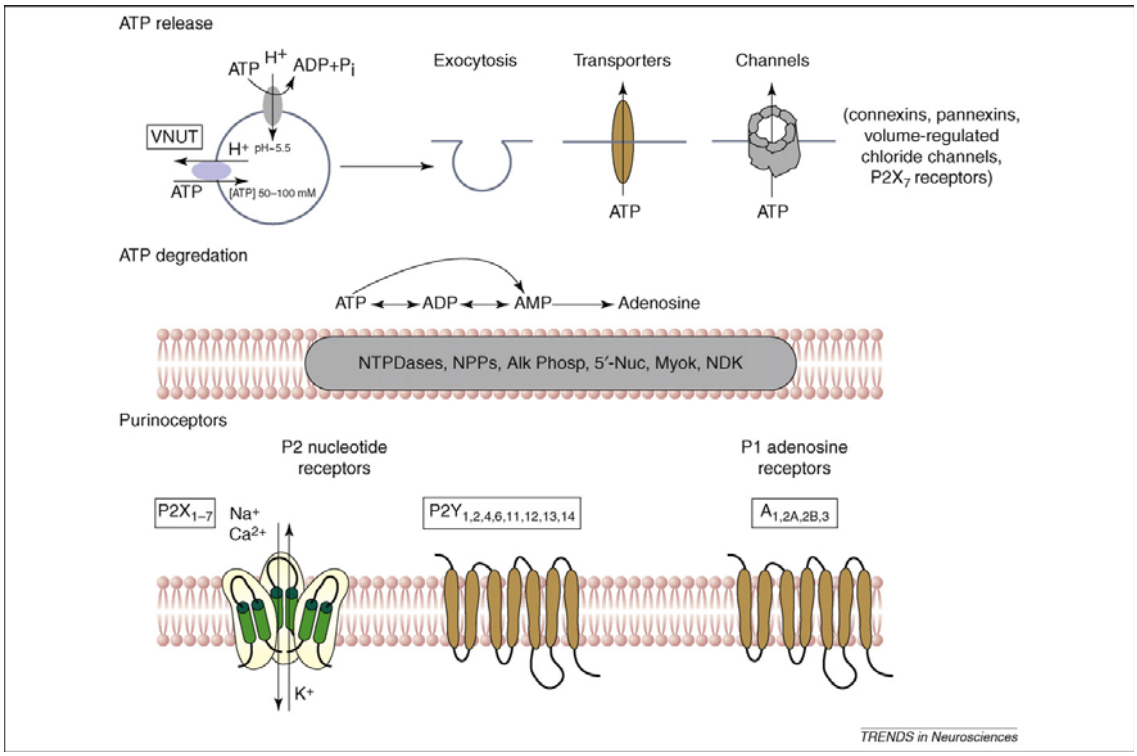

Figura 2. Mecanismo de liberação e degradação do ATP e os purinoceptores. Alk phos, fosfatase alcalina; Myok, mioquinase; NDK, nucleosídeo difosfoquinase; NPPs, fosfodiesterases; 5'-Nuc, 5'-nucleotidase; VNUT, transportador vesicular do nucleotídeo; E-NTPDases, ectonucleosideo trifosfato difosfohidrolases. Abbracchio *et al*., 2009.

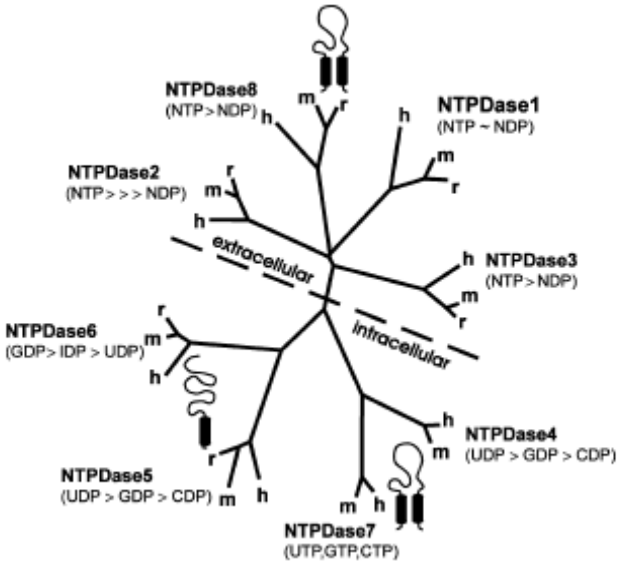

Figura 3. Árvore filogenética hipotética derivada dos 22 membros selecionados da família das E-NTPDases (NTPDase 1 a NTPDase 8) de rato (*r*), humano (*h*) e camundongo (*m*), seguindo o alinhamento da sequência de aminoácidos. O tamanho das linhas indica as diferenças entre as sequências de aminoácidos. A linha tracejada indica os tipos de NTPDases que apresentam o sítio catalítico voltado ao meio extra ou intracelular. Em adição, a preferência aos substratos de cada enzima e a topografia de membrana para cada grupo de enzimas (um ou dois domínios transmembrana, indicados com barris). Adaptado de Robson, Sévigny e Zimmermann, 2006.

A cascata de nucleotidases tem papel efetivo na regulação de vários processos, incluindo função protetora na manutenção de ATP (extracelular) e adenosina dentro de níveis fisiológicos (Agteresch *et al*., 1999). Nucleotídeos circulantes são importantes moléculas sinalizadoras, potencializando várias respostas fisiológicas (Brake & Julius, 1996). Em soro,

os nucleotídeos de adenina estão implicados em vários processos. ATP tem função em tônus vascular, função cardíaca e transporte epitelial renal (Chen *et al*., 1994, Inscho *et al*., 1994, Nuñez *et al*., 1995, Boarder *et al*., 1995, Ravelic, 2000). Adenosina é usada clinicamente como agente antiarrítmico ou vasodilatador. ADP é potente fator de recrutamento de plaquetas, e induz agregação plaquetária por meio da interação com dois tipos de receptores P2 plaquetários - $P2Y_1$ e $P2Y_T/P2Y_{12}$ (Burnstock & Williams, 2000). Hidrólise de ADP por ecto-nucleotidases presentes em soro inibe agregação plaquetária por remoção de ADP e formação de adenosina (também inibidora da agregação plaquetária) (Zimmerman, 1999). CD39-L4 solúvel no sangue melhora fluxo cerebral e reduz volume de infarto cerebral, quando administrada pré-operatoriamente (Pinsky *et al*., 2002).

A adenosina é particularmente usada como mensageiro transcelular para sinalizar desequilíbrio metabólico. Vários relatos têm documentado aumento na sua concentração em alterações metabólicas estressantes (Latini & Pedata, 2001), e o termo "*retaliatory metabolite*" tem sido utilizado para caracterizar a função homeostática da ADO que ocorre em virtualmente todos os tipos celulares (Cunha, 2001).

III. OBJETIVOS

3.1. OBJETIVO GERAL

O objetivo deste trabalho foi avaliar o efeito do padrão temporal sobre parâmetros comportamentais e bioquímicos na resposta ao estresse por restrição em ratos wistar. Foram verificadas as atividades das enzimas NTPDase e 5'nucleotidase, secreção de melatonina e corticosterona. Adicionalmente, foram avaliados parâmetros comportamentais no aparato de campo aberto (*Open-Field*) e no labirinto em cruz elevado (*Plus Maze*).

3.2. OBJETIVOS ESPECÍFICOS

Avaliou-se o efeito da exposição a uma única sessão de estresse por restrição em ZT 0, 6, 12 e 18. ZT0 equivalente a 7h, quando a luz do biotério era ligada e ZT12, equivalente a 19h, quando a luz do biotério era desligada. O estresse foi aplicado nesses horários e avaliado, imediatamente, 6 e 24h após o estresse. Para todos os horários, foram utilizados animais controles (sem aplicação de estresse). Os parâmetros avaliados foram:

- ✓ Atividades das enzimas NTPDase e 5'nucleotidase em soro de ratos Wistar;
- ✓ Níveis de melatonina, corticosterona e glicose em soro de ratos Wistar;
- ✓ Locomoção e ansiedade através dos apartos: Campo-aberto (*Open-field*) e Labirinto em Cruz Elevado (*Plus Maze*).

IV. REFERÊNCIAS DA REVISÃO DA LITERATURA

REFERÊNCIAS DA REVISÃO DA LITERATURA

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V. ARTIGOS CIENTÍFICOS

ARTIGO I: Effects of restraint stress upon temporal patterns of adenine nucleotides hydrolysis in rat blood serum in rats

Periódico: International Chronobiology

Status: Submetido

International Chronobiology: Submission confirmation

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EFFECTS OF RESTRAINT STRESS UPON TEMPORAL PATTERNS OF ADENINE NUCLEOTIDES HYDROLYSIS IN RAT BLOOD SERUM

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Running head: Stress effects on nucleotidases

ABSTRACT

Adenosine 5'-triphosphate (ATP) and its breakdown products, ADP, AMP and adenosine can act as extracellular messenger in a range of biological processes. Extracellular adenine nucleotides are metabolized to adenosine by a number of enzymes including NTPDases and ecto-5'-nucleotidase. Previous work of our group demonstrates that ATPase and ADPase activities exhibit a 24h temporal pattern in rat blood serum. Circadian rhythms represent an important mechanism for preparing the organism to environmental changes and stress can cause disruptions in this biological circadian rhythms. Therefore, the aim of the present study was to examine the influence of acute stress exposure upon temporal patterns in NTPDase and 5'-nucleotidase enzymes activities. The control groups showed significant higher ATPase and ADPase activities at ZT 12 and 18 when compared with control group. All stressed groups showed significant decrease in all enzymatic activities at ZT 12 and 18 when compared with control group. The acute stress provokes a decrease in the activities of nucleotidase enzymes dependent of the time that this stress occurs and this effect seems to persist at least 24 hours. It may be proposed that altered levels of nucleotides in serum can be involved in cardiovascular events, and with its etiology induced by stress.

Keywords: Adenine nucleotides hydrolysis; Circadian rhythm; Ecto-5'-nucleotidase; NTPDase; Rat blood serum; Restrain stress; Temporal pattern

INTRODUCTION

Extracellular adenosine 5'-triphosphate (ATP) and its breakdown products, adenosine 5'-diphosphate (ADP), adenosine 5'-monophosphate (AMP) and adenosine, can act as extracellular messenger in a range of biological processes through binding to the purinergic receptors. In addition to this, it has been shown to present pronounced effects on a variety of biological process as neurotransmission, regulation of cardiac function and platelet aggregation (Agteresch et al., 1999; Latini and Pedata, 2001), and pathological events including cerebral ischemia, neuroinflammatory and neuropsychiatric disorders, neurodegenerative and cardiovascular diseases (Burnstock, 2008; Erlinge and Burnstock, 2008). ATP can be release via stimulation of sympathetic nerves (Todorov et al., 1997) promoting vasoconstriction or vasodilatation (Burnstock, 2006a), and contributes to platelet aggregation (Rolf et al., 2001). Additionally, its breakdown produces the nucleotide diphosphate (ADP), which is the most important platelet aggregator (Kunapuli and Daniel, 1998) and it promotes vasoconstriction (Furukoji et al., 2008). The nucleoside adenosine, produced by ATP breakdown, is able to act as a vasodilator, inhibitor of platelet aggregation, and it may act as an endogenous cardioprotective substance (Jacobson and Gao, 2006).

Extracellular nucleotides can be hydrolyzed by a variety of enzymes that are located on the surface, or may be soluble in the interstitial medium or within body fluids (Zimmermann, 2001). Soluble nucleotidases, which can breakdown ATP and other adenosine nucleotides, have also been shown to be released from sympathetic nerves (Todorov et al., 1997). Nucleoside 5'tri- and diphosphates (NTP and NDP) may be hydrolyzed by the enzymes nucleoside triphosphate diphosphohydrolase family (NTPDases), nucleotide phosphate inhibitor/phosphodiesterase family (NPP), alkaline phosphatases and ecto-5'-nucleotidase (Zimmermann, 2000). Eight different NTPDases were described, NTPDase 1, 2, 3 and 8 are expressed on cell surface with catalytic site facing to the extracellular space, NTPDase 5 and 6

exhibit intracellular localization nevertheless may be soluble enzyme, and NTPDase 4 and 7 are entirely intracellular (Zimmerman, 2000). The ecto-5'-nucleotidase are attach on cell surface and may occur also in soluble form via cleavage of its glycosyl-phosphatidylinositol (GPI)-anchor by phospholipase C (Yegutkin et al., 2000; Zimmermann and Braun, 1999). These nucleotidases, together with 5'-nucleotidase, control the availability of ligands (ATP, ADP and adenosine) for both nucleotide and nucleoside receptors, and consequently, the duration and extent of receptor activation (Chen and Guidotti, 2001). Therefore, this cascade formed by nucleotidases and 5'nucleotidase is an enzymatic pathway with a double function of removing a signal of ATP and generating a second one, adenosine. These enzymes may also have a protective function by keeping extracellular ATP/ADP and adenosine levels within physiological conditions (Agteresch et al., 1999). Previous work of our group demonstrate that ATPase and ADPase activities exhibit a 24 hour temporal pattern in rat blood serum. The ATPase and ADPase activities were increased during the dark period while AMPase activity did not display circadian variation (Detanico et al., submitted).

