

UNIVERSIDADE FEDERAL DO PARANÁ

NATALIA FANTIN SARDI

MECANISMOS ENVOLVIDOS NO EFEITO PRÓ-NOCICEPTIVO INDUZIDO
PELA RESTRIÇÃO DE SONO EM RATOS

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NATALIA FANTIN SARDI

MECANISMOS ENVOLVIDOS NO EFEITO PRÓ-NOCICEPTIVO INDUZIDO PELA
RESTRIÇÃO DE SONO EM RATOS

Tese apresentada ao Curso de Pós-Graduação em Fisiologia, Setor de Ciências Biológicas, da Universidade Federal do Paraná, como requisito parcial à obtenção do grau de Doutora em Fisiologia.

Orientadora: Prof^ª. Dra. Luana Fischer.

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RESUMO

A diminuição do tempo de sono aumenta a sensibilidade à dor e predispõem ao desenvolvimento de condições dolorosas tanto em humanos quanto em animais. No entanto, pouco é conhecido a respeito dos mecanismos pelos quais a diminuição do tempo de sono afeta a dor. Dados do nosso laboratório mostram que a privação de sono impacta o sistema endógeno descendente de modulação da dor, aumentando a facilitação da dor e; o Núcleo Accumbens (NAc), que desempenha importante papel na modulação da dor, também medeia o efeito pró-nociceptivo da privação de sono REM. Sendo assim, este trabalho se dedicou a entender um pouco mais sobre os mecanismos pelos quais a diminuição do tempo de sono afeta a dor. No primeiro capítulo, investigamos o impacto da analgesia endógena no efeito pró-nociceptivo da privação do sono REM. Os resultados apresentados no primeiro capítulo demonstram que o estado hiperalgésico induzido pela privação de sono é totalmente suprimido pela ativação de mecanismos endógenos de modulação da dor desencadeados pela acupuntura, estresse ou estimulação nociva. Então, a privação de sono impacta a modulação endógena da dor, no entanto, não se sobrepõe a ela. No segundo capítulo, investigamos o envolvimento da substância cinzenta periaquedutal (PAG) – que compõe o sistema descendente de modulação da dor – e do NAc no aumento da sensibilidade à dor induzida por restrição crônica de sono. Neste capítulo, mostramos que a restrição crônica de sono induz um efeito pró-nociceptivo que aumenta progressivamente do dia 3 ao dia 12 permanecendo estável a partir daí até o dia 26. E que dois dias consecutivos de sono livre aos fins de semana não foram suficientes para reverter o efeito, nem mesmo para atenuá-lo. Esse efeito pró-nociceptivo depende da PAG e do NAc, pois foi prevenido por lesão excitotóxica. No terceiro capítulo, baseado em nossos dados anteriores e na literatura, que suportam o envolvimento do NAc e do córtex cingulado anterior (ACC) na modulação do ciclo sono-vigília e na modulação da dor, e sabendo que o ACC se conecta ao sistema descendente, investigamos o envolvimento do ACC, e os mecanismos dopaminérgicos no ACC e NAc envolvidos no efeito pró-nociceptivo da restrição de sono. Nossos achados neste terceiro capítulo sugerem que uma diminuição na neurotransmissão dopaminérgica no ACC e NAc contribui para o efeito pró-nociceptivo da restrição do sono. No quarto capítulo, devido ao importante envolvimento dos núcleos monoaminérgicos na modulação da dor e ciclo sono-vigília, investigamos o papel dos núcleos monoaminérgicos do tronco cerebral no efeito pró-nociceptivo da restrição de sono. Os nossos dados sustentam que o efeito pró-nociceptivo da restrição de sono depende do aumento da atividade GABAérgica na área tegmentar ventral, núcleo dorsal da rafe e locus coeruleus e, da diminuição da atividade monoaminérgica inibitória no ACC. Até o momento, concluímos que a privação de sono aumenta a facilitação descendente, no entanto, não impede a ativação do sistema endógeno de modulação da dor por mecanismos que o ativam, tais como a acupuntura, o estresse e a estimulação nociva. Podemos dizer também que o efeito pró-nociceptivo induzido pela redução do tempo de sono depende da integridade do NAc, da PAG e do ACC. Além disso, o efeito pró-nociceptivo induzido por restrição de sono parece depender de uma redução da atividade dopaminérgica no NAc e no ACC, sugerimos também que dependa do aumento da atividade GABAérgica na VTA, DRN e LC e, em adição, pode depender de uma diminuição da atividade monoaminérgica inibitória no ACC.

Palavras-chave: nocicepção; dor; hiperalgesia; perda de sono; privação de sono; restrição de sono; sistema descendente; modulação endógena da dor; dopamina; adenosina; serotonina; norepinefrina; núcleo accumbens; substância cinzenta periaquedutal; córtex cingulado anterior; área tegmentar ventral; locus coeruleus; núcleo dorsal da rafe.

ABSTRACT

Decreased sleep time increases sensitivity to pain and predisposes to the development of painful conditions in both humans and animals. However, the underlying mechanisms are largely unknown. Data from our laboratory show that sleep deprivation impacts the descending endogenous pain modulation system, increasing pain facilitation and; the Nucleus Accumbens (NAc), which plays an important role in pain modulation, also mediates the pronociceptive effect of REM sleep deprivation. Therefore, this work was dedicated to understanding a little more about the mechanisms by which the decrease in sleep time affects pain. In the first chapter, we investigated the impact of endogenous analgesia on the pronociceptive effect of REM sleep deprivation. The results presented in the first chapter demonstrate that the hyperalgesic state induced by sleep deprivation is totally suppressed by the activation of endogenous pain modulation mechanisms triggered by acupuncture, stress or noxious stimulation. Therefore, sleep deprivation impacts the endogenous modulation of pain, however, it does not overlap it. In the second chapter, we investigated the involvement of periaqueductal gray matter (PAG) – which composes the descending pain modulation system – and NAc in the increase in pain sensitivity induced by chronic sleep restriction. In this chapter, we show that chronic sleep restriction induces a pronociceptive effect that increases progressively from day 3 to day 12 and remains stable from then until day 26. In addition, two consecutive days of free sleep on weekends were not enough to reverse the effect, not even to attenuate it. This pronociceptive effect depends on PAG and NAc, as it was prevented by excitotoxic injury. In the third chapter, based on our previous data and in the literature, which support the involvement of the NAc and the anterior cingulate cortex (ACC) in the modulation of the sleep-wake cycle and pain, and knowing that the ACC connects to the descending system, we investigated the involvement of ACC, and the dopaminergic mechanisms in ACC and NAc involved in the pronociceptive effect of sleep restriction. Our findings in this third chapter suggest that a decrease in dopaminergic neurotransmission in the ACC and NAc contributes to the pronociceptive effect of sleep restriction. In the fourth chapter, due to the important involvement of monoaminergic nuclei in the modulation of pain and the sleep-wake cycle, we investigated the role of monoaminergic nuclei in the brainstem in the pronociceptive effect of sleep restriction. Our data support that the pronociceptive effect of sleep restriction depends on the increase in GABAergic activity in the ventral tegmental area, dorsal raphe nucleus and locus coeruleus, and a decrease in inhibitory monoaminergic activity in the ACC. So far, we have concluded that sleep deprivation increases the descending facilitation, however, it does not prevent the activation of the endogenous pain modulation system by mechanisms that activate it, such as acupuncture, stress and noxious stimulation. The pronociceptive effect induced by decrease sleep time depends on the integrity of NAc, PAG and ACC. Furthermore, the pronociceptive effect induced by sleep restriction seems to depend on a reduction of dopaminergic activity in the NAc and ACC, we also suggest that it depends on the increase in GABAergic activity in the VTA, DRN and LC and, in addition, it may depend on a decrease in inhibitory monoaminergic activity in the ACC.

Keywords: Nociception; Pain; Nucleus Accumbens; REM sleep deprivation; sleep restriction; Adenosine; Dopamine; Serotonin; noradrenaline; ventral tegmental area; locus coeruleus; dorsal raphe nuclei; anterior cingulate cortex.

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LISTA DE ABREVIATURAS

- REM** – Movimento rápido dos olhos, do inglês *“rapid eye movement”*
- SD** – Privação de sono, do inglês *“sleep deprivation”*
- REM-SD** – Privação de sono REM, do inglês *“rapid eye movement- sleep deprivation”*
- SR** – Restrição de sono, do inglês *“sleep restriction”*
- CSR** – Restrição cônica de sono, do inglês *“chronic sleep restriction”*
- NAc** – Núcleo Accumbens
- PAG** – Substância cinzenta periaquedutal, do inglês *“periaqueductal gray matter”*
- vlPAG** – Coluna ventrolateral da PAG
- RVM** – Bulbo rostro ventral, do inglês *“rostral ventral medulla”*
- ACC** – Córtex cingulado anterior, do inglês *“anterior cingulate cortex”*
- VTa** – Área tegmentar ventral, do inglês *“ventral tegmental area”*
- LC** – Locus coeruleus
- LH** – Hipotálamo lateral, do inglês *“lateral hypothalamus”*
- DRN** – Núcleo Dorsal da Rafe, do inglês *“dorsal raphe nucleus”*
- SNc** – Substância negra compacta
- IASP** - Associação Internacional para o Estudo da Dor, do inglês *“International Association for the Study of Pain”*
- DA** – Dopamina
- NE** – Norepinefrina
- 5-HT** – Serotonina, também conhecida como 5-hidroxitriptamina
- D₁** – Receptor de dopamina do tipo 1
- D₂** – Receptor de dopamina do tipo 2
- A₁** – Receptor de adenosina do tipo 1
- A_{2A}** – Receptor de adenosina do tipo 2A
- 5-HT_{1A}** – Receptor de serotonina do tipo 1A
- GABA** - Ácido gama-aminobutírico, do inglês *“Gamma-AminoButyric Acid”*
- GABA_A** – Receptor GABA do tipo A
- SIA** – Analgesia induzida por estresse, do inglês *“stress-induced analgesia”*
- ANC** – Controle nociceptivo ascendente, do inglês *“ascending nociceptive control”*
- NMDA** – N-metil D-Aspartato
- DAT** – Transportador de dopamina, do inglês *“dopamine transporter”*
- DOPAC** – Ácido 3,4-di-hidroxifenilacético, do inglês *“3,4-dihydro-xyphenylacetic acid”*
- HVA** – Ácido homovanílico, do inglês *“homovallinic acid”*
- 5-HIAA** – Ácido 5-hidroxi-indolacético do inglês *“5-hydroxyindoleacetic acid”*
- DHPG** – 3,4-di-hidroxifenilglicol do inglês *“3,4-dihydroxyphenylglycol”*
- HPLC** – Cromatografia líquida de alta eficiência do inglês *“High performance liquid chromatography”*
- kDa** – Quilodaltons - Unidade de massa molecular para proteínas, do inglês *“kiloDaltons”*
- DARPP-32** – Fosfoproteína regulada por dopamina e AMPc-32kDa do inglês *“dopamine and cyclic AMP-regulated phosphoprotein, 32 kDa”*
- DARPP-32-Thr34** – DARPP-32 fosforilada no resíduo Treonina 34
- DARPP-32-Thr75** – DARPP-32 fosforilada no resíduo Treonina 75

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INTRODUÇÃO

A dor é uma condição multifatorial e é sempre subjetiva, cada indivíduo aprende a usar o termo “dor” através de experiências prévias (IASP – Associação Internacional para Estudos da Dor, do inglês, International Association for the Study of Pain, 2020). O termo “dor” recentemente teve sua definição atualizada pela IASP como “uma experiência sensorial e emocional desagradável associada, ou semelhante àquela associada, a uma lesão tecidual real ou potencial” (Raja et al., 2020).

A dor aguda é um mecanismo de alerta que indica a presença de um estímulo lesivo ao organismo que acionará respostas protetoras apropriadas, ou seja, possui valor biológico (Apkarian et al., 2005, Basbaum et al., 2009, Baliki and Apkarian, 2015). O funcionamento adequado deste sistema é essencial para proteger o organismo contra danos teciduais. Entretanto, sob condições persistentes ou patológicas, este sistema pode tornar-se sensibilizado e a dor transforma-se em uma doença, perdendo, portanto, seu valor biológico (Apkarian et al., 2005, Basbaum et al., 2009, Baliki and Apkarian, 2015).

Um estudo sobre a epidemiologia de dor crônica no Brasil na última década revela que a prevalência de dor crônica pode variar de 29,3 a 73,3% no Brasil, afetando mais mulheres que homens e, a dor na região lombar foi a mais prevalente (Vasconcelos, 2018). Entre funcionários públicos brasileiros a prevalência de algum tipo de dor é de 62,4% (Machado et al., 2019). Em países em desenvolvimento latino americanos, asiáticos e africanos a prevalência de dor crônica varia entre 13 e 51% (Sa et al., 2019). No mundo, a prevalência geral de dor foi estimada em 27,5%, com variação significativa entre os países (variando de 9,9% a 50,3%), sendo que mulheres, idosos e residentes rurais foram significativamente mais propensos a relatar dor (Zimmer et al., 2021).

A dor certamente é um dos maiores problemas mundiais de saúde pública (Goldberg and McGee, 2011), que além de afetar diretamente a qualidade de vida de milhões de pessoas, está associado a um gigantesco impacto econômico. Só nos Estados Unidos, a estimativa de custo anual com tratamento e perda de produtividade relacionada à dor foi estimada entre 560 e 635 bilhões de dólares (Gaskin and Richard, 2012).

Assim como a dor, distúrbios de sono como a insuficiência na qualidade ou redução no tempo de sono têm se tornado um problema de saúde cada vez mais comum na sociedade moderna (Matsumoto and Chin, 2019). De fato, a prevalência subjetiva (questionário) de curta duração do sono (<6 h) é de 7,5–9,6%, enquanto a prevalência objetiva (actigrafia) de curta duração do sono (<6 h) é de 22,1–53,3% na população geral (Matsumoto and Chin, 2019). A

prevalência subjetiva de sono não restaurativo é de 19,2–31,0% em homens e 26,3–42,1% em mulheres (Matsumoto and Chin, 2019). Um estudo epidemiológico do sono na cidade de São Paulo revelou por actigrafia que 22,1% da população dorme menos de 6 horas por dia (Moraes et al., 2013). Estudos baseados nos dados do EPISONO – pesquisa epidemiológica realizada pelo Instituto do Sono na cidade de São Paulo – revelam que mais de 60% da população da cidade de São Paulo sofre com algum distúrbio relacionado ao sono, sendo que destes, a prevalência de apneia obstrutiva do sono é de 32,8% (Tufik et al., 2010) e de insônia 32% (Castro et al., 2013).

O sono parece afetar praticamente todos os aspectos da fisiologia, e numerosos processos dependem da ocorrência regular do sono para o restabelecimento e manutenção da homeostasia (Bringmann, 2019). Sendo assim, podem ser muitas as consequências relacionadas com a diminuição do tempo de sono, como memorização deficiente (Orzel-Gryglewska, 2010, Dos Santos et al., 2013, Proenca et al., 2014, Santos et al., 2017), erro na tomada de decisão, distúrbios emocionais como o aumento da agressividade (Orzel-Gryglewska, 2010), aumento da sensibilidade à dor e a prevalência e severidade de condições dolorosas (Morin et al., 1998, Lentz et al., 1999, Smith et al., 2000, Arima et al., 2001, Onen et al., 2001b, Nicholson and Verma, 2004, Smith and Haythornthwaite, 2004, Morin et al., 2006, Smith et al., 2007, Edwards et al., 2009, Tiede et al., 2010, O'Brien et al., 2011, Mork and Nilsen, 2012, Nitter et al., 2012, Paul-Savoie et al., 2012, Tang et al., 2012, Karaman et al., 2014, Afolalu et al., 2018, Eichhorn et al., 2018, Simpson et al., 2018, Bjurstrom et al., 2021, Soldatelli et al., 2021), entre outros problemas.

Claramente condições de dor persistente e distúrbios de sono são problemas de saúde pública, e existe uma relação bidirecional entre elas.

Recentemente vários estudos têm investigado a relação entre dor e distúrbios de sono (Smith and Haythornthwaite, 2004, Finan et al., 2013, Afolalu et al., 2018, Andersen et al., 2018, Haack et al., 2020, Herrero Babiloni et al., 2020). A partir desses estudos ficou claro que essa é uma relação bidirecional, uma vez que a dor pode causar distúrbios de sono (Affleck et al., 1996, Nicholson and Verma, 2004, Smith and Haythornthwaite, 2004, Edwards et al., 2008, Smith et al., 2008, Narita et al., 2011, O'Brien et al., 2011, Okifuji and Hare, 2011, Jansson-Frojmark and Boersma, 2012, Tang et al., 2012, Finan et al., 2013, Wang et al., 2015, Yin et al., 2016, Liu et al., 2019) e alterações do padrão normal de sono podem predispor ao desenvolvimento de condições dolorosas (Stone et al., 1997, Morin et al., 1998, Nicholson and Verma, 2004, Smith and Haythornthwaite, 2004, Gupta et al., 2007, Jones et al., 2009, O'Brien et al., 2011, Mork and Nilsen, 2012, Nitter et al., 2012, Agmon and Armon, 2014, Karaman et

al., 2014, Afolalu et al., 2018, Bjurstrom et al., 2021), aumentar a sensibilidade à dor em humanos (Lentz et al., 1999, Arima et al., 2001, Onen et al., 2001b, Smith and Haythornthwaite, 2004, Tiede et al., 2010, Matre et al., 2016, Eichhorn et al., 2018, Simpson et al., 2018), diminuir o limiar nociceptivo (Onen et al., 2000, Onen et al., 2001a, Onen et al., 2001b, Nascimento et al., 2007, Wei et al., 2007, Wei et al., 2008, Damasceno et al., 2009, Wei et al., 2010, Skinner et al., 2011, Damasceno et al., 2013, Tomim et al., 2016, Sardi et al., 2018a, Sardi et al., 2018b) e aumentar a dor existente em animais (Huang et al., 2014, Tomim et al., 2016, Hambrecht-Wiedbusch et al., 2017, Xue et al., 2018, Li et al., 2019).

O crescente número de estudos investigando a interação entre sono e dor e a unanimidade de resultados indicando que a diminuição do tempo de sono tem papel pró-nociceptivo contrasta com o pobre entendimento a respeito dos mecanismos pelos quais a diminuição do tempo de sono aumenta a dor. A maioria dos estudos que investigou tais mecanismos se concentrou em mecanismos espinhais, ou utilizaram intervenções sistêmicas. Alguns poucos estudos investigaram os mecanismos centrais pelos quais a redução do tempo de sono aumenta a sensibilidade à dor.

Por exemplo, já foi demonstrado que a privação de sono REM (movimento rápido dos olhos, do inglês, rapid eye movement) aumenta a atividade glutamatérgica (Wei et al., 2007), modula a atividade serotoninérgica, sendo que a atividade do receptor serotoninérgico 5-HT_{2C} é aumentada e a do 5-HT_{1A} é diminuída (Wei et al., 2008) e diminui a atividade GABAérgica (Wei et al., 2010) na medula espinhal aumentando a sensibilidade à dor. Também foi demonstrado que o efeito antinociceptivo induzido pela administração sistêmica de agonistas opióides (Nascimento et al., 2007, Skinner et al., 2011) ou agonista dopaminérgico é diminuído pela privação de sono REM (Skinner et al., 2011).

No entanto, os mecanismos centrais subjacentes a esse efeito pró-nociceptivo resultante da redução do tempo de sono são pouco compreendidos e aparentemente envolvem vários sistemas de neurotransmissores e regiões cerebrais (Sardi et al., 2018a, Sardi et al., 2018b, Haack et al., 2020). Portanto, a compreensão e conhecimento adequado sobre os mecanismos pelos quais a diminuição do tempo de sono aumenta a dor é essencial para incentivar o desenvolvimento de novos medicamentos e intervenções comportamentais que podem impactar positivamente o manejo clínico da dor e, mais amplamente, a saúde pública e a prevenção da dor. Sendo assim, objetivo geral deste trabalho foi investigar os mecanismos pelos quais a diminuição do tempo de sono aumenta a sensibilidade a dor; para melhor compreensão, apresentamos uma pequena revisão sobre o que se sabe até o momento sobre a interação entre sono e dor.

Modelos de privação/restrição de sono

As abordagens metodológicas de restrição parcial ou privação total do tempo de sono e privação de sono REM mimetizam distúrbios de sono em humanos e fornecem evidências experimentais necessárias para aumentar o conhecimento sobre a relação entre sono e dor (Araujo et al., 2013). As metodologias mais utilizadas são, a privação de sono REM e a restrição parcial de sono total ou privação total do tempo de sono. Como os nomes sugerem, a privação de sono REM priva o animal principalmente da fase REM do sono, já a restrição parcial de sono total é realizada por períodos menores, entre 3 a 12h de restrição em um dia e a privação total de sono, pode por exemplo, privar o animal de sono por um dia inteiro.

A maioria dos métodos utilizados para induzir privação de sono REM em animais são modificações de um método desenvolvido para gatos (Jouvet et al., 1964), conhecida como técnica “*flower pot*”. Em seguida, a técnica foi adaptada para ratos (Cohen and Dement, 1965). O método consiste em colocar um rato em uma plataforma de 6,5 cm de diâmetro e 10 cm de altura que fica dentro de um tanque completo com água até 1 cm antes da superfície da plataforma, nesse método, de plataforma única, o animal fica isolado em seu tanque; entretanto, a privação de sono REM também pode ser realizada pelo método de múltiplas plataformas, no qual, são colocadas várias plataformas dentro de um tanque, de modo que mais animais podem ser privados de sono simultaneamente; nos dois métodos, os animais têm comida e água disponíveis o tempo todo e, o tanque deve ter altura suficiente para que os animais não escapem (Morden et al., 1967, Machado et al., 2004). Sempre que os animais entram em sono REM, caem da plataforma por causa da atonia muscular ocasionada por essa fase do sono e acordam (Morden et al., 1967, Machado et al., 2004). A validação desse método por meio de registros polissonográficos (eletroencefalograma, EEG e eletromiograma, EMG) mostra que a privação de sono REM abole o sono REM e diminui significativamente o sono não-REM (Morden et al., 1967, Machado et al., 2004).

A restrição de sono total é realizada principalmente pelo método de “gentle handling” (em português, manipulação suave) que minimiza os efeitos do estresse (Tobler and Borbely, 1990, Alexandre et al., 2017). A restrição de sono total causa aumento significativo da atividade cerebral de ondas lentas (SWA do inglês, Slow Wave Activity) no EEG durante a recuperação de sono subsequente (Tobler and Borbely, 1990). A SWA é considerada uma boa medida da acumulação da pressão de sono (Tobler and Borbely, 1986). Recentemente, registros polissonográficos (EEG e EMG) validaram uma redução de aproximadamente 98% do sono de ondas lentas e supressão do sono REM em roedores (Alexandre et al., 2017). Este método

caracteriza-se por manter os animais acordados inserindo novos objetos em sua gaiola (Asikainen et al., 1997, Alexandre et al., 2017), por manipulação suave quando os animais mostram sinais de sono além de, escovar o dorso do animal com um pincel de cerdas macias, girar e bater levemente na gaiola (O'Hara et al., 1993, Asikainen et al., 1997).

Na literatura vários aparelhos automatizados foram descritos e validados por polissonografia (EEG e EMG) para privação ou restrição de sono total. Tais como, um aparelho totalmente automatizado que usa um déficit de sinais no EMG para acionar a roda de atividade, adaptar a velocidade de rotação, desativar ou alterar o sentido da roda de atividade (Fenzl et al., 2007). Outras rodas de atividade motorizadas são descritas, uma tem velocidade constante (Kim et al., 2007), outras são ativadas de maneira intermitente (Christie et al., 2008, Caron and Stephenson, 2010) e permitem interação social (Leenaars et al., 2011). Aparelhos tipo agitadores, impedem o sono por movimentos transitórios para cima e para baixo controlados por software (Chauveau et al., 2014), outro tipo de agitador quando a plataforma vai para baixo, sua superfície fica submersa por aproximadamente 2 cm de água o que tende a fazer os animais subirem em uma plataforma pequena fixa e seca (Everson et al., 1994). Outro sistema automatizado de privação de sono mantém os animais acordados por meio de sopros de ar (Gross et al., 2015).

Efeito da privação ou restrição de sono na dor

Dados da literatura mostram que tanto a privação de sono REM diminui o limiar nociceptivo em animais, em testes térmicos de imersão em água quente (Onen et al., 2001a) ou calor radiante aplicado na superfície plantar da pata (Nascimento et al., 2007, Damasceno et al., 2009, Skinner et al., 2011, Hirotsu et al., 2018), em testes mecânicos de pressão na pata (Onen et al., 2000, Onen et al., 2001a, Wei et al., 2007, Wei et al., 2008, Wei et al., 2010, Wei et al., 2011a, Wei et al., 2011b, Damasceno et al., 2013, Wei et al., 2013, Tomim et al., 2016, Sardi et al., 2018a, Sardi et al., 2018b) e em teste de choque elétrico na cauda (Onen et al., 2001a).

Ademais, a restrição ou privação de sono total diminui o limiar nociceptivo em animais, em teste térmico de calor radiante aplicado na superfície plantar da pata (Alexandre et al., 2017), em testes mecânicos de pressão na pata (Alexandre et al., 2017, Sardi et al., 2018a) e em teste químico com hiperalgesia induzida pela administração de capsaicina na pata (Alexandre et al., 2017)

A privação ou restrição de sono também aumenta a severidade da dor já existente em ratos, tais como, a dor induzida por injeção de formalina na pata (Tomim et al., 2016, Vanini,

2016), por incisão cirúrgica na pata (Hambrecht-Wiedbusch et al., 2017, Xue et al., 2018, Li et al., 2019), por ligadura ou constrição de nervo periférico (Wei et al., 2007, Huang et al., 2014).

Mecanismos envolvidos no efeito pró-nociceptivo induzido pela diminuição do tempo de sono

De maneira geral, é sabido que a privação de sono impacta o sistema descendente de modulação da dor, aumentando a atividade descendente facilitadora da dor, o que induz o efeito pró-nociceptivo (Tomim et al., 2016, Xue et al., 2018). Sabemos também que o efeito pró-nociceptivo induzido pela diminuição do tempo de sono depende da integridade do núcleo accumbens (NAc) e da substância cinzenta periaquedutal (PAG) (Sardi et al., 2018a, Sardi et al., 2018b) e que, a privação de sono parece aumentar a atividade adenosinérgica de receptores A_{2A} de adenosina e diminuir a atividade dopaminérgica de receptores D_2 de dopamina no NAc para induzir efeito pró-nociceptivo (Sardi et al., 2018b). Além disso, a privação de sono sabidamente reduz o potente efeito analgésico da morfina, um agonista opioide (Ukponmwan et al., 1984, Nascimento et al., 2007, Skinner et al., 2011, Tomim et al., 2016). No entanto, outras regiões e sistemas de neurotransmissores parecem estar envolvidos, a seguir, a descrição dos sistemas de neurotransmissores e regiões cerebrais possivelmente envolvidas no efeito pró-nociceptivo resultante da redução do tempo de sono até o momento.

Sistema dopaminérgico

O sistema dopaminérgico possui evidente influência sobre a modulação do comportamento, integrando informações sensoriais e gerando respostas motoras adequadas para os mais diversos estímulos (Marsden, 2006, Leknes and Tracey, 2008). Os receptores dopaminérgicos são divididos em duas classes, receptores tipo D_1 e receptores tipo D_2 , com ações pós-sinápticas de caráter excitatório e inibitório, respectivamente (Missale et al., 1998). Há também receptores D_2 no corpo celular, nos dendritos e no terminal axonal dos neurônios da substância negra compacta (SNc) e da área tegmentar ventral (VTA), exercendo a função de autoreceptor (Missale et al., 1998, Ford, 2014). Estes regulam o sistema dopaminérgico por meio de feedback negativo, interferindo na síntese (Pothos et al., 1998), liberação e recaptação da dopamina (Mayfield and Zahniser, 2001). Os núcleos da base são os locais de maior expressão dos receptores dopaminérgicos pós-sinápticos e estes recebem eferências dopaminérgicas da VTA e da SNc, formando as vias mesolímbica e nigroestriatal, respectivamente (Missale et al., 1998, Cadet et al., 2010).

A atividade dopaminérgica mesolímbica implica na atenuação da dor aguda (Dias et al., 2015), mas é essencial para a transição da dor aguda para a dor crônica (Apkarian, 2008, Chang et al., 2014, Dias et al., 2015, Vergara et al., 2020) e, uma vez que a dor se torna crônica, ocorre uma modulação no sistema dopaminérgico gerando um estado de hipofunção dopaminérgica (Wood et al., 2007, Chang et al., 2014, Taylor et al., 2016a). Em resumo, a dopamina parece ter efeitos diversos na modulação da dor, e a informação nociceptiva, através de alterações no tônus dopaminérgico, modula o comportamento frente a estímulos potencialmente nocivos (Herrero Babiloni et al., 2020).

A atividade dopaminérgica também participa da modulação do ciclo sono-vigília (Monti and Monti, 2007, Monti and Jantos, 2008, Qiu et al., 2010, Eban-Rothschild et al., 2016, Taylor et al., 2016b, Oishi and Lazarus, 2017, Oishi et al., 2017a, Scammell et al., 2017, Yang et al., 2021) e, o núcleo accumbens, cada vez mais, parece ser uma importante região para a transição entre o sono e a vigília (Qiu et al., 2010, Qu et al., 2010, Lazarus et al., 2012, Qiu et al., 2012, Lazarus et al., 2013, Oishi and Lazarus, 2017, Luo et al., 2018).

No entanto, o efeito da diminuição no tempo de sono sobre o sistema dopaminérgico possui uma literatura divergente, estudos demonstram tanto diminuição quanto aumento da sinalização dopaminérgica. Dois estudos proeminentes na área, porém antigos, utilizaram radioligantes antagonistas de receptores dopaminérgicos do tipo D₂ e avaliaram a marcação dos mesmos a fim de verificar a disponibilidade dos receptores dopaminérgicos do tipo D₂ no NAc (Tufik et al., 1978, Nunes Junior et al., 1994). Foi observado um aumento da ligação do radioligante [³H]spiperone no NAc após 96h de privação de sono REM (Nunes Junior et al., 1994) e, um aumento do efeito da apomorfina quando associado à privação de sono REM (Tufik et al., 1978), juntos concluíram que, a privação de sono REM gerou um estado de supersensibilidade dopaminérgica – *upregulation* do receptor dopaminérgico D₂ (Tufik et al., 1978, Nunes Junior et al., 1994). Já em Volkow et al., 2009, foi observado uma diminuição da ligação do radioligante [¹¹C]raclopride no NAc após uma noite de privação de sono total, gerando duas possíveis hipóteses: (1) aumento na disponibilidade sináptica de dopamina ou (2) *downregulation* do receptor dopaminérgico do tipo D₂ (Volkow et al., 2009). Para uma conclusão mais precisa, em Volkow et al., 2012 foi utilizado o metilfenidato a fim de se obter uma resposta de liberação de dopamina exacerbada, isso se a primeira hipótese fosse a mais condizente com a realidade (Volkow et al., 2012). Contudo, não foi observada diferença e a hipótese mais plausível que explique estes resultados, segundo Volkow e colaboradores, é de um *downregulation* do receptor dopaminérgico do tipo D₂ no NAc (Volkow et al., 2012). Estes

resultados contraditórios podem ser devido a diferenças na metodologia, no modelo de diminuição do tempo de sono ou nas análises empregadas, no entanto, é evidente que este tópico precisa de mais estudos para ser elucidado.

Com relação ao efeito pró-nociceptivo induzido por diminuição no tempo de sono, já foi demonstrado, por nosso laboratório, que a administração de agonista de receptores D₂ de dopamina no NAc bloqueia o efeito pró-nociceptivo induzido pela privação de sono REM (Sardi et al., 2018b), o que sugere, que a privação de sono promova uma redução na atividade dos receptores de dopamina inibitórios D₂ para aumentar a sensibilidade a dor. Este resultado vai de encontro com a hipótese de que a diminuição de sono gera um estado de hipofunção dopaminérgica (Volkow et al., 2012) e isto estaria favorecendo a pró-nocicepção. O que é fortalecido com o dado de que a administração sistêmica de modafinil, droga que aumenta - principalmente - a disponibilidade de dopamina, normaliza o aumento da sensibilidade a dor induzida por privação de sono, e o mesmo não ocorre com administração de ibuprofeno (inibidor não-seletivo de ciclooxigenases -COXs) ou morfina (Alexandre et al., 2017).

Sistema adenosinérgico

A base neural e celular da necessidade de sono pode ser conceituada como uma pressão homeostática que se acumula durante o período de vigília e se dissipa durante o sono (Lazarus et al., 2019). Um mecanismo potencial é que o acúmulo gradual de um ou mais fatores sonogênicos endógenos durante a vigília é a base da pressão homeostática do sono (Lazarus et al., 2019). A adenosina é até o momento, o fator sonogênico endógeno clássico, que promove o sono através dos receptores A₁ e A_{2A} (Lazarus et al., 2019).

A cafeína, é um antagonista do receptor de adenosina, que promove a vigília bloqueando os receptores excitatórios de adenosina A_{2A} no NAc (Lazarus et al., 2011). Usando estratégias de deleção seletiva de genes para silenciar a expressão de receptores A_{2A} em ratos, observou-se que os receptores A_{2A} no NAc são responsáveis pelo efeito da cafeína na vigília (Lazarus et al., 2011). A excitação induzida por cafeína não foi afetada em ratos quando os receptores A_{2A} foram removidos de outras áreas positivas para receptores A_{2A} dos gânglios da base (Lazarus et al., 2011). Por outro lado, a ativação quimiogénica ou optogénica de neurônios no NAc que expressam o receptor excitatório de adenosina A_{2A} induz fortemente o sono de ondas lentas (Oishi et al., 2017b). Mais recentemente, descobriu-se que níveis elevados de adenosina agindo em receptores A_{2A} no NAc promove o sono de ondas lentas (SWS, do inglês *slow-wave sleep*), o que fornece a primeira evidência de que a adenosina endógena ativa neurônios que expressam receptores A_{2A} no NAc e têm a capacidade de induzir o SWS (Zhou et al., 2019).

Os receptores adenosinérgicos são acoplados à proteína G, o que resulta em modulações intracelulares complexas, podendo contemplar: modulação de canais iônicos; alterações em segundos mensageiros e regulação da liberação de neurotransmissores (Sebastiao and Ribeiro, 2009). Devido à complexidade, a sinalização adenosinérgica parece ter um efeito dual na modulação da dor, podendo tanto facilitar como inibir, a depender da região e do receptor ativado (Ferre et al., 2007). A ativação farmacológica do receptor A_{2A} realizada de maneira sistêmica ou local (intratecal) pode promover tanto analgesia (Lee and Yaksh, 1996, Poon and Sawynok, 1998, Borghi et al., 2002) quanto hiperalgesia (Bastia et al., 2002), já a inibição do receptor A_{2A} parece ter efeito analgésico (Bastia et al., 2002, Godfrey et al., 2006) e a depleção de receptores A_{2A} promove hipoalgesia – diminuição da sensibilidade a dor (Godfrey et al., 2006, Hussey et al., 2007).

Estudos proeminentes que relacionaram sono e dor nos revelam alguns aspectos mecanísticos do envolvimento do receptor A_{2A} adenosinérgico no efeito pró-nociceptivo induzido pela redução do tempo de sono. Um estudo observou bloqueio da hipersensibilidade mecânica e redução parcial da hipersensibilidade térmica induzida por privação de sono total após tratamento sistêmico com cafeína em camundongos, sugerindo participação adenosinérgica no efeito pró-nociceptivo da diminuição do tempo de sono (Alexandre et al., 2017). Em outro estudo, a administração sistêmica de cafeína no início da privação do sono preveniu o aumento na hipersensibilidade a dor pós-operatória induzido pela diminuição do tempo de sono em ratos (Hambrecht-Wiedbusch et al., 2017). E, a microinjeção do antagonista do receptor de adenosina A_{2A} ZM 241385 no núcleo pré-óptico mediano bloqueou o aumento nos níveis e duração da dor cirúrgica causada pela privação prévia do sono e eliminou a hiperalgesia térmica induzida pela privação do sono em um grupo de ratos sem incisão cirúrgica (Hambrecht-Wiedbusch et al., 2017). Outro estudo recente, investigou o envolvimento de antagonista para o receptor A_{2A} no NAc, importante área para a indução de sono (Oishi and Lazarus, 2017, Oishi et al., 2017b, Zhou et al., 2019) e para a modulação da dor (Altier and Stewart, 1998, 1999, Gear and Levine, 2011, Haghparast et al., 2012, Sardi et al., 2018a, Sardi et al., 2018b, Tobaldini et al., 2019, Vergara et al., 2020), no efeito pró-nociceptivo da privação de sono. Este mostrou que, a administração intra-NAc do antagonista de receptor A_{2A} SCH-58261 preveniu o efeito pró-nociceptivo induzido por privação de sono REM em ratos, além disso, o efeito pró-nociceptivo foi prevenido por lesão excitotóxica do NAc e revertido por bloqueio agudo com anestésico local intra-NAc (Sardi et al., 2018b). Com isso, até o presente momento, o aumento na atividade de receptores adenosinérgicos A_{2A} parece ser fundamental para o aumento da sensibilidade à dor induzido por diminuição no tempo de sono.

Sistema noradrenérgico

Nenhuma evidência direta foi demonstrada até o momento sobre o envolvimento do sistema noradrenérgico no efeito pró-nociceptivo da redução do tempo de sono. No entanto, após privação de sono, foi encontrado aumento nos níveis de tirosina hidroxilase e de mRNA do transportador de norepinefrina no locus coeruleus (LC) (Basheer et al., 1998). Outro estudo realizou análise por autoradiografia, encontrou que, 96 h de privação de sono REM reduz significativamente a ligação do radioligante [^3H]Nisoxetine ao transportador de norepinefrina no córtex cingulado anterior (-16%), núcleo endopiriforme (-18%), núcleo olfatório anterior (-19%), camada glomerular do bulbo olfatório (-18%), pálido ventral (-14%), área pré óptica medial (-16%), hipotálamo retroquiasmático/arqueado (-18%), núcleo talâmico anteromedial (15%) e rafe rostral (-17%) (Hipolide et al., 2005). Mais recentemente, foi demonstrado que a estimulação optogenética do LC aumenta a vigília, diminui o sono não REM e facilita a transição do sono para a vigília (Koh et al., 2015), além disso, animais com dor neuropática induzida por ligadura parcial do nervo ciático, apresentam distúrbio de sono e tem a atividade dos neurônios noradrenérgicos do LC aumentada (Koh et al., 2015).

Sistema serotoninérgico

Embora não exista até o momento evidência da participação do sistema serotoninérgico no efeito pró-nociceptivo da diminuição do tempo de sono, existem algumas indicações de que esse sistema pode ser afetado pela privação de sono. Isso porque, em análise de autoradiografia, 96 h de privação de sono REM reduz significativamente a ligação do radioligante [^{11}C]DASB ao transportador de serotonina no núcleo olfatório anterior (- 22%) e na substância negra (-18%) (Hipolide et al., 2005). Além disso, já foi demonstrado que, a restrição crônica de sono reduz a sensibilidade do receptor de serotonina 1A (Meerlo et al., 2008), também que, a privação de sono aguda aumenta metabólitos de serotonina nos gânglios da base (Zant et al., 2011) e no plasma (Davies et al., 2014).