The circadian organization is important to enable an organism to keep equilibrium in response to the daily external environmental changes and prepare accordingly (Moore-Ede, 1986). In mammalians, a number of circadian patterns (~24h) have been described, including timing of endocrine hormone secretion (e.g. melatonin, corticosterone or cortisol, adrenocorticotropic), body temperature, respiratory rate, heart rate, blood pressure and effect of drugs (Haus, 2007; Lemmer, 2006; Smolensky and Peppas, 2007; Vieira et al., 2010). The temporal variation of nucleotidases enzymes (Detanico et al., submitted) can be of great importance in regulating the cardiovascular system, and it was suggested that temporal disturbances in these activities can be deleterious on the regulation of the physiological functions and may contribute to the occurrence of harmful changes in the cardiovascular system.

The extracellular adenosine concentrations could be increased in stressful challenges (Latini and Pedata, 2001) including exposure to inescapable shock (Minor et al., 2001). The physiological response to psychological or physical stress consists of an integration of endocrine and autonomic modulations. The psychological stress is caused by a stimulus that indicates immediate or future risk including social conflict, noise and restraint (Sawchenko et al., 1996). The stress response is triggered by activation of the hypothalamic-pituitary-adrenal (HPA) axis and sympathetic system with release of corticosterone (cortisol in humans), epinephrine, and norepinephrine (NE), respectively (Habib et al., 2001). The acute secretion of glucocorticoids is critical for responding to stress (Sapolsky, 1992). Some studies have demonstrated the influence of stress in modulating the purinergic system. Mild stress, such as a mild foot shock, is enough to promote specific changes in the ATP and ADP hydrolysis in some tissues such as cerebral cortex (Pereira et al., 2002). Moreover, alterations of enzyme activities involved in nucleotide hydrolysis have also been reported in spinal cord and blood serum after repeated restraint stress (Torres et al., 2002a; Torres et al., 2002b) and after acute restraint stress (Bohmer et al., 2003). Thus, it make sense to evaluate the effects of acute restraint stress on 24h temporal pattern of ATPase, ADPase and AMPase enzymes activities in blood serum of Wistar rats. More specifically, in this study we assess the relationship between the daytime in which the stress is apply and the temporal course of the nucleotidases activity of time after the stress.

MATERIALS AND METHODS

Animals: It was used 98 naive adult male Wistar rats (50-70 days old; 150-240 g of weight). The animals were housed in groups of 4-5 in home cages made of Plexiglass (65 x 25 x 15 cm) with the floor covered with sawdust. They were maintained under a standard 12 h-light/dark

cycle [lights on at 07:00 h, Zeitgeber time (ZT) 0, and off at 19:00 h, ZT 12], in a controlled environment (22±2°C, rat chow and water *ad libitum*). The ZT (Zeitgeber time) was used as reference to detect the rhythmicity of the variables under study. Declaration of Helsinki and the National Institutes of Health (NIH) "Guide for the Care and Use of Laboratory Animals" (NIH publication No. 80-23, revised 1996) was followed in all experiments. The protocol of this experimental study was approved by the Ethics Committee at the Institution where the work was conducted.

Stress model: Restraint was applied by placing the animal inside a 25 x 7 cm plastic tube, and fixing the tube with adhesive tape on the outside, so that the animal was unable to move. There was a 1 cm hole at the far end for breathing. The animals were submitted to only one procedure of restraint stress for one hour (Torres et al., 2002a). The apparatus was ventilated and did not cause physical compression, avoiding hyperthermia and sudoresis. Light was perceived through a transparent lid. The experimental controls animals were not experimentally manipulated.

Experimental Design: Rats were habituated to the maintenance room for two weeks before the beginning of experiment. On the experimental day, the animals were divided into 4 groups according to daytime (ZT 0, ZT 6, ZT 12 and ZT 18) and each of these was subdivided in 4 groups according to time of death (control group, 0 hours, 6 hours and 24 hours after acute stress) (see Figure 1). The rats were submitted to the model of acute restraint stress (Torres et al., 2002a). For this purpose, individual rats were quickly transferred to a separate room and decapitated within 1 min. Trunk blood was drawn and blood samples were centrifuged in plastic tubes for 5 min at 5000 x g at room temperature (Yegutkin, 1997). Serum was obtained and frozen at −20 °C until the enzymatic assays perform.

----------Figure 1----------

Enzymatic assay: ATP and ADP hydrolysis were performed using a modification of the method described by Oses and colleagues (2004). The reaction mixture, containing 0.5 to 1.0 mg serum protein in 112.5 mM Tris–HCl, pH 8.0, was preincubated for 10 min to equilibrate the mixture. The reaction was started by the addition of ATP or ADP (final concentration of 3.0 mM) and the incubation was performed at 37 °C in a final volume of 200 µl, for 40 min. The reaction was stopped by the addition of 200 µl 10% trichloroacetic acid (TCA). All samples were centrifuged at 5000 x g for 5 min to eliminate precipitated protein and the supernatant was used for the colorimetric assay. The inorganic phosphate (Pi) released was measured by the Malachite green method (Chan et al., 1986). The AMP hydrolysis was performed essentially as described above for ATP and ADP hydrolysis. The reaction mixture, containing 3.0 mM AMP as substrate in 100 mM Tris–HCl, pH 7.5, was incubated with 0.5 to 1.0 mg serum protein at 37 °C in a final volume of 200 µl. All other procedures were the same as described above for ATP and ADP hydrolysis.

For all enzyme assays, incubation times, substrate and protein concentrations were chosen in order to ensure the linearity of the reactions. All samples were run in triplicate. In order to correct non-enzymatic hydrolysis, we performed controls by adding the serum after the reaction was stopped with TCA. The protein concentration was measured by the Coomassie Blue method using bovine serum albumin as standard (Bradford, 1976). Enzyme activities were expressed as nmol of inorganic phosphate (Pi) released per minute per milligram of protein (nmol Pi/min/mg protein). All chemicals were purchased from Sigma Chemical Co. (St. Louis, MO, USA).

Statistical analysis: Data were expressed as mean ± standard error of the mean (S.E.M.). The comparison among groups was analyzed by One-Way ANOVA followed by the Tukey's test. Differences between groups were considered significant for $P < 0.05$. SPSS 17.0 for Windows was used for statistical analysis.

RESULTS

The summary of the all results of stress effects upon temporal patterns of ATPase, ADPase and AMPase activities are shown in Table 1, and graphical results are shown in Figure 2, 3 and 4.

----------Table 1----------

Effects of stress on ATPase-ADPase activities on blood serum during 24h

The hydrolysis of ATP and ADP were evaluated on blood serum at 0h, 6h and 24h after the stress procedure during 24 hour at ZT 0, 6, 12 and 18. One-Way ANOVA followed by Tukey's test revealed significant differences on effect of stress on 24h profile on ATPase ($F_{15,82}$=8.205, $P < 0.001$ – Table 1 and Figure 2) and ADPase activities ($F_{15,82}$=9.911, $P < 0.001$ – Table 1 and Figure 3). In agree with previous study of group, the control groups showed higher enzymatic activities at ZT 12 and 18 when compared to ZT 0 and 6. All stressed groups showed significant decrease in enzymatic activities at ZT 12 and 18 when compared with control group (Table 1, Figure 2 and 3).

----------Figure 2 and 3 ----------

Effects of stress on AMPase activity on blood serum during 24h

The hydrolysis of AMP was evaluated on blood serum at 0, 6 and 24h after the stress procedure during 24 hour at ZT 0, 6, 12 and 18. One-Way ANOVA followed by Tukey's test

revealed significant differences on effect of stress on 24h profile on AMPase activity ($F_{15,82}$=7.361, $P < 0.001$ – Table 1 and Figure 4). In agree with previous study of group no difference was observed between the control groups at different ZTs. All stressed groups showed significant decrease in enzymatic activities at ZT 12 and 18 when compared with control group (Table 1, Figure 4).

----------Figure 4----------

DISCUSSION

In the present study, ATPase, ADPase and AMPase activities in blood serum were decreased by acute (1h) restraint stress during the dark period (ZT 12 and ZT 18). In previous study we demonstrated that ATPase and ADPase activities (probably the soluble enzyme NTPDase 1-like) exhibit a 24h temporal pattern (Detanico et al., submitted). In the preset study was demonstrated that acute stress causes a loss of this temporal pattern of 24 hours in the nucleotidases activities lasts up to 6h and 24h after the stressor event, only in dark phase (ZT 12 and 18). This suggests that activities of nucleotidases enzymes suffer a higher influence during night hours than at daylight hours by acute stress and this influence seems to persist at least 24 hours. For our knowledge is the first investigation that verifies the effects of stress on temporal pattern of nucleotidases serum, responsible for adenine nucleotides hydrolyze.

This result is corroborated by previous finding in human (Grandin et al., 2006) and in rodents (Meerlo et al., 2002) that shown the disruptions in biological circadian rhythms induced by the acute stress. Thus, it is possible to hypothesize that the disruption of the circadian timing of the activities of nucleotidase enzymes explain, at least in part the physiological process of the cardiovascular events that display circadian pattern, such as the blood pressure, heart rate, vasodilating effects (Lemmer, 2006). Additionally, it is possible

point out that the disruption of orchestred activities of nucleotidase enzymes may be involved in the complex machinery of local oscillators in the heart, endothelium and vascular smooth muscle, endocrine interactions and their regulation by feeding, stress and energetic demands (Hastings et al., 2003). In this line, stress can deregulates the circadian timing presents in the cardiovascular system, since the acute stress might triggers of cardiovascular events by including myocardial infarction, ventricular dysfunction and dysrhythmia (Brotman et al., 2007).

Additionally, mechanisms of the acute stress response promotes the critical secretion of glucocorticoids that is linked in the modulation ATPase, ADPase and AMPase activities (Bohmer et al., 2003). Here, it is important to emphasize that in our study the reduced ATPase, ADPase and AMPase activities in blood serum by acute (1h) restraint stress during night hours (corresponding to wake period in humans) induced an increase in circulating levels of ATP, ADP, AMP and consequently decreased in the production of adenosine. As it is known, ATP constricts vascular smooth muscle via P2X receptor and together with NE stimulates platelet aggregation (Birk et al., 2003). ADP is platelet aggregator (Kunapuli and Daniel, 1998), and promotes vasoconstriction via P_2Y_{12} receptor on vascular smooth muscle cells (Furukoji et al., 2008). Taken together, ATP and ADP exert prothrombotic and proinflammatory effects. Moreover, the AMPase activity decreased after stress during night hours with consequent reduction in the production of adenosine. Effects like vasodilatation via smooth muscule P1 receptor and inhibition of platelet aggregation are mediated by adenosine, pointing to its cardioprotective action (Burnstock, 2006b). Additionally, stress causes release of epinephrine from the adrenal glands, and NE is released together with ATP by the terminus of the sympathetic nervous system (Habib et al., 2001). The elevation of NE and epinephrine in blood by itself can promote thrombosis through vasoconstrictive property and direct action on platelets via α2a receptor (Haft and Fani, 1973; Ikarugi et al., 1999). In

the similar way, under stress condition ATP and ADP act in combination with epinephrine and NE and thus can contribute to cardiovascular events including thrombosis (Birk et al., 2003). Mice NTPDase-1 knockout exhibited perturbation in vasculature and presented hemostatic and thromboregulatory disturbances (Enjyoji et al., 1999), and the recombinant soluble form of human NTPDase 1 was capable to inhibit thrombosis (Pinsky et al., 2002). Therefore, enzymes that degrade adenine nucleotides like E-NTPDase-1 and 5'-nucleotidase are very important in the regulation of cardiovascular system (Yegutkin, 2008). Accordingly, our results indicate a negative influence of acute stress upon nucleotidases (probably NTPDase1-like and 5'-nucleotidase), and it seems to act directly on the hydrolysis of adenine nucleotides only at night period (day in humans) where there is a significant physiological increase of NTPDase-1 activity (Detanico et al., submitted), suggesting a possible deregulation temporal of this enzyme. Even though, AMPase activity possibly mediated by 5'-nucleotidase enzyme does not have a temporal pattern (Detanico et al., submitted), the reduction in activity observed after acute stress only during the dark phase suggests a possible modulation of this enzyme.

In conclusion, acute stress decreased hydrolysis of nucleotides in rat blood serum during the night cycle, and this effect persists for up to 24 hours. It is tempting to propose that altered levels of nucleotides and nucleosides in serum may be involved in the occurrence of cardiovascular events more frequently during the day in humans (Maemura et al., 2007), and with its etiology induced by stress (Brotman et al., 2007).

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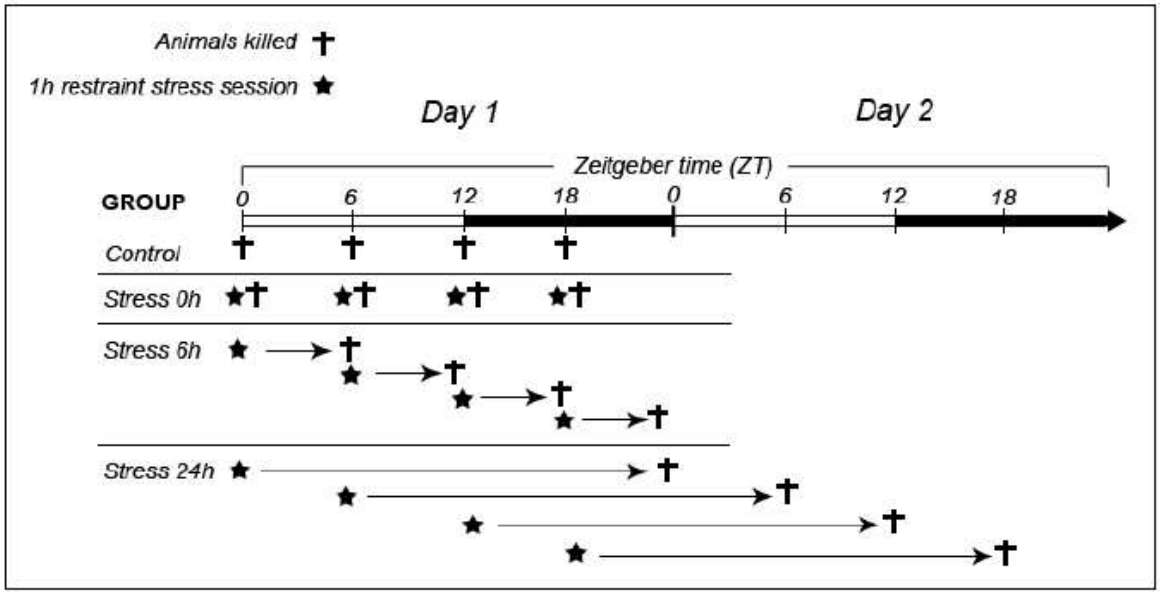
LEGENDS

Figure 1. Experimental design.

Figure 2. Effects of stress on 24-h temporal pattern on ATPase activity in blood serum. Values are expressed as mean ± S.E.M. specific activity (nmoles of Pi produced/mg protein/min). Number of animals per group = 5-10. * indicates significant differences (One-Way ANOVA/Tukey, $P < 0.05$) from ZT 0 and ZT 6 in control group. # indicates significant differences (One-Way ANOVA/Tukey, $P < 0.05$) from control group. Horizontal bar at the base of graph represent day (white) and night (black) phase, with Zeitgeber times (ZTs) indicated below.

Figure 3. Effects of stress on 24-h temporal pattern on ADPase activity in blood serum. Values are expressed as mean ± S.E.M. specific activity (nmoles of Pi produced/mg protein/min). Number of animals per group = 5-10. * indicates significant differences (One-Way ANOVA/Tukey, $P < 0.05$) from ZT 0 and ZT 6 in control group. # indicates significant differences (One-Way ANOVA/Tukey, $P < 0.05$) from control group. Horizontal bar at the base of graph represent day (white) and night (black) phase, with Zeitgeber times (ZTs) indicated below.

Figure 4. Effects of stress on 24-h temporal pattern on AMPase activity on blood serum. Values are expressed as mean ± S.E.M. specific activity (nmoles of Pi produced/mg protein/min). Number of animals per group = 5-10. # indicates significant differences (One-Way ANOVA/Tukey, $P < 0.05$) from control group. Horizontal bar at the base of graph represent day (white) and night (black) phase, with Zeitgeber times (ZTs) indicated below.



Experimental design.
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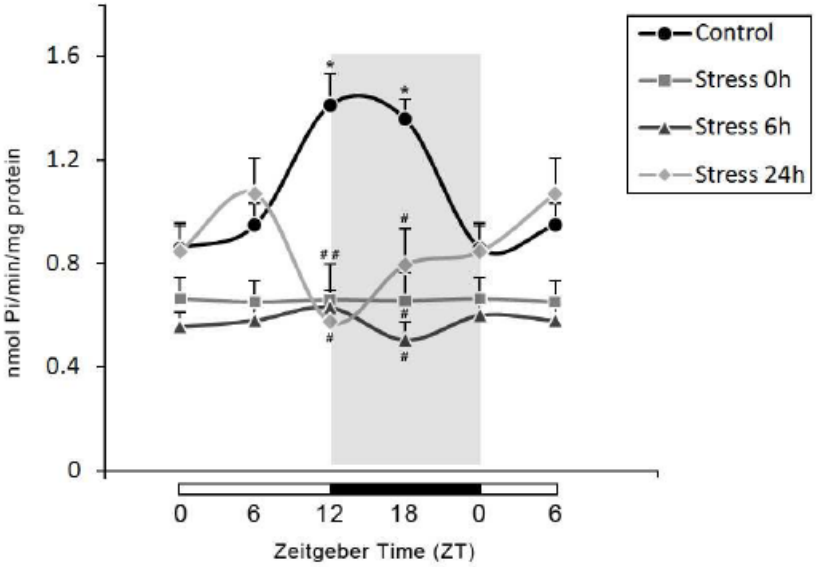

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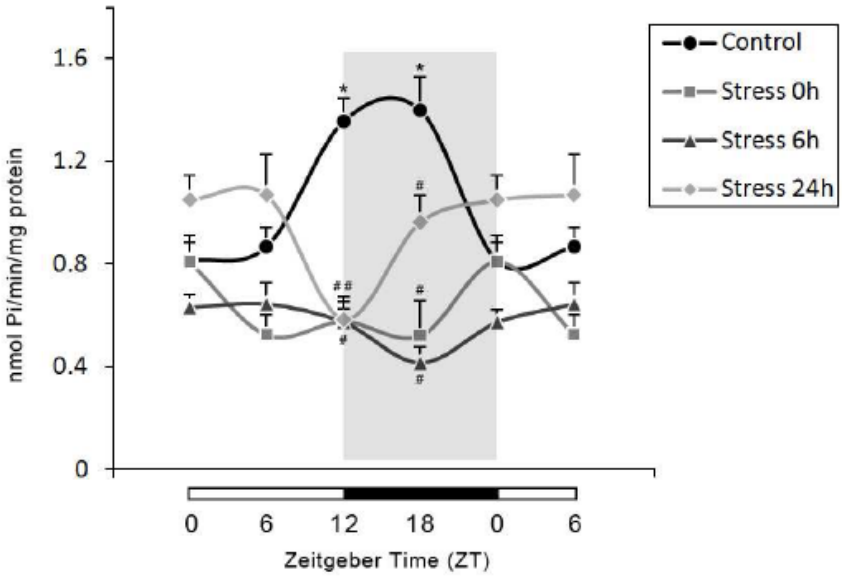

Effects of stress on 24-h temporal pattern on ADPase activity in blood serum. Values are expressed as mean ± S.E.M. specific activity (nmoles of Pi produced/mg protein/min). Number of animals per group = 5-10. * indicates significant differences (One-Way ANOVA/Tukey, P < 0.05) from ZT 0 and ZT 6 in control group. # indicates significant differences (One-Way ANOVA/Tukey, P < 0.05) from control group. Horizontal bar at the base of graph represent day (white) and night (black) phase, with Zeitgeber times (ZTs) indicated below.
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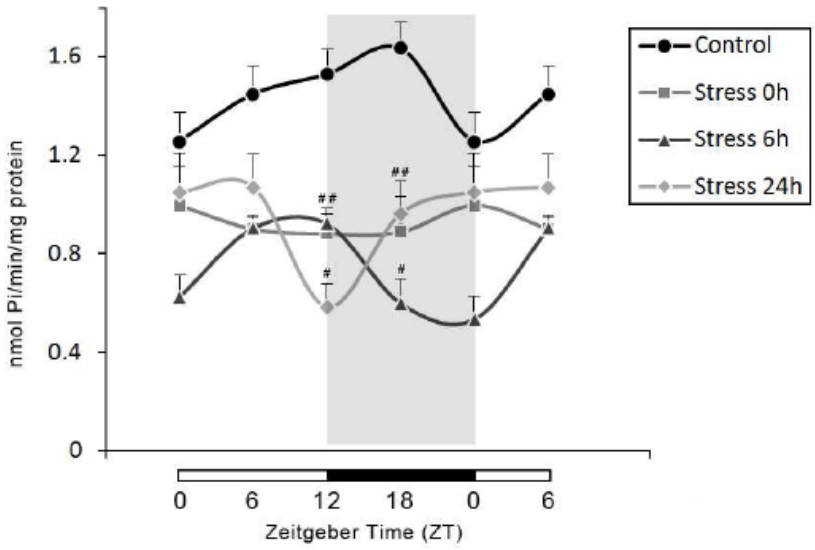

Effects of stress on 24-h temporal pattern on AMPase activity on blood serum. Values are expressed as mean ± S.E.M. specific activity (nmoles of Pi produced/mg protein/min). Number of animals per group = 5-10. # indicates significant differences (One-Way ANOVA/Tukey, P < 0.05) from control group. Horizontal bar at the base of graph represent day (white) and night (black) phase, with Zeitgeber times (ZTs) indicated below.
79x53mm (300 x 300 DPI)

Table 1 Summary of the results of stress effects on ATPase, ADPase and AMPase activities during 24 h.

		Zeitgeber time (h)			
Activity	**Group**	**ZT 0**	**ZT 6**	**ZT 12**	**ZT18**
ATPase	Control	0.85±0.08	0.94±0.05	1.40±0.12#	1.35±0.07#
	Stress 0h	0.66±0.08	0.65±0.08	0.66±0.13*	0.65±0.10*
	Stress 6h	0.55±0.05	0.58±0.05	0.63±0.06*	0.50±0.07*
	Stress 24h	0.84±0.10	1.06±0.13	0.57±0.09*	0.79±0.14*
ADPase	Control	0.81±0.09	0.86±0.07	1.35±0.08#	1.39±0.12#
	Stress 0h	0.80±0.07	0.52±0.07	0.57±0.07*	0.52±0.13*
	Stress 6h	0.63±0.04	0.64±0.08	0.57±0.09*	0.41±0.05*
	Stress 24h	0.92±0.09	1.19±0.15	0.35±0.04*	0.66±0.10*
AMPase	Control	1.25±0.12	1.44±0.11	1.52±0.10	1.63±0.10
	Stress 0h	0.99±0.15	0.90±0.05	0.88±0.10*	0.88±0.14*
	Stress 6h	0.62±0.08	0.90±0.03	0.92±0.03*	0.59±0.09*
	Stress 24h	1.05±0.15	1.06±0.13	0.58±0.09*	0.96±0.12*

The results were expressed as mean ± S.E.M. specific activity (nmoles of Pi produced/mg protein/min). * indicates significant differences (One-Way ANOVA/Tukey, $P < 0.05$) from ZT 0 and ZT 6 in control group. # indicates significant differences (One-Way ANOVA/Tukey, $P < 0.05$) from control group at ZT 12 and 18.