Sistema opioidérgico

Sabe-se que a privação de sono diminui o potente efeito analgésico do agonista mu-opioide, morfina (Ukponmwan et al., 1984, Nascimento et al., 2007, Skinner et al., 2011, Tomim et al., 2016). A primeira evidência observada, mostrou que 96 h de privação de sono REM antagoniza o efeito antinociceptivo de 20 μ g de morfina administrada intracerebroventricular em ratos avaliados no teste de Randall-Selitto (analgésimetro mecânico)

(Ukponmwan et al., 1984). Outros dois estudos observaram que, 96 h de privação de sono REM reduz o efeito antinociceptivo de uma alta dose de morfina (10 mg / kg) administrada pela via intraperitoneal em ratos avaliados em teste térmico nocivo (Nascimento et al., 2007, Skinner et al., 2011). Mais recentemente, foi demonstrado que 24 h de privação de sono REM é capaz de diminuir o efeito antinociceptivo máximo da morfina (0,9 - 9 nmol) administrada localmente, intra-PAG (substância cinzenta periaquedutal, do inglês periaqueductal gray matter) no teste mecânico com o analgesímetro Randall-Selitto (Tomim et al., 2016). Além disso, a administração de uma dose de morfina (5 mg / kg, administrada s.c.) que causa analgesia ao calor, tem seu efeito analgésico fortemente reduzido em camundongos privados de sono por 9 horas (Alexandre et al., 2017).

Sistema endocanabinoide

Embora não existam trabalhos que tenham estudado o envolvimento do sistema endocanabinoide no efeito da diminuição do tempo de sono na dor em animais, trabalhos com humanos mostram aumento nos níveis de endocanabinoides no plasma após restrição de sono (Cedernaes et al., 2016, Hanlon et al., 2016). Foi demonstrado também que o tratamento com canabinoides diminui a dor e melhora a qualidade de sono em pacientes com dor crônica (Serpell et al., 2014, Lynch and Ware, 2015). No entanto, esses dados são controversos e preliminares. Mais estudos são necessários para sugerir o envolvimento do sistema endocanabinoide no aumento da sensibilidade a dor induzida pela diminuição do tempo de sono.

Sistema descendente de modulação da dor

Um estudo do nosso laboratório contribuiu para melhorar o entendimento a respeito dos mecanismos pelos quais a privação de sono REM aumenta a dor. Este estudo demonstrou que o potente efeito pró-nociceptivo da privação de sono está associado à diminuição da ativação das vias descendentes de inibição da dor e aumento da ativação das vias descendentes de facilitação da dor (Tomim et al., 2016). 24 ou 48 h de privação de sono REM aumentou significativamente a nocicepção induzida pela formalina e diminuiu o limiar mecânico nociceptivo de retirada da pata (Tomim et al., 2016). Além disso, o efeito antinociceptivo máximo induzido pela morfina (intra-PAG) foi significativamente diminuído pela privação de sono REM (Tomim et al., 2016). Este estudo também demonstrou que a administração de uma dose baixa do antagonista do receptor GABA_A, bicuculina (30-300 pmol, intra-PAG), diminuiu a nocicepção em ratos controle, mas não em ratos privados de sono REM (Tomim et al., 2016). Além disso, a administração do antagonista do receptor de colecistocinina 2, YM022 (0,5-2

pmol) no bulbo rostroventral (RVM), diminuiu a nocicepção em ratos privados de sono REM, mas não nos controles (Tomim et al., 2016). Além disso, a injeção de um derivado do anestésico lidocaína (QX-314, 2%, intra-RVM) diminuiu a nocicepção em ratos privados de sono, mas não em ratos controle, enquanto a lesão do funículo dorsolateral impediu o efeito pró-nociceptivo da privação de sono REM (Tomim et al., 2016). Finalmente, a privação de sono aumentou significativamente a expressão de c-Fos no RVM (Tomim et al., 2016). Portanto, a privação de sono parece aumentar a dor diminuindo a atividade descendente inibitória da dor e aumentando a atividade descendente facilitadora da dor. Esta foi a primeira evidência de que a privação de sono aumenta a dor por modular o mais conhecido e potente sistema endógeno de modulação da dor, o sistema descendente. Lembrando que, o centro de tal sistema é a PAG, que recebe múltiplas aferências de diferentes regiões encefálicas - incluindo o núcleo accumbens (Zhang et al., 2013) - e as integra para controlar indiretamente, através de vias descendentes que partem do RVM, a transmissão da informação nociceptiva na medula espinhal (Millan, 2002, Fields, 2004, Heinricher et al., 2009).

Um estudo anterior já havia proposto que a hiperalgesia observada em ratos privados de sono estava associada ao aumento da atividade da via de sinalização da óxido nítrico sintase na PAG dorsolateral, o que presumivelmente influencia a via antinociceptiva descendente (Damasceno et al., 2013). Em um estudo, exposições repetidas à perda de sono no início da vida foram capazes de induzir alterações na resposta nociceptiva na adolescência em camundongos e isso estaria associado a diminuição na expressão de c-Fos na PAG, o que sugere que a restrição de sono diminui a modulação descendente a um estímulo nocivo (Araujo et al., 2018). Outro estudo recente indica que a privação de sono pré-operatória agrava e prolonga a hipersensibilidade à dor induzida por incisão via facilitação descendente mediada por sinalização de BDNF no RVM, em ratos (Xue et al., 2018). Além disso, a lesão do funículo dorsolateral reverte parcialmente o efeito pró-nociceptivo induzido por privação de sono na dor incisional e, microinjeção intra-RVM de um anticorpo de BDNF em 1 dia ou 4 dias após a incisão, reverteu parcialmente os efeitos pró-nociceptivos induzidos por privação de sono em ratos com incisão na pata, enquanto não alterou os escores de dor e os limiares de retirada da pata em ratos que receberam apenas incisão plantar (Xue et al., 2018).

De fato, a ideia de que a modulação endógena da dor se torna disfuncional com distúrbios do sono é apoiada por dados de estudos clínicos (Edwards et al., 2009, Haack et al., 2012, Paul-Savoie et al., 2012, Lee et al., 2013, Bulls et al., 2015, Bjurstrom et al., 2021) e experimentais em humanos (Smith et al., 2007, Edwards et al., 2009, Tiede et al., 2010, Eichhorn et al., 2018, Simpson et al., 2018, Carvalho et al., 2019).

Papel de estruturas chave no ciclo sono vigília

O núcleo accumbens (NAc) no estriado ventral, desempenha importante papel na modulação da dor (Altier and Stewart, 1998, 1999, Gear and Levine, 2011, Haghparast et al., 2012, Sardi et al., 2018a, Sardi et al., 2018b, Tobaldini et al., 2019, Vergara et al., 2020) e na regulação do ciclo sono-vigília (Bouyer et al., 1986, Satoh et al., 1999, Scammell et al., 2001, Qiu et al., 2010, Qu et al., 2010, Lazarus et al., 2011, Lazarus et al., 2012, Qiu et al., 2012, Lazarus et al., 2013, Oishi and Lazarus, 2017, Oishi et al., 2017b, Luo et al., 2018, Zhou et al., 2019) e recentemente, estudos do nosso grupo demonstram que o NAc medeia tanto o efeito pró-nociceptivo da privação de sono REM (Sardi et al., 2018b) quanto da restrição de sono total (Sardi et al., 2018a).

As evidências que indicam que o NAc tem papel importante na regulação do sono e da vigília estão aumentando. Já foi demonstrado que a infusão de agonista para o receptor A_{2A} de adenosina no espaço subaracnóide aumenta o sono e a expressão de c-Fos, um marcador de atividade neuronal no NAc (Scammell et al., 2001). A infusão de agonista para o receptor A_{2A} próximo do NAc também promove sono (Satoh et al., 1999). Lesão citotóxica do NAc em gatos (Bouyer et al., 1986) ou ratos (Qiu et al., 2010, Qiu et al., 2012) altera o ciclo sono-vigília e abole o efeito excitatório do modafinil (Qiu et al., 2012). E, a deleção dos receptores A_{2A} no NAc de ratos ou de camundongos bloqueia o efeito excitatório da cafeína (Lazarus et al., 2011). A ativação quimiogénica ou optogénica de neurónios excitatórios da via indireta que expressam o receptor de adenosina A_{2A} no NAc core induz fortemente o sono de ondas lentas e, que a inibição quimiogénica dos neurónios da via indireta no NAc impede a indução do sono (Oishi et al., 2017b). Mais recentemente foi demonstrada a primeira evidência de que níveis elevados de adenosina endógena no NAc promovem sono de ondas lentas pela ativação de receptores A_{2A} (Zhou et al., 2019). Por fim, a ativação optogénica dos neurónios que expressam o receptor D_1 de dopamina no NAc induz transições imediatas do sono não-REM para a vigília, e a estimulação quimiogénica prolonga a excitação, com diminuição da ingestão de alimentos (Luo et al., 2018).

O córtex pré-frontal tem papel de destaque no controle do ciclo sono vigília (Lazarus et al., 2012, Lazarus et al., 2013, Oishi and Lazarus, 2017, Scammell et al., 2017) e nos mecanismos de modulação da dor (De Felice and Ossipov, 2016). Vários sistemas neuroquímicos promovem a excitação e a rápida atividade cortical típica da vigília. Os grupos celulares monoaminérgicos subcorticais que estimulam a excitação, inervam difusamente o

córtex cerebral (Scammell et al., 2017), incluindo o córtex cingulado anterior (Lopez-Avila et al., 2004, Chandler et al., 2013). Essas regiões monoaminérgicas incluem neurônios noradrenérgicos do locus coeruleus, neurônios serotoninérgicos dos núcleos dorsal e mediano da rafe, neurônios dopaminérgicos da área tegmentar ventral (VTA) e neurônios histaminérgicos do núcleo tuberomamilar (Lopez-Avila et al., 2004, Chandler et al., 2013, Scammell et al., 2017).

Entre as regiões pré-frontais, o córtex cingulado anterior (ACC) tem se destacado como uma área especialmente envolvida nos mecanismos de facilitação da dor em roedores (Calejesan et al., 2000, Price, 2000, Gao et al., 2004, Lopez-Avila et al., 2004, Zhang et al., 2005, Zhuo, 2008, Ikeda et al., 2014, Bliss et al., 2016) e em humanos (Louis and Kowalski, 2002, Apkarian et al., 2005). O ACC se conecta amplamente com a PAG (Peyron et al., 2007, Ikeda et al., 2014, Bliss et al., 2016) e RVM (Calejesan et al., 2000), sua atividade está associada ao aumento da facilitação descendente (Calejesan et al., 2000, Zhang et al., 2005) e sofre ação modulatória de vias subcorticais monoaminérgicas (Lopez-Avila et al., 2004, Chandler et al., 2013). Portanto é possível que o ACC participe da modulação do efeito pró-nociceptivo induzido pela restrição de sono.

A área tegmentar ventral (VTA), localizada no tronco encefálico, participa da manutenção da ativação cortical e da vigília (Eban-Rothschild et al., 2016, Taylor et al., 2016b, Oishi and Lazarus, 2017, Oishi et al., 2017a) e, também têm reconhecida participação na modulação da dor (Morgan and Franklin, 1990, Altier and Stewart, 1998, Baliki and Apkarian, 2015). As projeções dopaminérgicas da VTA ao NAc formam a chamada via dopaminérgica mesolímbica (Russo and Nestler, 2013). Um estudo recente sugere que tais projeções sejam as responsáveis pelo potente efeito da dopamina na indução da vigília (Eban-Rothschild et al., 2016), mostrando que a estimulação optogenética de neurônios dopaminérgicos na VTA iniciam e mantêm a vigília (Eban-Rothschild et al., 2016). Além disso, Oishi e colegas descobriram que a ativação quimiogenética de neurônios dopaminérgicos na VTA induz fortemente a vigília com uma duração prolongada de episódios de vigília, enquanto o comportamento sono/vigília não é afetado pela ativação de neurônios produtores de dopamina na substância negra, área adjacente (Oishi et al., 2017a). Fortalecendo as evidências de que os neurônios dopaminérgicos da VTA em forte capacidade de induzir vigília, outro estudo demonstrou que a ativação optogenética dos neurônios dopaminérgicos da VTA produzem excitação em camundongos mesmo sob anestesia inalatória (Taylor et al., 2016b).

No entanto, as projeções dopaminérgicas da VTA se dirigem também a diversas áreas corticais e límbicas (Oades and Halliday, 1987, Ikemoto, 2007) desempenhando papel central

nos mecanismos de motivação e recompensa (Russo and Nestler, 2013) assim como na modulação da dor (Baliki and Apkarian, 2015). Por exemplo, a VTA contribui para a analgesia induzida pela administração sistêmica de morfina (Morgan and Franklin, 1990) ao aumentar a liberação de dopamina no NAc (Altier and Stewart, 1998) pois seus neurônios dopaminérgicos estão sob inibição tônica de neurônios GABAérgicos que expressam receptores opioides (Johnson and North, 1992). Portanto é possível que a VTA participe da modulação do efeito pró-nociceptivo induzido pela restrição de sono.

A substância cinzenta periaquedutal (PAG), localizada na ponte, participa da modulação do ciclo sono-vigília (Lu et al., 2006, Sapin et al., 2009, Hayashi et al., 2015, Weber et al., 2015, Scammell et al., 2017, Weber et al., 2018) e é amplamente conhecida por seu importante papel na modulação da dor (Millan, 2002, Fields, 2004, De Felice and Ossipov, 2016, Tomim et al., 2016, Tobaldini et al., 2018a, Tobaldini et al., 2019).

Os neurônios da PAG ventro lateral (vlPAG), parecem ser ativos durante a vigília e o sono não-REM e menos ativos durante o sono REM (Sapin et al., 2009). Já foi demonstrado que, a lesão ou inativação farmacológica da vlPAG aumenta o tempo de sono REM (Lu et al., 2006, Sapin et al., 2009). Da mesma forma, a ativação optogenética de neurônios GABAérgicos na vlPAG aumenta o sono REM e desencadeia transições do sono não-REM para o REM (Weber et al., 2015, Weber et al., 2018), enquanto a ativação ou inibição quimiogenética de neurônios GABAérgicos na vlPAG reduz ou aumenta o sono REM, respectivamente (Hayashi et al., 2015, Weber et al., 2015).

Estudos do nosso grupo já demonstraram que o potente efeito pró-nociceptivo da privação de sono está associado à diminuição da ativação das vias descendentes de inibição da dor e aumento da ativação das vias descendentes de facilitação da dor na PAG (Tomim et al., 2016). Além disso, recentemente, demonstramos que o efeito pró-nociceptivo da restrição de sono crônica (6 horas de restrição durante 12 dias) depende tanto do NAc quanto da PAG, uma vez que tal efeito foi prevenido por lesão excitotóxica de qualquer um deles (Sardi et al., 2018a).

O núcleo Dorsal da Rafe (DRN), localizado ao longo da linha média no tronco encefálico e formação reticular, é o principal núcleo serotoninérgico. Participa da modulação do sono e da vigília (Scammell et al., 2017) e tem papel modulatório no processamento da dor (Bannister and Dickenson, 2016, Cortes-Altamirano et al., 2018).

No geral, evidências atuais sugerem que a transmissão de serotoninérgica promove a vigília, inibe o sono REM e pode interferir no sono de ondas lentas (Monti, 2011, Monti and Jantos, 2014). Agonista para o receptor 5-HT₇ administrado sistemicamente, aumenta a vigília e reduz o sono REM, enquanto a administração local no DRN suprime o sono REM (Monti et

al., 2014) A supressão de receptores 5-HT_{1A} e 5-HT_{1B} em camundongos, aumentada a quantidade de sono REM (Boutrel et al., 1999, Boutrel et al., 2002) enquanto camundongos mutantes sem receptores 5-HT_{2A} ou 5-HT_{2C} apresentam tempo maior de vigília e menor tempo de sono não REM (Frank et al., 2002, Popa et al., 2005). A fotoativação (optogenética) de neurônios serotoninérgicos dobra a quantidade de vigília e fragmenta o sono não-REM (Ito et al., 2013). Recentemente foi demonstrado que a ativação optogenética de neurônios dopaminérgicos no DRN promove vigília e contribui para a regulação dos estados de sono-vigília (Cho et al., 2017). Portanto é possível que o DRN participe da modulação do efeito pró-nociceptivo induzido pela restrição de sono.

O Locus Coeruleus (LC), localizado na ponte, é o principal núcleo noradrenérgico para excitação cortical (Gompf et al., 2010, Scammell et al., 2017). Suas projeções noradrenérgicas ao ACC (Chandler and Waterhouse, 2012), tem papel não só em manter a vigília (Gompf et al., 2010), mas também em modular a dor (Bravo et al., 2016). Além de suas projeções ascendentes, o LC tem importantes projeções descendentes que modulam a transmissão da informação nociceptiva no corno dorsal da medula espinhal (Llorca-Torralba et al., 2016).

Agonistas noradrenérgicos para receptores do tipo α_1 aumentam a vigília e a ativação optogenética do LC rapidamente desperta camundongos do sono (Carter et al., 2010). Por outro lado, drogas que reduzem a liberação de noradrenalina, como agonistas para receptores noradrenérgicos do tipo α_2 , são sedativas, e a inibição optogenética de neurônios noradrenérgicos do LC promove transições para o sono não-REM e reduz levemente a vigília (Carter et al., 2010). De tal maneira, a atividade neuronal do LC está correlacionada com o estado de vigília, no entanto, lesões no LC produzem apenas pequenas alterações na vigília diária (Gompf et al., 2010). Sendo assim, é possível que o LC participe da modulação do efeito pró-nociceptivo induzido pela restrição de sono.

O hipotálamo lateral é outra área importante na ativação cortical (Lazarus et al., 2012, Oishi and Lazarus, 2017, Scammell et al., 2017) com papel na modulação da dor (Azhdari-Zarmehri et al., 2013, Yazdi-Ravandi et al., 2014, Rashidy-Pour et al., 2015). O hipotálamo lateral recebe projeções GABAérgicas do NAc (Lazarus et al., 2012, Zhang et al., 2013) e projeta neurônios orexinérgicos ao ACC (Kampe et al., 2009, Jin et al., 2016) liberando orexina para manter os níveis basais de excitabilidade dos neurônios piramidais (Li et al., 2010, Yan et al., 2012). Além disso, os neurônios da orexinérgicos estão espalhados pelo hipotálamo lateral, e as orexinas excitam fortemente todas as regiões cerebrais promotoras da vigília (Scammell et al., 2017). Portanto, é possível que o hipotálamo lateral contribua para o efeito pró-nociceptivo da restrição de sono.

Após todo o exposto no levantamento bibliográfico, este trabalho tem como objetivo investigar os mecanismos pelos quais a diminuição do tempo de sono induz aumento da sensibilidade a dor. A seguir estão apresentados 4 capítulos juntamente com seus objetivos específicos, cada capítulo é um artigo científico original.

CAPITULO 1

IMPACTO DA ANALGESIA ENDÓGENA DESENCADEADO POR ACUPUNTURA, ESTRESSE OU ESTIMULAÇÃO NOCIVA NO EFEITO PRÓ-NOCICEPTIVO DA PRIVAÇÃO DE SONO REM

Objetivo geral

Investigar se a ativação de mecanismos endógenos de modulação da dor é capaz de diminuir o efeito pró-nociceptivo induzido pela privação de sono REM, em caso afirmativo, como essa diminuição se compara à induzida em um modelo clássico de dor inflamatória animal.

Objetivos específicos

- Testar a capacidade da privação de sono REM intensificar a hiperalgesia induzida por um modelo clássico de dor inflamatória com carragenina.
- Comparar a intensidade e duração da analgesia endógena desencadeada por acupuntura em ratos tratados com carragenina e em ratos privados de sono REM.
- Comparar a intensidade e duração da analgesia endógena desencadeada por estresse agudo em ratos tratados com carragenina e em ratos privados de sono REM.
- Comparar a intensidade e duração da analgesia endógena desencadeada por estimulação nociva em ratos tratados com carragenina e em ratos privados de sono REM.

Apresentação:

Artigo científico 1: **Impact of endogenous analgesia triggered by acupuncture, stress or noxious stimulation on the pronociceptive effect of REM sleep deprivation**

Impact of endogenous analgesia triggered by acupuncture, stress or noxious stimulation on the pronociceptive effect of REM sleep deprivation

Abstract

Poor sleep increases the risk of developing pain conditions and pain decreases sleep quality. We have previously demonstrated that REM-SD (Rapid Eye Movement - Sleep Deprivation) increases pain by disrupting the descending pain modulation system. Indeed, the idea that endogenous pain modulation becomes dysfunctional with sleep disturbance is supported by clinical and experimental data in humans. Therefore, a major question raised in this article is whether the activation of endogenous pain modulation mechanisms is able to decrease the pronociceptive effect induced by REM-SD, if so, how this decrease compares to that induced in a classical animal inflammatory pain model - carrageenan model. First of all, we tested the ability of REM-SD to worsen carrageenan-induced hyperalgesia because the pronociceptive effect of REM-SD has never been demonstrated in the carrageenan model. Then, we choose three different ways of triggering endogenous analgesia (1) acupuncture; (2) acute stress and (3) noxious stimulation. The administration of a low dose of carrageenan (30 μ g) in sleep deprived animals resulted in a potentiated hyperalgesic effect that was more intense and longer lasting than that induced by a higher standard dose of carrageenan (100 μ g) or by REM-SD alone. This additive effect demonstrates that the hyperalgesic states induced by REM-SD or carrageenan are mediated by different mechanisms, otherwise a low dose of carrageenan would not change the pronociceptive effect of REM-SD. We found that (1) acupuncture, performed at ST36, completely reversed the pronociceptive effect of REM-SD, stabilizing the nociceptive threshold at baseline; and carrageenan-induced hyperalgesia was also completely reversed by acupuncture. In contrast, control animals (those submitted to the control procedure for SD or receiving a hind paw injection of saline) exhibited an intense and persistent increase in the nociceptive threshold. We found that (2) immobilization stress completely reversed the pronociceptive effect of REM-SD, stabilizing the nociceptive threshold at baseline. Interestingly, the analgesia induced by stress is characteristically transient, as we observe in control animals and in those receiving carrageenan, but the hyperalgesic state induced by REM-SD is not re-established when analgesia ends. This contrasts with carrageenan-induced hyperalgesia that was transiently inhibited by stress, and once analgesia ends, hyperalgesia returns to levels seen in animals not exposed to stress. We also tested the ability of pain-induced analgesia to decrease the pronociceptive effect of REM-SD. We found that (3) noxious stimulation of the forepaw by capsaicin reversed the hyperalgesic state induced by REM-SD, bringing the nociceptive threshold to baseline. In contrast, the nociceptive threshold was persistently increased above the baseline in control animals and in those treated with carrageenan. This study demonstrated that the hyperalgesic state induced by SD is totally suppressed by the activation of endogenous pain modulation mechanisms triggered by acupuncture, stress or noxious stimulation. Therefore, although SD disrupts endogenous pain modulation mechanisms, this does not prevent the recruitment of such mechanisms to induce analgesia in sleep-deprived subjects. In contrast to the homogeneous analgesic effect induced by acupuncture, stress or noxious stimulation in sleep-deprived animals, each intervention affected carrageenan-induced hyperalgesia differently.

Key words: sleep deprivation; pain; nociception; acupuncture; acute stress; stress-induced analgesia; capsaicin; ascending nociceptive control; pain-induced analgesia; analgesia; antinociception; pronociceptive; hyperalgesia; noxious stimulation; endogenous analgesia;

Introduction

Sleep disorders and persistent pain conditions are public health problems worldwide (Artner et al., 2013, Kim et al., 2015) and there is a clear bidirectional relationship between them. Poor sleep increases the risk of developing pain conditions and pain decreases sleep quality (Karaman et al., 2014, Sribastav et al., 2017). In fact, sleep disorders are present in up to 88% of patients with chronic pain (Morin et al., 2006, Cheatle et al., 2016) and at least 50% of insomnia subjects suffer from chronic pain (Taylor et al., 2007). The understanding about the mechanisms by which sleep impairment affects pain is essential to encourage the development of new drugs and behavioral interventions that would impact clinical pain management and most widely, public health and pain prevention.

In fact, our knowledge about how and how much sleep deficiency affects pain has advanced considerably in the last few years. Several experimental models of sleep restriction or deprivation have been used to unequivocally demonstrate that poor sleep lowers pain thresholds (Onen et al., 2001b, Smith and Haythornthwaite, 2004, Nascimento et al., 2007, Damasceno et al., 2009, Wei et al., 2010, Tomim et al., 2016, Sardi et al., 2018a, Sardi et al., 2018b) and increases already existing pain (Huang et al., 2014, Tomim et al., 2016, Hambrecht-Wiedbusch et al., 2017, Xue et al., 2018, Li et al., 2019). The mechanisms underlying this pronociceptive effect of sleep loss are poorly understood and apparently involves several neurotransmitter systems and brain regions (Sardi et al., 2018a, Sardi et al., 2018b, Haack et al., 2020). We have previously demonstrated that REM-SD (Rapid Eye Movement - Sleep Deprivation) increases pain by disrupting the most well-known endogenous pain modulation system, the PAG-RVM (Periaqueductal Gray - Rostral Ventral Medulla) descending system (Tomim et al., 2016). Indeed, the idea that endogenous pain modulation becomes dysfunctional with sleep disturbance is supported by clinical (Edwards et al., 2009, Paul-Savoie et al., 2012, Bulls et al., 2015, Bjurstrom et al., 2021) and experimental data in humans (Smith et al., 2007, Edwards et al., 2009, Tiede et al., 2010, Eichhorn et al., 2018, Simpson et al., 2018, Carvalho et al., 2019). But one thing is to increase pain by disrupting endogenous pain modulation and quite another is to induce a pronociceptive effect that is insensitive to endogenous analgesia. Therefore, a major question raised in this article is whether the activation of endogenous pain modulation mechanisms is able to decrease the pronociceptive effect induced by REM-SD, if so, how this decrease compares to that induced in a classical animal pain model.

To answer these questions, we chose the carrageenan model of inflammatory hyperalgesia as the standard model used to compare the intensity and duration of endogenous

analgesia in carrageenan-treated and in REM sleep deprived rats. First of all, we tested the ability of REM-SD to worsen carrageenan-induced hyperalgesia because the pronociceptive effect of REM-SD has never been demonstrated in the carrageenan model. Then, we choose three different ways of triggering endogenous analgesia (1) acupuncture; (2) acute stress and (3) noxious stimulation. Acupuncture is worldwide used in non-pharmacological pain control, both acute stress- and pain-induced analgesia are physiologically triggered during life-threatening situations. All of them depend on endogenous pain modulation mechanisms that, to some extent, overlap, but are not completely coincident (Tobaldini et al., 2014, Tobaldini et al., 2020).

Materials and Methods

Animals

Experiments were performed in male Wistar rats (270–330g), housed four or five per cage, in a temperature-controlled ($22^{\circ}\text{C} \pm 2$) on a 12-h/12-h light/dark cycle, with ad libitum access to food and water. In the present study, we used 141 animals. All experimental procedures and protocols were approved by The Committee on Animal Research of the Federal University of Parana, Brazil, and are in accordance with the guidelines proposed by the IASP (International Association for the Study of Pain) and the ARRIVE guidelines (Percie du Sert et al., 2020).

Drugs

Carrageenan- λ (30 or 100 μg injected into the hind paw) was freshly prepared and dissolved in 0.9% NaCl (sterile saline) to concentrations of 1 or 3.33 $\mu\text{g}/\mu\text{L}$ (Bonet et al., 2013). Capsaicin, a transient receptor potential vanilloid 1 (TRPV1) agonist (250 μg injected into the forepaw) was dissolved in 6% ethanol, 8% Tween 80, and 86% 0.9% NaCl at a concentration of 50 $\mu\text{g}/\mu\text{L}$ and then diluted in 0.9% NaCl to the concentration of 8.33 $\mu\text{g}/\mu\text{L}$ (Gear et al., 1999, Tobaldini et al., 2014, Tobaldini et al., 2019). The injection volume either in the hind paw or in the forepaw was 30 μL . Drugs were obtained from Sigma-Aldrich (St. Louis, MO).

Behavioral tests

Testing sessions took place during light phase (between 07:00 AM and 5:00 PM) in a quiet room maintained at $22^{\circ}\text{C} \pm 2$. Before experiments, each animal was manipulated for 7

days to be habituated to the experimental manipulation. Rats did not have access to food or water during the behavioral test and each animal was used once.

Carrageenan-induced inflammatory hyperalgesia

Carrageenan is an inflammatory agent classically used to induce inflammatory hyperalgesia, which peaks 3 h after injection and then progressively decreases, waning 24 hours later (Bonet et al., 2013). The rats were gently held and carrageenan (30 or 100 µg / 30 µL) or its vehicle (NaCl 0.9%) was subcutaneously injected into the dorsal surface of left hind paw using a 26-gauge needle connected to a PE-50 cannula and to a 50-µL Hamilton syringe, as previously described (Bonet et al., 2013). The mechanical nociceptive threshold (see below) was measured before and at different times after injection.

Rapid eye movement sleep deprivation-induced hyperalgesia

REM-SD is a model of sleep restriction widely used to investigate the physiological consequences of sleep disturbance (Tufik et al., 2009, Haack et al., 2020). In this study we used the single-platform method to abolish REM sleep and significantly decrease non-REM sleep (Morden et al., 1967), as previously described (Tomim et al., 2016, Sardi et al., 2018b). Briefly, the rats were kept for 24 hours in a cage (23×23×35 cm, available food and water) where a 6.5-cm-diameter, 10-cm-high circular platform is located. The cage was filled with water up to 1 cm from the surface of the platform, so whenever rats went into REM sleep, they fell off the platform due to muscle atony and woke up. Therefore, SD induced by the platform method involves numerous awakenings, which predominantly affect the REM stage of the sleep. Such procedure is considered to mimic sleep fragmentation due to repeated awakenings being a useful tool to investigate the effects of sleep loss (Machado et al., 2005). Control groups were submitted to exactly the same procedure, with the only difference being that they were placed on a platform large enough (18-cm-diameter) to allow normal sleep. The mechanical nociceptive threshold (see below) was measured before and at different times after REM-SD.

Antinociceptive procedures

Acupuncture

Acupuncture is an ancient Chinese technique in which specific body points (acupoints) are stimulated to treat a variety of diseases, including pain. In this study, acupuncture was performed at the right ST36 (Zusanli) acupoint, as previously described (Tobaldini et al., 2014). Briefly, a small area of skin overlying the hind limb region was shaved with an electric razor.

Manual acupuncture was performed by inserting a stainless steel needle (.18 x 8 mm) to a depth of about 4 to 5 mm at right ST36 acupoint, located, in the rat, below the capitulum fibulae and lateral to the tibia (Bing et al., 1990, Tobaldini et al., 2014). Adhesive tape was placed over the needle to protect the area during treatment. The animals were allowed to rest in their home cages, with the needle inserted into the acupoint during the 30 minutes of the acupuncture session. The rats were not restrained and no signs of distress were observed. Control animals (sham groups) underwent the same procedure but no needle was inserted. The 30 min acupuncture session was performed 2h 30min after carrageenan injection or 23h30 after the beginning of REM-SD, depending on the purpose of the experiment. The first post-acupuncture nociceptive threshold measurement was performed immediately after the end of the session (see figure 1).

Acute immobilization stress

Immobilization is a widely used and painless stress model (Buynitsky and Mostofsky, 2009), which induce a transient antinociceptive effect (Tobaldini et al., 2020). In this study, acute stress was induced by restriction of body movements for 30 minutes, as previously described (Tobaldini et al., 2020). Briefly, the rats were kept immobilized in an adjustable plastic tube with a 1-cm orifice at the anterior end to allow ventilation and a similar orifice at the posterior end to allow tail accommodation. Rats in the control group remained isolated into mice's plastic cages, without restriction. The 30 min immobilization session was started 2h 30min after carrageenan injection or 23h30 after the beginning of REM-SD, depending on the purpose of the experiment. The first post-immobilization nociceptive threshold measurement was performed immediately after the end of the session (see figure 1).

Capsaicin injection

The injection of capsaicin is a widely used nociceptive stimulus to elicit pain-induced analgesia. It is known to induce long-lasting (around 2 hours) analgesia demonstrated in a site remote from capsaicin injection, such as the orofacial region (Tambeli et al., 2003a, Tambeli et al., 2003b, Gear and Levine, 2009, Tambeli et al., 2009, Tobaldini et al., 2014) or the hind paw (Gear et al., 1999, Schmidt et al., 2002a, Ferrari et al., 2010, Tobaldini et al., 2019, Tobaldini et al., 2020).

In this study, we have used a forepaw injection of capsaicin to demonstrate pain-induced analgesia in the hind paw, as previously described (Tobaldini et al., 2019, Tobaldini et al., 2020). Briefly, the rats were gently held and a subcutaneous injection of capsaicin (250 µg, 30 µL) or vehicle (NaCl 0.9%) was performed using a 26-gauge needle connected to a PE-50 cannula and to a 50-µL Hamilton syringe. Capsaicin was injected in the forepaw 2h 30min

after the hind paw injection of carrageenan or 23h30 after the beginning of REM-SD, depending on the purpose of the experiment. The first post-capsaicin nociceptive threshold measurement was performed 30 minutes after capsaicin injection (see figure 1).

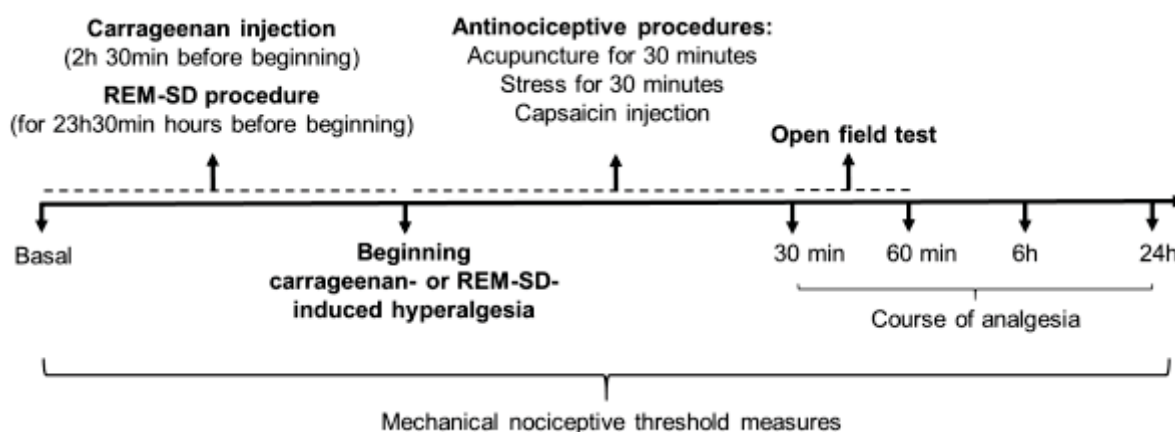


Figure 1 – Experimental design. The baseline mechanical nociceptive paw withdrawal threshold was measured and REM-SD was initiated or carrageenan (0, 30 or 100 μg , 30 μL) was injected into the left hind paw. The hyperalgesia induced by 23h30min of REM-SD or by a carrageenan injection performed 2h30min previously was measured. Then, depending on the group the animals: (1) received a capsaicin (250 μg , 30 μL) or a NaCl injection in the left forepaw; (2) went through 30 minutes of immobilization stress or social isolation; or (3) went through 30 minutes of acupuncture or sham treatment. The mechanical nociceptive paw withdrawal threshold was measured at different time points thereafter.

Mechanical Nociceptive Paw-withdrawal Test

The Randall–Selitto test (Randall and Selitto, 1957) was performed to assess the nociceptive mechanical paw withdrawal threshold, as a measure of the nociceptive response. In this test, a continuous pressure is applied to the dorsal surface of the rats' hind paw until the animal withdrew the paw. The mechanical nociceptive threshold was defined as the force in grams at which the rat withdrew its paw. The value was obtained from the mean of three readings made at intervals of 3 min. Data were expressed as mean $\pm$ standard error of the mean (SEM) of the mechanical paw withdrawal threshold (g) at each time point. The test was performed before any intervention (basal); 2 h and 30 min after carrageenan injection or 23h30 after the beginning of REM-SD; and repeated at different time intervals thereafter (see figure 1).

Locomotor activity in the Open Field Test

In order to determine the immediate effect of the different interventions on spontaneous locomotor/exploratory behavior in a novel environment, animals were tested in the open field arena between 30 and 60 minutes post-antinociceptive procedures (see figure 1). The open field

arena consists in a circular area (60 cm of diameter) limited by a 50 cm-high wall, divided into 12 squares (Insight®, Ribeirao Preto, SP, BR). The animals were placed at the center of the arena and allowed to explore it for 1 min. The exploratory behavior was measured by the quantification the number of crossed squares.

Statistical Analysis

Data were analyzed by analysis of variance (ANOVA) with repeated-measures (time) with REM-SD or carrageenan as within-subjects factor and treatments (stress, acupuncture or capsaicin) as between-subjects factors, when appropriate. The spontaneous locomotor activity was analyzed by One-way ANOVA. All post hoc contrasts, when appropriate, were performed using Tukey's test ($p < 0.05$). The software SigmaPlot® (Systat Software, San Jose, CA, USA) was used to perform data analysis and graphical representation. Data are plotted in figures as mean $\pm$ SEM.

Results

Effect of REM-SD on Carrageenan-induced hyperalgesia

The injection of carrageenan in the rats' hind paw induced a dose dependent decrease in the mechanical nociceptive threshold, characterizing the inflammatory hyperalgesia (Figure 2 - Two way RM ANOVA, $F_{\text{Treatment}} (4, 25) = 102.03$, $p < 0.001$; $F_{\text{Time}} (5, 125) = 144.13$, $p < 0.001$; $F_{\text{Treatment} \times \text{time}} (20, 125) = 16.00$, $p < 0.001$, followed by Tukey's test, symbols indicate $p < 0.05$). REM-SD for 23h30min also induced a significant decrease in the mechanical nociceptive threshold, characterizing the pronociceptive effect of SD ($p < 0.001$). This effect was similar in magnitude to that induced by the higher dose of carrageenan, but was more persistent remaining significant 24 h after SD ($p < 0.001$). Carrageenan at the lower dose (30 μg) potentiated the pronociceptive effect of REM-SD, inducing an even more pronounced and persistent reduction in the mechanical nociceptive threshold ($p \leq 0.001$).

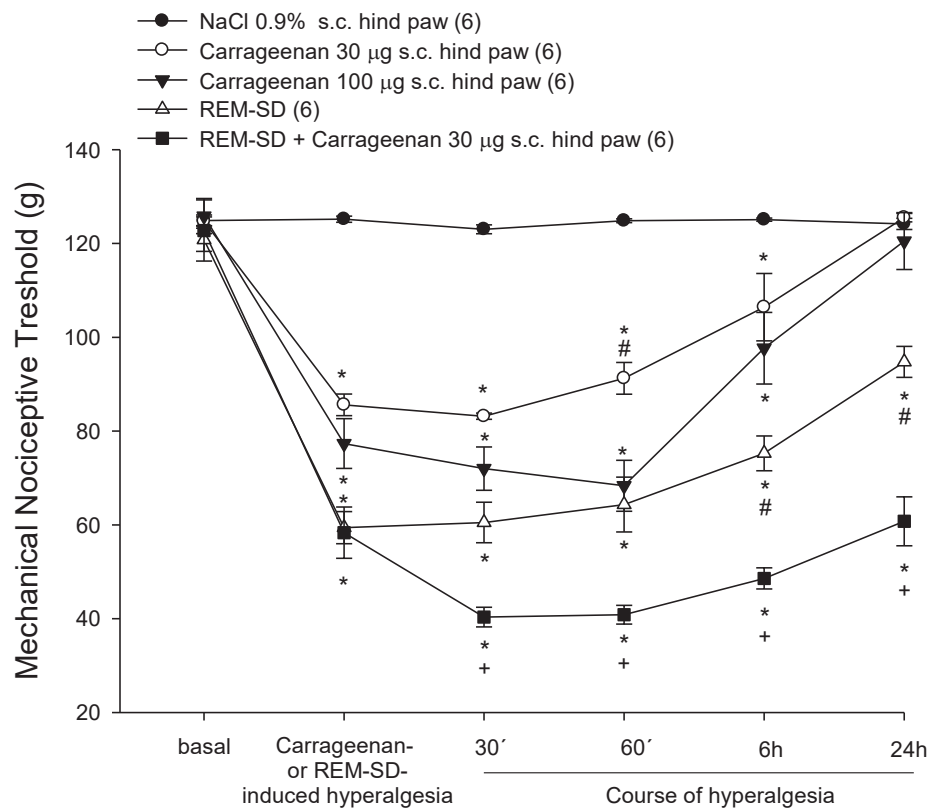


Figure 2- Effect of REM-SD on Carrageenan-induced hyperalgesia. Both carrageenan injection or REM-SD decreased the mechanical nociceptive threshold. The injection of carrageenan potentiated the pronociceptive effect of REM-SD, further decreasing the nociceptive threshold in sleep deprived animals. Two way RM ANOVA and Tukey post hoc test: The symbol, “*” indicates a statistically significant decrease in mechanical nociceptive threshold compared with the control group. The symbol, “+” indicates a statistically significant decrease in mechanical nociceptive threshold compared with all other experimental groups. The symbol, “#” indicates a statistically difference with all other groups. In this and in subsequent figures data are presented as mean $\pm$ SEM. Number in parenthesis indicate the number of animals in each group. See methods for additional details regarding data presentation and analysis. REM-SD = Rapid Eye Movement - Sleep Deprivation. s.c.= subcutaneous injection.