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ARTIGO II: Effect of the stress in the levels of systemic biomarkers of rhythm pattern

Periódico: Neuroendocrinology

Status: Submetido

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With kind regards,

Editorial office

EFFECT OF THE STRESS IN THE LEVELS OF SYSTEMIC BIOMARKERS OF TEMPORAL PATTERN

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Conflict of Interest: There was no financial interest between any of the authors or any commercial interest in the outcome of this study.

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Abstract

Objective: The aim of this study was to evaluate the rhythmic pattern of systemic biomarker in rats submitted to restraint stress. ***Methods:*** We used male Wistar rats with 70 days were divided into ZT0, ZT6, ZT12 and ZT18 groups, and it was subdivided into control, immediately, 6h and 24h after one hour of restraint stress. We used to test one-way ANOVA/SNK (P <0.05). ***Results:*** We observed that when animals were subjected to a single session of stress and analysed immediately after stress there is a loss of temporal pattern of corticosterone levels, and 6h after stress there is an early phase fromZT0 to ZT18, while 24h after the stress the levels returns to the temporal pattern. Immediately after stress, these same animals showed suppressed melatonin peak (ZT18), and it presented a melatonin peak early morning (ZT0). Six and 24 hours after stress the melatonin levels showed a loss of temporal pattern before observed. It is interesting that the glucose levels had the highest peak at ZT18 similar to the peak of melatonin. Regarding the temporal pattern of glucose immediately after stress, it was observed that there was a reversal of the rhythmic pattern associated with a decrease in ZT18 that remained until 6h after stress, returning to temporal pattern 24 hours after stress. ***Conclusion:*** These results confirmed that the temporal patterns of corticosterone, melatonin and glucose have a course of 24 hours, and it demonstrates that a single session of stress is capable of disrupting this temporal pattern.

Keywords: Restraint stress, chronobiology, biomarkers; hormones, blood serum rats.

Running Head: Biologic rhythms and stress.

Introduction

The physiological response to psychological or physical stress consists of an integration of endocrine and autonomic changes. In this situation, adrenomedullary epinephrine is released, and hormones such as CRH, ACTH and glucocorticoids are released by the hypothalamic–pituitary–adrenocortical (HPA) axis [1]. Besides controlling the HPA axis, the hypothalamus is also responsible for the integration of autonomic endocrine and even behavioural responses to stress [2]. Inputs from hypothalamic homeostasis get high priority, as the HPA system contributes to redistribution of bodily resources in times of physiologic need. Recent work showed that stressful conditions resulting in increasing glucocorticoid plasma levels should increase the nocturnal melatonin levels [3]. The melatonin secretion by pineal glad is a classic phase markers for measuring the timing of a mammal's circadian rhythm.

Circadian (~24 h) rhythms are present in physiological, biochemical and behavioural events in a wide variety of organisms, including mammals [4]. It has been proposed that this circadian organization is important to enable an organism to keep the balance in response to the daily external environmental changes and to be prepared accordingly [5]. In mammalians, a number of circadian patterns in biologic functions have been described, including hormonal levels (e.g. melatonin, corticosterone or cortisol, adrenocorticotropic), sleep/wake cycle, body temperature, respiratory rate and blood pressure [6,7].

The peak of melatonin secretion occurs at night and ebbs during the day and its presence provides information about night-length [8]. In the pineal gland, the synthesis of melatonin is triggered by sympathetic outflow in response to darkness-mediated activation of the suprachiasmatic nuclei, which sends information to the paraventricular nucleus (PVN) of the hypothalamus and then to the intermedial lateral column of the spinal cord [9], regulating

the activity of the key enzyme that converts serotonin (5-HT) into N-acetylserotonin (NAS), the immediate precursor of melatonin [10].

Glucocorticoids, typically under the control of the hypothalamic-pituitary-adrenal (HPA) axis, plays an important role in allowing an organism to rapidly adapt to changes in the environment and in maintaining general homeostasis. The HPA axis and circadian system interact at many levels with HPA hormones, following a circadian pattern of release [11,12] and the magnitude of a stress response being dependent upon time of day [13]. Furthermore, glucocorticoids are thought to play a role in maintaining circadian entrainment in peripheral tissues [14,15].

The hypothalamic–pituitary–adrenal (HPA) axis exhibits a pulsatile pattern of secretion of corticotrophin releasing hormone (CRH), adrenocorticotrophic hormone (ACTH) and glucocorticoids (cortisol in humans, corticosterone in rats and mice). This pulsatility has now been reported in approximately rhythm of corticosterone secretion hourly, which appears to be regulated by alternating activation and inhibition of the HPA axis. The nocturnal secretion of ACTH and cortisol in humans reaches its lowest level in the first half of the night, increasing rapidly when the dawn approaches andwhen its secretion is maximal (among 6.00 and 10.00 a.m.) [16]. In the rat, the secretion of corticosterone occurs in the turn round, with peaks in early evening [17].

A healthy response to acute stress is of great importance in our daily physical and psychological challenges. This response is mediated from the hypothalamus to the pituitary gland, which, in turn, it mediates various neuroendocrine signals. When the stressor is gone, the neuroendocrine responses are normally terminated. Failure to terminate this response is observed in various conditions of an overexpressed HPA axis [18] with a subsequent attenuation of the pituitary neuroendocrine response.

Dysregulation and hyperresponsiveness of the HPA axis have been linked to a number of pathological conditions such as increased insulin resistance [19], increased adiposity [20], immune suppression [21], depression [22], osteoporosis [23], muscle wasting [24], and cardiovascular disease [25].

Considering that brief restraint stress procedure is believed to be largely psychological in nature due to the feeling of confinement of the animal and has been [26] shown to affect various endocrine parameters, including the autonomic nervous system [27] and the HPA axis, the purpose of the present investigation is to evaluate the effect of the restraint stress in the levels of the systemic biomarkers of rhythm pattern, melatonin, corticosterone and glucose serum levels [28].

Materials and Methods

Animals

It was used 129 naive adult male Wistar rats (50-70 days old; 150-240 g of weight). The animals were housed in groups of 4-5 in home cages made of Plexiglas (65 x 25 x 15 cm) with the floor covered with sawdust. They were maintained under a standard 12 h-light/dark cycle [lights on at 07.00 h, Zeitgeber time (ZT) 0, and off at 19.00 h, ZT 12], in a controlled environment (22±2°C, rat chow and water ad libitum). Rats had free access to food (standard lab rat chow) and water. The animal's handling and experiments were performed in accordance with the international guidelines for animal welfare. The protocol of this experimental study was approved by the Ethics Committee at the Institution where the work was conducted and is adhered to the ethical and methodological standards for medical biological rhythm research, according to Portaluppi et al. (2008) [29].

Stress Model

Restraint was applied by placing the animal inside a 25 x 7-cm plastic tube, and fixing the tube with adhesive tape on the outside, so that the animal was unable to move. There was a 1 cm hole at the far end for breathing. The animals were submitted to only one procedure of restraint stress for one hour [30]. The apparatus was ventilated and did not cause physical compression, avoiding hyperthermia and sudoresis. Light was perceived through a transparent lid. The control in animals test was not experimentally manipulated.

Experimental Design

Rats were habituated to the maintenance room for two weeks before the beginning of experiment. The animals were divided into 4 groups (ZT0, ZT6, ZT12 and ZT18) according to time of day when the rats were submitted to the model of restraint stress [31]. The ZT (Zeitgeber time) was used as reference to detect the rhythmicity of the variables under study. Whereas when the lights are lit is ZT0 and the ZT12 is the time when the lights are off. As rats are nocturnal animals, the ZT0 roughly corresponds with the start of the home and ZT12 corresponds to the beginning of the active phase [32]. On the experimental day, animals were subdivided in 4 groups according to time of death (control group, immediately, 6 hours and 24 hours after stress). For this purpose, individual rats were quickly transferred to a separate room and decapitated within 1 min. Trunk blood was drawn and blood samples were centrifuged in plastic tubes for 5 min at 5000 x g at room temperature [33]. Serum was obtained and frozen at −20 °C until the assays perform.

---------- Insert Figure 1 ----------

Blood Serum Hormonal Assays

The hormonal levels in the blood serum samples were determined using a corticosterone ELISA kit (IBL-America #IB79112), melatonin ELISA kit (MP Biomedicals), and glucose kit PAP Liquiform (Labtest, ref. 84).

Statistical Analysis

Data was expressed as means ± standard error of the mean (SEM). The one-way ANOVA test was performed, followed by a test of multiple comparisons (Student's-Newman-Keuls). SPSS 17.0 for Windows was used for statistical analysis, and significance was set to $P < 0.05$.

Results

The animals' control (no disturb) and stress (submitted to one restraint stress) were analysed in the different time points (ZT0, ZT6, ZT12 and ZT18). All these groups were divided in 3 subgroups that were killed immediately, 6 and 24 hours after restraint stress section.

Effect of Temporal Patterns in Glucose level by Restraint Stress in Rats

Considering different time points evaluated in ZT18, the animal presented a significant increase in the level of serum glucose when comparing to ZT0 group. Immediately after stress exposure, the animals evaluated in ZT18 presented a significant decrease in the level serum glucose when compared to other time points (one way ANOVA/SNK, $P<0.05$, see horizontally in the table 1 and figure 2- panel A).

The groups of animals analysed immediately after stress presented in ZT0 point and in ZT6 point a significant increase in the level of serum glucose when compared to control

group and other groups, respectively. The groups of animals analysed in ZT18 presented a significant difference in the level of serum glucose in both groups, immediately after stress group and 6 hours after stress group when compared to other groups (one way ANOVA/SNK, $P<0.05$ to all, see vertically in the table 1).

Effect of Temporal Patterns in Melatonin level by Restraint Stress in Rats

In ZT18 point the control of animals presented a significant increase in the level of serum melatonin when compared to other groups. The animals evaluated immediately after the stress presented a significant increase in the level serum melatonin in ZT0, compared to other times points (one way ANOVA/SNK, $P<0.05$ to all, see horizontally in the table 1 and figure 2- panel B).

Only ZT0 point presented a significant increase in the level of serum melatonin in the groups of animals evaluated immediately after stress when compared to control group (one way ANOVA/SNK, $P<0.05$, see vertically in the table 1).

Effect of Temporal Patterns in Corticosterone level by Restraint Stress in Rats

In ZT6 and ZT12 the control groups presented a significant increase in the level of serum corticosterone when compared to other time points. Between the groups that were evaluated 24 hours after the stress, ZT0 and ZT12 points presented a significant difference in the level of serum corticosterone when compared to other time points, showing a decrease and an increase in the levels, respectively (one way ANOVA/SNK, $P<0.05$, see horizontally in the table 1 and figure 2- panel C).

In ZT0 point, the animals evaluated immediately after stress presented a significant increase in the level of serum corticosterone when compared to other groups, and 24 hours

after stress presented a significant decrease when compared to 6 hours after stressed group (one way ANOVA/SNK, $P<0.05$, see vertically in the table 1).

---------- Insert Table 1 ----------

---------- Insert Figure 2 ----------

Discussion

This work evaluates important biomarkers to maintenance of homeostasis of the body. The results obtained in this study have confirmed that the temporal pattern of corticosterone, melatonin and glucose have a course of 24 hours, and it demonstrates that a single session of stress is capable of disrupting this temporal pattern, and its effect lasts for at least 24 hours.

More specifically we observed that when the animals were subjected to a single session of stress and analysed immediately after stress, there is a loss of temporal pattern of corticosterone levels, and 6h after stress there is an early phase of ZT0 to ZT18, while 24h after the stress the levels returns to the temporal pattern. Immediately after stress, these same animals showed suppressed melatonin peak (ZT18), and it presented a melatonin peak early morning (ZT0). Six and 24 hours after stress the melatonin levels showed a loss of temporal pattern before observed. It is interesting to notice that glucose levels had the highest peak at ZT18 similar to the peak of melatonin. Regarding the temporal pattern of glucose immediately after stress, it was observed that there was a reversal of the rhythmic pattern associated with a decrease in ZT18 that remained until 6h after stress, returning to temporal pattern 24 hours after stress.

Many aspects of human physiology, metabolism and behaviour vary over the 24-hour day and can have a major impact on our health and well-being. For example, the risk for

adverse cardiovascular incidents shows a peak in the morning, temporal lobe epileptic seizures are more frequent in the late afternoon and asthma is generally worse at night [34,35]. This variation over 24-h can either be caused by changes in behavioural activity such as stress, the sleep/wake cycle, the fast/feeding cycle, the light/dark cycle, or be centrally orchestrated by an endogenous circadian system [36,37,38].

Immediately after the stress, it was observed an increase of corticosterone levels associated to a reversal of the rhythmic pattern, and both returned to temporal pattern 24 hours after stress. It is evident that glucocorticoids antagonise the effects of insulin at tissues where insulin performs its major storing actions (hepatocytes, adipocytes, and muscle tissue) by exerting opposite gluconeogenic effects to increase plasma glucose levels [39].

In addition, we observed the similar peaks of glucose and melatonin in ZT18, and the stress was capable of decreasing this level. The role of melatonin in human glucose regulation is poorly understood. Under normal conditions, glucose tolerance is modulated by circadian rhythmicity and sleep, two central nervous systems processed which may be influenced by melatonin. In the presence of a constant stimulus (e.g. intravenous glucose infusion), blood glucose levels increase from morning to evening and further increase until the middle of sleep, when a decline towards morning levels is initiated. This 24h variation is due to coordinated changes in insulin-dependent and non-insulin dependent glucose utilization (e.g. by the brain), in insulin sensitivity and in insulin secretion. Changes in sympatho-vagal balance at the level of the pancreas could also be implicated but have not been investigated. Melatonin is likely to play an indirect role in the mechanisms underlying glucose regulation via its actions on the suprachiasmatic nucleus and on sleep regulation [40]. The effect of melatonin in influencing the circadian rhythm of blood glucose was also studied in rabbits. Results showed that melatonin influences the circadian rhythm leading to a shift in the occurrence of minimums levels from 16.00 hr to 04.00 hr (next day) during fasting and

from 16.00 hr to 20.00 hr during feeding. The melatonin treatment also leads to a significant rise in blood glucose levels. It is probable that melatonin administration reduces glucose tolerance and influences the blood glucose circadian rhythm mainly through its effects on insulin release by pancreatic B cells [41]. The result set indicates importance physiological pulsations and the idea uses the analysis of kinetics of secretion of glucose as diagnostic tool, since these rhythmical changes of the oscillations of plasma glucose could be an early marker of development of diabetes [42,43].

In this study, we demonstrated that immediately after single session of stress there was a loss of temporal pattern of corticosterone, and suppression the melatonin peak in ZT18 that passing to be observed in ZT0 (early morning). 6 and 24 hours after stress there was a loss of temporal pattern of melatonin before observed. The interactions between the pineal melatonin secretion and the hypothalamic secretion of CRH are so far not fully understood. Most likely, melatonin regulates the binding sites of CRH [44]. As reported by Konakchieva et al. (1997) [45] exogenous melatonin administration in rats attenuated the adrenocortical response to both acute and chronic stress and prevented the decline in ACTH release resulting from chronic stress exposure. In this way, melatonin treatment may protect against disruptions caused by stress and thereby may reestablish homeostasis [46]. Moreover, as reported earlier, CRH may also inhibit secretion of melatonin in humans [47]. In addition, recent work with animal models showed that stressful conditions that result in increasing plasma glucocorticoid levels should increase the nocturnal melatonin levels [18]. However, the effect of corticosterone on NA-induced melatonin production follows a bell-shaped curve, therefore a mild but not severe stress increases corticosterone plasma levels and enhances the production of melatonin [48]. Thus, we may be suggested that the disturbances observed in the melatonin secretion in the stressed animals may reflect an apparent over expressed HPA axis.

In summary, this study demonstrated the importance of circadian variations in endogenous secretion of biomarkers, and they demonstrate pronounced oscillations, which show a high degree of temporal organization of organisms. In addition, these results showed that unique session of stress was able to produce a deregulation of temporal pattern these biomarkers. We may suggest that the temporal oscillations can also be used as a key element in the diagnosis and treatment of disorders involving these biomarkers.

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LEGENDS

Table 1. Effect of the Stress in the Levels of Systemic Biomarkers of Rhythm Pattern. Values are expressed as mean ± S.E.M.(corticosterone: ng/mL, melatonin: pg/mL, glucose: mg/dL), significantly different (One-Way ANOVA/SNK, $P<0.05$). Horizontally: * different to other groups; [a] different of ZT0; [b] different of ZT6; [c] different of ZT12. Vertically: # Different to other groups; [d] different of control; [e] different of 24 hours after stress.

Figure 1. Experimental design.

Figure 2. Effect of the Stress in the Levels of Systemic Biomarkers of Rhythm Pattern. Values are expressed as mean ± S.E.M., significantly different (One-Way ANOVA/SNK, P<0.05). Horizontal bar at the base of graph represent day (white) and night (black) phase, with Zeitgeber times (ZTs) indicated below.

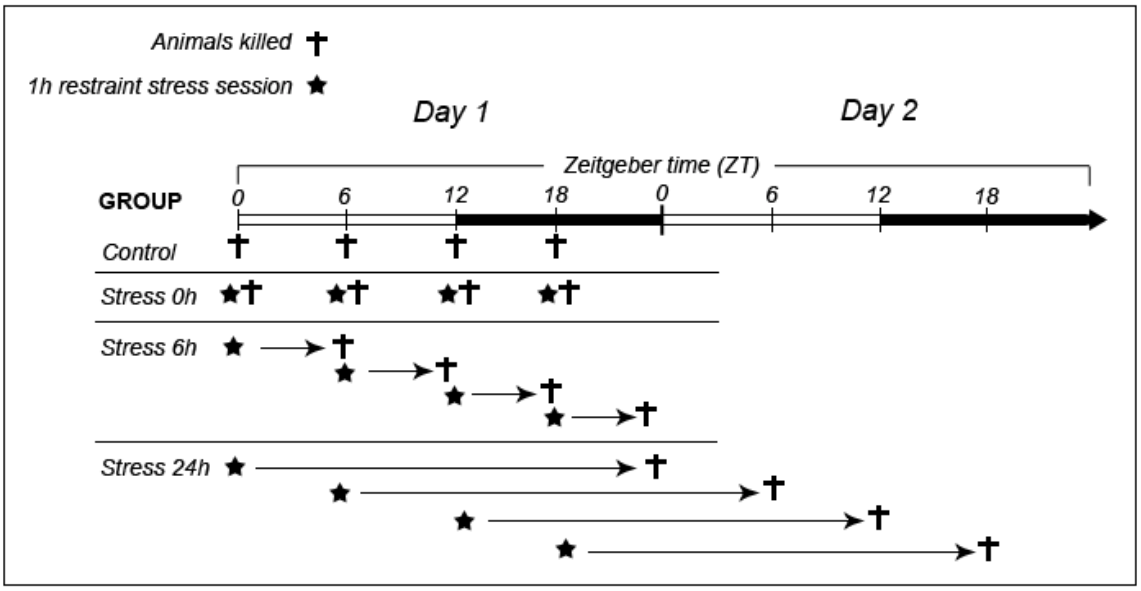
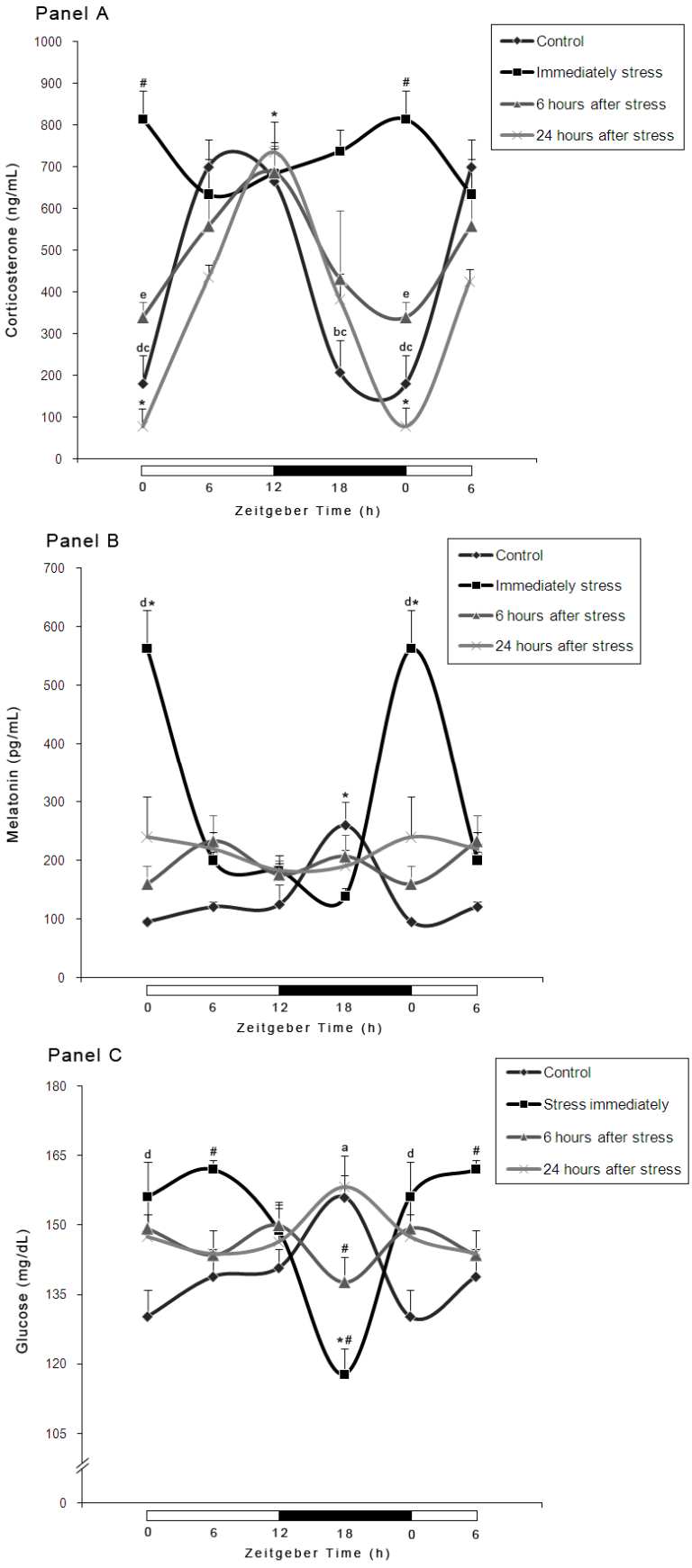


Table 1. Effect of the Stress in the Levels of Systemic Biomarkers of Rhythm Pattern.

	Group	*ZT0 (n)*	*ZT6 (n)*	*ZT12 (n)*	*ZT18 (n)*	*F*
C – Glucose	Control	130.28±5.75 (7)	138.91±4.85 (6)	140.80±4.03 (7)	156.01±4.68 (6)[a]	4.666
	Immediately stress	156.19±7.47 (7)[d]	162.04±1.92 (5)#	148.87±5.55 (7)	117.80±5.63 (7)*#	11.077
	6 hours after stress	149.26±6.68 (7)	143.57±1.28 (5)	149.95±4.94 (7)	137.70±6.69 (6)#	1.017
	24 hours after stress	147.46±4.89 (7)	143.80±4.99 (5)	146.39±7.17 (5)	158.27±5.36 (7)	1.389
	F	3.082	7.060	0.637	11.710	
B – Melatonin	Control	95.90±3.49 (4)	121.55±8.25 (4)	125.65±32.71 (4)	260.10±39.39 (4)*	8.117
	Immediately stress	562.93±66.09 (3)* [d]	200.54±14.41 (5)	182.60±26.71 (5)	139.02±13.25 (5)	37.539
	6 hours after stress	169.60±30.63 (5)	233.06±43.26 (5)	176.68±22.43 (5)	207.25±37.17 (4)	0.768
	24 hours after stress	240.08±69.52 (4)	219.70±29.01 (5)	183.00±14.75 (5)	190.38±27.07 (5)	0.503
	F	3.964	2.693	1.170	2.035	
A- Corticosterone	Control	179.93±68.07 (4)[b c]	700.08±65.47 (4)	665.95±94.19 (4)	207.00±78.30 (4)[b c]	13.411
	Immediately stress	814.70±67.59 (4)#	634.66±84.64 (5)	684.50±60.35 (4)	738.28±51.52 (4)	1.233
	6 hours after stress	339.88±37.01 (5)	558.40±87.92 (3)	685.98±65.30 (5)	431.78±162.79(4)	1.966
	24 hours after stress	77.53±44.98 (4)*	434.10±29.95 (4)	735.92±73.18 (5)*	381.70±63.39 (4)	21.249
	F	34.137	3.017	0.782	2.380	

Figure 1.