Effect of Acupuncture-induced analgesia on REM sleep deprived and Carrageenan treated animals

Acupuncture significantly and persistently increased the mechanical nociceptive threshold in control animals (submitted to control procedure for REM-SD, Fig. 3 A, $p < 0.001$; or those receiving injection of saline, Fig. 3 B, $p < 0.001$). In hyperalgesic animals, acupuncture restored the mechanical nociceptive threshold, reversing either REM-SD- (Figure 3 A – Two-way RM ANOVA, $F_{\text{Treatment}}(2, 14) = 240.48$, $p < 0.001$; $F_{\text{Time}}(5, 70) = 92.79$, $p < 0.001$; $F_{\text{Treatment} \times \text{time}}(10, 70) = 61.48$, $p < 0.001$, followed by Tukey’s test, symbols indicate $p < 0.05$) or carrageenan-induced hyperalgesia (Figure 3 B – Two-way RM ANOVA, $F_{\text{Treatment}}(2, 15) = 437.25$, $p < 0.001$; $F_{\text{Time}}(5, 75) = 86.62$, $p < 0.001$; $F_{\text{Treatment} \times \text{time}}(10, 75) = 39.88$, $p < 0.001$, followed by Tukey’s test, symbols indicate $p < 0.05$).

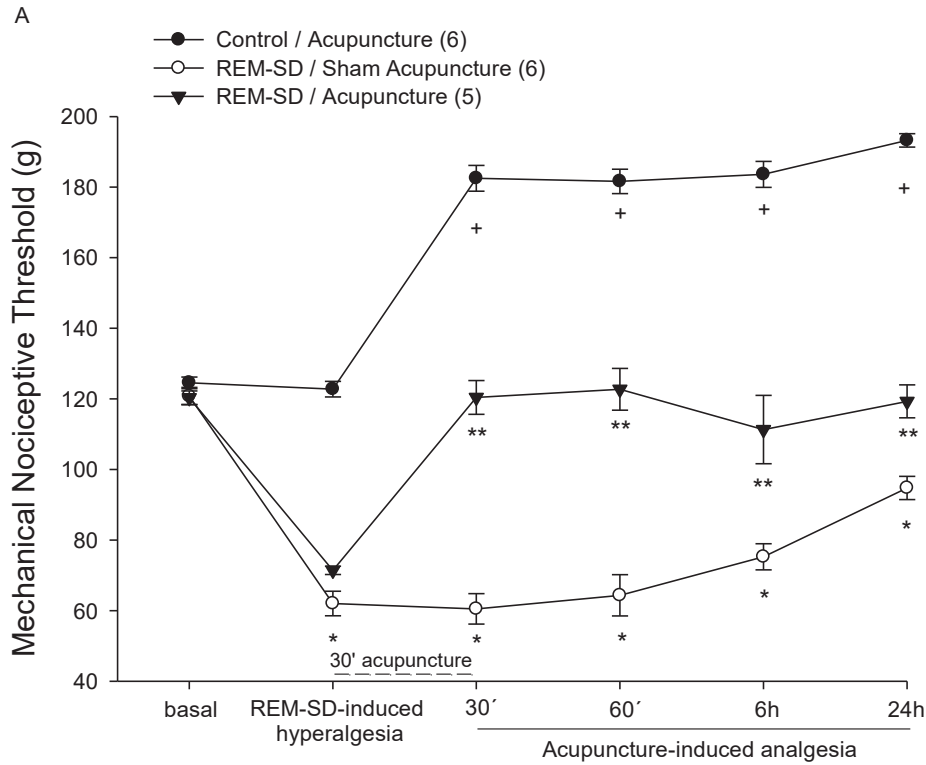


Figure 3- Effect of Acupuncture-induced analgesia on REM sleep deprived animals. A. Acupuncture significantly and persistently increased the mechanical nociceptive threshold in animals submitted to control procedure for REM-SD and restored the mechanical nociceptive threshold, reversing REM-SD-induced hyperalgesia in sleep deprived animals. Two way RM ANOVA and Tukey post hoc test: the symbol, “*” indicates a statistically significant decrease in mechanical nociceptive threshold compared to all other groups; the symbol “+” indicates a statistically significant increase above in basal mechanical nociceptive threshold compared to within-subjects and to all other groups; the symbol “**” indicates a statistically significant increase in the mechanical threshold compared to REM-SD / sham acupuncture group. Dashed line indicates when the 30' of acupuncture session.

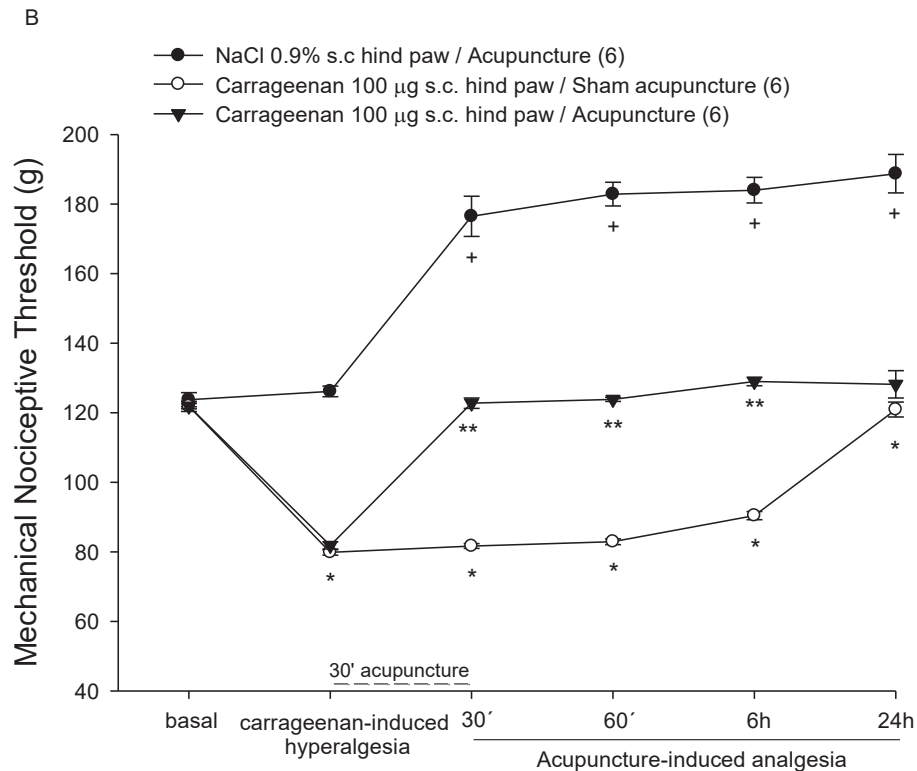


Figure 3- Effect of Acupuncture-induced analgesia on Carrageenan treated animals. B. Acupuncture significantly and persistently increased the mechanical nociceptive threshold in animals that received a NaCl injection and restored the mechanical nociceptive threshold, reversing carrageenan-induced hyperalgesia in animals that received a carrageenan injection. Two way RM ANOVA and Tukey post hoc test: the symbol “*” indicates a statistically significant decrease in mechanical nociceptive threshold compared to all other groups; the symbol “+” indicates a statistically significant increase above in basal mechanical nociceptive threshold compared to within-subjects and to all other groups; the symbol “**” indicates a statistically significant increase in the mechanical threshold compared to carrageenan / sham acupuncture group. Dashed line indicates when the 30' of acupuncture session.

Effect of Acute stress-induced analgesia on REM sleep deprived and Carrageenan treated animals

Acute immobilization stress transiently increased the mechanical nociceptive threshold in control animals (Fig. 4 A, $p < 0.001$; Fig. 4 B, $p < 0.001$). In animals which received a carrageenan injection, stress transiently reversed the hyperalgesia, but the antinociceptive effect wore off within 30 minutes, after which hyperalgesia ran its standard course (Figure 4 A – Two-way RM ANOVA, $F_{\text{Treatment}}(2, 15) = 141.63$, $p < 0.001$; $F_{\text{Time}}(5, 75) = 54.34$, $p < 0.001$; $F_{\text{Treatment} \times \text{time}}(10, 75) = 39.49$, $p < 0.001$, followed by Tukey's test, symbols indicate $p < 0.05$). In contrast, acute immobilization stress blocked the pronociceptive effect of REM-SD, permanently restoring the mechanical nociceptive threshold to the basal level (Figure 4 B – Two-way RM ANOVA, $F_{\text{Treatment}}(2, 17) = 74.25$, $p < 0.001$; $F_{\text{Time}}(5, 85) = 61.66$, $p < 0.001$; $F_{\text{Treatment} \times \text{time}}(10, 85) = 42.17$, $p < 0.001$, followed by Tukey's test, symbols indicate $p < 0.05$).

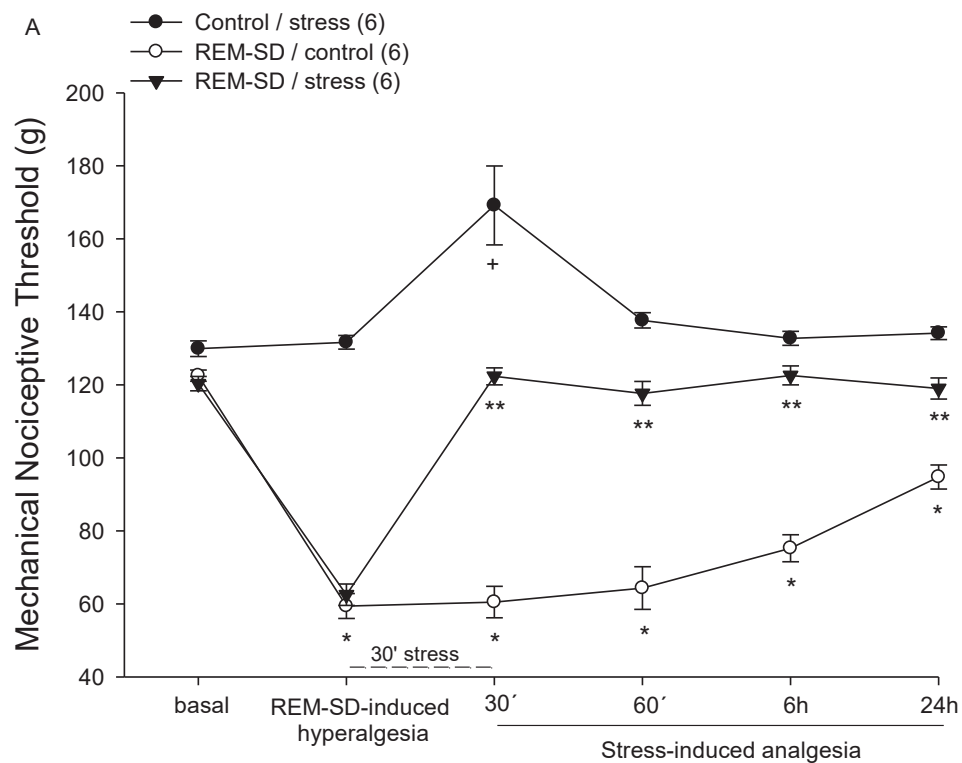


Figure 4- Effect of Acute stress-induced analgesia on REM sleep deprived animals. A. Acute immobilization stress transiently increased the mechanical nociceptive threshold in control animals and, was blocked the pronociceptive effect of REM-SD, permanently restoring the mechanical nociceptive threshold to the basal level. Two way RM ANOVA and Tukey post hoc test: the symbol “*” indicates a statistically significant decrease in mechanical nociceptive threshold compared to all other groups; the symbol “+” indicates a statistically significant increase above in basal mechanical nociceptive threshold compared to within-subjects and to all other groups; the symbol “**” indicates a statistically significant increase in the mechanical threshold compared to REM-SD / control group. Dashed line indicates 30' immobilization session.

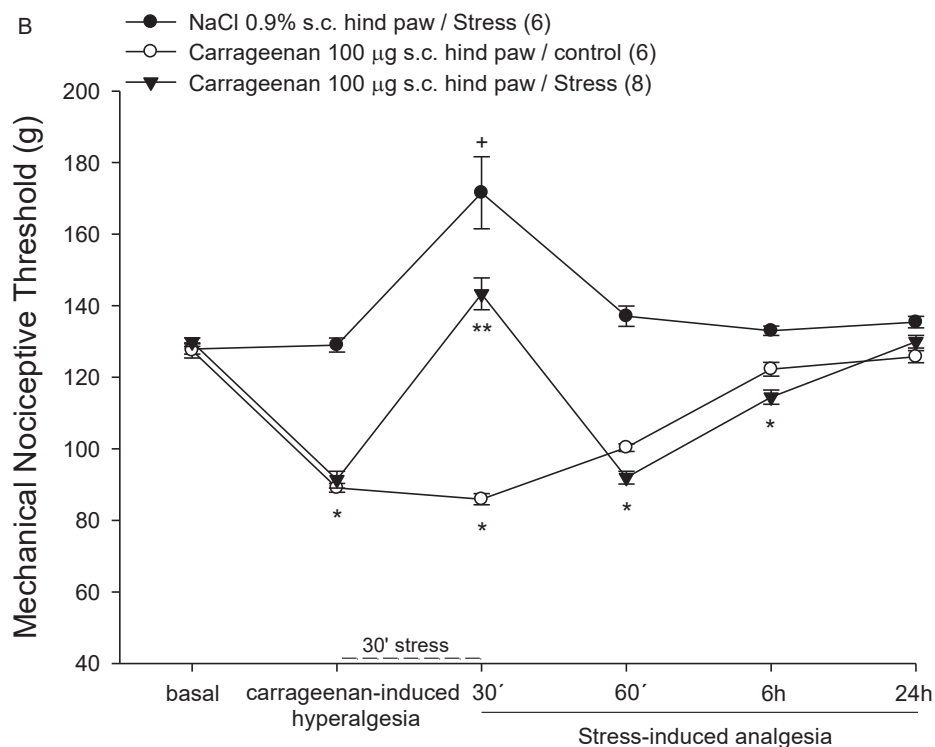


Figure 4- Effect of Acute stress-induced analgesia on Carrageenan treated animals. B. Acute immobilization stress transiently increased the mechanical nociceptive threshold in animals that received a NaCl injection and, in animals which received a carrageenan injection the antinociceptive effect wore off within 30 minutes, after which hyperalgesia ran its standard course. Two way RM ANOVA and Tukey post hoc test: the symbols “*” indicates a statistically significant decrease in mechanical nociceptive threshold compared with NaCl group; the symbol “+” indicates a statistically significant increase above in basal mechanical nociceptive threshold compared to within-subjects and to all other groups; the symbol “**” indicates a statistically significant increase in the mechanical threshold compared to carrageenan / control group.. Dashed line indicates the 30' immobilization session.

Effect of pain-induced analgesia on REM sleep deprived and Carrageenan treated animals

The injection of capsaicin in the forepaw was used as noxious stimulus to induce analgesia detected in the hind paw and it induced potent and long-lasting increase in the mechanical nociceptive threshold in control animals (Fig. 5 A, $p < 0.001$; Fig. 5 B, $p < 0.001$). Noxious stimulation of the forepaw not only blocked carrageenan-induced hyperalgesia in the hind paw, but persistently increased the nociceptive threshold keeping it well above the basal level, despite carrageenan injection (Figure 5 A – Two-way RM ANOVA, $F_{\text{Treatment}} (2, 15) = 28.15$, $p < 0.001$; $F_{\text{Time}} (5, 75) = 12.83$, $p < 0.001$; $F_{\text{Treatment} \times \text{time}} (10, 75) = 10.18$, $p < 0.001$, followed by Tukey's test, symbols indicate $p < 0.05$).

The injection of capsaicin in the forepaw blocked the pronociceptive effect of REM-SD, permanently restoring the mechanical nociceptive threshold to the basal level (Figure 5 B – Two-way RM ANOVA, $F_{\text{Treatment}} (2, 17) = 47.81$, $p < 0.001$; $F_{\text{Time}} (5, 85) = 57.13$, $p < 0.001$; $F_{\text{Treatment} \times \text{time}} (10, 85) = 31.94$, $p < 0.001$, followed by Tukey's test, symbols indicate $p < 0.05$).

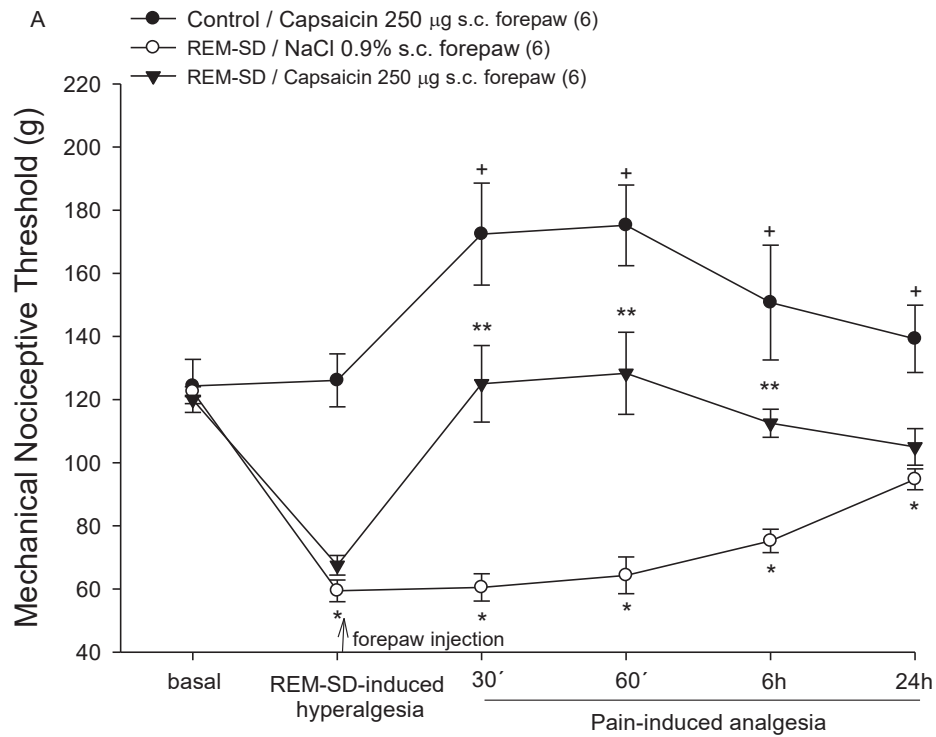


Figure 5- Effect of pain-induced analgesia on REM sleep deprived animals. A. The injection of capsaicin in the forepaw was used as noxious stimulus to induce analgesia detected in the hind paw and it induced potent and long-lasting increase in the mechanical nociceptive threshold in control animals. The injection of capsaicin in the forepaw blocked the pronociceptive effect of REM-SD, permanently restoring the mechanical nociceptive threshold to the basal level. Two way RM ANOVA and Tukey post hoc test: the symbol “*” indicates a statistically significant decrease in mechanical nociceptive threshold to other groups; the symbol “+” indicates a statistically significant increase above in basal mechanical nociceptive threshold compared to within-subjects and to all other groups; the symbol “**” indicates a statistically significant increase in the mechanical threshold compared to REM-SD / NaCl group. Arrow indicates the capsaicin or NaCl forepaw injection.

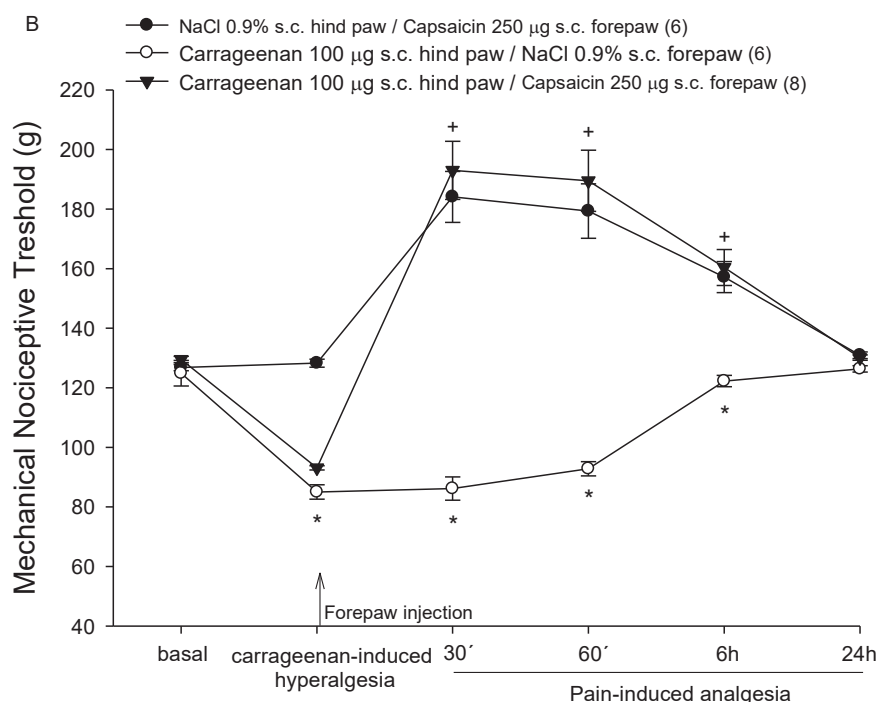


Figure 5- Effect of pain-induced analgesia on Carrageenan treated animals. B. The injection of capsaicin in the forepaw persistently increased the nociceptive threshold in both saline and carrageenan treated animals. Two way RM ANOVA and Tukey post hoc test: the symbol “*” indicates a statistically significant decrease in mechanical nociceptive threshold compared to other groups; the symbol “+” indicates a statistically significant increase above in basal mechanical nociceptive threshold compared to within-subjects and to carrageenan / NaCl group. Arrow indicates the capsaicin or NaCl forepaw injection.

Effect of the different experimental manipulations on the locomotor behavior

The inflammatory agent carrageenan injected into the hind paw of animals does not affect locomotor activity in the open field test, either at a dose of 30 µg ($p = 0.60$) or 100 µg ($p = 0.30$) compared to animals that received saline injection.

REM-SD increases the locomotor activity of animals in the open field test compared to control animals ($p = 0.04$).

The antinociceptive procedures acupuncture ($p = 0.59$), stress ($p = 0.71$) and capsaicin injection ($p = 0.15$) did not alter the locomotor activity of the animals compared to saline injection in the hind paw. The association of inflammation by carrageenan and acupuncture ($p = 0.44$) or capsaicin injection ($p = 0.44$) did not change locomotion, however, the association of inflammation with stress ($p = 0.007$) increased locomotion compared to the group that received just carrageenan injection.

The antinociceptive interventions acupuncture ($p = 0.04$) and capsaicin injection ($p = 0.03$) associated with REM-SD reduced animal locomotion compared to REM-SD animals without antinociceptive interventions, while this association with stress did not change animal locomotion ($p = 0.32$), as well as the association of REM-SD with carrageenan ($p = 0.10$).

Table 1**Effect of experimental manipulations on locomotor activity in the open field test.**

Experimental manipulations	Mean $\pm$ SEM	p value
NaCl 0.9% s.c. hind paw	19.33 $\pm$ 2.51	0.60
Carrageenan 30 μ g s.c. hind paw	17.66 $\pm$ 1.84	
Carrageenan 100 μ g s.c. hind paw	15.5 $\pm$ 2.53	
Control	21.56 $\pm$ 1.56	0.04*
REM-SD	27.83 $\pm$ 2.18	
REM-SD	27.83 $\pm$ 2.18	0.02*
Carrageenan 30 μ g s.c. hind paw	17.66 $\pm$ 1.84	
REM-SD / Carrageenan 30 μ g s.c. hind paw	21 $\pm$ 3.09	
NaCl 0.9% s.c. hind paw	19.33 $\pm$ 2.51	0.15
NaCl 0.9% s.c. hind paw / Acupuncture	21 $\pm$ 1.63	
NaCl 0.9% s.c. hind paw / Stress	18.37 $\pm$ 1.22	
NaCl 0.9% s.c. hind paw / Capsaicin 250 μ g s.c. forepaw	15.57 $\pm$ 0.75	
Carrageenan 100 μ g s.c. hind paw	15.5 $\pm$ 2.53	0.44
Carrageenan 100 μ g s.c. hind paw / Acupuncture	17.83 $\pm$ 1.51	
Carrageenan 100 μ g s.c. hind paw / Stress	23.62 $\pm$ 1.10	
Carrageenan 100 μ g s.c. hind paw / Capsaicin 250 μ g s.c. forepaw	18.37 $\pm$ 2.50	
REM-SD	27.83 $\pm$ 2.18	0.03*
REM-SD / Carrageenan 30 μ g s.c. hind paw	21 $\pm$ 3.09	
REM-SD / Acupuncture	19.0 $\pm$ 3.27	
REM-SD / Stress	24.66 $\pm$ 2.16	
REM-SD / Capsaicin 250 μ g s.c. forepaw	20.33 $\pm$ 2.03	

The symbols “*” indicates statistically significant difference compared to appropriate control in the one-way ANOVA test. REM-SD = Rapid Eye Movement - Sleep Deprivation; s.c.= subcutaneous injection; SEM = standard error of the mean.

Discussion

This study demonstrated that the pronociceptive effect of REM-SD is sensitive to endogenous analgesia, in spite of the consistent evidence that SD disrupts endogenous pain modulation (Smith et al., 2007, Edwards et al., 2009, Tiede et al., 2010, Paul-Savoie et al., 2012, Bulls et al., 2015, Tomim et al., 2016, Eichhorn et al., 2018, Simpson et al., 2018, Xue et al., 2018, Carvalho et al., 2019, Bjurstrom et al., 2021). Acupuncture-, stress- and pain-induced analgesia are known to be dependent on endogenous pain modulation mechanisms that, to some extent, overlap, but do not fully coincide (Tobaldini et al., 2014, Tobaldini et al., 2019, Tobaldini et al., 2020). Both acupuncture; stress immobilization and noxious stimulation reversed the pronociceptive effect of REM-SD, bringing the nociceptive threshold back to baseline. These findings contrast with those obtained with carrageenan-induced hyperalgesia,

in which each intervention induced an antinociceptive effect that was different in magnitude and duration.

Although numerous studies have unequivocally demonstrated that sleep restriction or deprivation lowers pain thresholds (Onen et al., 2001b, Smith and Haythornthwaite, 2004, Nascimento et al., 2007, Damasceno et al., 2009, Wei et al., 2010, Tomim et al., 2016, Sardi et al., 2018a, Sardi et al., 2018b) and increases already existing pain (Huang et al., 2014, Tomim et al., 2016, Hambrecht-Wiedbusch et al., 2017, Xue et al., 2018, Li et al., 2019), this study demonstrated for the first time the effect of sleep deprivation on carrageenan-induced hyperalgesia. The administration of a low dose of carrageenan in sleep deprived animals resulted in a potentiated hyperalgesic effect that was more intense and longer lasting than that induced by a higher standard dose of carrageenan or by REM-SD alone (Fig 2). This additive effect demonstrates that the hyperalgesic states induced by REM-SD or carrageenan are mediated by different mechanisms, otherwise a low dose of carrageenan would not change the pronociceptive effect of REM-SD. In fact, the mechanisms by which sleep loss increases pain are dependent on central changes in neural processing (Haack et al., 2020), such as in pain modulation systems (Tomim et al., 2016, Sardi et al., 2018b) and although similar central mechanisms may contribute to the effect of carrageenan (Gao et al., 2001), it is mostly dependent on peripheral inflammatory responses (Bonet et al., 2013). This difference in underlying mechanisms highlights the relevance of comparing the endogenous analgesia obtained in each model.

Acupuncture is an ancient Chinese technique that is now widely used in the western world as complementary pain therapy (Vickers et al., 2018, Lv et al., 2019). Consistent evidence demonstrated that acupuncture-induced analgesia depends on the activation of the PAG-RVM descending pain modulation system (Takeshige et al., 1992, Bai et al., 2010, Lv et al., 2019) and we have recently demonstrated that it is also dependent on an opioidergic inhibition of the nucleus accumbens (Tobaldini et al., 2014). Interestingly, sleep deprivation increases pain, apparently, by inducing exactly opposite effects on these brain regions. We have shown that the pronociceptive effect of REM-SD disrupts descending pain modulation (Tomim et al., 2016) and increases the activity of the nucleus accumbens (Sardi et al., 2018b), which made us wonder if the pronociceptive effect of REM-SD would be sensitive to acupuncture. Indeed, although acupuncture-induced analgesia has been broadly demonstrated in different animal models, it had never been demonstrated in sleep-deprived animals. We found that acupuncture, performed at ST36, a widely used acupoint located in the hind limb (Han, 2011), completely reversed the pronociceptive effect of REM-SD, stabilizing the nociceptive threshold at baseline

(Fig 3 A). This finding indicates that SD can even disrupt the physiological activity of the PAG (Tomim et al., 2016) and NAc (Sardi et al., 2018a, Sardi et al., 2018b), but this does not prevent their recruitment by external stimulus (acupuncture) to induce analgesia. Carrageenan-induced hyperalgesia was affected in a quite similar way; it was completely reversed by acupuncture (Fig 3 B), which is in line with previous studies showing that acupuncture or electroacupuncture attenuates carrageenan-induced hyperalgesia (Zhang et al., 2002, Oh et al., 2006, Taguchi et al., 2010, Chen et al., 2011). In contrast, control animals (those submitted to the control procedure for SD or receiving a hind paw injection of saline) exhibited an intense and persistent increase in the nociceptive threshold. Therefore, it seems that acupuncture reverses painful conditions (nullifies the hyperalgesic effect of SD or carrageenan) and reduces the basal nociceptive activity. Anyway, these data support the use of acupuncture for pain control in patients with sleep disturbance.

Pain is essential for the preservation of life; but equally essential is the ability to inhibit pain perception during life-threatening situations, when pain may be disabling, compromising survival strategies. For example, any animal facing a predator goes into an acute stress reaction, which among other changes induces transient analgesia. Stress-induced analgesia is part of the fight-or-flight response (Butler and Finn, 2009) and is dependent on the activation of the neuroendocrine stress axes (Filaretov et al., 1996, Butler and Finn, 2009, Lafrance et al., 2010) as well as on the PAG-RVM descending system (Yilmaz et al., 2010, Lau and Vaughan, 2014, Yesilyurt et al., 2015, Ferdousi and Finn, 2018). Recently, we have demonstrated that stress-induced analgesia is also dependent on opioidergic mechanisms in the nucleus accumbens (Tobaldini et al., 2020). Therefore, as with acupuncture, stress seems to induce analgesia by recruiting regions that are disrupted by SD. However, stress-induced analgesia differs from acupuncture-induced analgesia in several ways. First, acupuncture decreases stress reaction, (Wang et al., 2014, Hu and Wang, 2021), while stress-induced analgesia depends on it, evidently (Filaretov et al., 1996, Butler and Finn, 2009, Lafrance et al., 2010); second, analgesia induced by acupuncture is long-lasting (Vickers et al., 2018), while that induced by stress is characteristically transient (Butler and Finn, 2009, Buynitsky and Mostofsky, 2009), which is consistent with its biological function; third, acupuncture depends on the activation of C-fibers in the acupoints (Tobaldini et al., 2014, Vickers et al., 2018); while stress-induced analgesia is triggered by a disturbance of the physiological state (Butler and Finn, 2009, Buynitsky and Mostofsky, 2009). The pronociceptive effect of SD is also triggered by a disturbance of the physiological state and the ability of stress in decreasing this pronociceptive effect had never been demonstrated. In this study, we found that immobilization stress completely reversed the

pronociceptive effect, stabilizing the nociceptive threshold at baseline (Fig 4 A). Thus, SD can even disrupt the physiological activity of regions involved in stress-induced analgesia, but this does not prevent their recruitment by stress. Interestingly, the analgesia induced by stress is characteristically transient, as we can see in control animals and in those receiving carrageenan, but the hyperalgesic state induced by REM-SD is not re-established when analgesia ends. This contrasts with carrageenan-induced hyperalgesia that was transiently inhibited by stress, and once analgesia ends, hyperalgesia returns to levels seen in animals not exposed to stress (Fig 4 B). This is to be expected, since the peripheral inflammatory mechanisms recruited by carrageenan remain active for several hours after its injection.

Pain-induced analgesia is another endogenous analgesia modality recruited during life-threatening situations. Painful wounds are common during life-threatening situations and may require more intense and long-lasting analgesia than that induced by stress may be required. There is a form of pain-induced analgesia that results in potent and long-lasting analgesia dependent on the inhibition of the nucleus accumbens, which has been called *ascending nociceptive control* (Gear et al., 1999, Schmidt et al., 2002a, Tobaldini et al., 2019). The injection of capsaicin in a body region far from where analgesia is measured is the most commonly used experimental strategy to trigger this type of pain-induced analgesia (Gear et al., 1999, Schmidt et al., 2002a, Tobaldini et al., 2019). We have previously demonstrated that the mechanisms underlying the ascending nociceptive control are also recruited by acupuncture (Tobaldini et al., 2014) and stress (Tobaldini et al., 2020) and that the resultant analgesia ultimately depends on the PAG-RVM descending system (Tobaldini et al., 2019). Although these data demonstrate that there is considerable overlap in the mechanisms underlying the analgesia induced by acupuncture; stress and noxious stimulation, the remarkable differences in the magnitude and duration of their effects could not be explained if they depended on the exact same mechanism. Therefore, we tested the ability of pain-induced analgesia to decrease the pronociceptive effect of REM-SD. We found that noxious stimulation of the forepaw by capsaicin reversed the hyperalgesic state induced by REM-SD, bringing the nociceptive threshold to baseline (Fig 5 A). In contrast, the nociceptive threshold was persistently increased above the baseline in control animals and in those treated with carrageenan (Fig 5 B). The increase in the nociceptive threshold above baseline in animals treated with carrageenan is a paradoxical result, since it suggests that carrageenan is potentializing the analgesic effect. This is because control and carrageenan-treated animals start from different nociceptive thresholds, which are leveled after analgesia. The long-term duration of pain-induced analgesia is compatible with its biological role, because in a life-threatening environment, persistent

analgesia is adaptive for a wounded animal. This may even help to explain why analgesia is potentiated by carrageenan. However, why noxious stimulation just reverses the pronociceptive effect of SD and not increases the nociceptive threshold above baseline, as it did in non-sleep deprived animals, is an open question.

Taking the data as a whole, acupuncture, stress and noxious stimulation induced analgesia with different in magnitude and duration in sleep deprived and in carrageenan treated animals. Carrageenan-induced hyperalgesia was permanently reversed by acupuncture, which is consistent with a therapeutic effect that counteracts a damage to the nociceptive system. Either stress or noxious stimulation not only reversed carrageenan-induced hyperalgesia but also increased the nociceptive threshold above baseline, which is consistent with their role in fight-or-flight responses, when potent analgesia is required. However, notably stress-induced analgesia is transient; while pain-induced analgesia is longer-lasting, which is also consistent with their role. Stress-induced analgesia anticipates the need for analgesia, if there is no need, the effect fades. On the other hand, pain-induced analgesia is triggered when there is a real need for analgesia, so that the animal is free to engage in defensive behaviors, avoiding recuperative behaviors. Analgesia must be long lasting to fulfill such a role. In remarkable contrast, in sleep-deprived animals, acupuncture, stress or noxious stimulation induced a quite similar effect, reversing the hyperalgesic state induced by REM-SD and bringing the nociceptive threshold back to baseline. It seems that endogenous analgesia reverses the sensitization of the nociceptive system induced by SD, just like turning off a switch. This draws special attention in stress-induced analgesia that despite being short-term, permanently abolishes the hyperalgesic state.

In summary, this study demonstrated that the hyperalgesic state induced by SD is totally suppressed by the activation of endogenous pain modulation mechanisms triggered by acupuncture, stress or noxious stimulation. Therefore, although SD disrupts endogenous pain modulation mechanisms (Smith et al., 2007, Edwards et al., 2009, Tiede et al., 2010, Paul-Savoie et al., 2012, Bulls et al., 2015, Tomim et al., 2016, Eichhorn et al., 2018, Simpson et al., 2018, Xue et al., 2018, Carvalho et al., 2019, Bjurstrom et al., 2021), this does not prevent the recruitment of such mechanisms to induce analgesia in sleep-deprived subjects. In contrast to the homogeneous analgesic effect induced by acupuncture, stress or noxious stimulation in sleep-deprived animals, each intervention affected carrageenan-induced hyperalgesia differently.

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CAPÍTULO 2

A RESTRIÇÃO CRÔNICA DE SONO AUMENTA A SENSIBILIDADE À DOR AO LONGO DO TEMPO DE MANEIRA DEPENDENTE DA SUBSTANCIA CINZENTA PERIAQUEDUTAL E DO NUCLEUS ACCUMBENS

Objetivo geral

Caracterizar o efeito pró-nociceptivo da restrição crônica de sono e verificar o envolvimento da substancia cinzenta periaquedutal (PAG) e do núcleo accumbens (NAc) no aumento da sensibilidade à dor induzida por restrição crônica de sono.

Objetivos específicos

- Investigar se o efeito pró-nociceptivo da restrição do sono aumenta com o tempo, e verificar quando atinge a intensidade máxima.
- Investigar se dois dias de sono normal (imitando o sono livre nos finais de semana) são suficientes para normalizar a sensibilidade à dor aumentada.
- Testar se o efeito pró-nociceptivo da restrição crônica do sono também depende da PAG e do NAc.
- Verificar se a atividade neuronal nessas regiões aumenta com a restrição crônica de sono.

Apresentação:

Artigo Científico 2: **Chronic sleep restriction increases pain sensitivity over time in a periaqueductal gray and nucleus accumbens dependent manner.**

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Chronic sleep restriction increases pain sensitivity over time in a periaqueductal gray and nucleus accumbens dependent manner

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ABSTRACT

Painful conditions and sleep disturbances are major public health problems worldwide and one directly affects the other. Sleep loss increases pain prevalence and severity; while pain disturbs sleep. However, the underlying mechanisms are largely unknown. Here we asked whether chronic sleep restriction for 6 h daily progressively increases pain sensitivity and if this increase is reversed after two days of free sleep. Also, whether the pronociceptive effect of chronic sleep restriction depends on the periaqueductal gray and on the nucleus accumbens, two key regions involved in the modulation of pain and sleep-wake cycle. We showed that sleep restriction induces a pronociceptive effect characterized by a significant decrease in the mechanical paw withdrawal threshold in rats. Such effect increases progressively from day 3 to day 12 remaining stable thereafter until day 26. Two consecutive days of free sleep were not enough to reverse the effect, not even to attenuate it. This pronociceptive effect depends on the periaqueductal gray and on the nucleus accumbens, since it was prevented by their excitotoxic lesion. Complementarily, chronic sleep restriction significantly increased c-Fos protein expression within the periaqueductal gray and the nucleus accumbens and this correlates with the intensity of the pronociceptive effect, suggesting that the greater the neural activity in this regions, the greater the effect. These findings may contribute not only to understand why painful conditions are more prevalent and severe among people who sleep poorly, but also to develop therapeutic strategies to prevent this, increasing the effectiveness of pain management in this population.

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1. Introduction

Pain conditions and sleep disorders are major public health problems worldwide (Appleton et al., 2018; Murphy et al., 2017) and there is a clear bidirectional relationship between them. There is no doubt that pain impairs sleep (Artner et al., 2013; Karaman et al., 2014) and different types of sleep impairment increase pain sensitivity (Okifuji and Hare, 2011). However, the underlying mechanisms are largely unknown.

The reason why sleep disorders are great predictors of pain development (Mørk and Nilsen, 2012; Okifuji and Hare, 2011) may rely on the ability of sleep loss to disrupt endogenous pain modulation, as suggested by clinical findings (Paul-Savoie et al., 2012;

Tiede et al., 2010). In fact, several brain regions that play a key role in pain modulation, such as the ventrolateral periaqueductal gray (vlPAG) (Fields, 2004), and the Nucleus Accumbens (NAC), (Gear et al., 1999), also contribute to control sleep-wake cycle (Lu et al., 2006; Oishi et al., 2017; Weber et al., 2018). Since most sleep disorders are characterized by impairment mainly in rapid eye movement (REM) sleep (Brown et al., 2012; Naiman, 2017), we have performed REM sleep deprivation (REM-SD) in rats to provide a mechanistic basis for these clinical observations. According to our previous data, REM-SD increases nociceptive responses by disrupting the PAG-RVM (periaqueductal gray – rostral ventral medulla) descending pain modulation system (Tomim et al., 2016) as well as by increasing NAC adenosinergic activity and by decreasing NAC dopaminergic activity (Sardi et al., 2018).

However, insomnia (Calhoun et al., 2014) and restriction of sleep time due to occupational or recreational reasons have become increasingly frequent in the modern society (Owens et al., 2014). In

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order to mimic this decrease in sleep duration in laboratory animals, some types of gentle stimulation have been used to keep them awake for long periods of time. Like selective REM-SD (Damasceno et al., 2009; Nascimento et al., 2007; Sardi et al., 2018; Tomim et al., 2016; Wei et al., 2013), sleep restriction (SR) has been associated with increased pain sensitivity in both humans (Okifuji and Hare, 2011; Tiede et al., 2010) and animals (Alexandre et al., 2017). However, the underlying mechanisms are largely unknown and many unanswered questions remain. For example: Does the pronociceptive effect of sleep restriction increase over time? If yes, when does it reach the maximum intensity? Are two days of normal sleep (mimicking free sleep on weekends) enough to normalize pain sensitivity? Is the pronociceptive effect of chronic sleep restriction also dependent on the PAG and on the NAc? If yes, the neuronal activity within these regions increases with chronic sleep restriction? This study aimed to answer these questions.

2. Materials and methods

2.1. Animals

The experiments were performed in male Wistar rats (270–300 g). The animals were housed five per cage in a room with controlled 12:12-h light/dark cycle and temperature ($23 \pm 2^\circ\text{C}$), with free access to food and water. All animal experimental procedures and protocols were approved by the Committee on Animal Research of the Federal University of Paraná, Brazil, and followed the guidelines of the Ethics Standards of the International Association for the Study of Pain in animals (Zimmermann, 1983).

2.2. Stereotaxic surgery and NMDA lesion

In this study we have focused in the ventrolateral column of the PAG (vlPAG) and in the core region of the NAc for two reasons: first, both vlPAG (Fields, 2004; Reynolds, 1969) and NAc core (Gear et al., 1999; Gear and Levine, 2011; Tobaldini et al., 2014) have key roles in pain modulation. vlPAG for example, controls the descending pain modulation system in an opioid dependent manner (Fields, 2004). Second, previous work from our lab have implicated either vlPAG or NAc core (as well as NAc shell) in the pronociceptive effect of REM-sleep deprivation (Sardi et al., 2018; Tomim et al., 2016).

Stereotaxic surgery was performed 20 days before experiments under general anesthesia (ketamine, 60 mg/kg and xylazine, 10 mg/kg, i.p.). NMDA (N-Methyl-D-aspartic acid; from Sigma, St Louis, MO, USA) or its vehicle was microinjected during the surgery procedure under the following coordinates: NAc - from bregma: anteroposterior +1.3 mm; lateral ± 1.8 mm; and dorsoventral -7.2 mm; PAG - from lambda: anteroposterior 0 mm; lateral -2 mm; dorsoventral -5.4 mm in a 18° angle (Paxinos and Watson, 2007).

Selective bilateral lesions were performed within the NAc core and unilateral lesions within the ventrolateral PAG by injecting NMDA dissolved in NaCl 0.9% (pH 7.2–7.4) (Jongen-Reijo et al., 2002) at a dose of 5.5 μg . The rats assigned to the sham-lesion group were infused with NaCl 0.9%. Microinjections were performed through injectors (stainless steel needle, 30 gauge, Misawa Medical Industry, Japan) connected with a polyethylene tubing (PE-20) in a 5 μl Hamilton syringe coupled to an injection pump (model KDS-100 kdsScientific Holliston, MA, USA). Total microinjection volume was 0.3 μl infused within 2 min. Injectors were left on site for an additional period of 1 min to minimize backflow along the cannulae tract.

After surgery, the rats received dipyrone (30 mg/kg) and gentamicin (0.5 mg/kg) and were observed for 2 h after they regain consciousness. Experiments were carried out 20 days later (Jongen-

Reijo et al., 2002). Lesion location and extension were histologically assessed.

2.3. Chronic sleep restriction (CSR)

The animals were deprived of total sleep during six hours per day by the gentle handling method, which reduces approximately 98% of slow-wave sleep and abolishes REM sleep in rodents (Alexandre et al., 2017). This method is characterized by keeping the animals awake by inserting novel objects in their home cage (Alexandre et al., 2017), rotating and tapping on the cage (O'Hara et al., 1993) and, if necessary, by brushing their back with a soft bristle brush. This procedure was performed 5 days per week (from Monday to Friday); 6 h per day starting at the first light of day (7:00 a.m. to 1:00 p.m.), during 12 or 26 days depending on the group, with free access to food and water. After daily CSR, the animals were free for sleeping. The control groups were housed in their cages under the same conditions and in the same room, but have not had their sleep disturbed.

2.4. Mechanical paw-withdrawal test

The mechanical paw-withdrawal test (Randall and Selitto, 1957) was performed and the nociceptive threshold was used as a measure of pain sensitivity (Sardi et al., 2018; Tomim et al., 2016). The test was performed in a blinded fashion, always before the start of the CSR procedure (07:00). In this test, an increasing pressure is applied to the dorsal surface of the rat's hind paw, the nociceptive mechanical threshold is defined as the force (mean of three readings) in grams at which the rat withdrew its paw. The test was performed before experiments (basal) and repeated at different time points thereafter (every Monday, Wednesday and Friday along the experiment).

2.5. Qualitative assessment of home cage activity

Decreased activity measured by actimetry has been used as an indirect behavioral measure of sleep in both rodents (Tang and Sanford, 2002) and humans (Townhill et al., 2016). Therefore, home cage activity was monitored in order to indirectly assess the effect of CSR and NAc or PAG lesion in the sleep-wake cycle. Since social isolation affects behavioral nociceptive measures (Meng et al., 2010), the animals were maintained in standard environmental conditions (community, 4 animals per cage). This avoids an additional variable to the pain tests, but makes statistical tests unfeasible, since each experimental group of 8 animals originates data from two home cages. Therefore these data were qualitatively presented.

Passive infrared motion captors placed over the cages were connected to a computerized data acquisition system (National Instruments, Austin, TX, USA). Home cage activity was continuously monitored throughout the experiment (12 or 26 days), except when animals were being either sleep deprived or submitted to the control condition (control groups were maintained in the same room where sleep deprivation was being conducted, but rats were free to sleep). Activity records were analyzed with the LabVIEW software package.

2.6. Histological sample preparation

The rats were anesthetized (ketamine, 60 mg/kg and xylazine, 10 mg/kg, i.p.) and transcardially perfused with saline 0.9%, pH 7.4, followed by 4% paraformaldehyde in 0.1-Mphosphate buffer, pH 7.4. Brains were removed and immersed in paraformaldehyde at 4°C for a week, in 30% sucrose solution for another week and stored

at -80°C until sectioning. Eight sections ($30\ \mu\text{m}$) were sliced per animal, between bregma $+1.44$ and $+1.20$ mm for the NAc and -8.04 and -8.28 for the PAG.

2.7. Cresyl violet staining

Cresyl Violet, a cationic dye that stains Nissl corpuscles present in the cell body and dendrites of neurons (Ovalle, 2013) was used in order to determine the extension of NMDA lesions. The sections were mounted on gelatin-coated slides, passed through a series of ethanol solutions of descending concentration (100%, 95%, and 70% ethanol in water, 3 min each) and stained for approximately 1 min with cresyl violet (0.05% aqueous cresyl violet, 2 mM acetic acid, and 5 mM formic acid in water). After staining, sections were rinsed in water and 70% ethanol; differentiated in 95% ethanol with acetic acid; dehydrated in ascending concentrations of ethanol-xylene and cover slipped.

2.8. c-Fos immunohistochemistry

c-Fos protein is rapidly and transiently expressed in stimulated neurons in response to elevation of intracellular calcium (Coderre et al., 1993; Leroy et al., 1992). Therefore, we have quantified c-Fos expression in an attempt to indirectly estimate the effect of CSR on neuronal activation within the NAc and PAG. Free-floating sections were rinsed in 0.1 M phosphate-buffered saline (PBS) and treated with 0.5% H₂O₂ in 0.1 M PBS for 30 min to suppress endogenous peroxidase activity. Tissue sections were incubated overnight at 4°C with rabbit anti-c-Fos primary antibody (#AB038; Chemicon, Temecula, CA; 1:500 in phosphate-buffered saline plus 0.3% Triton X-100) and then incubated with a biotin-conjugated secondary antibody (#PK4001; Vector Laboratories, Burlingame, CA; 1:200) for 2 h at room temperature. After several washes with phosphate-buffered saline, the antibody complex was localized using the ABC system (#PK4001; Vectastain ABC Elite kit, Vector Laboratories, Burlingame, CA) followed by reaction with 3,3'-diaminobenzidine with nickel enhancement. The sections were then mounted on gelatin-coated slides and cover slipped after dehydration in ascending concentrations of ethanol-xylene solutions.

2.9. Quantification of excitotoxic lesion and c-Fos immunoreactive cells

The slices were digitized with a microscope scanner (Axio Imager Z2, Carl Zeiss, Jena, DE) coupled to an imaging system (Metasystems, Altusheim, DE). Quantification of the NAc and PAG lesions and c-Fos immunoreactive (c-Fos-ir) cells was performed automatically by optical density using ImageJ 1.37c (Schneider et al., 2012) image analysis software.

2.10. Statistical analysis

Data from nociceptive tests were analyzed by repeated-measures (time) analysis of variance (ANOVA) with sleep condition (CSR or control procedure) as between-subject factor for naïve animals and sleep condition and treatment (NMDA lesion or sham-lesion) as between-subject factors for all other groups. Data from histological analysis of the excitotoxic lesion with the NMDA were analyzed by two-way ANOVA with sleep condition and treatment (NMDA lesion or sham-lesion) as factors. Data from c-Fos immunoreactivity were analyzed by one-way ANOVA. The correlation between c-Fos immunoreactivity and nociceptive threshold was determined by the Pearson's Correlation test. All post hoc contrasts, when appropriate, were performed using Tukey's test ($p < 0.05$). The software SigmaPlot® (Systat Software, San Jose, CA, USA) was

used to perform data analysis and graphical representation. Data are plotted in figures as mean $\pm$ SEM. The number of animals in each group ranges from 6 to 8, except data from c-Fos expression which were obtained with 4–5 animals per group.

3. Results

3.1. The pronociceptive effect of chronic sleep restriction and its temporal evolution

Chronic sleep restriction (CSR) for six hours daily progressively increased nociceptive response, as demonstrated by the decrease in mechanical nociceptive paw-withdrawal threshold (Fig. 1, repeated-measures (time) ANOVA – sleep condition (CSR or control procedure): $F(1,14) = 248.15$, $p < 0.001$; sleep condition $\times$ time: $F(11,154) = 16.208$, $p < 0.001$). Post hoc analysis using Tukey's test indicated that CSR decreased mechanical nociceptive threshold during the overall experiment, $p < 0.003$. Only two previous days of sleep restriction were sufficient to significantly increase the nociceptive response, as demonstrated by the decrease in mechanical nociceptive paw-withdrawal threshold in the third experimental day (within subject comparison in the CSR group, $p = 0.001$). The nociceptive response kept increasing progressively until the twelfth day, when it reached its maximum level, not changing significantly thereafter (within subject comparison in the CSR group, $p = 0.999$ for days 12 vs. 26). Important, along this chronic experiment, the animals were three times allowed to sleep freely for two consecutive days (indicated in figures as FS). Each two-day-periods of free sleep was neither enough to normalize pain sensitivity, nor to even significantly change the nociceptive response (within subject comparison in the CSR group, $p = 0.460$; $p = 0.999$; $p = 0.705$, respectively).

Qualitative assessment of home cage activity performed throughout the 26 days of the experiment suggests that general motor activity was decreased in chronically sleep restricted animals during the dark phase, which is compatible with an increase in

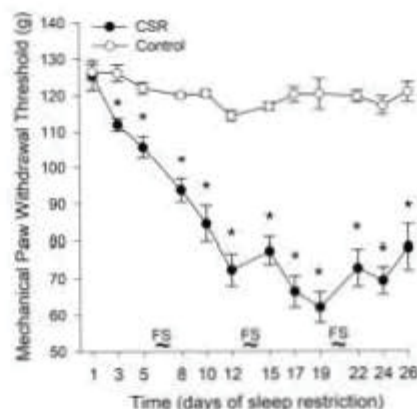


Fig. 1. The pronociceptive effect of chronic sleep restriction. The mechanical nociceptive threshold decreased progressively from day 3 to day 12 when it stabilized and no longer significantly changed until day 26. The symbol *** indicates a mechanical nociceptive threshold significantly lower than that of the control group (repeated-measures ANOVA followed by the Tukey's post hoc test, $p < 0.05$). In this and in subsequent figures data are presented as mean $\pm$ SEM. The number of animals in each group ranges from 4 to 8 per group. See methods for additional details regarding data presentation and analysis. CSR = chronic sleep restriction; FS = free sleep, it indicates the two-day-periods of free sleep.

sleep time (Supplementary Figure 1A).

3.2. The role of the nucleus accumbens in the pronociceptive effect of chronic sleep restriction

Excitotoxic NAc lesion induced by NMDA prevented the pronociceptive effect of CSR (Fig. 2A, repeated-measures (time) ANOVA – sleep condition (CSR or control procedure): $F(1,19) = 1195.7$, $p < 0.0001$; treatment (NMDA or sham-lesion): $F(1,19) = 957.71$, $p < 0.0001$; sleep condition $\times$ treatment: $F(1,19) = 1257.1$, $p < 0.0001$; sleep condition $\times$ treatment $\times$ time: $F(5,95) = 68.872$, $p < 0.0001$; Tukey test $p < 0.001$) and significantly decreased the number of the NAc neurons (Fig. 2B, two-way ANOVA – sleep condition (SD or control procedure): $F(1,14) = 1.805$, $p = 0.20$; treatment (NMDA or sham-lesion): $F(1,14) = 27.878$, $p < 0.001$; sleep condition $\times$ treatment: $F(1,14) = 1.029$, $p = 0.32$; Tukey test $p < 0.01$). Representative images of lesioned tissue clearly indicate a decreased number of neurons within the NAc (Fig. 2C). Schematic anatomical reconstruction of the lesions shows their location and extension (Fig. 2D).

3.3. The role of the periaqueductal gray in the pronociceptive effect of chronic sleep restriction

Excitotoxic PAG lesion induced by NMDA prevented the

pronociceptive effect of CSR (Fig. 3A, two-way repeated measures ANOVA – sleep condition (SD or control procedure): $F(1,19) = 445.12$, $p < 0.0001$; treatment (NMDA or sham-lesion): $F(1,19) = 361.56$, $p < 0.0001$; sleep condition $\times$ treatment: $F(1,19) = 392.52$, $p < 0.0001$; sleep condition $\times$ treatment $\times$ time: $F(5,95) = 103.00$, $p < 0.0001$; and Tukey test $p < 0.001$) and significantly decreased the number of the PAG neurons (Fig. 3B, two-way ANOVA – sleep condition (SD or control procedure): $F(1,14) = 0.0311$, $p = 0.86$; treatment (NMDA or sham-lesion): $F(1,14) = 15.312$, $p = 0.002$; sleep condition $\times$ treatment: $F(1,14) = 0.546$, $p = 0.47$; Tukey test $p < 0.04$). Representative images of lesioned tissue clearly indicate a decreased number of neurons within the PAG (Fig. 3C). Schematic anatomical reconstruction of the lesions shows their location and extension (Fig. 3D).

3.4. Chronic sleep restriction increases c-Fos expression in the nucleus accumbens and in the periaqueductal gray

Chronic sleep restriction for 12 days ($p < 0.001$), significantly increased c-Fos expression within the NAc. Excitotoxic PAG lesion did not change c-Fos expression within the NAc either in control or in sleep restricted animals (Fig. 4A, one-way ANOVA – between groups: $F(3, 14) = 21.823$, $p < 0.001$). Representative images clearly indicate increased c-Fos expression within the NAc with 12 days of CSR (Fig. 4B). There is a strong negative correlation between

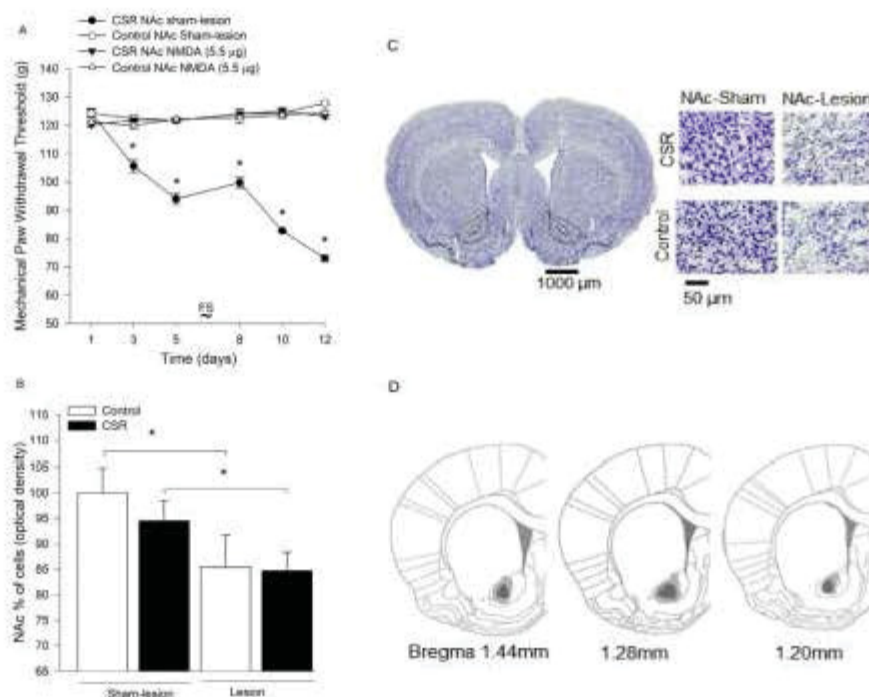


Fig. 2. The role of the nucleus accumbens in the pronociceptive effect of chronic sleep restriction. **A**—The excitotoxic lesion of the NAc with NMDA (5.5 µg) prevented the pronociceptive effect of CSR. The symbol *** indicates a mechanical nociceptive threshold significantly lower than that of the other groups [repeated-measures ANOVA followed by the Tukey's post hoc test, $p < 0.05$]. **B**—The administration of NMDA into the NAc significantly decreased the number of local neurons, characterizing the excitotoxic lesion. The symbol *** indicates a significantly lower number of neurons (two-way ANOVA followed by the Tukey's post hoc test, $p < 0.05$). **C**—Representative images of the NAc lesion. **D**—Anatomical reconstruction of the NAc lesions based on the atlas of Paxinos and Watson (2007).

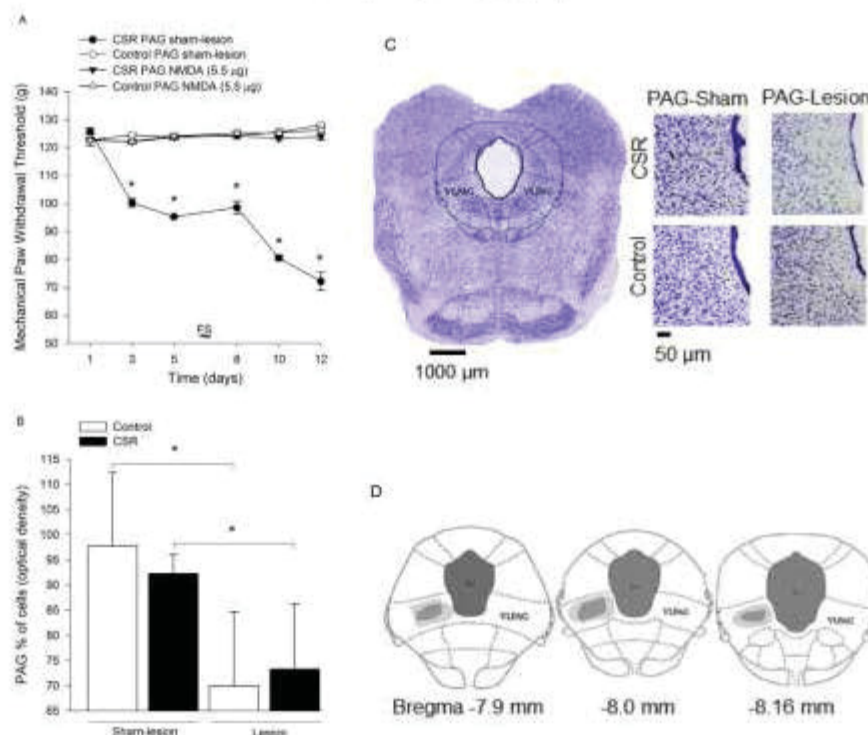


Fig. 3. The role of the periaqueductal grey in the pronociceptive effect of chronic sleep restriction. **A**– The excitotoxic lesion of the PAG with NMDA (5.5 µg) prevented the pronociceptive effect of CSR. The symbol **** indicates a mechanical nociceptive threshold significantly lower than that of the other groups (repeated-measures ANOVA followed by the Tukey's post hoc test, $p < 0.05$). **B**– The administration of NMDA into the PAG significantly decreased the number of local neurons, characterizing the excitotoxic lesion. The symbol *** indicates a significantly lower number of neurons (two-way ANOVA followed by the Tukey's post hoc test, $p < 0.05$). **C**– Representative images of the PAG lesion. **D**– Anatomical reconstruction of the PAG lesions based on the atlas of Paxinos and Watson (2007).

nociceptive threshold and c-Fos expression within the NAc in animals with no lesion (Fig. 4C $r = -0.915$; $p = 0.00143$). When the PAG is lesioned, this correlation is no longer observed (Fig. 4D $r = -0.548$; $p = 0.101$).

Chronic sleep restriction for 12 days ($p < 0.001$) significantly increased c-Fos expression within the PAG. Excitotoxic NAc lesion did not change c-Fos expression within the PAG in sleep restricted animals ($p = 0.996$), but significantly increased it in control ones ($p = 0.002$) (Fig. 5A, one-way ANOVA – between groups: $F(3, 15) = 28.569$, $p < 0.001$). Representative images clearly indicate increased c-Fos expression within the PAG with 12 days of CSR (Fig. 5B). There is a strong negative correlation between nociceptive threshold and c-Fos expression within the PAG in animals with no lesion (Fig. 5C $r = -0.910$; $p = 0.00168$). When the PAG is lesioned, this correlation is no longer observed (Fig. 5D $r = -0.100$; $p = 0.769$).

Qualitative assessment of home cage activity performed throughout the 12 days of the experiment suggests that neither NAc nor PAG lesion affect general motor activity in chronically sleep restricted animals (Supplementary Figure 1B) or control animals (Supplementary Figure 1C).

4. Discussion

This study demonstrated that sleep restriction for 6 h daily

induces a pronociceptive effect that increases progressively from day 3 to day 12 remaining stable thereafter. Repeated two-day-periods of free sleep were neither enough to normalize pain sensitivity, nor to even attenuate the increased nociceptive response. The pronociceptive effect of CSR depends on both the NAc and the PAG, since it was prevented by the excitotoxic lesion of any one of them. Complementarily, CSR significantly increased c-Fos protein expression within the NAc and the PAG and this correlates with the intensity of the pronociceptive effect, suggesting that the greater the neural activity in these regions, the greater the pronociceptive effect.

These findings largely extended a limited literature about the ability of sleep loss to increase pain sensitivity and the underlying mechanisms. The majority of the studies on this field have used selective REM sleep deprivation in animals (Damasceno et al., 2009; Nascimento et al., 2007; Sardi et al., 2018; Tomim et al., 2016; Wei et al., 2013). However, nowadays people worldwide sleep less than they would like or need due to occupational/recreational reasons or insomnia disorders (Calhoun et al., 2014; Owens et al., 2014). Understanding to what extent and how sleep restriction impacts pain sensitivity would certainly contribute to increase the effectiveness of pain management in this population. In order to advance our understanding of this issue, we have used a four week protocol in which the animals were prevented from

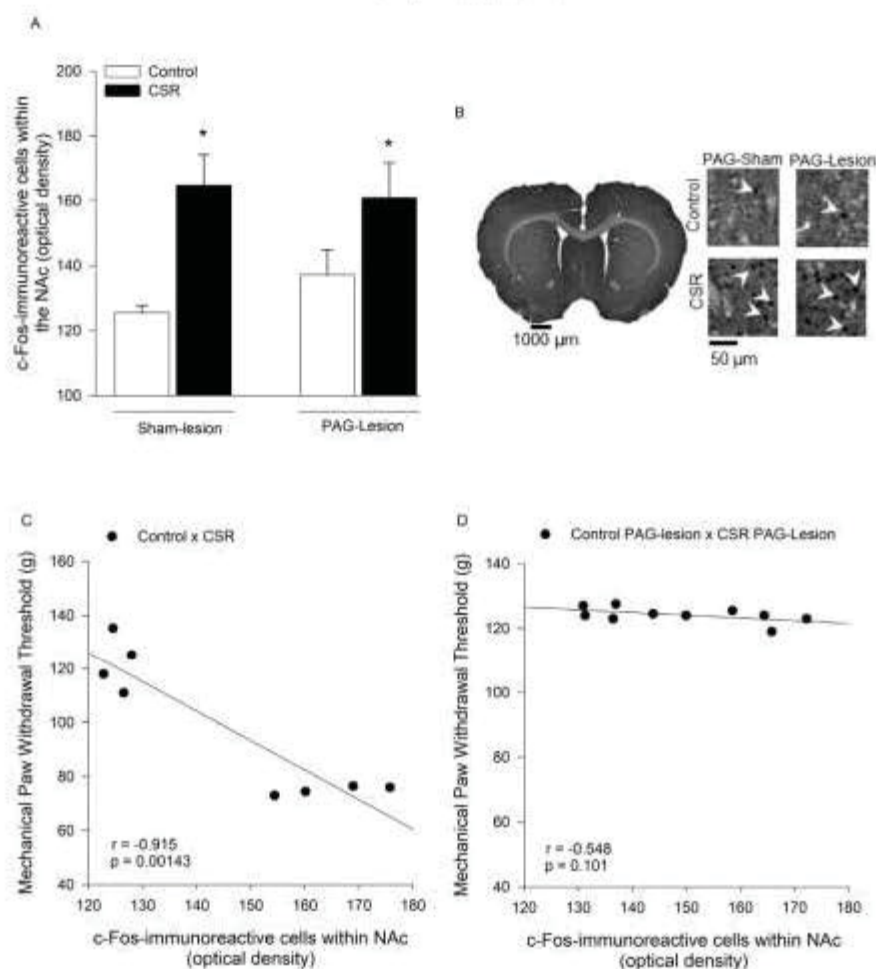


Fig. 4. The effect of chronic sleep restriction on c-Fos protein expression within the nucleus Accumbens. **A**–CSR significantly increased c-Fos expression within the NAc and this is not affected by PAG lesion. The symbol *** indicates a significantly higher number of c-Fos-immunoreactive cells within the NAc (two-way ANOVA followed by the Tukey's post hoc test, $p < 0.05$). **B**–Representative images of c-Fos immunoreactive cells (indicated by arrows) within the NAc. **C**–There is a strong negative correlation between mechanical paw withdrawal threshold and c-Fos-immunoreactive cells within the NAc of non-lesioned animals (Pearson's Correlation test). **D**–In animals with PAG lesion this correlation is lost.

sleeping during the first six hours of the light phase, when sleep pressure is highest in rodents. Although similar procedures for sleep restriction in both animals (Alexandre et al., 2017) and humans (Tiede et al., 2010) have already been associated with increased pain sensitivity, this study extends these previous ones, in part, by showing the temporal evolution of the pronociceptive effect of CSR and its unchanged persistence after two days of free sleep (Fig. 1). Our data showed a progressive increase in nociceptive responses, demonstrated by the decrease in mechanical nociceptive threshold, until day 12, after which the nociceptive response stabilized and significant changes were no longer observed until day 26, when the experiment ended. Important, along this four-week chronic experiment, three two-day-periods of free sleep

were designed in order to mimic free sleep during weekends. Their complete inability to restore normality in pain sensitivity suggests that extended periods of sleep recovery are demanded to reverse the effects of CSR. From a translational perspective, this suggests that the impact of sleep loss on the prevalence and intensity of pain conditions is even greater than could be supposed, since free sleep on weekends may not be enough to reverse the changes in pain processing imposed during the working week.

The mechanisms by which sleep loss increases pain sensitivity (Alexandre et al., 2017; Nascimento et al., 2007; Okifuji and Hare, 2011; Sardi et al., 2018; Schuh-Hofer et al., 2013; Tinnum et al., 2016) and the prevalence of pain conditions (Mork and Nilsen, 2012; Paul-Savoie et al., 2012; Tiede et al., 2010) are largely

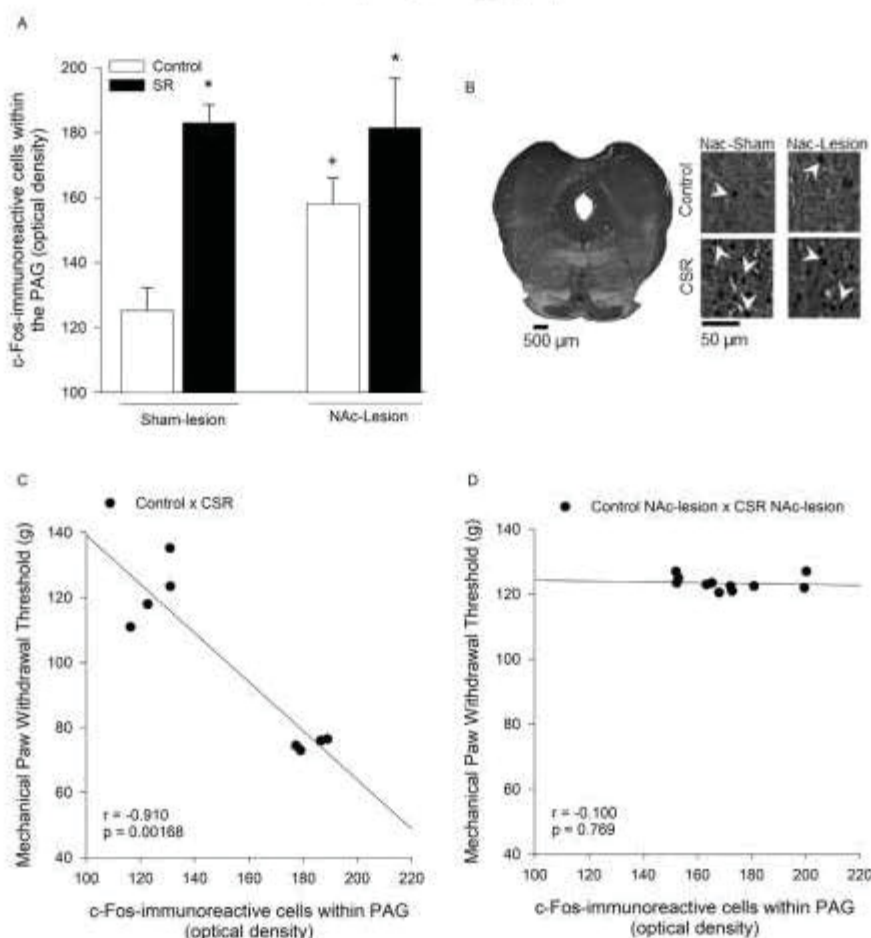


Fig. 5. The effect of chronic sleep restriction on c-Fos protein expression within the periaqueductal grey. **A**—c-Fos expression within the PAG was significantly increased by CSR or NAc lesion. The significantly higher number of c-Fos-immunoreactive cells within the PAG is indicated by the symbol *** comparison with all other groups and * comparison with control animals (two-way ANOVA followed by the Tukey's post hoc test, $p < 0.05$). **B**—Representative images of c-Fos immunoreactive cells (indicated by arrows) within the PAG. **C**—There is a strong negative correlation between mechanical paw withdrawal threshold and c-Fos-immunoreactive cells within the PAG of non-lesioned animals (Pearson's Correlation test). **D**—In animals with NAc lesion this correlation is lost.

unknown. This study also advances our understanding in this area by showing that the potent pronociceptive effect of CSR depends on both the NAc and the PAG. To our knowledge no previous studies have investigated the central mechanisms underlying the pronociceptive effect of CSR, being this the first evidence of specific brain nuclei mediating such effect. The experimental support to this evidence was provided by data showing that the excitotoxic lesion of either the NAc (Fig. 2) or of the PAG (Fig. 3) prevented the pronociceptive effect induced by CSR. The indirect estimation of neural activity by c-Fos expression within the NAc and the PAG further support their role in such effect. The increased c-Fos expression in both the NAc (Fig. 4A and B, two bars/images at left) and the PAG (Fig. 5A and B, two bars/images at left) of chronic sleep restricted animals is consistent with their increased activity in response to

CSR. In fact, findings from nociceptive activity and c-Fos expression correlate well in non-lesioned animals. The higher the nociceptive response (decrease in mechanical nociceptive threshold), the greater the expression of c-Fos either in the NAc (Fig. 4C) or in the PAG (Fig. 5C). As expected, the high significant correlation between nociceptive response and c-Fos expression is lost in animals with lesion either in the PAG (Fig. 4D) or in the NAc (Fig. 5D). This is because although the excitotoxic lesion of the PAG prevented the pronociceptive effect of CSR, it did not change the increased c-Fos expression within the NAc of chronic sleep restricted animals (Fig. 4A and B, two bars/images at right). Similarly, the excitotoxic lesion of the NAc prevented the pronociceptive effect of CSR without affecting the increased c-Fos expression within the PAG of chronic sleep restricted animals (Fig. 5A and B, two bars/images at

right). One possible explanation for the extinction of the effect and the maintenance of c-Fos expression is that the pronociceptive effect depends on the integrity of the underlying neural circuitry, thus the lesion of one nucleus in the circuitry is able to prevent the overall effect without necessarily affecting CSR-induced changes in neural activity of other nuclei. Worthy of note is also the fact that the NAc lesion significantly increased c-Fos expression in the PAG of control (not sleep restricted) animals (Fig. 5A, compare the white bars). This may result from decreased inhibitory activity from the NAc to the PAG, since efferent activity from the NAc is predominantly GABAergic (Mogenson et al., 1983) and some of them project directly to the PAG (Zhang et al., 2013).

The NAc, in the ventral striatum, is a key component of the mesolimbic dopaminergic system, with recognized role in the modulation of both pain (Gear and Levine, 2011; Tobaldini et al., 2014) and sleep wake cycle (Oishi and Lazarus, 2017). Sleep pressure is believed to increase the NAc efferent activity in order to inhibit wake-promoting nuclei in the brainstem and hypothalamus (Lazarus et al., 2013). We have recently demonstrated that increased NAc activity mediates the pronociceptive effect of acute selective REM sleep deprivation (Sardi et al., 2018). The present study extends this previous one by showing that the pronociceptive effect of CSR, which develops progressively over 12 days, is also dependent on the NAc, where c-Fos expression correlates with the intensity of such effect. Acute selective REM sleep deprivation is a worldwide used method to mimic the effects of several sleep disturbances that affect primarily REM sleep (Brown et al., 2012; Naiman, 2017). On the other hand, restriction of total sleep by some hours per day is supposed to mimic the decrease in sleep time due to occupational or recreational reasons (Iacovides et al., 2017). Together, the present study and that previous one suggest that sleep loss, no matter the model used, increases nociceptive responses by increasing the NAc activity, which is in accordance with the suggested pronociceptive role of this nucleus (Gear and Levine, 2011). Important, sleep loss is known to increase the prevalence of chronic pain conditions (Mork and Nilsen, 2012; Okifuji and Hare, 2011) and recent evidences from both human (Baliki et al., 2012) and animal (Dias et al., 2015; Miranda et al., 2015) studies support a key role of the NAc in pain chronification. Therefore, the NAc may be a major player of pain chronification in patients who sleep poorly.

The PAG is the center of the most known mechanism for pain modulation, the PAG-RVM descending system. In this system, inputs from multiple forebrain regions are integrated within the vPAG, which projects to the rostral ventromedial medulla (RVM), from where descending pathways target the dorsal horn to facilitate or inhibit nociceptive transmission (Millan, 2002). In addition to its key role in pain modulation, the PAG also contributes to control sleep-wake cycle (Lu et al., 2006). PAG dopaminergic neurons induce wakefulness (Lu et al., 2006), while its GABAergic neurons suppresses REM sleep and consolidate NREM sleep (Weber et al., 2018). We have previously demonstrated that acute selective REM sleep deprivation increases pain by increasing the pain facilitatory activity of the PAG-RVM descending system (Tonum et al., 2016). The present study shows that the pronociceptive effect of CSR also depends on the PAG and that local c-Fos expression correlates with the intensity of such effect. Therefore, sleep loss in response to either CSR or acute REM sleep deprivation appears to increase pain by disrupting the way by which the PAG controls pain processing. The key role of the PAG in pain processing has been consistently demonstrated by several studies showing that the intensity of acute pain (Bee and Dickenson, 2008; Burgess et al., 2002) and the development of chronic pain (Granovsky, 2013; Martel et al., 2013) are strongly influenced by changes in the PAG activity. Therefore, an increase in pain facilitatory activity from the

PAG may lead to the increased pain intensity and vulnerability associated to sleep loss.

One limitation of this study is that we could not determine whether and how CSR and the NAc or PAG lesion affects sleep-wake cycle. A qualitative measurement of home cage activity by actimetry performed throughout the experiment suggested that CSR increases sleep time during the dark phase (Supplementary Figure S1A), while the lesion of neither NAc nor PAG seems to have affected home cage motor activity either in chronically sleep restricted (Supplementary Figure S1B) or control (Supplementary Figure S1C) animals. Another limitation of this study is that we have focused in the core region of the NAc and in the ventrolateral column of the PAG, so that we could not determine if the role of NAc and PAG in the pronociceptive effect of CSR is limited to these specific regions. We believe that it is not for the NAc, since our previous study demonstrated that both the core and shell regions of the NAc contribute to the pronociceptive effect of REM-sleep deprivation (Sardi et al., 2018). However, we believe that the contribution of PAG is limited to its ventrolateral column, because this and not the adjacent columns of the PAG controls the descending pain modulation system (Basbaum and Fields, 1979; Behbehani, 1995), which is implicated in the pronociceptive effect of REM-sleep deprivation (Tomini et al., 2016). Future studies would contribute to overcome the limitations of the present study definitively supporting or refuting our suggestions.

In summary, this study showed that the daily restriction of sleep time progressively increases pain sensitivity, which is not reversed by two consecutive days of free sleep. The pronociceptive effect of CSR depends on the NAc and the PAG, where the neural activity, indirectly estimated by c-fos expression, correlates with the intensity of such effect. Both the NAc and the PAG have key roles in pain chronification and in the modulation of pain sensitivity. Since it is known that chronic pain conditions are more prevalent (Appleton et al., 2018; Karaman et al., 2014; Mork and Nilsen, 2012; Murphy et al., 2017; Okifuji and Hare, 2011) and pain sensitivity is greater (Okifuji and Hare, 2011; Paul-Saviole et al., 2012) among people who sleep poorly, these findings may contribute to understanding why this happens and how it can be prevented.