Panel A
Control
Immediately stress
6 hours after stress
24 hours after stress
Corticosterone (ng/mL)
Zeitgeber Time (h)
Panel B
Control
Immediately stress
6 hours after stress
24 hours after stress
Melatonin (pg/mL)
Zeitgeber Time (h)
Panel C
Control
Stress immediately
6 hours after stress
24 hours after stress
Glucose (mg/dL)
Zeitgeber Time (h)

ARTIGO III: Evaluation of effect of temporal patterns in behavioral response by restraint stress in rats

Periódico: International Chronobiology

Status: a ser submetido

EVALUATION OF EFFECT OF TEMPORAL PATTERNS IN BEHAVIORAL RESPONSE BY RESTRAINT STRESS IN RATS

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Conflict of Interest: There was no financial relationship between any of the authors or any commercial interest in the outcome of this study.

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Abstract

Considering the hypothesis that there is a correlation between temporal patterns and effect of stress exposure, this work aimed to answer two questions: (i) Is there a diurnal variation in the temporal patterns in locomotion and anxiety-like behaviors according the day time in which the stress is applied (ZT0, ZT6, ZT12 and ZT18)?; (ii**)** What is the duration of the stress effect in the rhythmic pattern in locomotion and anxiety-like behaviors (immediately, 6 and 24h after stress procedure)? 121 male Wistar rats with 70 days of age (150-230g) were randomized and kept 5 animals /box. Immediately after the stress session, in each time listed above, the animals were exposed to the apparatus of open-field or plus-maze. The parameters analyzed in the open field were the total locomotion, the rearing and number of fecal boluses. In plus maze test were evaluated: number of protected head-dipping (PHD*)*; number of non-protected head-dipping (NPHD); total number of entries in the open arms and in the closed arms (TA); the time spent on the open arms (TOA); the time spent on the closed arms (TCA). The data were analyzed by ANOVA followed by a Student-Newmann-Keuls test when indicated, considered significantly different with $P < 0.05$. The behavioral response evaluated in the plus maze test showed a temporal pattern on the number of PHD and NPHD. It was also observed a rhythmic pattern in time spent in the open and close arms. The open-field test only the number of fecal boluses presented rhythm pattern. In addition, the stress exposure was able of disrupting the rhythm observed in behaviors in both Plus-maze test and open-field test. In addition, rearing and total locomotion behaviors showed alterations after stress. It is important to note that the stress effect was more pronounced after 6 and 24 h after the exposure. The present findings suggest that behavioral results may be directly influenced by the daytime that it was performed and it was performed may be lead to misinterpretation of results.

Keywords: ***restraint stress, chronobiology, open field; plus maze, rats.***

Running head: Biologic rhythms and stress.

1. Introduction

Biological rhythms are endogenous in origin cyclic changes that correspond to the temporal organization of the environment (Goldbeter, 2008; Roenneberg et al., 2003; Duguay & Cermakian, 2009). In mammals, a number of circadian patterns have been described, including hormones levels (e.g. melatonin, corticosterone or cortisol, adrenocorticotropic), sleep/wake cycle, body temperature, respiratory rate, heart rate, and blood pressure (Smolensky and Peppas, 2007; Haus, 2007; Vieira et al., 2010). Also, the potency and/or toxicity of drugs changes according to the time of their administered during the 24 hours of the day (Ohdo, 2003; Debon et al., 2004; Pan et al., 2005).

The body's endogenous circadian clock receives information from the external and internal environments, and its function is the timing organization of the physiological and behavioral processes in a circadian pattern (Cardoso et al., 2009). The environmental cues that each day reset the rhythms are called *Zeitgebers* (from the German, Time Givers). The main environment *Zeitgebers* is the cycle light/dark that regulated the melatonin secretion by pineal glad, which is a classic phase markers for measuring the timing of a mammal's circadian rhythm (Murphy et al., 2007). The peak of melatonin secretion occurs at night and ebbs during the day and its presence provides information about night-length (Hastings et al., 2003). The melatonin secretion may be affected by several factors, such as endogenous fluctuations and external influences, which affect touch-evoked pain (Ambriz-Tututi, et al., 2009) and levels of physical activity and the stress level (Couto Moraes et al., 2009).

Thus, it is plausible suppose that the activation of hypothalamus–pituitary–adrenal (HPA) axis by the stress, leading to a sustained tonus of corticosterone release that in together with the activation of the sympathetic nervous system produce a cascade events (Steiner, et al., 1990). Take into account that the stress influenced strongly the routine of modern life, its

effect in the biological regulation could be more widely studied, including the intensity of stress and time of day in which it was applied. Considering the hypothesis that there is a correlation between temporal patterns and effect of stress exposure, this work aimed to answer two questions: (i) Is there a diurnal variation in the temporal patterns in locomotion and anxiety-like behaviors according the day time in which the stress is applied (ZT0, ZT6, ZT12 and ZT18)?; (ii**)** What is the duration of the stress effect in the rhythmic pattern in locomotion and anxiety-like behaviors (immediately, 6 and 24h after stress procedure)?.

2. Materials and Methods

2.1. Animals

It was used 121 naive adult male Wistar rats (50-70 days old; 150-240 g of weight) were used. The animals were housed in groups of 4-5 in home cages made of Plexiglas (65 x 25 x 15 cm) with the floor covered with sawdust. They were maintained under a standard 12 h-light/dark cycle [lights on at 07:00 h, Zeitgeber time (ZT) 0, and off at 19:00 h, ZT 12], in a controlled environment (22±2°C, rat chow and water ad libitum). Rats had free access to food (standard lab rat chow) and water. The animal's handling and experiments were performed in accordance with the international guidelines for animal welfare. The protocol of this experimental study was approved by the Ethics Committee at the Institution where the work was conducted and is adhered to the ethical and methodological standards for medical biological rhythm research according with Portaluppi et al. (2008).

2.2. Stress model

Restraint was applied by placing the animal inside a 25 x 7-cm plastic tube, and fixing the tube with adhesive tape on the outside, so that the animal was unable to move. There was

a 1 cm hole at the far end for breathing. The animals were submitted to only one procedure of restraint stress for one hour (Torres et al., 2003). The apparatus was ventilated and did not cause physical compression, avoiding hyperthermia and sudoresis. Light was perceived through a transparent lid. The experimental controls animals were not experimentally manipulated.

The animals were divided into 4 groups (ZT0, ZT6, ZT12 and ZT18) according to time of day when the rats were submitted to the model of restraint stress (Torres et al., 2002). All experiments were done immediately, 6 and 24 hours after stress was applied. The ZT (Zeitgeber time) was used as reference to detect the rhythmicity of the variables under study. Whereas when the lights are lit is ZT0 and the ZT12 is the time when the lights are off. As rats are nocturnal animals the ZT0 roughly corresponds with the start of the home and ZT12 corresponds to the beginning of the active phase (Pelegri, 2003).

……..…..insert figure 1……

2.3 Behavioral Procedures

2.3.1 Open Field Test

The evaluation of behavior was performed in a varnished wood cage, measuring 60 x 40 x 50 cm, with the inside lined with glass. The floor was recovered with linoleum divided into 12 rectangles of 13.0 x 13.0 cm with dark lines. The animal was gently placed in the left back corner and left free to explore the surroundings for 5 min (Bianchin et al., 1993; Carlini et al., 2002). The number of crossings performed by each animal was taken as locomotor activity (Roesler et al., 1999). The latency to leave of the first quadrant was taken as the anxiety measurement (Britton and Britton, 1981; Lister, 1990). Rearing was defined as the moment the rat rose up on its hind legs and ended when one or both front paws touched the floor again (Wells et al., 2008) and it was evaluated as exploratory activity (Silveira et al., 2005). The

start of a trial was immediately after the rat was placed in the environment for scoring purposes. In this test, the animal was recorded as entering a new area when all four of the animal's paws crossed the boundary into a different marked-out area. Five measures were taken during the 5 min test sessions: (1) the number of line crossings (i.e. horizontal activity), outer and inner crossings in the OF; (2) number of fecal boluses; (3) the number of rearing behaviors (i.e. vertical activity). The box was cleaned between each trial.

2.3.2 Elevated plus-maze test (EPM)

The elevated plus-maze test was used to assess the anxiety-like behavior state. At the start, the animal was placed into the central area of the EPM, facing one of the open arms, and the behavior was recorded for 5 minutes. The apparatus comprised two open arms and two closed arms (50cm x 40cm x 10 cm) that extended from a common central platform (10 x 10 cm). The maze was constructed from black PVC synthetic material and elevated to a height of 50 cm above floor level. The measures were taken during the 5 min test sessions: (1) number of protected head-dipping (PHD*)*; (2) number of non-protected head-dipping (NPHD); (3) total number of entries in the open arms and in the closed arms (EA); (4) the time spent on the open arms (TOA); (5) the time spent on the closed arms (TCA). Protected head dips included dipping the head over the sides of the maze from within the center platform or a closed arm, whereas unprotected head dips were considered when the animal dipped its head over the sides of the maze while on an open arm. In the EPM, entering a new area was recorded when all four paws crossed onto a new arm or into the central area (Lynn, 2009). Each subject was only tested once in each novel environment. After each test, the apparatus was cleaned to remove any odor.

2.4. Statistical analysis

Data was expressed as means $\pm$ standard error of the mean (SEM). The one-way ANOVA test was performed, followed by a multiple comparisons test (Student's-Newman-

Keuls). SPSS 17.0 for Windows was used for statistical analysis, and significance was set to $P < 0.05$.

3. Results

3.1. Effect of Temporal Patterns in Open Field Test by Restraint Stress in Rats

The animals control (no disturb) and stress (submitted to one restraint stress) were analyzed in the different time points (ZT0, ZT6, ZT12 and ZT18). All these groups were subdivided in 3 groups that were evaluated immediately, 6 hour and 24 hour after restraint stress section. The behaviors evaluated were rearing, total locomotion and fecal boluses.

3.1.1. Rearing Behavior

There was no significant difference between control groups and stress immediately groups in the rearing in all times analyzed. There was a significant increase in number of rearing in ZT6 and ZT12 between the groups that were evaluated 6 hours after stress when compared to ZT0 and ZT18 groups. Between the groups that were evaluated 24 hours after stress, it was observed a significant decrease in number of rearing in the ZT0 when compared to other groups (see horizontally in the table 1).

All groups (no disturb and stress) analyzed in ZT0 time point showed no differences among the groups. In ZT6, the animals evaluated 6 hours after stress presented a significant increase in number of rearing when compared to other groups, and in ZT12, the group analyzed immediately after stress presented a significant decrease in number of rearing when compared to group analyzed 6 hours after stress. In ZT18 time point, the group evaluated 24 hours after stress presented a significant increase in number of rearing when compared to other groups (see vertically in the table 1).

3.1.2. Total locomotion behavior

There was no significant difference in all times analyzed between the control groups. When the groups were evaluated immediately after stress, it was observed in ZT12 time point a significant increase in when compared to ZT0 and ZT6 groups. In 6 hours after stress at ZT18 presented decrease when compared to other groups, and ZT6 showed a significant increase when compared to ZT0 time point. In groups evaluated 24 h after stress at ZT18 group presented decrease in relation to ZT0 group (see horizontally in the table 1).

At ZT0 time point, the 24 hours after stress group presented a significant increase in total time locomotion compared to other groups. At ZT6, the groups evaluated 6 hours and 24 hours after stress showed a significant increase in of total locomotion when compared to other groups. At ZT12 time point here were no differences among the groups. At ZT18 the groups evaluated immediately and 24 hours after stress showed a significant increase when compared to 6 hours after stress group (see vertically in the table 1).

3.1.3. Fecal boluses behavior

The animals of control groups evaluated at ZT18 time point presented a significant decrease when compared to ZT12 group. At immediately after stress the ZT6 showed a significant increase in when compared to ZT12 and ZT18 groups. At 6 hours after stress, ZT18 time point presented a significant increase when compared to ZT0 and ZT12. At ZT6 the animals presented a significant increase when compared to other groups evaluated 24 hours after stress (see horizontally in the table 1).