Conflicts of interest

The authors have no conflicts of interest to declare.

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Appendix A. Supplementary data

Supplementary data related to this article can be found at <https://doi.org/10.1016/j.neuropharm.2018.06.022>.

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CAPÍTULO 3

MECANISMOS DOPAMINÉRGICOS NO CÓRTEX CINGULADO ANTERIOR E NO NÚCLEO ACCUMBENS ENVOLVIDOS NO EFEITO PRÓ-NOCICEPTIVO DA RESTRIÇÃO DE SONO

Objetivo geral

Investigar o envolvimento do córtex cingulado anterior (ACC) no efeito pró-nociceptivo da restrição de sono e os mecanismos dopaminérgicos no ACC e no núcleo accumbens (NAc) envolvidos no efeito pró-nociceptivo da restrição de sono.

Objetivos específicos

- Investigar se seis horas por dia de privação total de sono por três dias afetaria a sensibilidade à dor de maneira diferente em ratos machos e fêmeas.
- Verificar se a integridade do ACC é essencial para o efeito pró-nociceptivo da restrição do sono.
- Investigar se a ativação farmacológica dos receptores de dopamina D₁ ou D₂ no NAc e no ACC previne o efeito pró-nociceptivo da restrição de sono.
- Estimar a atividade dopaminérgica no ACC e no NAc após a restrição de sono, após rebote de sono e em animais controle.
- Estimar indiretamente o impacto da restrição de sono na resposta ao estresse.

Apresentação:

Artigo científico 3: **Dopaminergic mechanisms in the anterior cingulate cortex and nucleus accumbens involved in the pronociceptive effect of sleep restriction**

Dopaminergic mechanisms in the anterior cingulate cortex and nucleus accumbens involved in the pronociceptive effect of sleep restriction

Abstract

Sleep impairment increases pain sensitivity and the risk of developing pain conditions in both animals and humans. However, the mechanisms are poorly understood. Therefore, the first question we address in this article is whether six hours a day of total sleep deprivation for three days would affect pain sensitivity differently in male and female rats. The second question is whether the integrity of ACC is essential to the pronociceptive effect of sleep restriction. The third question is whether the pharmacological activation of dopamine D₁ or D₂ receptors in the NAc (Nucleus Accumbens) and ACC (Anterior Cingulate Cortex) prevents the pronociceptive effect of sleep restriction. Dopaminergic activity at both regions was indirectly estimated. The findings demonstrate that sleep restriction increased pain sensitivity in a very similarly way in males and females, without inducing a significant stress response. This pronociceptive effect of sleep restriction depends on the integrity of the ACC, in addition to the NAc, as previously demonstrated. The pharmacological activation of dopamine D₂, but not D₁ receptors in the ACC or NAc prevented the increase in pain sensitivity induced by sleep restriction. Our findings show that dopamine levels in the ACC and NAc were not significantly affected by sleep restriction, however the levels of DOPAC, the major intraneuronal dopamine metabolite, as well as the DOPAC/dopamine ratio were decreased in the NAc and ACC of sleep restricted animals. Complementary, the expression of non-glycosylated-DAT increased and the ratio glycosylated/non-glycosylated DAT decreased in the NAc after sleep restriction, suggesting that sleep restriction reduces the efficiency of dopamine reuptake. A decreased in DAT glycosylation and dopamine transport is compatible with decreased dopaminergic neuronal activity and may contribute to explain the decreased DOPAC levels. Additional support to a decrease in dopaminergic neurotransmission is given by data showing that the phosphorylation of DARPP-32 at Thr-75 increases after sleep deprivation in both NAc and ACC. Taken together, these findings suggest that a decrease in dopaminergic neurotransmission in the ACC and NAc contributes to the pronociceptive effect of sleep restriction.

Keywords: dopamine; pain; sleep loss; sleep disorders; short sleep; pain sensitivity; hyperalgesia; anterior cingulate cortex; nucleus accumbens; DAT; DARPP-32.

Introduction

Persistent pain conditions and sleep disorders are public health problems worldwide (Mathias et al., 2018) and there is a clear, but poorly understood bidirectional relationship between them. In recent years, evidence has accumulated to unequivocally state that pain disrupts sleep (Sun et al., 2021), while disturbed or short sleep increases pain sensitivity and the risk of developing pain conditions (Haack et al., 2020). The understanding of the mechanisms by which sleep impairment affects pain would contribute to clinical pain management and prevention.

There is a broad consensus that sleep impairment increases pain sensitivity in both animals (Araujo et al., 2011, Page et al., 2014, Yamashita et al., 2014, Tomim et al., 2016, Alexandre et al., 2017, Hambrecht-Wiedbusch et al., 2017, Sardi et al., 2018a, Sardi et al., 2018b) and humans (Edwards et al., 2009, Bulls et al., 2015, Larson and Carter, 2016, Eichhorn et al., 2018, Smith et al., 2019, Sun et al., 2020, Lenert et al., 2021) and that painful conditions are more prevalent and severe in women than in men (Bartley and Fillingim, 2013, Mogil, 2020). However, the consensus ends here, any possible gender difference in the effect of sleep disruption on pain is still under debate. The increased pain sensitivity in women appears to result, at least in part, from a less-efficient endogenous pain modulation (Bulls et al., 2015, Lenert et al., 2021). We and others have demonstrated that sleep deprivation disrupts endogenous pain modulation (Edwards et al., 2009, Tomim et al., 2016) and females seem to be more sensitive to this disruption than males (Bulls et al., 2015, Eichhorn et al., 2018). This would support a greater sensitivity of females to the effects of sleep impairment on pain, but this is not what the mixed results of the literature indicate (Araujo et al., 2011, Bulls et al., 2015, Alexandre et al., 2017, Eichhorn et al., 2018, Smith et al., 2019). Part of this divergence results from different methodologies used for sleep deprivation and pain assessment. Therefore, the first question we address in this article is whether six hours a day of total sleep deprivation for three days would affect pain sensitivity differently in male and female rats.

Although the understanding of the mechanisms underlying the strong influence of sleep disruption on pain has undergone major advances in recent years (Haack et al., 2020), it is far from being satisfactory. For example, evidence has accumulated to support the mesodopaminergic system as a key player in the increased pain sensitivity that results from sleep disruption (Finan and Smith, 2013, Haack et al., 2020). In this system, the dopaminergic neurons of the midbrain ventral tegmental area (VTA), project, on one hand, to the nucleus accumbens (NAc) by the mesolimbic tract and, on the other hand, to frontal cortical regions by

the mesocortical tract (Yang et al., 2020). We have previously demonstrated that NAc mediates the pronociceptive effect of either Rapid eye movement (REM)-sleep deprivation (Sardi et al., 2018b) or chronic partial (6 hours a day/12-26 days) sleep restriction (Sardi et al., 2018a). However, no cortical region has been implicated in the increased pain sensitivity that results from sleep impairment. Among the cortical areas densely innervated by VTA dopaminergic projections, the Anterior Cingulate Cortex (ACC) is one with the most prominent role in pain modulation (Xiao et al., 2021). Therefore, the second question we address in this article is whether the integrity of ACC is essential to the pronociceptive effect of sleep restriction.

How sleep disruption affects mesolimbic dopaminergic signaling and with what consequences on pain sensitivity is an issue under intense investigation. The decrease in mesolimbic dopaminergic activity in response to sleep restriction has been suggested by recent studies (Volkow et al., 2012, Sardi et al., 2018b, Haack et al., 2020), which may be explained by the depressed neuronal activity in the VTA during sleep deprivation (Fifel et al., 2018). Indeed, we have recently demonstrated that decreased dopaminergic D₂ activity within the NAc underlie the pronociceptive effect of REM-sleep deprivation (Sardi et al., 2018b). However, it is not known whether similar mechanisms are seen in the ACC, with D₁ receptors and in the pronociceptive effect induced by total sleep restriction. Therefore, the third question we address in this article is whether the pharmacological activation of dopamine D₁ or D₂ receptors in the NAc or ACC prevents sleep restriction from increasing pain sensitivity. Dopaminergic activity at both regions was indirectly estimated by measuring the levels of dopamine and its metabolites; the relative state of phosphorylation of DARPP-32 (dopamine and cyclic AMP-regulated phosphoprotein, 32 kDa) and of glycosylation of DAT (dopamine transporter). The impact of sleep restriction on stress response was indirectly estimated by glucocorticoid metabolites excretion in feces.

Methods

Animals

The experiments were performed in male and female Wistar rats $\pm$ 60 days old, weighing between 270-330 g. The animals were kept in plastic cages (4 per cage) containing wood shavings, in a controlled 12 hours light / dark cycle, with food and water, ad libitum. All experimental procedures were approved by the animal research ethics committee of the Federal University of Parana (protocol # 1126) and followed the guidelines proposed by the IASP (International Association for the Study of Pain) committee for research in conscious animals

and the ARRIVE guidelines (Percie du Sert et al., 2020). In particular, the duration of the experiments was as short as possible, the nociceptive stimulus and the number of animals were kept to the minimum necessary to allow statistical analysis.

Drugs

The dopamine D₂ receptor agonist, piribedil 6 µg (Sardi et al., 2018b, Tobaldini et al., 2018a); the dopamine D₁ receptor agonist, SKF-38393 1 µg (Tobaldini et al., 2018a); N-Methyl-D-Aspartate (NMDA), 5.5 µg, pH 7.4 (Sardi et al., 2018a, Sardi et al., 2018b) were purchased from Sigma-Aldrich, São Paulo, SP, Brazil and diluted in 0.9% sterile saline.

Stereotaxic surgery and drug infusion

The animals were anesthetized with an intraperitoneal injection of xylazine (10 mg / kg) and ketamine (60 mg / kg) and a 25-gauge stainless steel cannula was positioned, according to the stereotactic plan, above the site of interest and fixed with acrylic. Stereotaxic coordinates were as follow, from bregma, incisor bar – 3.3 mm: For the ACC, in the rostro-caudal direction + 1 mm, latero-lateral, ± 0.6 mm and dorso-ventral - 1.4 mm (Paxinos and Watson, 2007); for the NAc, in the rostro-caudal direction + 1.30, latero-lateral ± 1.80 mm and dorso-ventral - 6.20 mm (Paxinos and Watson, 2007, Sardi et al., 2018a, Sardi et al., 2018b). After surgery, the animals received dipyrone (50 mg / kg) and enrofloxacin (0.5 mg / kg).

Experiments were performed seven days after implantation of the cannulas. The injection volume was 0.25 µL in all experiments. The drugs were administered slowly by a mechanical infusion pump (model KDS-100 KD Scientific Holliston, MA, USA) over a period of one minute. Injection cannula was connected to a PE-10 polyethylene tube and also to a 5-µl Hamilton syringe. After the injection, the cannula remained in place for 30 seconds to prevent retrograde flow of the drug.

For lesion in the ACC, bilateral injections of NMDA were performed at the day of surgery, 21 days before experiments, to allow the complete recovery of behavioral outcomes. The stereotactic coordinates were those described above and injection sites are represented in the supplementary figure 1.

In all experiments, mapping was performed in order to confirm the injection site. At the end of experiments, the animals were anesthetized and Evans Blue dye (1%, 0.25 µl) was injected through the guide cannula. The rats were perfused transcardiacally with 0.9% saline followed by 4% formalin solution and the brains were removed and stored in 4% formaldehyde.

Coronal sections (50 μm) were performed to determine the location of the dye. Injection sites were mapped according to the Atlas of Paxinos and Watson (Paxinos and Watson, 2007).

Sleep Restriction enriched gentle handling method

The rats were submitted to sleep restriction for a period of 6 hours a day for 3 days, started 7 a.m., using the enriched gentle handling method (Alexandre et al., 2017, Sardi et al., 2018a). Briefly, the method consists of using gentle manipulation to prevent the animal from settling to sleep. To keep the animals awake we can touch the animals with a soft bristle brush, move the experimentation cage gently in a seesaw movement, introduce small objects into the experimentation cage so that the animals are distracted by these objects while remaining alert and consequently awake (Alexandre et al., 2017, Sardi et al., 2018a). During sleep restriction, the animals were kept in their usual cages with wood shavings beddings and with water and food ad libitum. Control groups were kept in the same condition, but were free to sleep.

Randall-Selitto Test – Nociceptive evaluation

The nociceptive behavioral test was performed before and immediately after the end of the sleep restriction procedure on the three days of sleep restriction and at different times (18, 24 and 48 hours) during the sleep rebound period, always during the light phase (at 7:00 am and 1:00 pm), in a quiet room maintained at $22\text{ }^{\circ}\text{C} \pm 1\text{ }^{\circ}\text{C}$ (see figure 1). Before the experiments, each animal was handled for 7 days to get used to the experimental handling. The rats did not have access to food or water during the test and each animal was used once.

The Randall–Selitto test (Randall and Selitto, 1957) was used to assess the mechanical nociceptive paw withdrawal threshold, as a measure of the nociceptive response. For the test, we used an analgesimeter (Insight, Brazil), which applies a mechanical force in grams of gradual increase in the dorsal surface of the rats' paw. When the animal exhibits the paw withdrawal behavior, the applied force is immediately interrupted, this value in grams is recorded and used as a measure of the mechanical nociceptive threshold. The value was obtained from the mean of three readings made at intervals of 3 min. Data were expressed as mean $\pm$ SEM of the mechanical paw withdrawal threshold (g) at each time point.

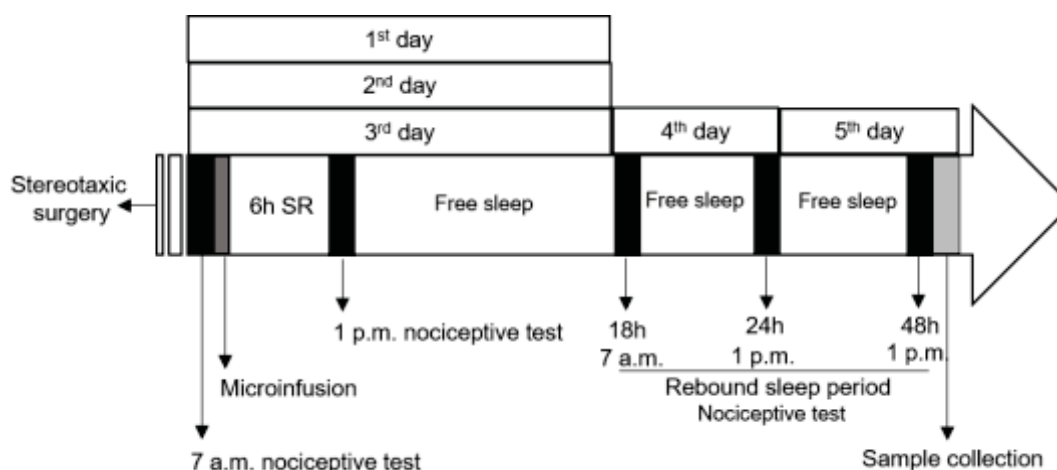


Figure 1 – Experimental design. Stereotaxic surgery was performed 21 (excitotoxic lesion) or 7 (implantation of the guide cannula) days before the beginning of the experiments. The mechanical nociceptive paw withdrawal threshold was measured and sleep restriction was initiated (7 a.m.). Depending on the group, the animals received a 0.25 μ L microinjection (see methods) into the ACC or NAc. The mechanical nociceptive paw withdrawal threshold was measured again 6h after the beginning of sleep restriction (1 p.m.). Then the animals were free to sleep until next day at 7 a.m. The procedure detailed above was repeated for three days (from the 1st to 3rd day) after which the sleep rebound period began. The mechanical nociceptive paw withdrawal threshold was measured at different time points in the rebound sleep period. The animals in the control groups were submitted to the same procedures, however, they were free to sleep. SR = sleep restriction.

Histological sample preparation and Cresyl violet staining

The rats were anesthetized by an intra-peritoneal injection of xylazine (10 mg / kg) and ketamine (60 mg / kg) and transcardially perfused with saline followed by 4% paraformaldehyde both in 0.1-M phosphate buffer, pH 7.4. Brains were removed and immersed in paraformaldehyde (4 °C for a week), in 30 % sucrose solution (another week) and stored at –80°C until sectioning. Four sections (30 μ m), were sliced per animal.

Cresyl violet, a cationic dye that stains Nissl corpuscles in neurons (Ovalle, 2013), was used to determine the extension of NMDA lesion in the ACC, as described previously (Sardi et al., 2018a, Sardi et al., 2018b). The sections were mounted on to gelatin-coated slides, passed through a series of ethanol solutions of descending concentration (3 min in each of 100%, 95%, and 70% ethanol in water) and stained for ~2 min with cresyl violet at 37 °C (0.05% aqueous cresyl violet, 2mM acetic acid, and 5mM formic acid in water). After staining, sections were rinsed in water and 70% ethanol; differentiated in 95% ethanol with acetic acid; dehydrated in ascending concentrations of ethanol–xylene and cover slipped. Quantification of ACC lesion was performed automatically by optical density using ImageJ 1.37c (Public Domain) image analysis software.

High performance liquid chromatography (HPLC)

For determining DA, DOPAC (3,4-dihydro-xyphenylacetic acid), HVA (homovallinic acid), norepinephrine, DHPG (3,4-dihydroxyphenylglycol), 5-HT (5-hydroxytryptamine) and HIAA (5-hydroxyindoleacetic acid) contents in brain, the rats were killed by decapitation at different sleep conditions (control, sleep restriction and sleep rebound). The brains were immediately removed, and the NAc and the ACC were freshly dissected, frozen in liquid nitrogen, and stored at -80°C . The concentration of DA, DOPAC, HVA, norepinephrine, DHPG, 5-HT and HIAA was assayed by reverse-phase HPLC with electrochemical detection.

The system consisted of a Synergi Fusion-RPC-18 reverse-phase column (150×4.6 mm i.d., $4\ \mu\text{m}$ particle size) fitted with a 4×3.0 mm precolumn (SecurityGuard Cartridges Fusion-RP), an electrochemical detector (ESA Coulochem III Electrochemical Detector) equipped with a guard cell (ESA 5020), with the electrode set at 350 mV and a dual electrode analytical cell (ESA 5011A); and a LC-20AT pump (Shimadzu) equipped with a manual Rheodyne 7725 injector with a $20\ \mu\text{L}$ loop. The column was maintained inside a temperature-controlled oven (25°C , Shimadzu). The cell had two chambers in series: each chamber included a porous graphite colorimetric electrode, a double counter electrode, and a double reference electrode. Oxidizing potentials were set at 100 mV for the first electrode and at 450 mV for the second electrode. DA, DOPAC, HVA, norepinephrine, DHPG, 5-HT and HIAA were detected at the second electrode. The tissue samples were homogenized with an ultrasonic cell disrupter (Sonics) in 0.1 M perchloric acid containing sodium metabisulfite 0.02% and internal standard. After centrifugation at $10000 \times g$ for 30 minutes at 4°C , $20\ \mu\text{L}$ of the supernatant was injected into the chromatograph. The mobile phase used at a flow rate of 1 ml/min had the following composition: 20 g citric acid monohydrate (Merck), 200 mg octane-1-sulfonic acid sodium salt (Merck), 40 mg ethylenediaminetetraacetic acid (EDTA) (Sigma), and 900 ml HPLC-grade water. The pH of the buffer running solution was adjusted to 4.0 then filtered through a $0.45\ \mu\text{m}$ filter. Methanol (Merck) was added to give a final composition of 10% methanol (v/v). The peak areas of the external standards were used to quantify the sample peaks. The data was expressed as ng/g of tissue and the turnover of DA, was expressed accordingly to the equations $(\text{DOPAC} + \text{HVA})/\text{DA}$, based on previous works (Vergara et al., 2020).

Western blot

The relative state of phosphorylation of the Dopamine and cyclic AMP-regulated phosphoprotein, 32 kDa (DARPP-32) and the relative state of glycosylation of the dopamine transporter (DAT) were quantified by Western Blotting and used as indirect measures of

dopaminergic signaling. DARPP-32 is a cytosolic protein with dual-function, when phosphorylated at threonine 34 (Phospho-Thr 34 DARPP-32) it is a potent inhibitor of protein phosphatase-1 (PP-1) and leads to potentiation of dopaminergic signaling at multiple steps (Nishi et al., 2000, Walaas et al., 2011). While when phosphorylated at threonine 75 (Phospho-Thr 75 DARPP-32) it acts as a cAMP-dependent protein kinase (PKA) inhibitor, reducing the efficacy of dopaminergic signaling (Nishi et al., 2000, Walaas et al., 2011). DAT glycosylation is a marker of its efficiency, the greater the proportion of glycosylated DAT, the greater the dopamine reuptake efficiency (Li et al., 2004).

For western blotting analysis, the brains were removed and the NAc and the ACC tissue freshly dissected, frozen in liquid nitrogen and stored in a -80°C freezer. The samples were processed with a lysis buffer containing protease inhibitor and phosphatase inhibitor on ice. Then the samples were homogenized with a sonicator and centrifuged at 4°C, 14,000 rpm for 20 minutes. Total proteins were quantified by Bradford reagent. The proteins were denatured in a water bath for 10 minutes at 95°C in sample buffer, separated by electrophoresis in 12% polyacrylamide (SDS-page) and transferred to a nitrocellulose membrane, blocked with 2% BSA (bovine serum albumin) for 1h30min, incubated overnight with the specific primary antibody: anti-phospho-thr75-DARPP-32 (1:1000) (#2301s, cell signaling; rabbit), anti-phospho-thr34-DARPP-32 (1:1000) (#12438s, rabbit, cell signaling) or anti-dopamine transporter (1:1000) (DAT, #22524-1-ap, proteintech; rabbit). Then the membranes were incubated for 1 hour with the secondary anti-rabbit antibody (1:50,000). The analysis of the bands were performed by chemiluminescence in a photodocumenter. The membranes undergo a mild membrane stripping process and were again blocked with 2% BSA, incubated overnight with the primary anti- β -actin antibody (mouse, 1:10,000) and incubated for 1h with the anti-mouse-specific secondary antibody (1:40,000) and again revealed by chemiluminescence.

Fecal glucocorticoid metabolites

To determine the impact of sleep restriction on stress response, we measured glucocorticoid metabolites in feces, a noninvasive approach that allows monitoring of persistent stressful conditions without the additional stress of blood collection. The measurement of fecal glucocorticoid metabolites reflects the overall plasmatic hormone fluctuation of the past 24 hours, minimizing the impact of circadian changes in hormone levels (Bamberg et al., 2001, Pihl and Hau, 2003, Muller et al., 2013).

Feces were collected directly from the animals' cages at different time points (0, 24, 48, 54, 72, 78 and 96h) during the experiment and kept at -20°C until analysis. Steroid extraction

was performed as previously described (Muller et al., 2013). Briefly, an aliquot of 0.5 g of thawed, well-mixed, and dry feces was extracted in 5mL of 90% ethanol (vol:vol; ultrapure water), by shaking for 30 minutes (multipulse vortexer; Glas-Col, Terre Haute, IN, USA). After centrifuging (1000 g for 15 minutes), the supernatant was recovered and stored at -20°C. Fecal extracts were analysed for fecal glucocorticoid metabolites by enzyme immunoassay (Watson et al., 2013). We used the antibody against corticosterone (CJM006; 1: 15,000) and the corticosterone horseradish peroxidase conjugate (1: 40,000), obtained from Coralie Munro at the University of California, Davis (Davis, CA, USA). The intra- and inter-assay coefficients of variation were less than 5%. The data were corrected for the dilution factor and are expressed as ng/g of dry feces.

Statistical analysis

Data from behavioral experiment and fecal glucocorticoid were analyzed by Two-Way ANOVA with repeated measures, followed by Student-Newman-Keuls (SNK) post-hoc test. Dependent measures include nociceptive behavior (time), and independent measures include different treatments and sleep conditions. Data from histology were analyzed by Two-Way ANOVA and data from HPLC, and western blot were analyzed by One-way ANOVA. All post hoc contrasts, when appropriate, were performed using SNK test. The level for statistical significance was $p < 0.05$. STATISTICA® software (StatSoft, Tulsa, OK, USA) was used to perform data analysis. SigmaPlot® software (Systat Software, San Jose, CA, USA) was used to perform graphical representation. Data are plotted in figures as mean $\pm$ SEM.

Results

Gender, stress and the pronociceptive effect of sleep restriction

Six hours a day of total sleep deprivation over three days progressively lowers the mechanical nociceptive threshold (Fig. 2 A). Afterwards, the animals were allowed to sleep freely and the nociceptive threshold increased progressively, returning to baseline within 48 h. Both the decrease of nociceptive threshold during sleep restriction and its return to baseline during free sleep period were similar in males and females, with the exception of a slight difference at the beginning of the experiment (Fig. 2 A – Two-way repeated measures (time) ANOVA – $F_{\text{sleep condition}} (1, 49) = 935.53, p < 0.001$; $F_{\text{sex}} (1, 49) = 10.790, p = 0.001$; $F_{\text{sleep condition} \times \text{sex}} (1, 49) = 0.628, p = 0.431$; $F_{\text{time}} (8, 392) = 128.07, p < 0.001$; $F_{\text{time} \times \text{sleep condition}} (8, 392) = 127.03, p < 0.001$; $F_{\text{time} \times \text{sex}} (8, 392) = 0.900, p = 0.515$; $F_{\text{time} \times \text{sleep condition} \times \text{sex}} (8, 392) =$

0.514, $p = 0.845$). Post hoc analysis using SNK test indicated that sleep restriction decreased mechanical nociceptive threshold during the overall experiment, $p < 0.001$. The nociceptive threshold was lower in females than in males only at the end of the first day ($p = 0.018$) and at the beginning of the second day ($p = 0.033$) of sleep restriction. As the development of the pronociceptive effect and its recovery was similar between males and females, subsequent experiments were performed only with male rats.

The levels of fecal glucocorticoid metabolites are similar between animals submitted to sleep restriction and their controls throughout the experiment (Fig. 2 B – Two-way repeated measures (time) ANOVA - $F_{\text{sleep condition}} (1, 11) = 0.296$, $p = 0.597$; $F_{\text{time}} (6, 66) = 5.993$, $p < 0.001$; $F_{\text{sleep condition} \times \text{time}} (6, 66) = 0.569$, $p = 0.752$). This data supports that sleep restriction did not induce a significant change in the fecal corticosterone levels.

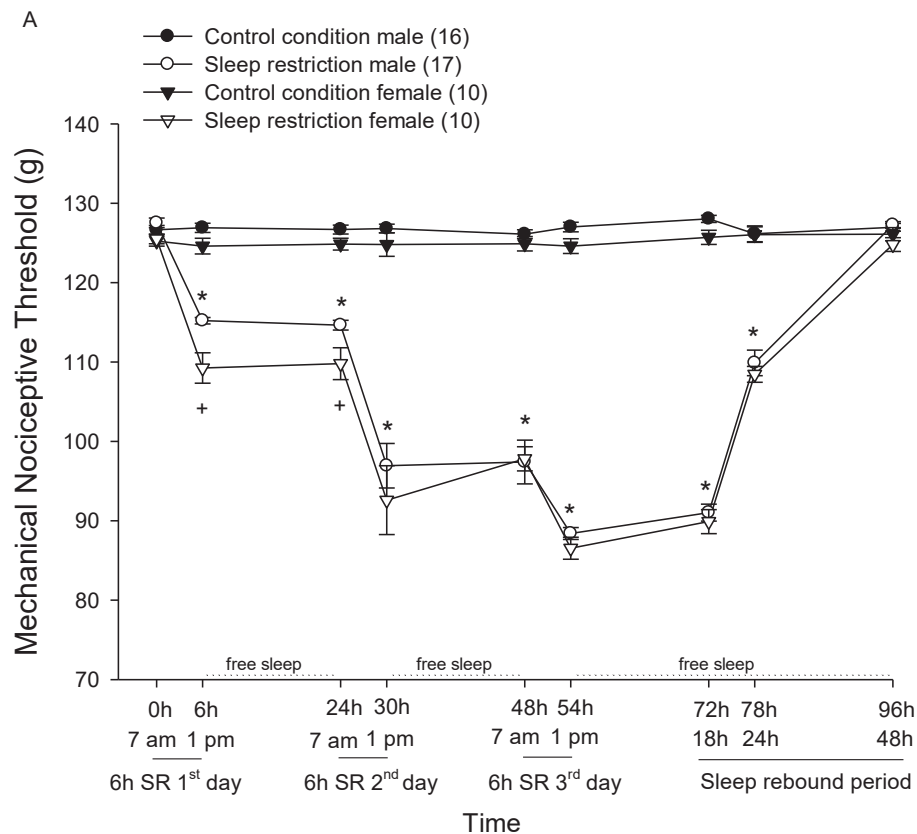


Figure 2 – Gender, stress and the pronociceptive effect of sleep restriction. A - Six hours a day of total sleep deprivation over three days progressively lowers the mechanical nociceptive threshold. Afterwards, the animals were allowed to sleep and the nociceptive threshold progressively increased, returning to baseline within 48 h. Both the decrease of nociceptive threshold during sleep restriction and its return to baseline during free sleep period were similar in males and females, with the exception of the first two measurements, when females showed a slightly greater decrease in the nociceptive threshold (indicated by “+”). Two way RM ANOVA and Newman-Keuls post hoc test: the symbol, “*” indicates a statistically significant decrease in mechanical nociceptive threshold, and “+” indicates a statistically significant decrease in mechanical nociceptive threshold in sleep restricted females compared to sleep restricted males. In this and in subsequent experiments, animals were prevented from sleeping during six hours a day (from 7 a.m. to 1 p.m.) over three days. After that, they were able to sleep freely for two days, when the experiment ended. In this and subsequent figures representing nociceptive tests, dotted lines indicate free sleep period; numbers in parenthesis indicate the number of animals in each group. See methods for additional details regarding data presentation and analysis. SR = Sleep restriction.

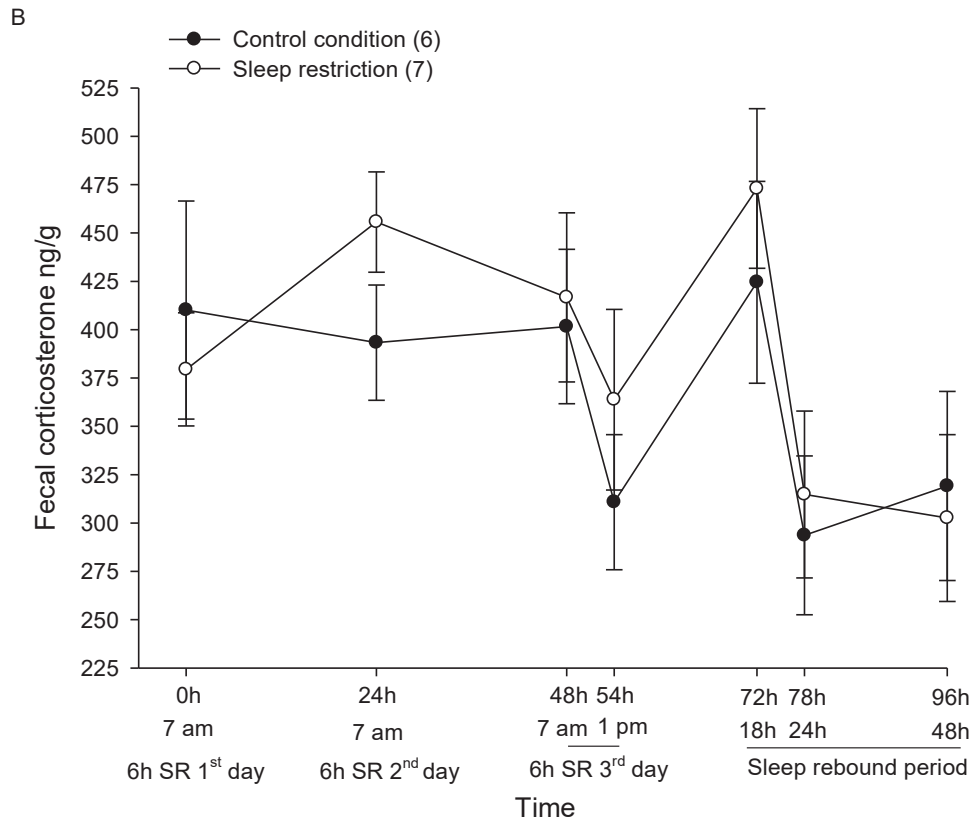


Figure 2 B - Sleep restriction did not significantly change the level of fecal glucocorticoid metabolites throughout the experiment. In this figure, numbers in parenthesis indicate the number of cages (4 or 5 animals each).

The pronociceptive effect of sleep restriction depends on the Anterior Cingulate Cortex

Excitotoxic lesion of the ACC by NMDA administration prevented the pro-nociceptive effect of sleep restriction (Fig. 3 A – Two-way repeated measures (time) ANOVA – $F_{\text{sleep condition}} (1, 20) = 323.89, p < 0.001$; $F_{\text{treatment}} (1, 20) = 299.92, p < 0.001$; $F_{\text{sleep condition} \times \text{treatment}} (1, 20) = 315.68, p < 0.001$; $F_{\text{time}} (8, 160) = 103.16, p < 0.001$; $F_{\text{time} \times \text{sleep condition}} (8, 160) = 95.25, p < 0.001$; $F_{\text{time} \times \text{treatment}} (8, 160) = 94.908, p < 0.001$; $F_{\text{time} \times \text{sleep condition} \times \text{treatment}} (8, 160) = 100.81, p < 0.001$; SNK test $p < 0.01$) and significantly decreased the number of ACC neurons (Fig. 3 B - Two-way ANOVA – $F_{\text{sleep condition}} (1, 16) = 0.49, p = 0.49$; $F_{\text{treatment}} (1, 16) = 23.32, p = 0.001$; $F_{\text{sleep condition} \times \text{treatment}} (1, 16) = 2.60, p = 0.126$; SNK test $p < 0.03$). The finding that the nociceptive threshold did not change in sleep-deprived lesioned animals indicates that the ACC is necessary for sleep restriction to increase pain sensitivity.

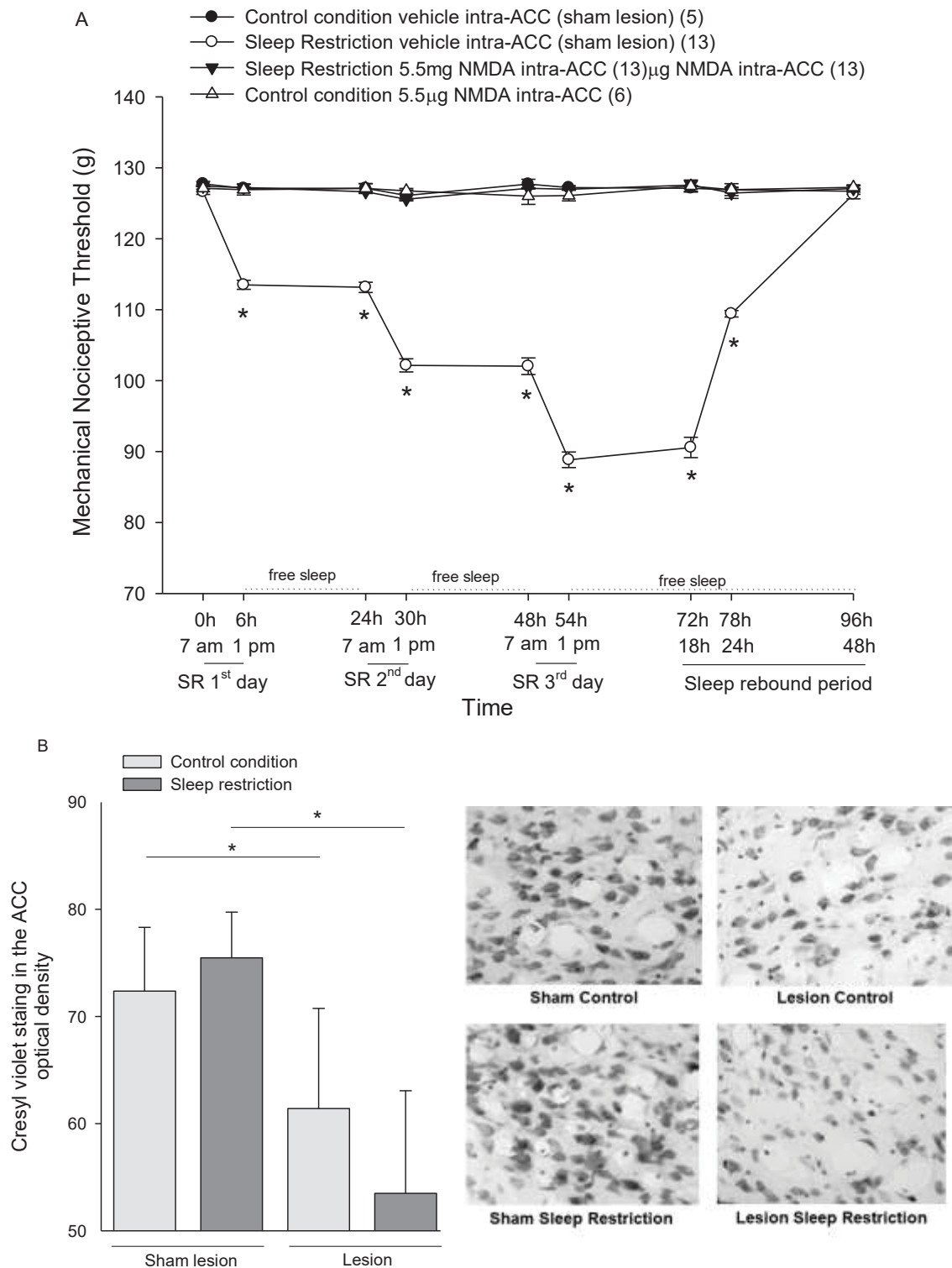


Figure 3 – The pronociceptive effect of sleep restriction depends on the Anterior Cingulate Cortex.
A- Excitotoxic lesion of the ACC by local NMDA administration prevented the pronociceptive effect of sleep restriction. Two way RM ANOVA and Newman-Keuls post hoc test: the symbol, “*” indicates a statistically significant decrease in mechanical nociceptive threshold. NMDA administration was performed 21 days before experiments. SR = Sleep restriction. **B, left-** Excitotoxic lesion of the ACC by local NMDA administration significantly decreased the number of ACC neurons. Two-way ANOVA and Newman-Keuls post hoc test: the symbol, “*” indicates a statistically significant decrease in the number of ACC neurons. **B, right-** representative images of excitotoxic lesion of the ACC by local NMDA administration. Slice images from bregma 1.08 mm with 40x magnification.

The pronociceptive effect of sleep restriction is prevented by the activation of dopamine D₂, but not D₁, receptors in the Anterior Cingulate Cortex or Nucleus Accumbens

The administration of the dopamine D₂ receptor agonist piribedil into the ACC prevented the pronociceptive effect of sleep restriction and did not affect the basal nociceptive threshold in control animals (Fig. 4 A, Two-way repeated measures (time) ANOVA – F_{sleep condition} (1, 22) = 97.986, $p < 0.001$; F_{treatment} (1, 22) = 101.43, $p < 0.001$; F_{sleep condition x treatment} (1, 22) = 51.589, $p < 0.001$; F_{time} (7, 154) = 23.1642, $p < 0.001$; F_{time x sleep condition} (7, 154) = 33.196, $p < 0.001$; F_{time x treatment} (7, 154) = 35.534, $p < 0.001$; F_{time x sleep condition x treatment} (7, 154) = 24.220, $p < 0.001$; SNK test $p < 0.001$). The administration of the dopamine D₁ receptor agonist SKF-38393 into the ACC slightly increased the pronociceptive effect of sleep restriction at the first six hours (Fig. 4 B, $p = 0.0008$). However, it decreased the mechanical nociceptive threshold in control animals, inducing a pronociceptive effect *per se* (Two-way repeated measures (time) ANOVA – F_{sleep condition} (1, 20) = 137.38, $p < 0.001$; F_{treatment} (1, 20) = 212.29, $p < 0.001$; F_{sleep condition x treatment} (1, 20) = 126.63, $p < 0.001$; F_{time} (8, 160) = 126.48, $p < 0.001$; F_{time x sleep condition} (8, 160) = 13.905, $p < 0.001$; F_{time x treatment} (8, 160) = 21.454, $p < 0.001$; F_{time x sleep condition x treatment} (8, 160) = 21.627, $p < 0.001$; SNK test $p < 0.001$).

The administration of the dopamine D₂ receptor agonist piribedil into the NAc prevented the pronociceptive effect of sleep restriction, while it did not affect the basal nociceptive threshold in control animals (Fig. 5 A, Two-way repeated measures (time) ANOVA – F_{sleep condition} (1, 21) = 113.53, $p < 0.001$; F_{treatment} (1, 21) = 124.29, $p < 0.001$; F_{sleep condition x treatment} (1, 21) = 1.942, $p < 0.001$; F_{time} (7, 147) = 31.991, $p < 0.001$; F_{time x sleep condition} (7, 147) = 27.905, $p < 0.001$; F_{time x treatment} (7, 147) = 26.565, $p < 0.001$; F_{time x sleep condition x treatment} (7, 147) = 23.797, $p < 0.001$; SNK test $p < 0.001$). The administration of the dopamine D₁ receptor agonist SKF-38393 into the NAc affect neither the pronociceptive effect of sleep restriction nor the basal nociceptive threshold in control animals (Fig. 5 B, Two-way repeated measures (time) ANOVA – F_{sleep condition} (1, 21) = 1100.3, $p < 0.001$; F_{treatment} (1, 21) = 0.001, $p = 0.966$; F_{sleep condition x treatment} (1, 21) = 0.786, $p = 0.385$; F_{time} (8, 168) = 160.63, $p < 0.001$; F_{time x sleep condition} (8, 168) = 156.41, $p < 0.001$; F_{time x treatment} (8, 168) = 2.184, $p = 0.03$; F_{time x sleep condition x treatment} (8, 168) = 0.539, $p = 0.825$; SNK test $p < 0.001$).

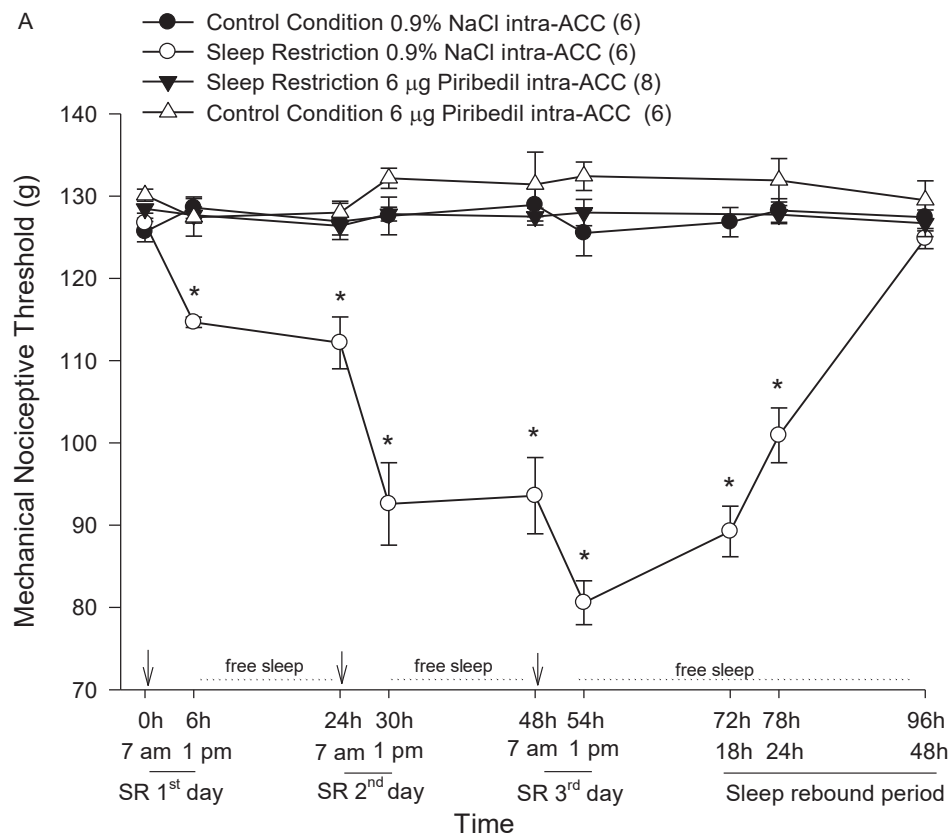


Figure 4 – The pronociceptive effect of sleep restriction is prevented by the activation of dopamine D₂, but not D₁, receptors in the Anterior Cingulate Cortex. A- The administration of the dopamine D₂ receptor agonist piribedil into the ACC prevented the pronociceptive effect of sleep restriction and did not affect the basal nociceptive threshold in control animals. Two way RM ANOVA and Newman-Keuls post hoc test: the symbol, “*” indicates a statistically significant decrease in mechanical nociceptive threshold. In this and subsequent figures, arrows indicate when dopaminergic agonists were microinjected (daily, before sleep restriction). SR = Sleep restriction.

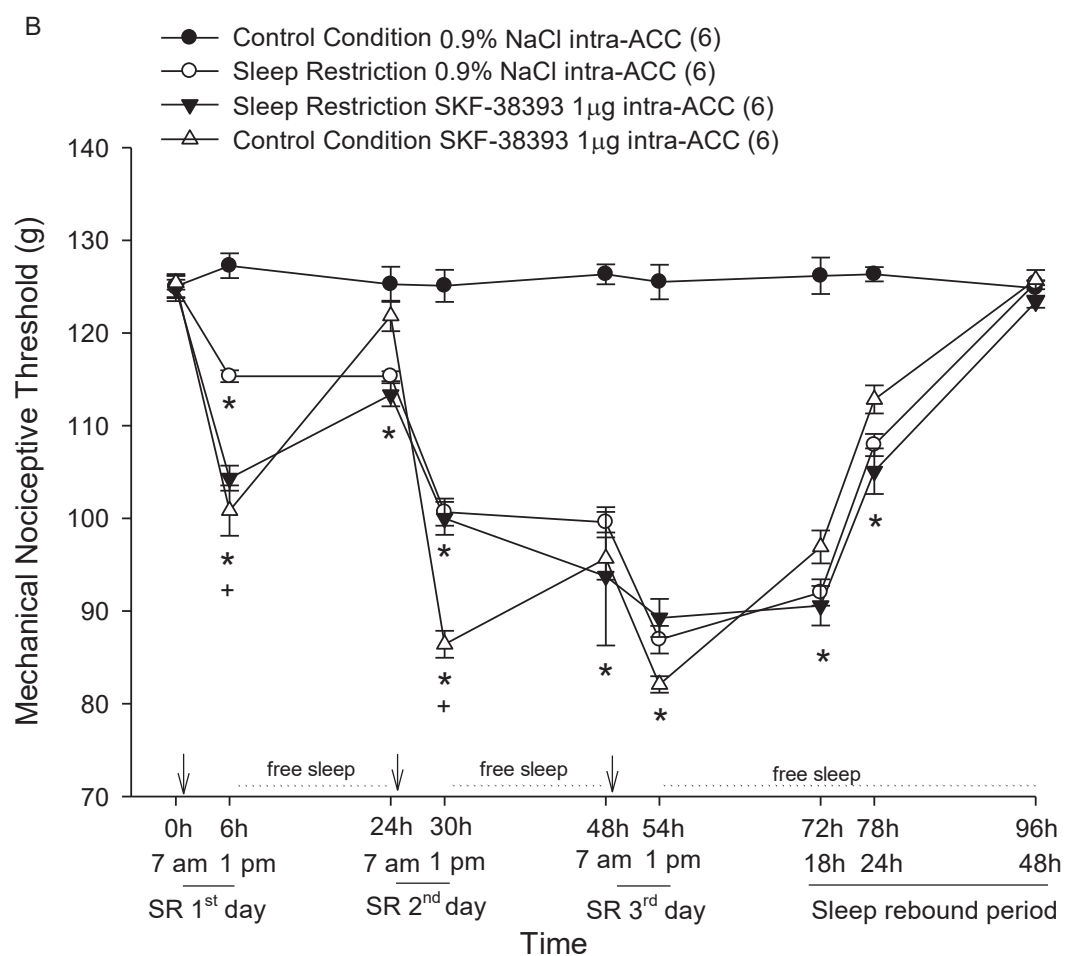


Figure 4 B- The administration of the dopamine D1 receptor agonist SKF-38393 into the ACC slightly increased the pronociceptive effect of sleep restriction at the first six hours and decreased the mechanical nociceptive threshold in control animals, inducing a pronociceptive effect by itself. Two way RM ANOVA and Newman-Keuls post hoc test: the symbol, “*” indicates a statistically significant decrease in mechanical nociceptive threshold compared to control; the symbol, “+” indicates significant decrease in mechanical nociceptive threshold compared to other all groups. SR = Sleep restriction.

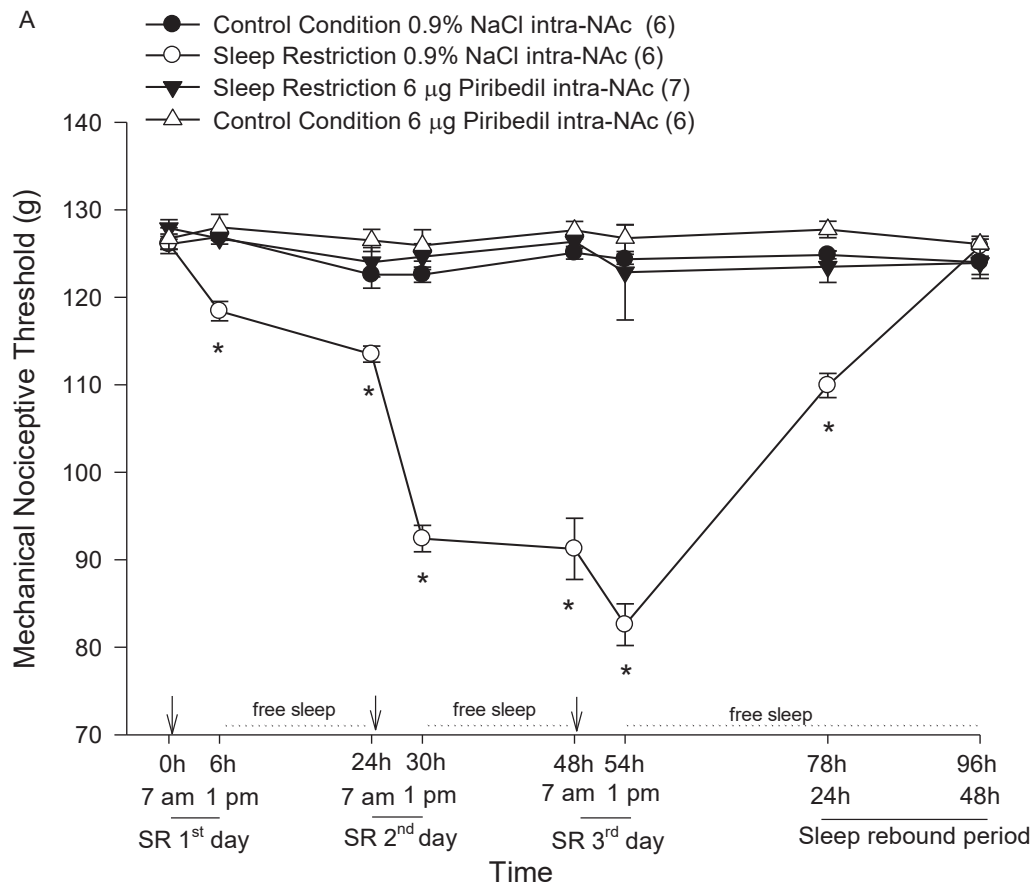


Figure 5 – The pronociceptive effect of sleep restriction is prevented by the activation of dopamine D₂, but not D₁, receptors in the Nucleus Accumbens. A- The administration of the dopamine D₂ receptor agonist piribedil into the NAc prevented the pronociceptive effect of sleep restriction, while it did not affect the basal nociceptive threshold in control animals. Two way RM ANOVA and Newman-Keuls post hoc test: the symbol, “*” indicates a statistically significant decrease in mechanical nociceptive threshold. SR = Sleep restriction.

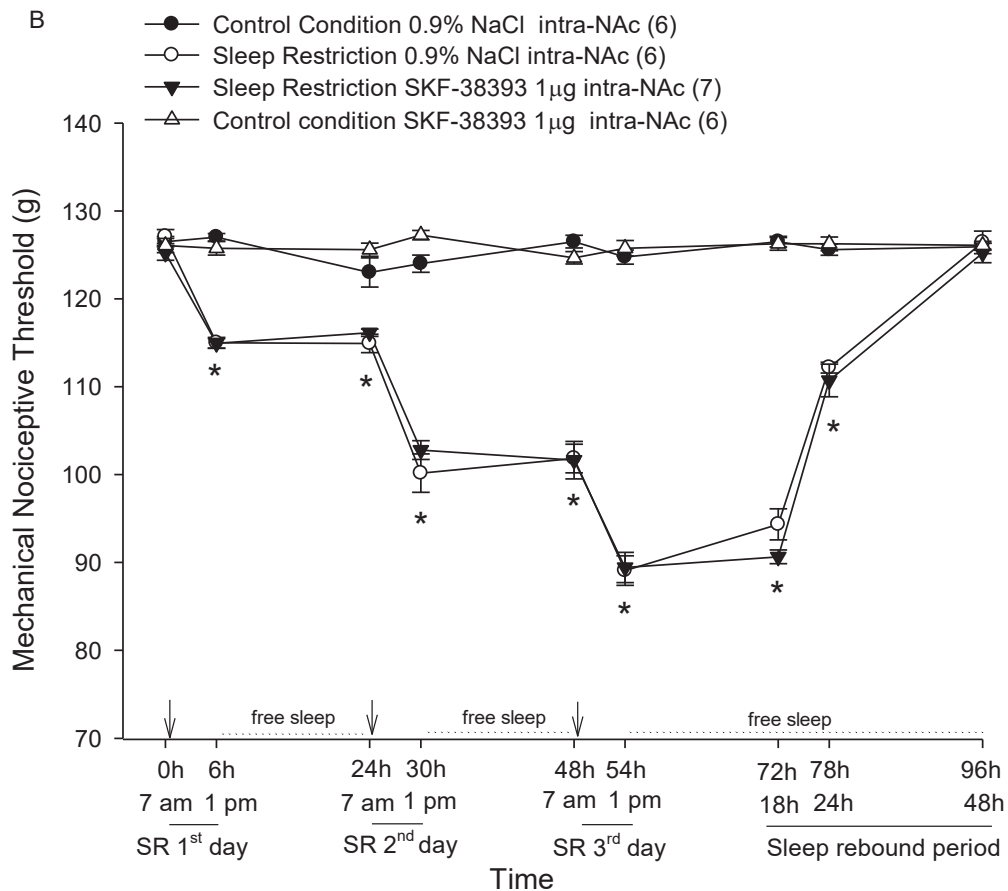


Figure 5 B- The administration of the dopamine D₁ receptor agonist SKF-38393 into the NAc affect neither the pronociceptive effect of sleep restriction nor the basal nociceptive threshold in control animals. Two way RM ANOVA and Newman-Keuls post hoc test: the symbol, “*” indicates a statistically significant decrease in mechanical nociceptive threshold. SR = Sleep restriction.

The levels of dopamine, 5-HT, norepinephrine and their metabolites changes in the Anterior Cingulate Cortex and Nucleus Accumbens after sleep restriction and rebound

No significant changes in dopamine levels were detected in the ACC (Fig. 6.1 A) or NAc (Fig. 6.1 B) after sleep restriction, but a significant decrease was revealed in both regions after sleep rebound (One Way ANOVA followed by SNK post hoc test, $p < 0.001$ and $p < 0.005$, respectively).

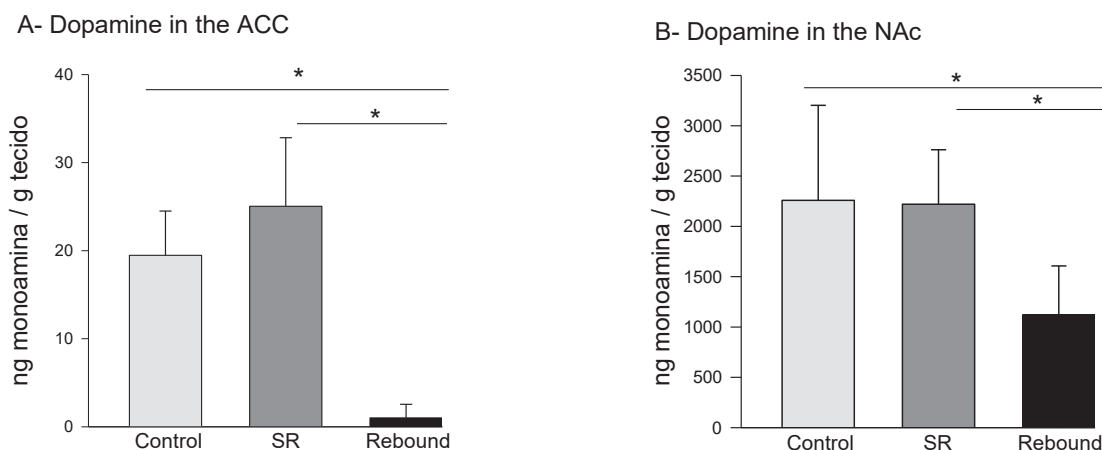


Figure 6 – The levels of dopamine, serotonin, norepinephrine and their metabolites changes in the Anterior Cingulate Cortex and Nucleus Accumbens after sleep restriction and rebound. 6.1 Dopamine and metabolites: a significant decreased in dopamine levels was detected in the ACC (6.1 A) and NAc (6.1 B) after sleep rebound. One-way ANOVA and Newman-Keuls post hoc test: statistically significant differences are indicated by the symbol “*”. SR = Sleep restriction.

The levels of DOPAC (3,4-dihydroxyphenylacetic acid), the major intraneuronal dopamine metabolite, decreased with sleep deprivation, but increased with sleep rebound both in the ACC (Fig. 6.1 C, One Way ANOVA followed by SNK post hoc test, $p < 0.05$ and $p < 0.05$, respectively) and in the NAc (Fig. 6.1 D, One Way ANOVA followed by SNK post hoc test, $p = 0.023$ and $p = 0.005$, respectively), while no significant changes were detected in the levels of HVA (homovanillic acid), the major intraneuronal dopamine metabolite (Fig. 6.1 E, One Way ANOVA, $p = 0.096$ and F, $p = 0.45$).

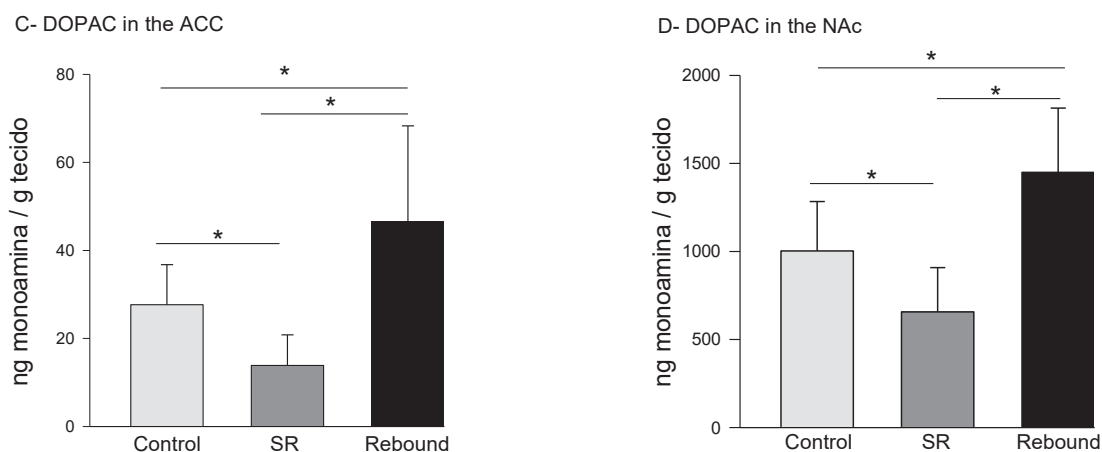


Figure 6 –The levels of DOPAC decreased with sleep restriction, but increased with sleep rebound both in the ACC (6.1 C) and in the NAc (6.1 D). One-way ANOVA and Newman-Keuls post hoc test: statistically significant differences are indicated by the symbol “*”.

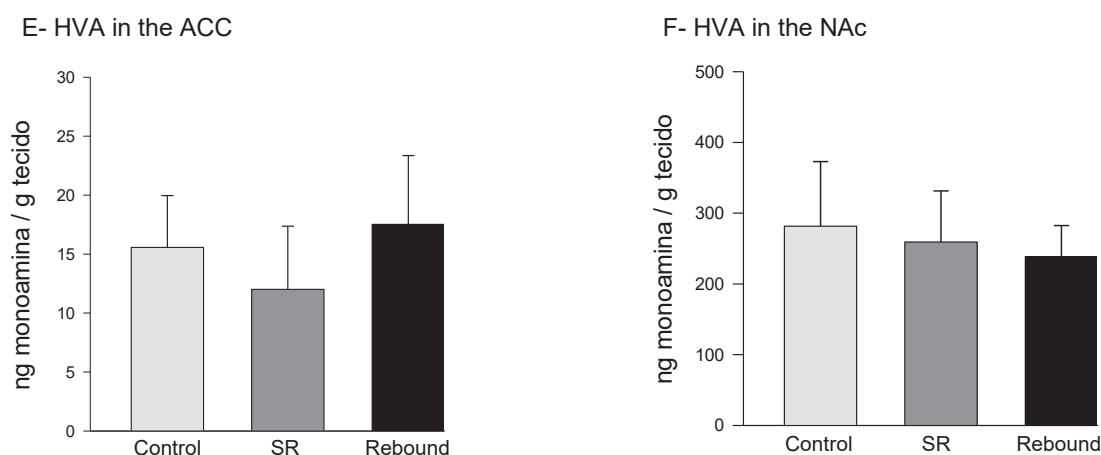


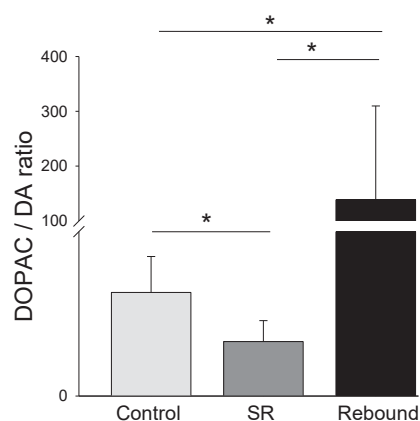
Figure 6 – No significant changes were detected in the levels of HVA in the ACC (6.1 E) and NAc (6.1 F).

Both the ratio DOPAC/Dopamine and the general dopamine turnover were decreased with sleep deprivation, but increased with sleep rebound in the ACC (Fig. 6.1 G and I, One Way ANOVA followed by SNK post hoc test, $p < 0.05$) and in the NAc (Fig. 6.1 H and J, One Way ANOVA followed by SNK post hoc test, $p < 0.05$).

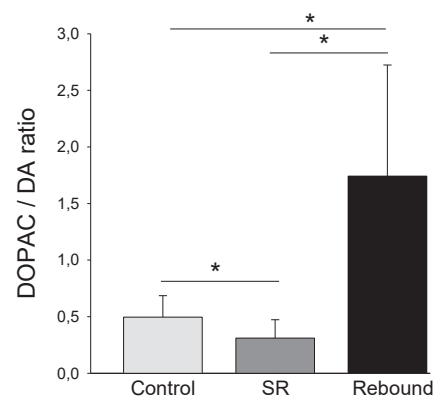
The levels of 5-HT decreased after sleep rebound in the ACC (Fig. 6.2 A, One Way ANOVA followed by SNK post hoc test, $p < 0.001$) and increased with sleep restriction in the NAc (Fig. 6.2 B, One Way ANOVA followed by SNK post hoc test, $p = 0.034$), while the levels of its metabolite 5-HIAA increased with sleep rebound in the ACC (Fig. 6.2 C, One Way ANOVA followed by SNK post hoc test, $p = 0.004$), and did not change in the NAc (Fig. 6.2 D, One Way ANOVA, $p = 0.79$). The ratio 5-HIAA/5-HT increase after sleep rebound in the ACC (Fig. 6.2 E, One Way ANOVA followed by SNK post hoc test, $p < 0.05$) and decreased after sleep restriction in the NAc (Fig. 6.2 F, One Way ANOVA followed by SNK post hoc test, $p < 0.05$).

The levels of norepinephrine decreased after sleep rebound in the ACC (Fig. 6.3 A, One Way ANOVA followed by SNK post hoc test, $p = 0.046$) and increased with sleep restriction in the NAc (Fig. 6.3 B, One Way ANOVA followed by SNK post hoc test, $p < 0.006$), while the levels of its metabolite DHPG decreased with sleep restriction in the ACC (Fig. 6.3 C, One Way ANOVA followed by SNK post hoc test, $p = 0.012$), and did not change in the NAc (Fig. 6.3 D, One Way ANOVA, $p = 0.055$). The ratio DHPG/ norepinephrine increase after sleep rebound in the ACC (Fig. 6.3 E, One Way ANOVA followed by SNK post hoc test, $p < 0.003$) and did not change in the NAc (Fig. 6.3 F, One Way ANOVA, $p = 0.326$).

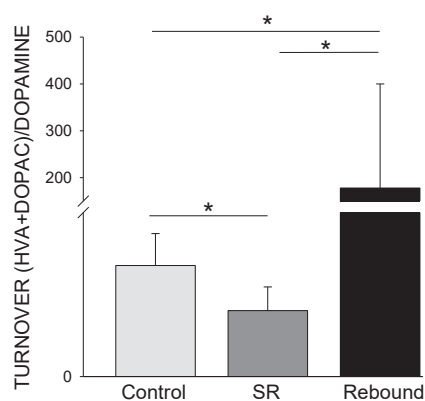
G- DOPAC / DA ratio in the ACC



H- DOPAC / DA ratio in the NAc



I- Turnover (HVA+DOPAC)/DA in the ACC



J- Turnover (HVA+DOPAC)/DA in the NAc

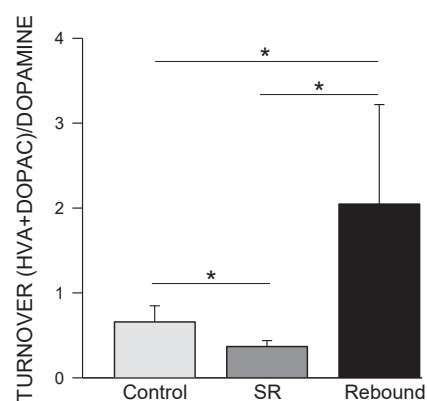


Figure 6 –DOPAC/Dopamine ratio and the general dopamine turnover decreased with sleep restriction and increased with sleep rebound in the ACC (6.1 G and I) and in the NAc (6.1 H and J). One-way ANOVA and Newman-Keuls post hoc test: statistically significant differences are indicated by the symbol “*” DA = dopamine.

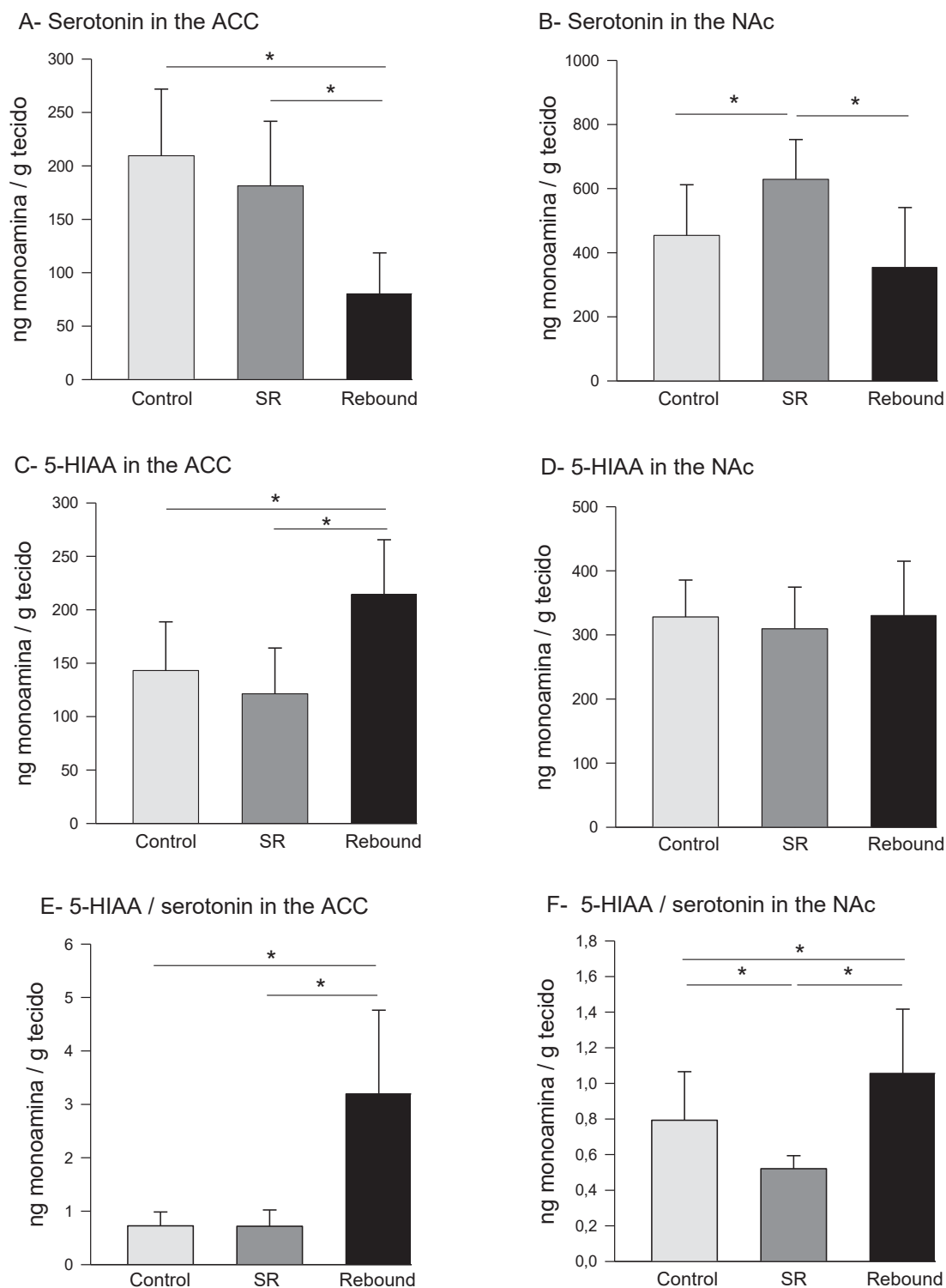


Figure 6.2 Serotonin: the levels of serotonin decreased after sleep rebound in the ACC (6.2 A) and increased with sleep restriction in the NAc (6.2 B), while the levels of its metabolite 5-HIAA increased with sleep rebound in the ACC (6.2 C), and did not change in the NAc (6.2 D). The ratio 5-HIAA/Serotonin increase after sleep rebound in the ACC (6.2 E) and decreased after sleep restriction in the NAc (6.2 F). One-way ANOVA and Newman-Keuls post hoc test: statistically significant differences are indicated by the symbol “*” SR = Sleep restriction.

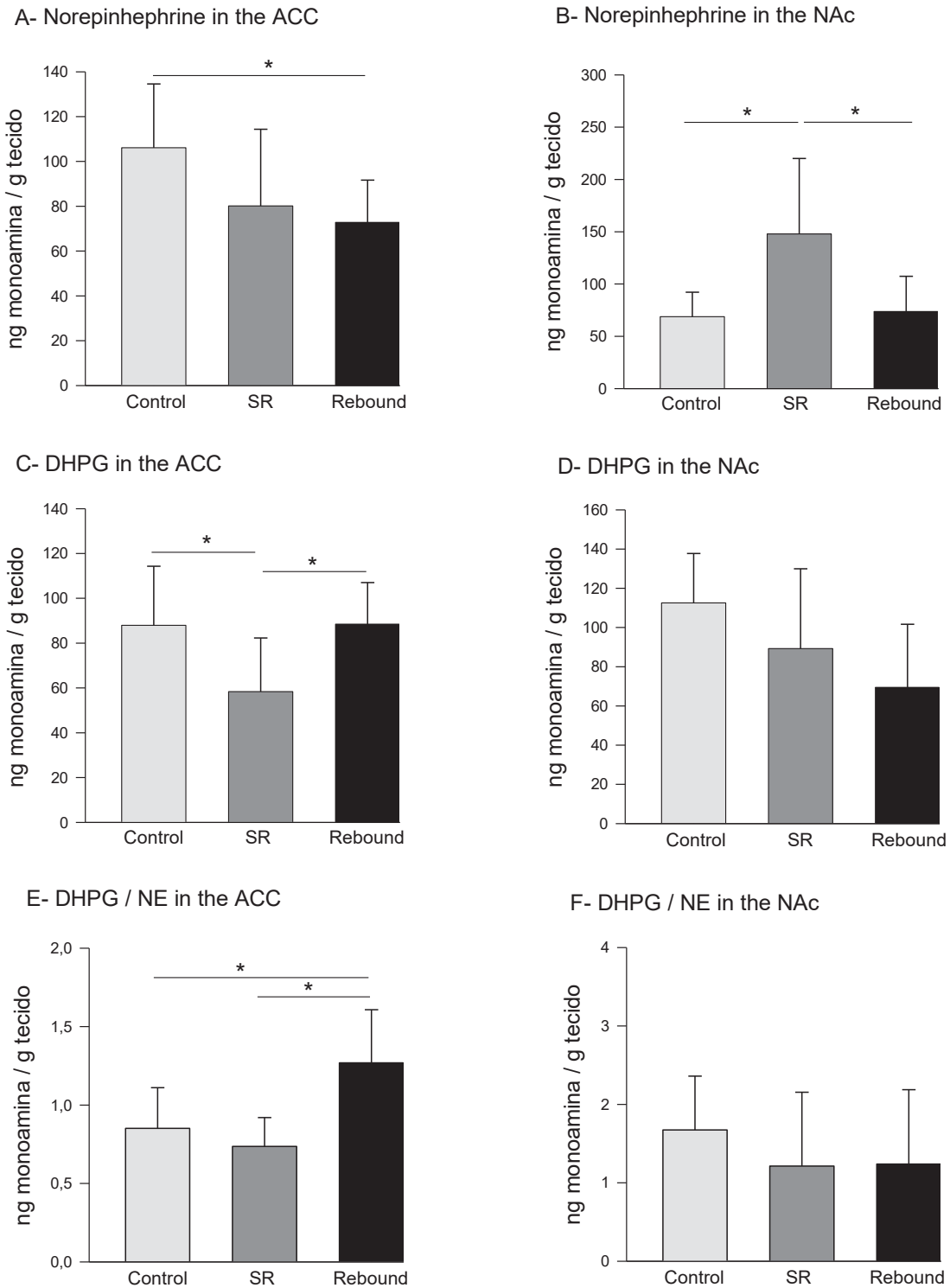


Figure 6.3 Norepinephrine: the levels of norepinephrine decreased after sleep rebound in the ACC (6.3 A) and increased with sleep restriction in the NAc (6.3 B), while the levels of its metabolite DHPG decreased with sleep restriction in the ACC (6.3 C), and did not significantly change in the NAc (6.3 D). The ratio DHPG/Norepinephrine increase after sleep rebound in the ACC (6.3 E) and did not change in the NAc (6.3 F). One-way ANOVA and Newman-Keuls post hoc test: statistically significant differences are indicated by the symbol “*”. SR = sleep restriction. NE = norepinephrine.

Relative DAT glycosylation and DARPP-32 phosphorylation changes in the Anterior Cingulate Cortex and Nucleus Accumbens after sleep restriction and rebound

The levels of glycosylated-DAT ~80 KDa in the ACC did not change after sleep restriction, but decreased after sleep rebound (Fig. 7 A, One-way ANOVA, $F_{\text{sleep condition}} (2, 23) = 4.173$, $p = 0.028$, SNK test $p < 0.04$), while no significant changes were found in the NAc (Fig. 7 B, One-way ANOVA, $F_{\text{sleep condition}} (2, 21) = 1.306$, $p = 0.292$).

The levels of non-glycosylated-DAT ~50 KDa did not change in the ACC (Fig. 7 C, One-way ANOVA, $F_{\text{sleep condition}} (2, 20) = 0.558$, $p = 0.581$), but significantly increased in the NAc after sleep restriction (Fig. 7 D, One-way ANOVA, $F_{\text{sleep condition}} (2, 19) = 4.658$, $p = 0.023$, SNK test $p < 0.03$).

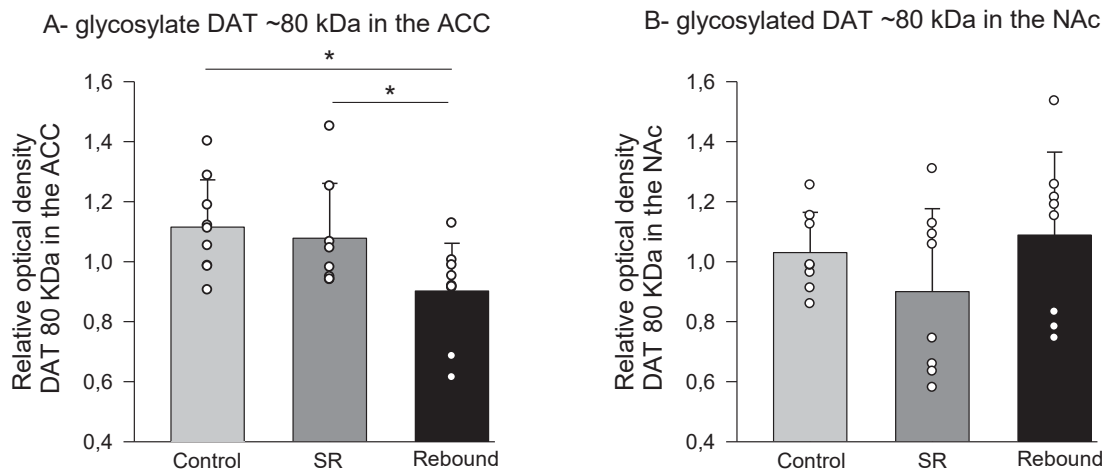


Figure 7 – Relative levels of glycosylated and non-glycosylated DAT in the Anterior Cingulate Cortex and Nucleus Accumbens after sleep restriction and rebound. The level of glycosylated-DAT ~80 KDa decreased after sleep rebound in the ACC (A), while it did not significantly change in the NAc (B). One-way ANOVA and Newman-Keuls post hoc test: statistically significant differences are indicated by the symbol “*”. SR = sleep restriction.

The ratio DAT 80:50 KDa did not change in the ACC (Fig. 7 E, One-way ANOVA, $F_{\text{sleep condition}} (2, 20) = 0.688$, $p = 0.514$), but significantly decreased in the NAc after sleep restriction (Fig. 7 F, One-way ANOVA, $F_{\text{sleep condition}} (2, 19) = 11.075$, $p < 0.001$, SNK test $p < 0.006$).