When the animals were analyzed in ZT0 time point, the all stressed groups presented a significant decrease when compared to control group. At ZT6 time point, the groups evaluated immediately after stress presented a significant decrease when compared to 24 hours after stress group. At ZT12 the groups evaluated 6 hours and 24 hours after stress

presented a significant decrease when compared to control group, and the groups evaluated immediately after stress presented a significant decrease when compared to other groups. At ZT18 time point, the groups evaluated 6 hours after stress presented a significant increase when compared to other groups (see vertically in the table 1).

……..……..insert table 1……

3.2. Effect of Temporal Patterns in Plus Maze Test by Restraint Stress in Rats

The control (no disturb) and stressed groups were analyzed in the different time points (ZT0, ZT6, ZT12 and ZT18). All these groups were subdivided in 3 groups were evaluated immediately, 6 hour and 24 hour after restraint stress section. The behaviors evaluated were: number of protected head-dipping (PHD); number of non-protected head-dipping (NPHD); total number of entries in the open arms and in the closed arms (EA); the time spent on the open arms (TOA); the time spent on the closed arms (TCA).

3.2.1. Number of protected head-dipping (PHD)

At ZT12 the control group presented a significant decrease when compared to other control groups evaluated at different time points of day. At ZT18 the group evaluated immediately after stress presented a significant increase when compared to ZT0 and ZT12 groups, and the ZT18 group evaluated 6 hours after stress presented a significant decrease when compared to ZT6 time point. At ZT12, the group evaluated 24 hours after stress presented a significant increase when compared to ZT0 and ZT6 groups (see horizontally in the table 2).

The evaluation of PHD behavior at ZT0 and ZT6 did not show differences among the groups. At ZT12 time point, the groups evaluated 6 hours after stress presented a significant increase when compared to control group, and the group evaluated 24 hours after stress

presented significant increase when compared to immediately after stress and control groups. At ZT18 time point, the 6h after stress group presented decrease when compared to immediately stress group (see vertically in the table 2).

3.2.2. Number of non-protected head-dipping (NPHD)

At ZT12 and ZT18 control groups presented a significant increase when compared to ZT0 group. The groups that were evaluated immediately after stress showed no difference in all times point evaluated. At ZT18 time point, the groups evaluated 6 hours after stress presented a significant decrease when compared to other groups. The groups evaluated 24 hours after stress did not show significant difference in all point times evaluated (see horizontally in the table 2).

At ZT0 there were no differences among the groups, but at ZT6 the groups evaluated 24 hours after stress presented a significant decrease when compared to control and 6 hours after stress groups. At ZT12, the groups evaluated immediately and 24 hours after stress presented a significant decrease when compared to control group, and at ZT18 the stressed groups presented a significant decrease independently of time point when compared to control group (see vertically in the table 2).

3.2.3. Total number of entries in the open arms and in the closed arms (EA)

At ZT0 the control group presented a significant decrease when compared to other control groups. The groups evaluated immediately and 24 hours after stress did not show significant difference. At ZT18, the groups evaluated 6 hours after stress presented a significant decrease when compared to ZT6 and ZT12 groups (see horizontally in the table 2).

At ZT0 there were no differences between the groups. At ZT6, the groups evaluated 24 hours after stress presented a significant decrease when compared to control and 6 hours

after stress groups. At ZT12 the groups evaluated immediately after stress presented a significant decrease when compared to control group. At ZT18 the groups evaluated immediately and 6 hours after stress presented a significant decrease when compared to control group, and the groups evaluated 6 hours after stress presented a significant decrease when compared to groups evaluated 24 hours after stress (see vertically in the table 2).

3.2.4. Time spent on the open arms (TOA)

Considering the different time points, in relation the control groups, ZT18 the groups presented significant decrease when compared to ZT0 group. There were no differences between the groups evaluated immediately and 24 hours after stress. At ZT18 the groups evaluated 6 hours after stress presented a significant increase when compared to other groups (see horizontally in the table 2).

At ZT0 and ZT12 there were no differences between the all groups analyzed. At ZT6 the group analyzed 24 hours after stress presented a significant increase when compared to control and 6 hours after stress groups. At ZT18, the stressed groups presented a significant decrease when compared to control group in all time point of day evaluated (see vertically in the table 2).

3.2.5. The time spent on the closed arms (TCA)

When analyzed the control groups, at ZT18 presented a significant increase when compared to ZT0 time point. The groups evaluated immediately and 24h after stress did not show difference between the all time points of day. When the groups were evaluated 6 hours after stress, at ZT18 showed a significant decrease when compared to other groups (see horizontally in the table 2).

At ZT0, the control and stressed groups did not show differences amongst themselves, and at ZT6 the group evaluated 24 hours after stress presented a significant decrease when compared to other groups. At ZT12, the group analyzed immediately after stress presented a significant decrease when compared to control group, and at ZT18 the all stressed groups presented a significant decrease when compared to control group (see vertically in the table 2).

……..…..insert table 2……

4. Discussion

The present study showed that the behavioral response evaluated in the plus maze test showed a temporal pattern on the number of PHD and NPHD behaviors. It was also observed a rhythmic pattern, the time spent in the open and close arms. These results showed increase anxiety symptoms in nocturnal period in male rats. In addition, the unique stress exposure also was able of disrupting the rhythm observed in behavior evaluated on Plus-maze test.

When the animals were evaluated in the open field test, the only one behavior that presented rhythm pattern it was the number of fecal boluses, and the stress was able of altered this behavior rhythm. In addition, rearing and total locomotion behaviors showed alterations after stress. It is important to note that the stress effect was more pronounced after 6 and 24 h after stress exposure. Therefore, it is necessary some hours for that effect of stress appear. This suggests that these alterations in behavioral responses may be explained by the synthesis of neurotransmitters. Our results agree with what was described by Turek (1998) that demonstrated that the body adapts physiologically to behavioral alteration such as challenges associated to it, resulting in a synchronization between the organism and external environment. The instability of circadian rhythmicity with repeated desynchronization

/resynchronization, can weaken the homeostatic mechanisms. An example of this phenomenon is the exposure to stress increasingly in the organization of society. The consequence of this weakness is possibly implicated in the genesis or acceleration of the health-disease (Kern, 1996).

In this work showed that rats in middle of the night remained lower open arms when compared with other times of the day suggesting ansiogenic effect time-dependent. However when induced stress, there is a lost rhythmic with different answers at all times of day. Circadian rhythms can be regulated by a variety of external indicators, such as pulses of ambient temperature, meals and bedtime and wake up (Gronfier et al., 2007). But the pace light and dark is a key variable in the synchronization (or desynchronization) (Gronfier et al., 2007).

Daan et al. (2001) suggested the presence of two genetic oscillators activated by radiation level of light produced by the seasons and the intensity of light could be measured by the central nervous system and acts on two pairs of circadian genes (per1, cry1, per2 and cry2). The oscillator per1/cry1 would be accelerated by light and decelerated by darkness, while the oscillator per2/cry2 would be slowed by light and accelerated by the dark, providing a perfect molecular mechanism for measuring the intensity of motor activity. The mechanisms of these endogenous rhythms provide an anticipatory capacity, enabling animals to organize physiological and behavioral resources before they are needed, providing a more efficient response to environmental changes (Lambec and Shine, 1990; Paranjpe and Sharma, 2005).

Our results agree with previous work that it showed the circadian system is influenced by the degree of brightness allowed the emergence of rhythmic oscillators able to prepare adequately the behavior and physiology of animals, preceding the changes of the environment (Daan et al., 2001).

In conclusion, it is noteworthy that chronobiology is an area that is developing rapidly and that experimental studies should be encouraged, because the results of these can contribute to future studies. Since, the present findings suggests that the behavioral results may be directly influenced by the daytime that it was performed and it introduces a new question in the researches area: the hour in which the experiment was performed may be lead to misinterpretation of results. Conversely, additional experiments are needed to understand the neurophysiologic mechanism underline these behavioral responses.

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Legends

Figure 1. Experimental design.

Table 1. Temporal Patterns in Open Field Test by Restraint Stress in Rats. Values are expressed as mean ± S.E.M. (absolute number), significantly different (One-Way ANOVA/SNK, P<0.05). Horizontally: * different to other groups; ❶ different de ZT0; ❷ different of ZT6; ❸ different of ZT 12; vertically: # = Different to other groups; ① different of control; ② different of immediately after stress; ③ different of 6 hours after stress; ④ different of 24 hours after stress.

Table 2. Temporal Patterns in Plus Maze Test by Restraint Stress in Rats. Values are expressed as mean ± S.E.M. (absolute number), significantly different (One-Way ANOVA/SNK, P<0.05). Horizontally: * different to other groups; ❶ different de ZT0; ❷ different of ZT6; ❸ different of ZT 12; vertically: # = Different to other groups; ① different of control; ② different of immediately after stress; ③ different of 6 hours after stress; ④ different of 24 hours after stress.

Figure 1

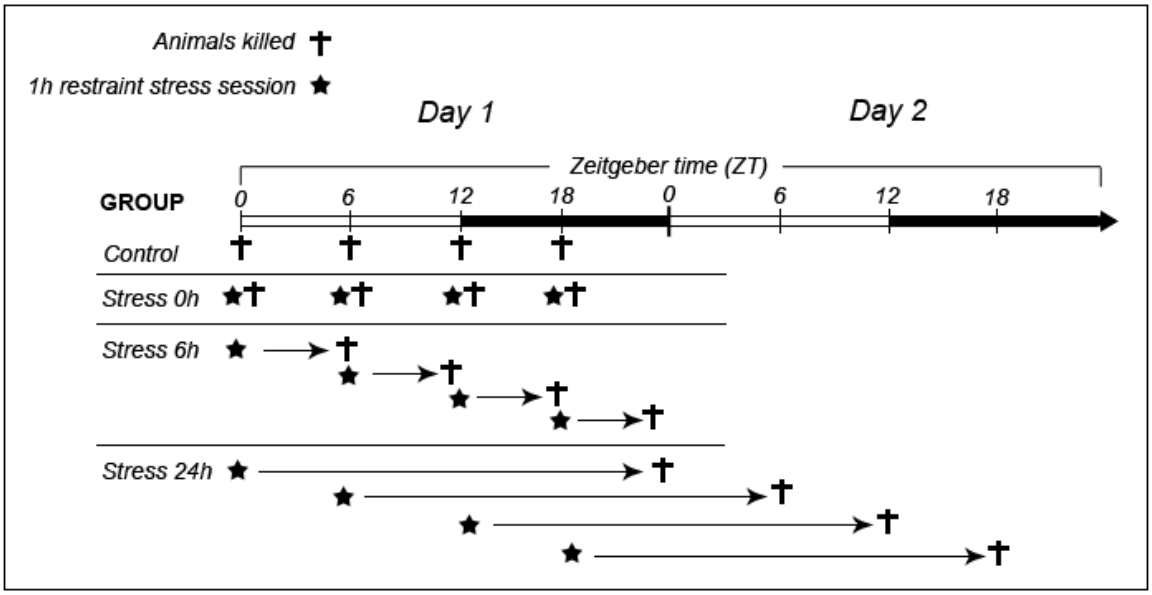

Table 1

	Group	ZT0 (n)	ZT6 (n)	ZT12 (n)	ZT18 (n)	F
Rearing	Control	37.25±4.63 (8)	38.88±3.31 (8)	47.75±2.06 (8)	37.00±2.63 (8)	2.378
	Immediately stress	24.00±2.21 (6)	34.25±6.06 (8)	35.18±2.75 (11) ③	34.29±5.08 (7)	1.266
	6 hours after stress	28.57±3.56 (7)	60.75±3.76 (8) ❶#	52.1±4.72 (10) ❶	31.13±4.45 (8) ❷❸	12.815
	24 hours after stress	27.43±3.73 (7)*	46.50±5.50 (8)	42.00±4.07 (10)	50.00±4.89 (7)#	4.091
	F	2.258	5.839	4.247	3.598	
Total locomotion	Control	76.00±7.21 (8)	77.50±6.24 (8)	89.63±5.25 (8)	75.88±3.38 (8)	1.351
	Immediately stress	77.00±5.63 (6)	76.88±6.59 (8)	112.0±7.96 (11) ❶❷	96.14±10.11 (7)	4.878
	6 hours after stress	83.29±9.91 (7)	110.63±7.57 (8) ❶①②	97.00±3.82 (10)	61.00±7.59 (8)* ②④	8.856
	24 hours after stress	114.29±5.2 (7) #	101.88±4.87 (8) ①②	105.0±4.49 (10)	90.43±5.75 (7) ❶	3.369
	F	6.022	7.202	2.668	5.093	
Number fecal boluses	Control	5.00±0.71 (8)	4.13±1.01 (8)	6.50±0.75 (8)	2.13±1.01 (8) ❸	4.512
	Immediately stress	0.83±0.31 (6) ①	1.50±0.50 (8)	0.00±0.00 (11)# ❷	0.00±0.00 (7) ❷	7,283
	6 hours after stress	2.29±1.48 (7) ①	4.13±1.19 (8)	1.80±0.66 (10) ①	6.00±0.93 (8) ❶❸#	3.461
	24 hours after stress	0.57±0.57 (7) ①	5.25±0.56 (8)* ②	2.40±0.81 (10) ①	0.43±0,20 (7)	11.767
	F	5.420	3.390	19.263	13.926	