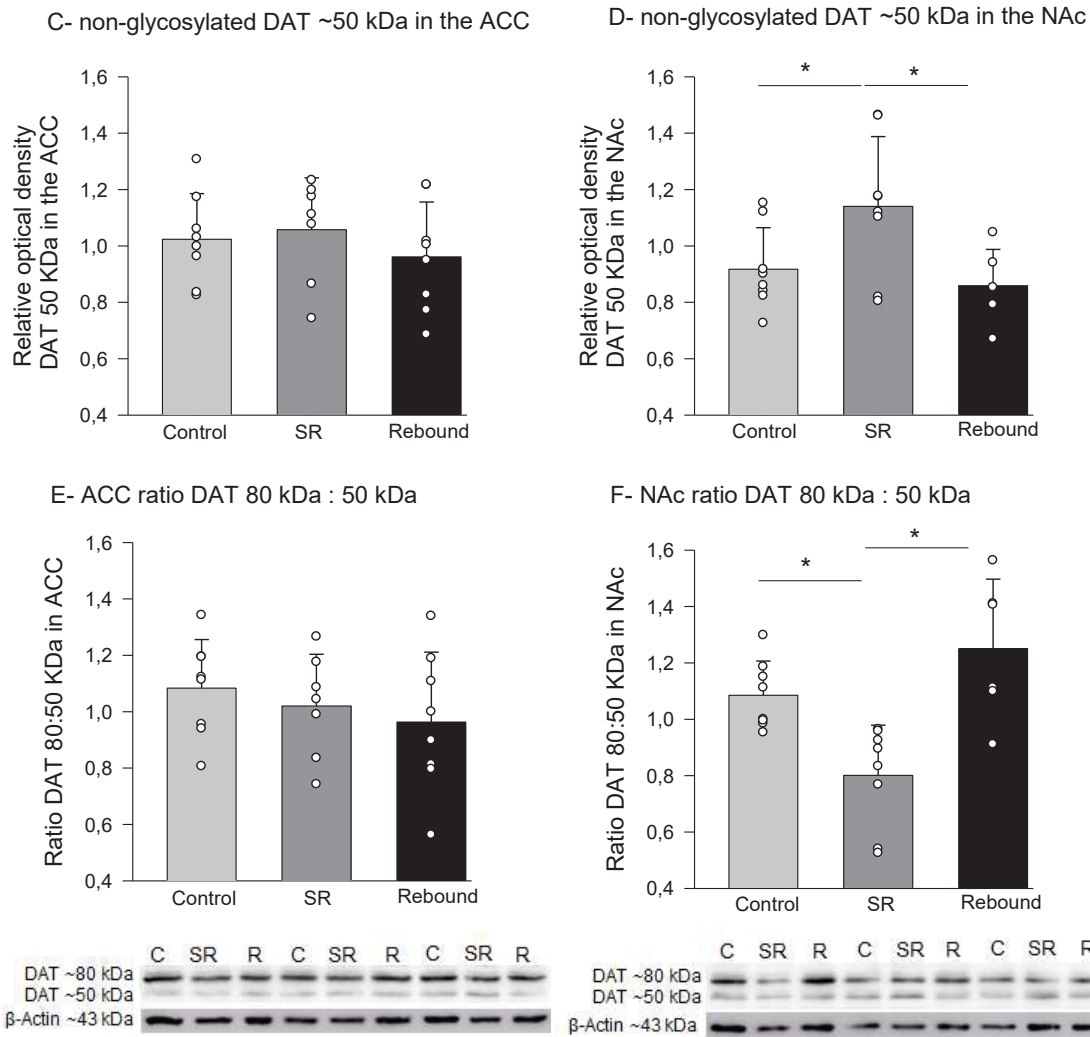


Figure 7 - The level of non-glycosylated-DAT ~50 KDa did not significantly change in the ACC (C), while it significantly increased with sleep restriction in the NAc (D). The ratio DAT 80:50 KDa did not significantly change in the ACC (E), but it was decreased in the NAc after sleep restriction. One-way ANOVA and Newman-Keuls post hoc test: statistically significant differences are indicated by the symbol “*”. Bottom panel: representative figures of western blot gels for detection and quantification of glycosylated (~80 kDa) and non-glycosylated (~50 kDa) DAT and β-actin (~43 kDa) in the ACC and NAc, respectively. C = control; SR = sleep restriction; R = rebound.

The levels of Phospho-Thr 34 DARPP-32 in the ACC did not change after sleep restriction, but significantly increased after sleep rebound (Fig. 8 A, One-way ANOVA, $F_{\text{sleep condition}}(2, 18) = 4.791$, $p = 0.021$, SNK test $p < 0.036$). Very similar findings were found in the NAc (Fig. 8 B, One-way ANOVA, $F_{\text{sleep condition}}(2, 21) = 7.072$, $p = 0.004$, SNK test $p < 0.009$).

The levels of phospho-Thr75-DARPP-32 in the ACC were significantly increased with sleep restriction and sleep rebound (Fig. 8 C, One-way ANOVA, $F_{\text{sleep condition}}(2, 18) = 4.643$, $p = 0.024$, SNK test $p < 0.035$). In the NAc, the levels of phospho-Thr75-DARPP-32 were increased with sleep restriction (Fig. 8 D, One-way ANOVA, $F_{\text{sleep condition}}(2, 20) = 5.613$, $p = 0.012$, SNK test $p < 0.026$).

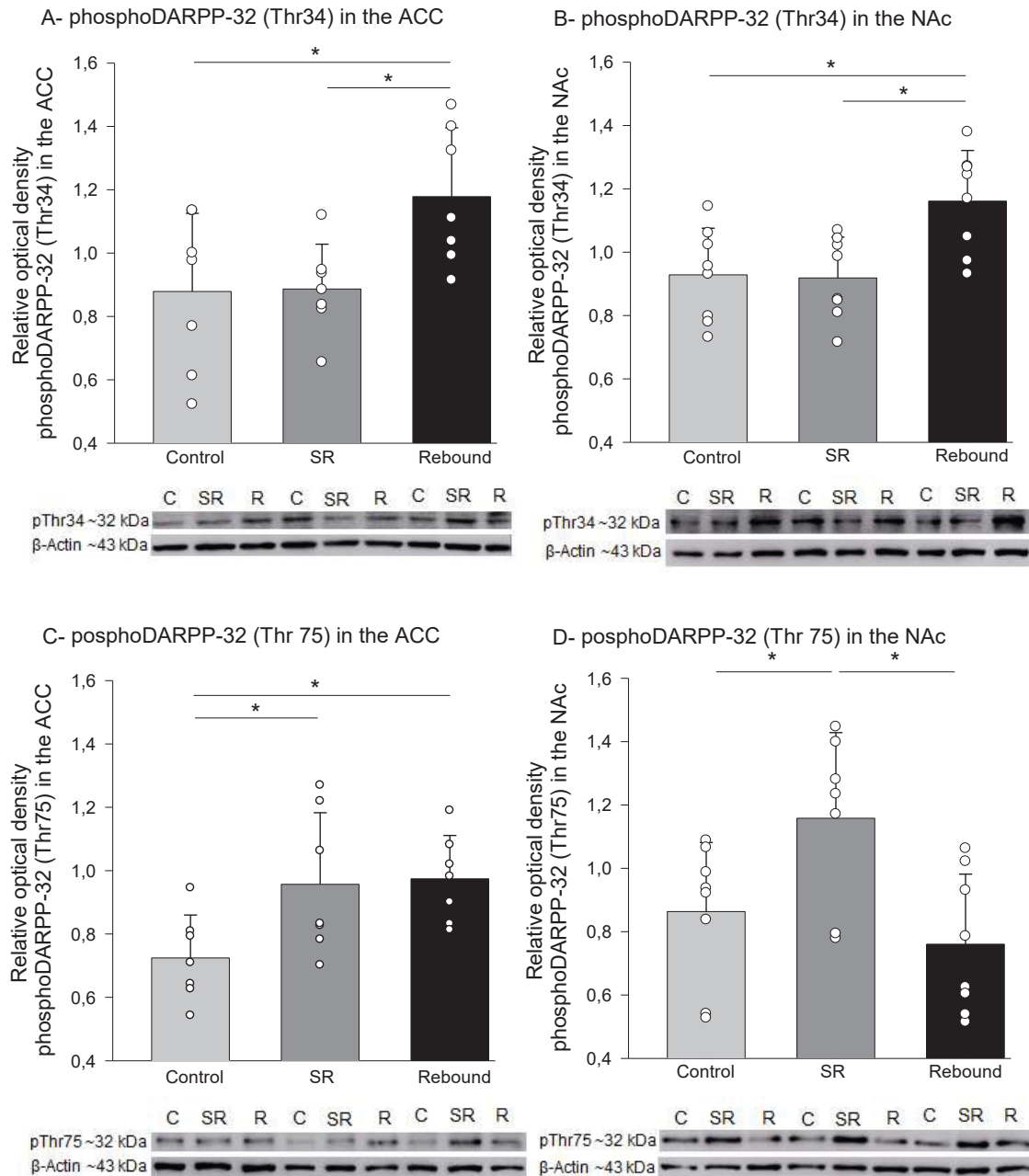


Figure 8 – Relative levels of DARPP-32 phosphorylated at residues thr 34 and 75 in the Anterior Cingulate Cortex and Nucleus Accumbens after sleep restriction and rebound. The levels of phospho-DARPP-32 Thr34 were significantly increased with sleep restriction and rebound in the ACC (A) and in the NAc (B). The level of phosphor-DARPP-32 Thr75 was significantly increased with sleep restriction and sleep rebound in the ACC (C), while it was significantly increased with sleep restriction in the NAc (D). One-way ANOVA and Newman-Keuls post hoc test: statistically significant differences are indicated by the symbol “*”. Bottom panels: representative figures of western blot gels for detection and quantification of phosphor-DARPP-32-Thr34 or Thr75 (~32 kDa) and β-actin (~43 kDa) for ACC and NAc respectively. C = control; SR = sleep restriction; R = rebound.

Discussion

This study demonstrate that sleep restriction increases pain sensitivity in a very similarly way in males and females, without inducing a significant stress response. The pronociceptive effect of sleep restriction depends on the integrity of the ACC, in addition to the NAc, as

previously demonstrated (Sardi et al., 2018a, Sardi et al., 2018b). Pharmacological activation of D₂, but not D₁ dopamine receptors in the ACC or NAc prevents sleep restriction from increasing pain sensitivity. Data on dopaminergic parameters suggest that dopaminergic activity, transport and signaling decrease in the NAc and ACC of sleep deprived animals.

Both clinical pain conditions and sleep disturbances affect females and males differently. Although data on sex differences in sleep architecture and disorders are contradictory (Hajali et al., 2019), the literature clearly indicates that clinical pain conditions are more prevalent and severe in women than in men (Bartley and Fillingim, 2013). Experimental pain is also more intense in females than in males (Mogil, 2020). This increased pain sensitivity appears to result, at least in part, from a less-efficient endogenous pain modulation in females (Bulls et al., 2015, Lenert et al., 2021). Endogenous pain modulation is disrupted by sleep deprivation (Edwards et al., 2009, Tomim et al., 2016) and females seem to be more sensitive to this disruption than males (Bulls et al., 2015, Eichhorn et al., 2018). Therefore, it could be expected that the pronociceptive effect of sleep restriction would be more intense in females, but this was not what we found. Six hours a day of total sleep deprivation over three days similarly increased pain sensitivity in female and male rats. The recovery towards the baseline was also similar, at the end of two days of sleep rebound, pain sensitivity was normalized in both sexes (Fig. 2 A). This is the first time that the pronociceptive effect of persistent (three days) partial restriction of total sleep was compared between the sexes, but some previous studies have used other sleep disruption protocols to investigate this issue in both humans and animals. Most of them support our findings that sleep deficiency affects pain thresholds in females and males in a similar way (Larson and Carter, 2016, Hambrecht-Wiedbusch et al., 2017, Smith et al., 2019). However, other studies have found mixed results, ranging from no difference between the sexes to greater sensitivity in females, depending on experimental conditions (Araujo et al., 2011, Page et al., 2014, Eichhorn et al., 2018). It is important to note that these studies differed largely with respect to their methodology and outcome measures, therefore any sex difference in the effects of sleep loss on pain remains an open question.

Any physiologically significant decrease in sleep time is potentially stressful (Hirotsu et al., 2015), however glucocorticoid excretion was similar between sleep-restricted animals and their controls throughout the experiment (Fig. 2 B). This finding indicates that our protocol of six hours a day of total sleep restriction over three days does not trigger a significant stress response, discarding stress as determinant of the increased pain sensitivity observed in sleep restricted animals.

Consistent literature on both humans and animals demonstrate that ACC is overactivated during pain and this overactivation mediates descending pain facilitation (Xiao et al., 2021). A growing number of studies have also associated this overactivation with sleep impairments resultant from pain (Yamashita et al., 2014, Sun et al., 2020). This study extended the understanding on the function of ACC in the interplay between sleep and pain by showing for the first time that ACC mediates the pronociceptive effect of sleep restriction. The excitotoxic lesion of the ACC prevented the decrease in mechanical nociceptive threshold induced by six hours a day of total sleep deprivation over three days (Fig. 3 A), showing that ACC integrity is essential for sleep restriction to increase pain sensitivity.

ACC and NAc are major targets of the dopaminergic projections arising from the VTA (Yang et al., 2020). The role of dopamine in the control of sleep wake cycle (Oishi and Lazarus, 2017, Scammell et al., 2017) and pain (Mitsi and Zachariou, 2016) has long been recognized. But in recent years, evidence has accumulated to support a contribution of dopaminergic neurotransmission in the increased pain sensitivity that results from sleep disruption (Finan and Smith, 2013, Sardi et al., 2018b, Haack et al., 2020). In the present study we tested whether dopamine D₂ or D₁ receptor agonists administered in the ACC or NAc prevent the pronociceptive effect of partial restriction of total sleep. Pharmacological activation of dopamine D₂ receptors either in the ACC (Fig. 4 A) or in the NAc (Fig. 5 A) prevented the pronociceptive effect of sleep restriction with no effect in control (not sleep restricted) animals. On the other hand, pharmacological activation of dopamine D₁ receptors had little effect. The most striking effect of the D₁ agonist was in the ACC of control animals, it decreased the nociceptive threshold, inducing a pronociceptive effect by itself (Fig. 4 B). In sleep restricted animals, the administration of the D₁ receptor agonist into the ACC slightly increased the pronociceptive effect in the sixth hour. In the NAc, the dopamine D₁ receptor agonist had no effect either in sleep deprived or in control animals (Fig. 5 B). We have previously demonstrated that the intra-NAc administration of a D₂ receptor agonist prevented the pronociceptive effect of REM sleep deprivation, while that of a D₂ receptor antagonist had no effect (Sardi et al., 2018b). The present findings demonstrated that the role of NAc dopamine D₂ receptors in the increased pain sensitivity resultant of sleep impairment is not limited to REM sleep deprivation and extends to partial restriction of total sleep. However, this is the first demonstration that the activation of dopamine D₂ receptors in the ACC prevents the pronociceptive effect of sleep restriction, while that of D₁ receptors either in the ACC or in the NAc had little effect.

Dopamine is a well-known wake-promoting neurotransmitter and its signaling decreases with sleep pressure (Oishi and Lazarus, 2017, Scammell et al., 2017, Menon et al., 2019). However, our findings show that dopamine levels in the ACC and NAc were not significantly affected by sleep restriction (Fig. 6.1 A and B). In contrast, the levels of DOPAC, the major intraneuronal dopamine metabolite, as well as the DOPAC/dopamine ratio were decreased in the NAc and ACC of sleep restricted animals (Fig. 6.1 C, D, G and H). A decrease in DOPAC associated with the maintenance of dopamine levels may reflect a decrease in dopaminergic activity in consequence of sleep restriction. In fact, the DOPAC/dopamine ratio has been used as an index of dopaminergic neuronal activity (Storch et al., 2013, Del-Bel et al., 2014) thus, its decrease suggest that dopamine neurons in the NAc and ACC of sleep restricted animals are less active. Complementary, the expression of non-glycosylated-DAT (~50 kDa) increased and the ratio glycosylated/non-glycosylated DAT decreased in the NAc after sleep restriction (Fig. 7 D and F, respectively). These findings indicate that sleep restriction reduces the efficiency of dopamine reuptake, because the lower the level of DAT glycosylation, the lower the efficiency of dopamine reuptake (Li et al., 2004). A decreased in DAT glycosylation and dopamine transport is compatible with decreased dopaminergic neuronal activity and may contribute to explain the decreased DOPAC levels. Additional support to a decrease in dopaminergic neurotransmission is given by data showing that the phosphorylation of DARPP-32 at Thr-75 increases after sleep deprivation in both NAc and ACC (Fig. 8). Phospho-Thr 75 DARPP-32 act as a PKA inhibitor, reducing the efficacy of dopaminergic signaling (Nishi et al., 2000, Walaas et al., 2011) and its levels are increased during low dopaminergic states (Nishi et al., 2000, Greengard, 2001, Brown et al., 2005, Santini et al., 2007, Yamashita et al., 2014).

Overall cortical monoaminergic neurotransmission is increased during wake and decreased during sleep (Menon et al., 2019) (with an important exception for REM sleep, as discussed below). However, sleep restriction associates high sleep pressure with extended watch. Sleep pressure decreases alertness and cortical monoaminergic function, however, forced wakefulness, in animals under sleep restriction, recruits monoaminergic function. The conflicting action of forced wakefulness and sleep pressure on monoamine levels may prevent their significant decrease in the ACC during sleep restriction (Fig. 6.1 A, 6.2 A and 6.3 A). But once the animals are free to sleep, during sleep rebound period, the levels of dopamine, 5-HT and norepinephrine in the ACC fall (Fig. 6.1 A, 6.2 A and 6.3 A). In fact, the metabolism of all the three monoamines is increased during sleep rebound in the ACC (Fig. 6.1 G, I, 6.2 E and 6.3 E), suggesting an attempt to compensate their deficits (Asikainen et al., 1995, Storch et al., 2013, Del-Bel et al., 2014). Unlike the cortex, NAc activity increases with sleep pressure (Oishi

et al., 2017b, Sardi et al., 2018a, Zhou et al., 2019) and the increased levels of norepinephrine and 5-HT found in the NAc of sleep-restricted animals (Fig. 6.2 B and 6.3 B) may contribute to this increase (Barnes and Sharp, 1999, Mallick and Singh, 2011). However, unlike other monoamines, dopamine levels do not increase and its activity appears to decrease in the NAc of sleep-restricted animals. This could result from increased adenosinergic activity, which is characteristic of sleep pressure (Lazarus et al., 2019) and antagonizes dopamine activity in the NAc (Oishi et al., 2017b, Zhou et al., 2019).

During the so-called sleep rebound period, at the end of the three-day partial sleep restriction period, the animals are free to sleep and the changes induced by sleep restriction are expected to be reversed. Our data indicate that the aforementioned decrease in dopamine levels after sleep rebound are associated with a decrease in DAT glycosylation in the ACC (Fig. 7 A) and with an increase in phospho-Thr 34 DARPP-32 both in the ACC and NAc (Fig. 8 A and B). The decrease in DAT glycosylation indicates a decrease of dopamine reuptake (Li et al., 2004) and probably results from the low dopamine levels, since DAT activity is regulated by extracellular dopamine levels (Ikawa et al., 1993, Salvatore et al., 2003, Zahniser and Sorkin, 2004). However, the increase in phospho-Thr 34 DARPP-32 indicates an increase in dopaminergic signaling (Nishi et al., 2000, Walaas et al., 2011). Phosphorylation of DARPP-32 at Thr 34 is regulated by PKA, which activity increases with dopamine signaling (Nishi et al., 2000, Walaas et al., 2011). In a positive feedback loop, phospho-Thr 34 DARPP-32 potentializes dopaminergic signaling by inhibiting PP1 (Nishi et al., 2000, Walaas et al., 2011). This apparent paradox between decreased dopamine levels and increased dopamine signaling may be explained by the changes in sleep architecture during the rebound period. Evidently, total sleep time increases during the rebound period, but this increase is more pronounced for REM sleep with a more modest increase in non-REM sleep (Borbely et al., 1984, Feinberg and Campbell, 1993, Kim et al., 2007, Stephenson et al., 2015). While monoamine levels are low during sleep, dopaminergic activity increases specifically during REM sleep (Menon et al., 2019, Van Erum et al., 2019). Therefore, the increase in total sleep time, with a proportionally greater increase in REM sleep may help to explain why dopaminergic signaling increases during sleep rebound.

Taken together, our data contribute to advance the understand of the mechanisms by which sleep deficiency affects pain. Dopamine has long been implicated in the control of sleep wake cycle (Oishi and Lazarus, 2017, Scammell et al., 2017) and pain (Mitsi and Zachariou, 2016), however our understanding on its role in the interplay between sleep and pain is far from being satisfactory. Changes in the *in vivo* activity pattern of mesocorticolimbic dopaminergic

projections during sleep restriction has not been studied, but here we presented some indirect evidence to suggest that sleep restriction decreases dopaminergic neurotransmission in the ACC and NAc. This is in line with previous studies suggesting that sleep restriction decreases neuronal activity in the VTA (Fifel et al., 2018) as well as striato-cortical dopaminergic transmission (Brock et al., 1995, Nijima et al., 2010, Volkow et al., 2012, Sardi et al., 2018b, Haack et al., 2020). Our finding that pharmacological activation of D₂, but not D₁ receptors prevents the pronociceptive effect of sleep restriction, without affecting nociceptive responses in control animal, suggest that D₂ signaling in the ACC and NAc contributes for sleep restriction to increase pain sensitivity. Although dopaminergic neurotransmission can either increase or decrease neural activity, depending on the brain region studied (Beaulieu and Gainetdinov, 2011), evidence indicate that dopamine at basal tonic levels, acts on high-affinity D₂ receptors to inhibit neural function in the ACC and NAc (Richfield et al., 1989, Dreyer et al., 2010, Darvish-Ghane et al., 2016, Hunger et al., 2020). Therefore, by decreasing dopaminergic neurotransmission, sleep restriction may disinhibit ACC and NAc, increasing their neural activity. Indeed, increased neural activity in ACC and NAc increases pain (Calejesan et al., 2000, Schmidt et al., 2002b, Gao et al., 2004, Apkarian et al., 2005, Kuo and Yen, 2005, Becerra and Borsook, 2008, Wang et al., 2009, Baliki et al., 2010, Gear and Levine, 2011, Narita et al., 2011, Becerra et al., 2013, Yamashita et al., 2014, Vatthauer et al., 2015, Wang et al., 2015, Sardi et al., 2018a, Sardi et al., 2018b, Sun et al., 2020, Xiao et al., 2021) and activation of D₂ receptors in these regions decreases pain (Altier and Stewart, 1998, 1999, Schmidt et al., 2002a, Schmidt et al., 2002b, Taylor et al., 2003, Haghparast et al., 2012, Sogabe et al., 2013, Dias et al., 2015, Sardi et al., 2018b, Liu et al., 2020, Sardari et al., 2021).

Persistent pain conditions and sleep disturbances are public health problems around the world, and the understand of the mechanisms by which sleep disruption increases pain will contribute to pain management and prevention. This study supports that despite the greater prevalence and severity of pain in females than in males, sleep restriction increases pain sensitivity similarly in both sexes. Although evidence has accumulated in the past few years to support a role of dopamine in the pronociceptive effect of sleep restriction (Finan and Smith, 2013, Alexandre et al., 2017, Sardi et al., 2018b, Haack et al., 2020), the mechanisms are still poorly understood. The present study contributes to add some new pieces to this puzzle. We focus on the ACC and NAc, which are targets of mesodopaminergic projections (Yang et al., 2020) with a key role in pain modulation (Gear and Levine, 2011, Mitsi and Zachariou, 2016, Yang et al., 2020, Xiao et al., 2021). Our findings suggest that a decrease in dopaminergic neurotransmission in the ACC and NAc mediates the pronociceptive effect of sleep restriction.

Future studies should be conducted to determine whether sleep restriction decreases D₂ dopaminergic signaling, increasing neural activity in ACC and NAc to increase pain sensitivity.

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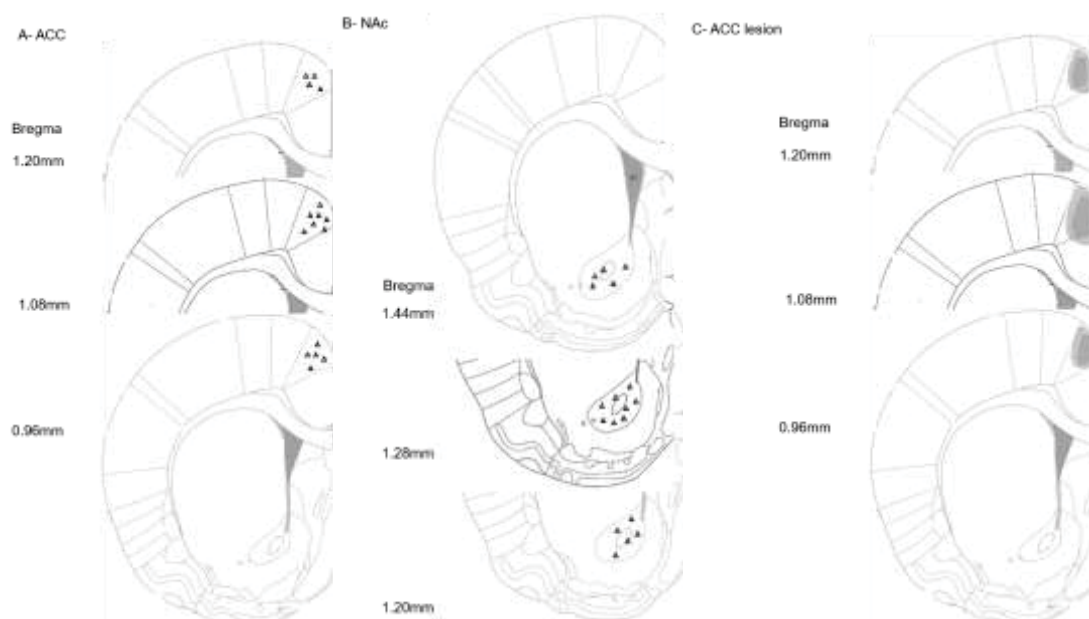
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Supplementary material

Supplementary figure:



Supplementary Figure 1- Representative figures of microinjections in the Anterior Cingulate Cortex and Nucleus Accumbens and extension of excitotoxic lesion of the Anterior Cingulate Cortex. **A** and **B**-representative figures of microinjections in the ACC and NAc. The triangles represent the microinjection sites. **C**- Representative figure of lesion extent in the ACC by local administration of NMDA. The light gray shading represents the greater spread of the lesion and the dark gray shading represents the lesion concentration. The images were adapted from the atlas by Paxinos and Watson (2007).

CAPÍTULO 4

PAPEL DOS NÚCLEOS MONOAMINÉRGICOS DO TRONCO CEREBRAL NO EFEITO PRÓ-NOCICEPTIVO DA RESTRIÇÃO DE SONO

Objetivo geral

Investigar o envolvimento de áreas com conhecido papel na modulação da dor e do ciclo sono-vigília no efeito pró-nociceptivo induzido pela restrição de sono em ratos.

Objetivos específicos

- Testar se a inibição GABAérgica da area tegmentar ventral (VTA), do núcleo dorsal da rafe (DRN), do locus coeruleus (LC) e do hipotálamo lateral previne o efeito pró-nociceptivo da restrição de sono e, em caso afirmativo,
- Investigar como a manipulação monoaminérgica no córtex cingulado anterior (ACC) afeta o efeito pró-nociceptivo induzido pela restrição de sono.
- Estimar indiretamente a atividade neuronal no ACC, no núcleo accumbens e nas regiões monoaminérgicas após restrição de sono, após rebote de sono e em animais controles.

Apresentação:

Artigo científico 4: **Role of monoaminergic brainstem nuclei in the pronociceptive effect of sleep restriction**

Role of monoaminergic brainstem nuclei in the pronociceptive effect of sleep restriction

Abstract

Acute or chronic sleep restriction increases pain sensitivity, but the mechanisms are poorly understood. We recently demonstrated the importance of the Nucleus Accumbens (NAc) and Anterior Cingulate Cortex (ACC) for the pronociceptive effect of sleep restriction. In this study we tested whether the GABAergic inhibition of brainstem monoaminergic nuclei prevents the pronociceptive effect of sleep restriction and if so, how monoaminergic manipulation of the ACC affects such effect. Neuronal activation was indirectly estimated by c-Fos expression. The findings demonstrate that sleep restriction increased pain sensitivity, without inducing a significant stress response. This pronociceptive effect of sleep restriction depends on the A_{2A} receptors in the NAc, as we have previously demonstrated in a REM (rapid eye movement)-sleep deprivation model. The pharmacological blockade of GABAA receptors located in the Ventral Tegmental Area (VTA), Dorsal Raphe nuclei (DRN) or Locus Coeruleus (LC), but not in the lateral hypothalamus, prevented the pronociceptive effect of sleep restriction. In contrast, GABAergic blockade of each of these nuclei only transiently decreased carrageenan-induced hyperalgesia. The pharmacological activation of dopamine D₂, serotonin 5-HT_{1A} and norepinephrine alpha-2 receptors located in the ACC also prevented the pronociceptive effect of sleep restriction, while their pharmacological blockade rescued the pronociceptive effect that had been blocked by GABAergic blockade of the VTA, LC or DRN. Data on c-Fos expression suggest that sleep restriction increases neural activity in the NAc and ACC, but not in the VTA, LC and DRN. Overall, the data support that the pronociceptive effect of sleep restriction depends on increased adenosinergic activity in the NAc and GABAergic activity in the VTA, DRN and LC and on decreased inhibitory monoaminergic activity in the ACC.

Keywords: sleep loss, sleep quality, sleep poor, pain, pronociceptive, hyperalgesia, analgesia, nucleus accumbens, adenosine, GABA, dopamine, norepinephrine, serotonin, anterior cingulate cortex, sleep and pain.

Introduction

Sleep disorders and persistent pain conditions are public health problems around the world (Artner et al., 2013, Murphy et al., 2017, Mathias et al., 2018) and there is a clear, but still poorly understood relationship between them (Okifuji and Hare, 2011, Karaman et al., 2014). Sleep impairment increases the risk of developing pain conditions while pain decreases sleep quality (Karaman et al., 2014, Kim et al., 2015, Sribastav et al., 2017, Haack et al., 2020). This study was designed to contribute to understand how sleep restriction increases pain sensitivity. Adequate knowledge of the mechanisms by which sleep impairment increases pain is essential to encourage the development of new drugs and behavioral interventions that would impact pain prevention and management.

There seems to be a coincidence, at least in part, between the mechanisms for inducing sleep and those for increasing pain sensitivity in response to sleep impairment. For example, the nucleus accumbens (NAc), in the ventral striatum has long been implicated in pain modulation (Gear and Levine, 2011, Tobaldini et al., 2018b, Vergara et al., 2020) and more recently in sleep-wake cycle control (Lazarus et al., 2012, Oishi and Lazarus, 2017). As sleep pressure increases, the endogenous somnogen adenosine accumulates in the in the basal forebrain and NAc has emerged as a key site for adenosine to induce sleep (Lazarus et al., 2011, Oishi and Lazarus, 2017). Adenosinergic activity at A_{2A} receptors activates a subset of GABAergic NAc projections to inhibit wake-promoting nuclei in the basal forebrain and brainstem, inducing sleep (Oishi and Lazarus, 2017, Oishi et al., 2017b). Recently, we have demonstrated that NAc integrity is essential for sleep deprivation to increase pain sensitivity (Sardi et al., 2018a, Sardi et al., 2018b) and that an increase in adenosinergic A_{2A} activity in the NAc mediates the pronociceptive effect of 24 h rapid eye movement (REM) sleep deprivation (Sardi et al., 2018b). However, whether NAc A_{2A} receptors also mediate the pronociceptive effect of persistent partial restriction of total sleep is not known.

The monoaminergic brainstem nuclei receive GABAergic projections from the NAc A_{2A} -expressing neurons (Zhang et al., 2013). Dopamine, 5-hydroxytryptamine (5-HT) and norepinephrine neurons, originating respectively from the Ventral tegmental area (VTA), dorsal raphe nucleus (DRN) and locus coeruleus (LC) make up most of the monoaminergic system, which have prominent role in sleep-wake cycle (Scammell et al., 2017) and pain modulation (Wang and Nakai, 1994, Baliki and Apkarian, 2015, Bannister and Dickenson, 2016, Bravo et al., 2016, Haack et al., 2020). However, whether the GABAergic inhibition of VTA, DRN and LC contribute to the pronociceptive effect of sleep restriction has never been tested.

The ascending projections of the monoaminergic systems target diffusely cortical regions and some basal forebrain nuclei (Matsuzaki et al., 1993, Delfs et al., 1998, Ikemoto, 2007, Zhang et al., 2013, Beliveau et al., 2015, Scammell et al., 2017). Among other modulatory functions, the monoaminergic projections to the cortex have a well-known role in sleep wake cycle (Oishi and Lazarus, 2017, Scammell et al., 2017) and a less understood but, crucial, role in pain modulation (Haack et al., 2020). The Anterior Cingulate Cortex (ACC) is one of the cortical areas densely innervated by monoaminergic projections (Chandler et al., 2013) and plays a prominent role in pain modulation (Xiao et al., 2021). We have previously demonstrated that its integrity is essential for sleep restriction to increase pain sensitivity (Sardi et al., 2022a; unpublished data), but if monoaminergic mechanisms in the ACC underlie the pronociceptive effect of sleep restriction has never been tested.

Therefore, in this study we used pharmacological approaches to investigate whether the GABAergic inhibition of VTA, DRN or LC prevents the pronociceptive effect of sleep restriction and if so, how monoaminergic manipulation of the ACC affect such effect. Neuronal activation in the ACC, NAc and monoaminergic nuclei was indirectly estimated by c-Fos expression.

Methods

Animals

Experiments were performed in male Wistar rats $\pm$ 60 days old, weighing between 270-330 g, housed 4 per cage, in a temperature-controlled ($22^{\circ}\text{C} \pm 1$) in an environment with luminosity control (12 hours light / dark cycles) with food and water, ad libitum. All experimental procedures were approved by the Committee on Animal Research of the Federal University of Parana, Brazil, under protocol # 1126 and followed the guidelines proposed by the IASP (International Association for the Study of Pain) committee for research in conscious animals and the ARRIVE guideline (Percie du Sert et al., 2020). In particular, the duration of the experiments was as short as possible, the nociceptive stimulus and the number of animals were kept to the minimum necessary to allow statistical analysis.

Drugs

Adenosinergic antagonist for the A_{2A} receptor, SCH-58260 7 ng (Sardi et al., 2018b). Dopaminergic agonist and antagonist for the D_2 receptor, piribedil 6 μg and raclopride 5 μg (Sardi et al., 2018b), respectively. GABA $_A$ receptor antagonist, bicuculline 20 ng (Chen et al., 2016). Noradrenaline alpha-2 receptor agonist, clonidine 4 μg (Zuo et al., 2015) and antagonist, idazoxan 9 μg (Ortiz et al., 2007). Serotonin 5-HT $_{1A}$ receptor agonist, 8-OH-DPAT 0.1 μg (Hui

et al., 2015) and antagonist, WAY-100135 1 μ g (Sardari et al., 2015). All drugs were purchased from Sigma -Aldrich, São Paulo, SP, Brazil, except WAY-100135 which was acquired from Tocris, São Paulo, SP, Brazil. All drugs were diluted in 0.9% sterile saline.

Stereotactic surgery and drug infusion

The animals were anesthetized by intraperitoneal injection of xylazine (10 mg / kg) and ketamine (60 mg / kg) and then a 25-gauge stainless steel cannula was positioned according to the stereotactic plan established so that its lower extremity located 2 mm above the site of interest. The cannulas were fixed with acrylic resin in the following region. From bregma, NAc in the rostro-caudal direction +1.30, dorso-ventral -6.20 mm and latero-lateral $\pm$ 1.80 mm (Paxinos and Watson, 2007, Sardi et al., 2018a, Sardi et al., 2018b); VTA, rostro-caudal - 4.8 mm; dorso-ventral -8.2 mm and latero-lateral $\pm$ 1 mm (Paxinos and Watson, 2007, Vergara et al., 2020); ACC, rostro-caudal +1 mm, latero-lateral $\pm$ 0.6 mm, dorso-ventral -1.4 mm (Paxinos and Watson, 2007), LH, rostro-caudal -3.2 mm, latero-lateral $\pm$ 1.6 mm, dorso-ventral -8.8 mm (Paxinos and Watson, 2007). From the interaural line, LC, rostro-caudal -0.8 mm, dorso-ventral + 5.1 and latero-lateral $\pm$ 1.4 mm (Paxinos and Watson, 2007), for all regions above incisor bar -3.3mm. From lambda, DRN, 28° angle, incisor bar -2.5mm, rostro-caudal +2 mm, latero-lateral -3 mm and dorso-ventral -4.3 mm (Paxinos and Watson, 2007).

After surgery, the animals received dipyrone (50 mg / kg) and enrofloxacin (0.5 mg / kg). The animals were submitted to the experiment seven days after implantation of the cannulas. The injection volume was 0.25 μ L in all experiments. The drugs were administered slowly by mechanical infusion pump (model KDS-100 KD Scientific Holliston, MA, USA) coupled injection cannula was connected to a PE-10 polyethylene tube and also to a 5- μ L Hamilton syringe over a period of one minute. After the injection, the needles remained in place for 30 seconds to prevent retrograde flow of the drug.

In all experiments, the mapping was carried out in order to confirm the injection site. For this, the animals will receive inhalation anesthesia (isoflurane) and were perfused transcardiacally with 0.9% saline followed by 4% formalin solution. The injection sites were verified by injection of Evans Blue (1%, 0.25 μ l), then the brains were removed and stored in 4% formaldehyde. Coronal 50 μ m sections were performed to determine the location of the Evans marking. Injection sites were mapped according to the Atlas of Paxinos and Watson (Paxinos and Watson, 2007).

Sleep restriction enriched gentle handling method

The rats were submitted to sleep restriction for a period of 6 hours a day for 3 days (see figure 1) using the enriched gentle handling method (Alexandre et al., 2017, Sardi et al., 2018a).

Briefly, the method consists of using gentle manipulation to prevent the animal from settling to sleep. To keep the animals awake we can touch the animals with a soft bristle brush, introduce small objects into the experimentation box so that the animals are distracted by these objects while remaining alert and consequently awake, move the experimentation box gently in a seesaw movement as well is included in this method (Alexandre et al., 2017, Sardi et al., 2018a). During sleep restriction the animals will be kept in their usual cages with wood shavings and will have water and food ad libitum. Control groups will be kept in the same condition, but will be free to sleep.

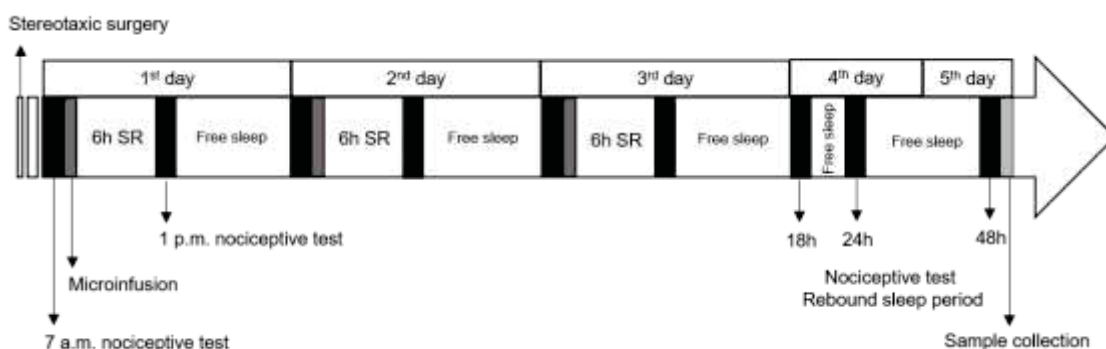


Figure 1 – Experimental design. Stereotaxic surgery for implantation of the guide cannula was performed 7 days before the beginning of the experiments. The baseline mechanical nociceptive paw withdrawal threshold was measured and sleep restriction was initiated (7 a.m.). Microinfusions were performed daily at the beginning of the 6 h sleep restriction period. The nociceptive test was repeated daily, before (7 a.m.) and after (1 p.m.) sleep restriction. After three days with six hours of total sleep deprivation daily, the animals were allowed to sleep freely during the two-day sleep rebound period. The mechanical nociceptive paw withdrawal threshold was measured at different time points in the sleep rebound period. The control groups are submitted to the same procedures, however, they are free to sleep. SR = sleep restriction.

Nociceptive mechanical threshold test - Randall-Selitto test

The nociceptive behavioral test was performed before and immediately after the end of the sleep restriction procedure on the three days of sleep restriction and at different times during the sleep rebound period, always during the light phase (at 7:00 am and 1:00 pm, see Figure 1). Before the experiments, each animal was handled for 7 days to get used to the experimental handling. The rats did not have access to food or water during the test and each animal was used only once.