Table 2

	Group	*ZT0 (n)*	*ZT6 (n)*	*ZT12 (n)*	*ZT18 (n)*	*F*
PHD	Control	4.50±0.65 (8)	5.71±0.68 (7)	2.00±0.42 (8)*	5.00±0.85 (8)	6.887
	Immediately stress	2.86±1.03 (7)	4.63±0.53 (8)	3.38±0.94 (8)	7.00±1.35 (7)❶❸	0.547
	6 hours after stress	3.14±0.96 (7)	6.33±1.45 (6)	5.29±0.64 (7)①	2.13±0.55 (8)❷②	3.740
	24 hours after stress	2.67±0.67 (9)	3.50±0.38 (8)	6.57±1.17 (7) ❶❷①②	4.63±0.75 (8)	1.570
	F	1.231	4.774	4.727	21.234	
NPHD	Control	8.00±2.01 (8)	14.86±2.57 (7)	21.75±2.60 (8) ❶	20.00±2.36 (8) ❶	5.829
	Immediately stress	5.57±2.64 (7)	8.75±3.03 (8)	5.75±1.06 (8)①	4.86±2.27 (7)①	3.379
	6 hours after stress	13.43±2.26 (7)	16.00±5.38 (6)	12.57±4.31 (7)	1.75±0.73 (8)*①	4.534
	24 hours after stress	8.89±3.60 (9)	1.50±0.76 (8)①③	9.14±4.49 (7)①	4.38±1.56 (8)①	4.745
	F	1.074	2.596	5.891	4.950	
EA	Control	8.38±1.66 (8)*	14.43±1.28 (7)	18.50±1.67 (8)	14.25±1.33 (8)	7.822
	Immediately stress	7.57±2.86 (7)	9.25±1.32 (8)	8.13±1.32 (8)①	9.57±1.56 (7)①	0.260
	6 hours after stress	8.29±2.01 (7)	14.17±2.48 (6)	12.57±1.62 (7)	5.75±0.92 (8) ❷❸①④	4.930
	24 hours after stress	10.44±2.24 (9)	5.50±0.82 (8) ①③	12.71±2.26 (7) ❷	10.88±1.44 (8)	2.808
	F	0.334	8.571	6.507	7.284	
TOA	Control	192.38±31.58 (8)	148.71±20.66 (7)	118.13±19.46 (8)	99.13±10.95 (8) ❶	3.118
	Immediately stress	249.86±21.20 (7)	210.13±25.06 (8)	216.13±16.02 (8)	221.43±21.09 (7)①	0.878
	6 hours after stress	207.43±14.30 (7)	175.17±19.13 (6)	172.86±26.67 (7)	270.38±6.27 (8)*①	4.178
	24 hours after stress	207.56±26.82 (9)	267.75±10.04 (8)①③	188.43 ±28.72 (7)	229.75±21.19 (8)①	1.146
	F	0.962	5.153	1.442	17.279	
TCA	Control	78.00±26.49 (8)	98.00±16.55 (7)	109.63±2.83 (8)	151.50±10.62 (8) ❶	3.509
	Immediately stress	29.43±12.94 (7)	51.13±17.42 (8)	57.38±11.82 (8)①	31.71±16.34 (7)①	0.672
	6 hours after stress	64.86±10.30 (7)	77.00±21.17 (6)	83.14±24.41 (7)	12.38±6.22 (8)*①	7.148
	24 hours after stress	61.56±22.45 (9)	14.38±9.35 (8)#	64.71±28.80 (7)	51.38±22.26 (8)①	2.140
	F	0.892	7.076	3.518	22.294	

V. CONSIDERAÇÕES GERAIS

CONSIDERAÇÕES GERAIS

Os resultados obtidos com esta dissertação de mestrado permitem emitir as seguintes conclusões:

- ✓ de acordo com o horário do dia em que o animal é avaliado observou-se distintas respostas comportamentais e bioquímicas;
- ✓ o padrão de respostas comportamentais e bioquímicas ao modelo de estresse por restrição está sob influência do ritmo circadiano;
- ✓ o modelo de estresse por restrição é um desincronizador do padrão temporal de respostas comportamentais e bioquímicas;
- ✓ as atividades das enzimas NTPDase apresentam um padrão temporal ao longo do dia;
- ✓ A exposição ao estresse por restrição foi capaz de desregular no padrão temporal da NTPDases;
- ✓ a exposição ao estresse induziu maiores alterações no padrão temporal das atividades das enzimas NTPDase e a 5'nucleotidase no período da noite e essas alterações persistem por pelo menos 24 horas.
- ✓ em condições circadianas basais os níveis de glicose, de melatonina e de corticosterona séricas são regulados pelo ciclo claro/escuro, no entanto, em situações de estresse observou-se uma mudança no padrão temporal desses hormônio (Artigo II);
- ✓ os organismos se adaptam fisiologicamente apresentando respostas alteradas a desafios associados com fatores ambientais, tais como exposição a estresse. Podendo resultar em um processo de dessincronização entre o organismo e o ambiente externo.

VI. DIVULGAÇÕES

DIVULGAÇÕES

2009:

a) SOUZA, A. ; MEDEIROS, L. F. ; SANTOS, V. S. ; ROZISKY, J.R. ; HIDALGO, M.P. ; TORRES, Iraci Lucena da Silva . **Efeito do Horário sobre a resposta ao estresse**. In: XXIV Reunião Anual da Federação de Sociedades de Biologia Experimental - FeSBE, 2009, São Paulo. XXIV Reunião Anual da Federação de Sociedades de Biologia Experimental - **FeSBE**. São Paulo, 2009.

b) SOUZA, Andressa. ; MEDEIROS, L. F. ; SANTOS, V. S. ; ROZISKY, J.R. ; HIDALGO, M.P. ; CALMO, W. ; TORRES, Iraci Lucena da Silva. **Alteração comportamental no modelo de estresse agudo no ciclo de 24 horas**. In: 29 Semana Científica do Hospital de Clínicas de Porto Alegre, 2009, Porto Alegre. Anais da 29 Semana Científica do Hospital de Clínicas de Porto Alegre. Porto Alegre : **HCPA**, 2009. v. 29. p. 253-254.

c) SANTOS, V. S. ; SOUZA, Andressa. ; ROZISKY, J.R. ; MEDEIROS, L. F. ; HIDALGO, M.P. ; CALMO, W. ; TORRES, Iraci Lucena da Silva. **Investigação do efeito do horário na resposta comportamental**. In: XXI Salão de Iniciação Científica da UFRGS, 2009, Porto Alegre. Anais do XXI Salão de Iniciação Científica da **UFRGS**. Porto Alegre : UFRGS, 2009. v. XXI.

2010 (trabalhos já submetidos que serão apresentados):

a) Souza A., Detanico B.C., Medeiros L.F., Rozisky J.R., Caumo W., Hidalgo M.O.L., Battastini A.M.O., Torres I.L.S. **Effects of Restraint Stress upon Temporal Patterns of Adenine Nucleotides Hydrolysis in Rat's Blood Serum**. In: XXV Reunião Anual da Federação de Sociedades de Biologia Experimental - FeSBE, 2010, São Paulo. XXV Reunião Anual da Federação de Sociedades de Biologia Experimental - **FeSBE**. São Paulo, 2010.

b) Andressa de Souza , Liciane Medeiros, Joanna Ripoll Rosisky, Vanessa Scarabelot, Ionara Siqueira1; Wolnei Caumo, Iraci Lucena da Silva Torres. **O Estresse como dessincronizador do Padrão Rítmico de Biomarcadores Sistêmicos em Ratos**. In: XXV Reunião Anual da Federação de Sociedades de Biologia Experimental - FeSBE, 2010, São Paulo. XXV Reunião Anual da Federação de Sociedades de Biologia Experimental - **FeSBE**. São Paulo, 2010.

c) Stefânia Cioato, Andressa de Souza, Liciane Medeiros, Joanna Ripoll Rosisky, Vanessa Scarabelot, Bernardo Detânico; Wolnei Caumo, Iraci Lucena da Silva Torres. **Efeito do Estresse por Restrição no Padrão Temporal da resposta comportamental**. In: XXV Reunião Anual da Federação de Sociedades de Biologia Experimental - FeSBE, 2010, São Paulo. XXV Reunião Anual da Federação de Sociedades de Biologia Experimental - **FeSBE**. São Paulo, 2010.

d) Stefânia Cioato; Andressa de Souza, Liciane Medeiros, Vinicius Souza dos Santos, Bernardo Detânico, Wolnei Caumo, Iraci Lucena da Silva Torres. **Avaliação do Padrão Temporal sobre Parâmetros Comportamentais em Ratos submetidos a Estresse por Restrição**. In: XXV Reunião Anual da Federação de Sociedades de Biologia Experimental -

FeSBE, 2010, São Paulo. XXV Reunião Anual da Federação de Sociedades de Biologia Experimental - **FeSBE**. São Paulo, 2010.

VII. ANEXOS

A) Aprovação do Comitê de Ética

HCPA - HOSPITAL DE CLÍNICAS DE PORTO ALEGRE
Grupo de Pesquisa e Pós-Graduação
COMISSÃO CIENTÍFICA E COMISSÃO DE PESQUISA E ÉTICA EM SAÚDE

A Comissão Científica e a Comissão de Pesquisa e Ética em Saúde, que é reconhecida pela Comissão Nacional de Ética em Pesquisa (CONEP)/MS como Comitê de Ética em Pesquisa do HCPA e pelo Office For Human Research Protections (OHRP)/USDHHS, como Institucional Review Board (IRB0000921) analisaram o projeto:

Projeto: 08-148

Pesquisadores:
IRACI LUCENA DA SILVA TORRES
ANA MARIA OLIVEIRA BATTASTINI
WOLNEI CAUMO
IONARA RODRIGUES SIQUEIRA
CARLOS ALEXANDRE NETTO
MARIA PAZ LOAYZA HIDALGO
LICIANE FERNANDES MEDEIROS
ANDRESSA DE SOUZA

Título: EFEITO DO FOTOPERÍODO SOBRE A RESPOSTA AO ESTRESSE

Este projeto foi Aprovado em seus aspectos éticos e metodológicos, de acordo com as Diretrizes e Normas Internacionais e Nacionais, especialmente as Resoluções 196/96 e complementares do Conselho Nacional de Saúde. Toda e qualquer alteração do Projeto deverá ser comunicada ao CEP/HCPA.
Os membros do CEP/HCPA não participaram do processo de avaliação dos projetos onde constam como pesquisadores.

Porto Alegre, 09 de junho de 2008.

Profª Nadine Clausell
Coordenadora do GPPG e CEP-HCPA

B) OUTROS ARTIGOS CIENTÍFICOS REALIZADOS EM CO-AUTORIA DURANTE O PERÍODO DE MESTRADO:

1. Detanico BC, **SOUZA A**, Medeiros LF, Rozisky JR, Caumo W, Hidalgo MPL, Battastini AMO, Torres ILS. 24-Hour Temporal Pattern of NTPDase and 5'-nucleotidase enzymes in rat blood serum. International Chronobiology, **submetido**, 2010.

2. Medeiros LF, Rozisky JR, **SOUZA A**, Hidalgo MP, Netto CA, Caumo W, Battastini AMO, Torres ILS. Surgery and/or Anesthetics Exposure in Infant Rats Alter Behavior in Lifetime of Rats. Behavioural Brain Research. **Submetido**: 2010.

3. Rozisky JR, Medeiros LF, Adachi LS, Espinosa J, **SOUZA A**, Bonan CD, Caumo W, Torres ILS. Long term effect of morphine exposure in early life upon nociceptive and inflammatory response in adult life. International Developmental Brain Research. **A ser submetido**.