The Randall–Selitto test (Randall and Selitto, 1957) was used to assess the nociceptive mechanical paw withdrawal threshold, as a measure of the nociceptive response. For the test we used a mechanical analgesimeter (Insight, Brazil), which applies a mechanical force in grams of gradual increase in the dorsal surface of the rats' paw. When the animal exhibits the paw withdrawal behavior, the applied force is immediately interrupted, this value in grams is recorded and used as a measure of the mechanical nociceptive threshold. The value was

obtained from the mean of three readings made at intervals of 3 min. Data were expressed as mean $\pm$ standard error of the mean (SEM) of the mechanical paw withdrawal threshold (g) at each time point.

Immunohistochemistry sample preparation

The rats were anesthetized by an intra-peritoneal injection of xylazine (10 mg / kg) and ketamine (60 mg / kg) and transcardially perfused with saline followed by 4 % paraformaldehyde both in 0.1-M phosphate buffer, pH 7.4. Brains were removed and immersed in paraformaldehyde (4 °C for a week), in 30 % sucrose solution (another week) and stored at -80°C until sectioning. Four sections (30 μm), were sliced per animal.

Immunohistochemistry

The c-Fos protein is expressed in neurons stimulated in response to elevated intracellular calcium (Lerea et al., 1992, Coderre et al., 1993). Therefore, we quantified the expression of c-Fos in an attempt to indirectly estimate the effect of sleep restriction on neuronal activation of ACC, NAc, VTA, LC and DRN. The tissue sections will be incubated overnight at 4 °C with primary rabbit anti-c-Fos antibody (#PC38; Chemicon, Temecula, CA; 1:10.000 in PBS and 0.3% Triton X-100) and then incubated with the secondary antibody conjugated with biotin (#PK4001; Vector Laboratories, Burlingame, CA; 1:200) for 2 hours at room temperature. After several washes with PBS, the antibody complex was located using the ABC system (#PK4001; Vectastain ABC Elite kit, Vector Laboratories, Burlingame, CA). To reveal the antigen-antibody complex, the sections were incubated in a solution containing diaminobenzidine (DAB, 0.05%) and H₂O₂ (0.015%) in PBS, pH 7.4, for 5-10 minutes. The reaction was stopped by a minimum of three washes in PBS. Finally, the free-floating sections were mounted on gelatinized glass slides, air-dried, dehydrated in a series of alcohols, cleared in xylene, and placed under a coverslip with entellan® and analyzed under a microscope.

Quantification of c-Fos immunoreactive cells

The slices were digitized with a microscope scanner (Axio Imager Z2, Carl Zeiss, Jena, DE) coupled to an imaging system (Metasystems, Altlussheim, DE). Quantification of c-Fos immunoreactive cells in the ACC, NAc, VTA, DRN and LC was performed automatically by optical density using ImageJ 1.53k (Public Domain) image analysis software.

Fecal glucocorticoid metabolites

To determine the impact of sleep restriction on the stress response, we measured glucocorticoid metabolites in feces, a noninvasive approach that allows monitoring of persistent stressful conditions without the additional stress of blood collection. The measurement of fecal glucocorticoid metabolites reflects the overall plasmatic hormone fluctuation of the past 24

hours, minimizing the impact of circadian changes in hormone levels (Bamberg et al., 2001, Pihl and Hau, 2003, Muller et al., 2013).

Feces were collected directly from the animals' cages at different points (0, 24, 48, 54, 72, 78 and 96h) over time during the experiment and kept at -20°C until analysis. Steroid extraction was performed as previously described (Muller et al., 2013). Briefly, an aliquot of 0.5 g of thawed, well-mixed, and dry feces was extracted in 5mL of 90% ethanol (vol:vol; ultrapure water), by shaking for 30 minutes (multipulse vortexer; Glas-Col, Terre Haute, IN, USA). After centrifuging (1000 g for 15 minutes), the supernatant was recovered and stored at -20°C. Fecal extracts were analyzed for fecal glucocorticoid metabolites by enzyme immunoassay (Watson et al., 2013). We used the antibody against corticosterone (CJM006; 1: 15,000) and the corticosterone horseradish peroxidase conjugate (1: 40,000), obtained from Coralie Munro at the University of California, Davis (Davis, CA, USA). The intra- and inter-assay coefficients of variation were less than 5%. The data were corrected for the dilution factor and are expressed as ng/g of dry feces.

Statistical analysis

The nociceptive behavior and fecal glucocorticoid collected data were evaluated by ANOVA of repeated measures, with dependent measures include nociceptive behavior (time), and independent measures include different treatments and sleep conditions. c-Fos data were analyzed by One-way ANOVA. All post hoc contrasts, when appropriate, were performed using Student-Newman-Keuls (SNK) test. The level for statistical significance was $p < 0.05$. STATISTICA® software (StatSoft, Tulsa, OK, USA) was used to perform data analysis. SigmaPlot® software (Systat Software, San Jose, CA, USA) was used to perform graphical representation. Data are plotted in figures as mean $\pm$ SEM.

Results

A_{2A} receptors in the Nucleus Accumbens mediates the pronociceptive effect of partial restriction of total sleep

We have previously demonstrated that adenosine A_{2A} receptors in the NAc mediate the pronociceptive effect of 24 h REM sleep deprivation (Sardi et al., 2018b). In this study we asked whether these receptors also mediate the pronociceptive effect of persistent partial restriction of total sleep. Six hours a day of total sleep deprivation over three days progressively lowers the mechanical nociceptive threshold. The blockade of adenosine A_{2A} receptors by its selective antagonist, SCH-58261, microinjected into the NAc prevented the pronociceptive effect of sleep restriction (Fig. 2). In control animals (not sleep restricted), the A_{2A} receptor

antagonist did not affect the basal nociceptive threshold (Figure 2 – ANOVA with repeated measures – $F_{\text{sleep condition}} (1, 22) = 340.94, p < 0.0001$; $F_{\text{Treatment}} (1, 22) = 308.64, p < 0.0001$; $F_{\text{Sleep condition} \times \text{Treatment}} (1, 22) = 369.73, p < 0.0001$; $F_{\text{Time}} (7, 154) = 106.94, p < 0.0001$; $F_{\text{Time} \times \text{Sleep condition}} (7, 154) = 92.516, p < 0.0001$; $F_{\text{Time} \times \text{Treatment}} (7, 154) = 87.832, p < 0.0001$; $F_{\text{Time} \times \text{Sleep condition} \times \text{Treatment}} (7, 154) = 83.581, p < 0.0001$; post-hoc SNK test $p < 0.001$). These findings demonstrated that the activation of A_{2A} receptors in the NAc is necessary for sleep restriction to increase pain sensitivity.

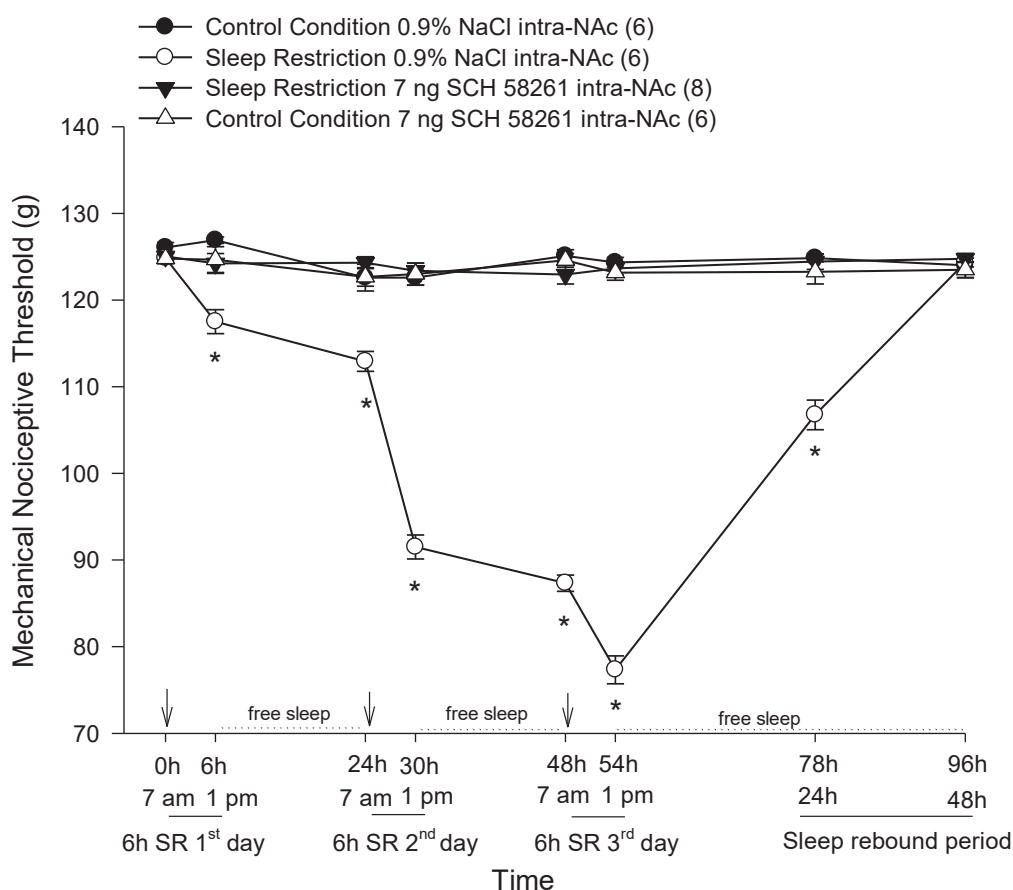


Figure 2 – A_{2A} receptors in the NAc mediates the pronociceptive effect of persistent partial restriction of total sleep. The blockade of adenosine A_{2A} receptors by its selective antagonist, SCH-58261, microinjected into the NAc prevented the pronociceptive effect of sleep restriction. Two way RM ANOVA and Newman-Keuls post hoc test: the symbol, “*” indicates a statistically significant decrease in mechanical nociceptive threshold. In this and subsequent experiments animals were prevented from sleeping during six hours a day (from 7 a.m. to 1 p.m.) over three days. After that, they were able to sleep freely for two days when the experiment ended. Microinjections were performed daily, before sleep restriction, as indicated by the arrows. Dotted line indicates free sleep period. Numbers in parenthesis indicate the number of animals in each group. See methods for additional details regarding data presentation and analysis. SR = Sleep restriction.

The blockade of $GABA_A$ receptors located in the brainstem monoaminergic nuclei prevents the pronociceptive effect of sleep restriction

It is known that the monoaminergic nuclei of the brainstem receive $GABA_A$ ergic projections from NAc A_{2A} expressing neurons (Zhang et al., 2013). In this study we asked

whether the blockade of GABA_A receptors in these nuclei prevents the pronociceptive effect of total sleep restriction.

The selective GABA_A receptor antagonist, bicuculline, microinjected into the VTA (Figure 3 A – ANOVA with repeated measures – $F_{\text{sleep condition}} (1, 18) = 92.778, p < 0.0001$; $F_{\text{Treatment}} (1, 18) = 21.827, p < 0.0001$; $F_{\text{Sleep condition} \times \text{Treatment}} (1, 18) = 64.766, p < 0.0001$; $F_{\text{Time}} (7, 126) = 29.148, p < 0.0001$; $F_{\text{Time} \times \text{Sleep condition}} (7, 126) = 29.195, p < 0.0001$; $F_{\text{Time} \times \text{Treatment}} (7, 126) = 16.174, p < 0.0001$; $F_{\text{Time} \times \text{Sleep condition} \times \text{Treatment}} (7, 126) = 17.803, p < 0.0001$; post-hoc SNK test $p < 0.05$); DRN (Figure 3 B – ANOVA with repeated measures – $F_{\text{sleep condition}} (1, 16) = 419.30, p < 0.0001$; $F_{\text{Treatment}} (1, 16) = 361.06, p < 0.0001$; $F_{\text{Sleep condition} \times \text{Treatment}} (1, 16) = 427.91, p < 0.0001$; $F_{\text{Time}} (8, 128) = 39.120, p < 0.0001$; $F_{\text{Time} \times \text{Sleep condition}} (8, 128) = 38.438, p < 0.0001$; $F_{\text{Time} \times \text{Treatment}} (8, 128) = 28.221, p < 0.0001$; $F_{\text{Time} \times \text{Sleep condition} \times \text{Treatment}} (8, 128) = 30.831, p < 0.0001$; SNK test $p < 0.05$) and LC (Figure 3 C – ANOVA with repeated measures – $F_{\text{sleep condition}} (1, 20) = 293.09, p < 0.0001$; $F_{\text{Treatment}} (1, 20) = 146.72, p < 0.0001$; $F_{\text{Sleep condition} \times \text{Treatment}} (1, 20) = 326.77, p < 0.0001$; $F_{\text{Time}} (7, 140) = 40.675, p < 0.0001$; $F_{\text{Time} \times \text{Sleep condition}} (7, 140) = 45.115, p < 0.0001$; $F_{\text{Time} \times \text{Treatment}} (7, 140) = 38.228, p < 0.0001$; $F_{\text{Time} \times \text{Sleep condition} \times \text{Treatment}} (7, 140) = 41.456, p < 0.0001$; SNK test $p < 0.05$) prevented the pronociceptive effect of sleep restriction, with no effect in control (not sleep restricted) animals. Indeed, sleep restricted animals receiving bicuculline behaved like non-private controls.

Since the lateral hypothalamus is an extremely important region for the control of sleep-wake cycle, which is also a target of GABAergic inhibition as sleep pressure increases (Lazarus et al., 2012), we asked whether the blockade of its GABA_A receptors affects the pronociceptive effect of sleep restriction. The selective GABA_A receptor antagonist, bicuculline, microinjected into the lateral hypothalamus did not decrease the pronociceptive effect, but rather slightly increased it (Figure 3 D – ANOVA with repeated measures – $F_{\text{sleep condition}} (1, 20) = 180.65, p < 0.0001$; $F_{\text{Treatment}} (1, 20) = 13.359, p = 0.0015$; $F_{\text{Sleep condition} \times \text{Treatment}} (1, 20) = 8.041, p = 0.1022$; $F_{\text{Time}} (7, 140) = 32.639, p < 0.0001$; $F_{\text{Time} \times \text{Sleep condition}} (7, 140) = 28.996, p < 0.0001$; $F_{\text{Time} \times \text{Treatment}} (7, 140) = 3.7323, p = 0.0009$; $F_{\text{Time} \times \text{Sleep condition} \times \text{Treatment}} (7, 140) = 5.026, p < 0.0001$; SNK test $p < 0.05$).

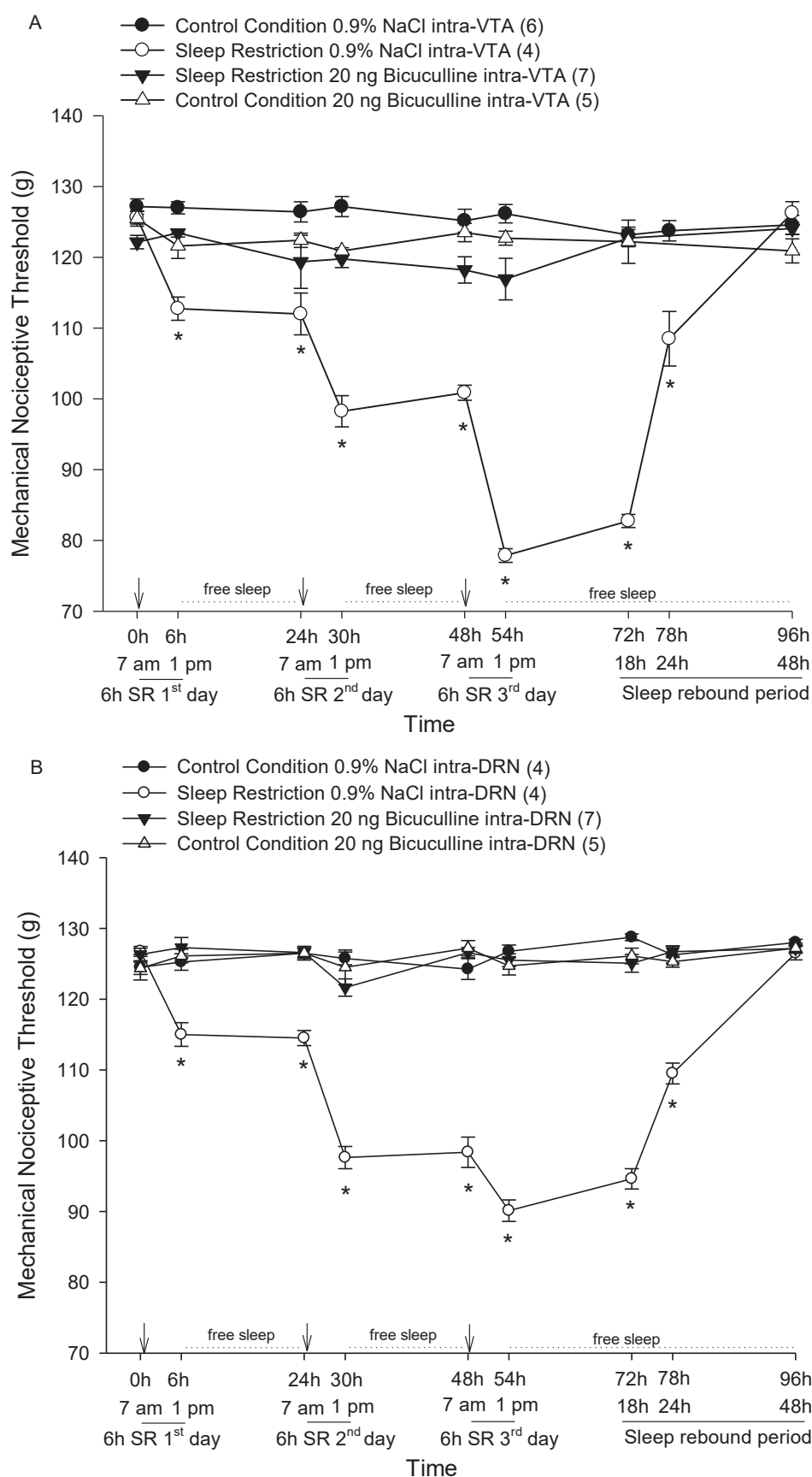
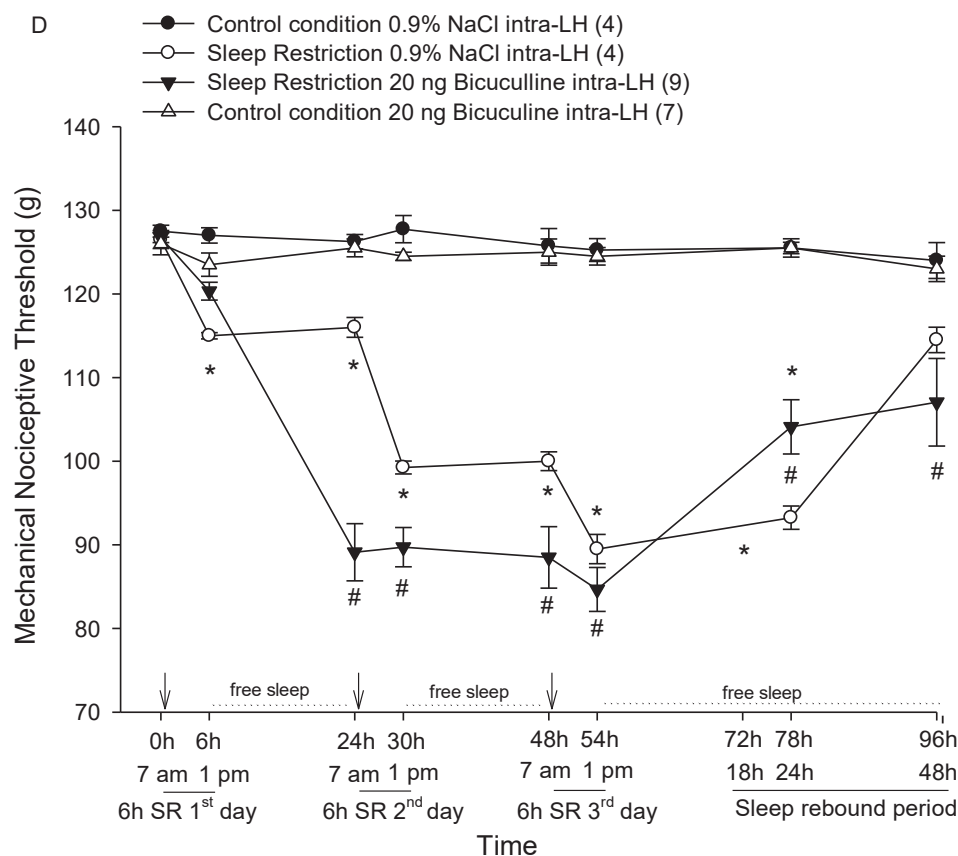
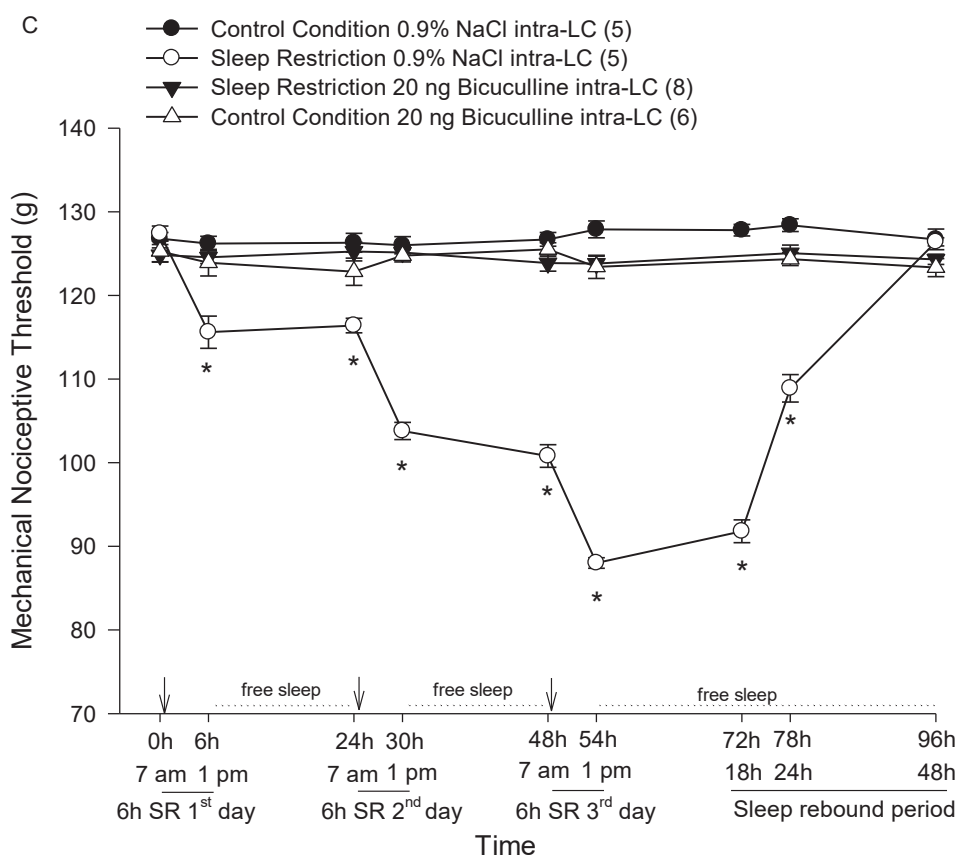


Figure 3 – The blockade of GABA_A receptors located in the brainstem monoaminergic nuclei prevents the pronociceptive effect of sleep restriction. The selective GABA_A receptor antagonist, bicuculline, microinjected into the VTA (A), DRN (B), or LC (C) prevented the pronociceptive effect of sleep restriction. Bicuculline microinjected into the lateral hypothalamus (LH) slightly increased the pronociceptive effect of

sleep restriction (D). Two way RM ANOVA and Newman-Keuls post hoc test: the symbol, “*” indicates a statistically significant decrease in mechanical nociceptive threshold. The symbol “#” indicates a statistically significant decrease in mechanical nociceptive threshold compared to sleep restricted animals that received vehicle.



Both VTA (Baliki and Apkarian, 2015); DRN (Wang and Nakai, 1994) and LC (Bravo et al., 2016) have well-known role in pain modulation, for this reason we sought to test the effect of their GABAergic blockade in a classical animal model of pain. We choose the carrageenan model of inflammatory pain because as sleep restriction, carrageenan induces an increase in pain sensitivity, defined as hyperalgesia. A single microinjection of the selective GABA_A receptor antagonist, bicuculline, into the VTA (Supplementary fig.1A – ANOVA with repeated measures – $F_{\text{Treatment}} (1, 8) = 12.749, p = 0.0072$; $F_{\text{Time}} (7, 56) = 30.151, p < 0.0001$; $F_{\text{Time} \times \text{Treatment}} (7, 56) = 14.898, p < 0.0001$; SNK test $p < 0.0004$); DRN (Supplementary fig. 1B – ANOVA with repeated measures – $F_{\text{Treatment}} (1, 9) = 14.835, p = 0.0039$; $F_{\text{Time}} (7, 63) = 12.341, p < 0.0001$; $F_{\text{Time} \times \text{Treatment}} (7, 63) = 6.183, p < 0.0001$; SNK test $p < 0.001$) or LC (Supplementary fig. 1C – ANOVA with repeated measures – $F_{\text{Treatment}} (1, 10) = 162.57, p < 0.0001$; $F_{\text{Time}} (7, 70) = 52.716, p < 0.0001$; $F_{\text{Time} \times \text{Treatment}} (7, 70) = 25.639, p < 0.0001$; SNK test $p < 0.001$) transiently inhibited carrageenan-induced hyperalgesia. Importantly, in all cases, the effect of bicuculline did not last longer than 180 minutes, after which the hyperalgesia followed its regular course (with no difference between animals receiving bicuculline or its vehicle).

The activation of inhibitory monoaminergic receptors located in the ACC prevents the pronociceptive effect of sleep restriction

The previous findings obtained with the GABA_A receptor antagonist suggest that for sleep restriction to increase pain sensitivity the monoaminergic brainstem nuclei should be inhibited. These nuclei densely project to the ACC where monoamines act mostly to inhibit its neural activity (Matsuzaki et al., 1993, Delfs et al., 1998, Ikemoto, 2007, Zhang et al., 2013, Beliveau et al., 2015). In this study we asked whether the pharmacological activation of dopaminergic D₂; noradrenergic alpha-2 or serotonergic 5-HT_{1A} receptors in the ACC prevents the pronociceptive effect of sleep restriction.

The microinjection of both piribedil (Figure 4 A – ANOVA with repeated measures – $F_{\text{sleep condition}} (1, 21) = 101.30, p < 0.0001$; $F_{\text{Treatment}} (1, 21) = 93.375, p < 0.0001$; $F_{\text{Sleep condition} \times \text{Treatment}} (1, 21) = 54.488, p < 0.0001$; $F_{\text{Time}} (7, 147) = 20.987, p < 0.0001$; $F_{\text{Time} \times \text{Sleep condition}} (7, 147) = 31.44, p < 0.0001$; $F_{\text{Time} \times \text{Treatment}} (7, 147) = 32.253, p < 0.0001$; $F_{\text{Time} \times \text{Sleep condition} \times \text{Treatment}} (7, 147) = 23.060, p < 0.0001$; SNK test $p < 0.05$) and clonidine (Figure 4 C – ANOVA with repeated measures – $F_{\text{sleep condition}} (1, 22) = 60.895, p < 0.0001$; $F_{\text{Treatment}} (1, 22) = 51.340, p < 0.0001$; $F_{\text{Sleep condition} \times \text{Treatment}} (1, 22) = 34.473, p < 0.0001$; $F_{\text{Time}} (7, 154) = 16.987, p < 0.0001$; $F_{\text{Time} \times \text{Sleep condition}} (7, 154) = 20.523, p < 0.0001$; $F_{\text{Time} \times \text{Treatment}} (7, 154) = 24.382, p < 0.0001$; $F_{\text{Time} \times \text{Sleep condition} \times \text{Treatment}} (7, 154) = 24.382, p < 0.0001$).

Time x Sleep condition x Treatment (7, 154) = 15.498, $p < 0.0001$; SNK test $p < 0.05$), a D₂ and an alpha-2 receptor agonist, respectively, into the ACC prevented the pronociceptive effect of sleep restriction. The microinjection of the 5-HT_{1A} receptor agonist 8-OH-DPAT (Figure 4 B – ANOVA with repeated measures – F_{Treatment} (1, 39) = 84.609, $p < 0.0001$; F_{Sleep condition x Treatment} (1, 39) = 139.58, $p < 0.0001$; F_{Time x Treatment} (8, 312) = 17.524, $p < 0.0001$; F_{Time x Sleep condition x Treatment} (8, 312) = 16.975, $p < 0.0001$; SNK test $p < 0.05$) in the ACC induced a dose-dependent effect, in which the higher dose (0.5 ug) prevented the pronociceptive effect, while the lower ones tended to increase it. This dual effect may result from the complex role of 5-HT_{1A} receptors in presynaptically regulating serotonergic neurotransmission (Popova and Naumenko, 2013).

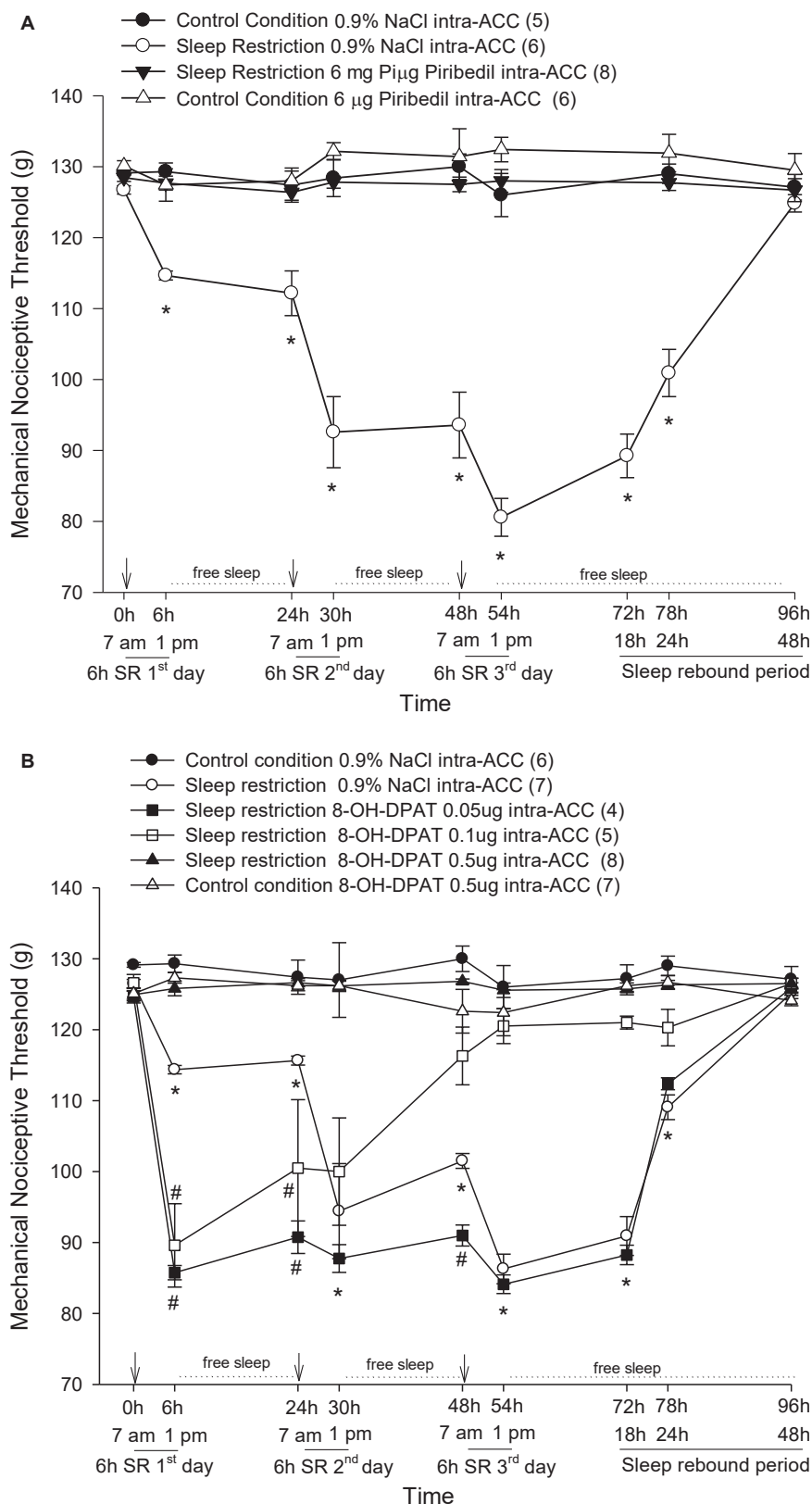
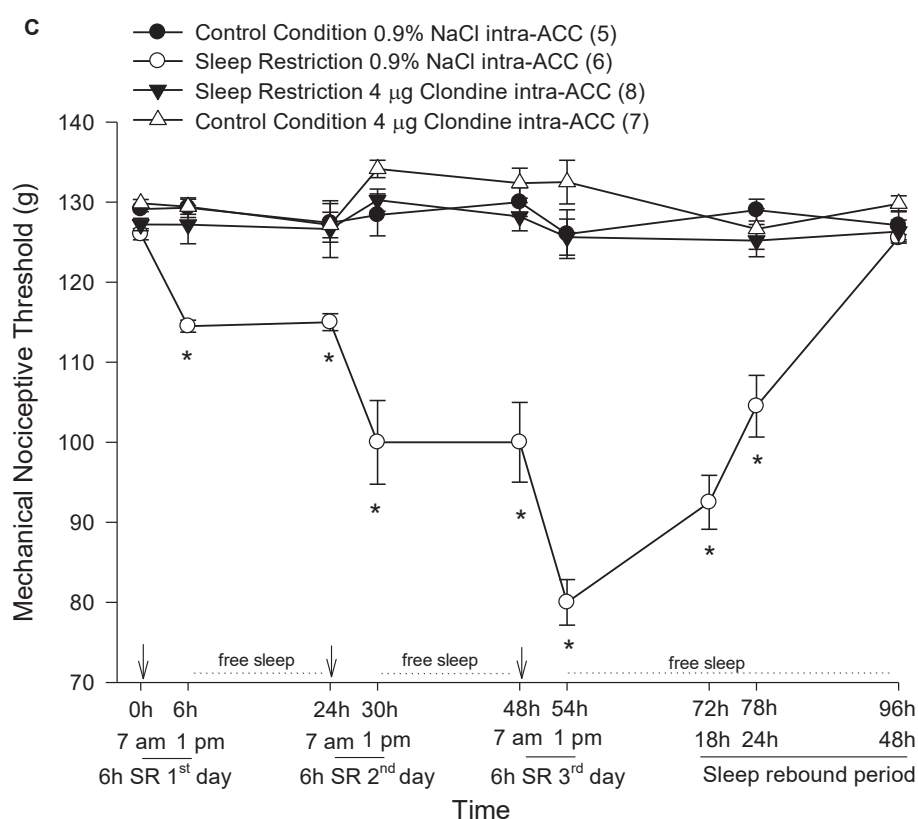


Figure 4 – The pharmacological activation of inhibitory monoaminergic receptors located in the ACC prevents the pronociceptive effect of sleep restriction. The microinjection of dopamine D_2 (A, piribedil); serotonin 5- HT_{1A} (B, 8-OH-DPAT, dose dependently) or noradrenaline α -2 (C, clonidine) receptors in the ACC prevented the pronociceptive effect of sleep restriction. Two way RM ANOVA and Newman-Keuls post hoc test: the symbol, “*” indicates a statistically significant decrease in mechanical nociceptive threshold; the symbol, “#” indicates a statistically significant decrease in mechanical nociceptive threshold compared to sleep restricted animals that received vehicle.



None of the agonists affected the nociceptive threshold in control (not sleep deprived) animals. However, as clonidine is used for clinical pain control in some instances, we sought to test whether its microinjection in the ACC decreases carrageenan-induced hyperalgesia. As expected, the microinjection of clonidine in the ACC decreases carrageenan-induced hyperalgesia (Supplementary fig. 2 – ANOVA with repeated measures – $F_{\text{Treatment}} (1, 11) = 25.972$, $p = 0.0003$; $F_{\text{Time}} (7, 77) = 16.759$, $p < 0.0001$; $F_{\text{Time} \times \text{Treatment}} (7, 77) = 10.513$, $p < 0.0001$; SNK test $p < 0.0007$). But this analgesic effect was transitory and lasted for about two hours, after which hyperalgesia followed its normal course.

The blockade of inhibitory monoaminergic receptors located in the ACC restored the pronociceptive effect of sleep restriction that had been prevented by GABAergic inhibition of brainstem monoaminergic nuclei

The findings accumulated so far have shown that the pronociceptive effect of sleep restriction depends on GABAergic inhibition of brainstem monoaminergic nuclei and is prevented by the activation of monoaminergic receptors in the ACC. Therefore, we tested whether the blockade of these monoaminergic receptors in the ACC would rescue the pronociceptive effect in animals that have received bicuculline in the monoaminergic nuclei of the brainstem.

Animals receiving microinjections of bicuculline in the VTA and of raclopride (selective D₂ receptor antagonist) in the ACC (Figure 5 A – ANOVA with repeated measures – $F_{\text{sleep condition}}(1, 19) = 566.47, p < 0.0001$; $F_{\text{Treatment}}(1, 19) = 2.927, p = 0.10$; $F_{\text{Sleep condition} \times \text{Treatment}}(1, 19) = 5.521, p = 0.029$; $F_{\text{Time}}(8, 152) = 47.917, p < 0.0001$; $F_{\text{Time} \times \text{Sleep condition}}(8, 152) = 46.917, p < 0.0001$; $F_{\text{Time} \times \text{Treatment}}(8, 152) = 1.179, p = 0.31$; $F_{\text{Time} \times \text{Sleep condition} \times \text{Treatment}}(8, 152) = 0.801, p = 0.60$; SNK test $p < 0.05$) behaved like those receiving vehicle in both regions, that is, the pronociceptive effect developed normally in these animals. Similar result was obtained with animals receiving microinjections of bicuculline in the DRN and Way 100,135 (selective 5-HT_{1A} receptor antagonist) in the ACC (Figure 5 B – ANOVA with repeated measures – $F_{\text{sleep condition}}(1, 17) = 144.30, p < 0.0001$; $F_{\text{Treatment}}(1, 17) = 1.641, p = 0.21$; $F_{\text{Sleep condition} \times \text{Treatment}}(1, 17) = 0.91, p = 0.35$; $F_{\text{Time}}(8, 136) = 96.286, p < 0.0001$; $F_{\text{Time} \times \text{Sleep condition}}(8, 136) = 81.825, p < 0.0001$; $F_{\text{Time} \times \text{Treatment}}(8, 136) = 0.696, p = 0.69$; $F_{\text{Time} \times \text{Sleep condition} \times \text{Treatment}}(8, 136) = 0.945, p = 0.48$; SNK test $p < 0.05$), or bicuculline in the LC and idazoxan (selective alpha-2 receptor antagonist) in the ACC (Figure 5 C – ANOVA with repeated measures – $F_{\text{sleep condition}}(1, 19) = 623.52, p < 0.0001$; $F_{\text{Treatment}}(1, 19) = 1.109, p = 0.30$; $F_{\text{Sleep condition} \times \text{Treatment}}(1, 19) = 0.007, p = 0.93$; $F_{\text{Time}}(8, 152) = 128.98, p < 0.0001$; $F_{\text{Time} \times \text{Sleep condition}}(8, 152) = 122.31, p < 0.0001$; $F_{\text{Time} \times \text{Treatment}}(8, 152) = 0.657, p = 0.72$; $F_{\text{Time} \times \text{Sleep condition} \times \text{Treatment}}(8, 152) = 0.297, p = 0.96$; SNK test $p < 0.05$).

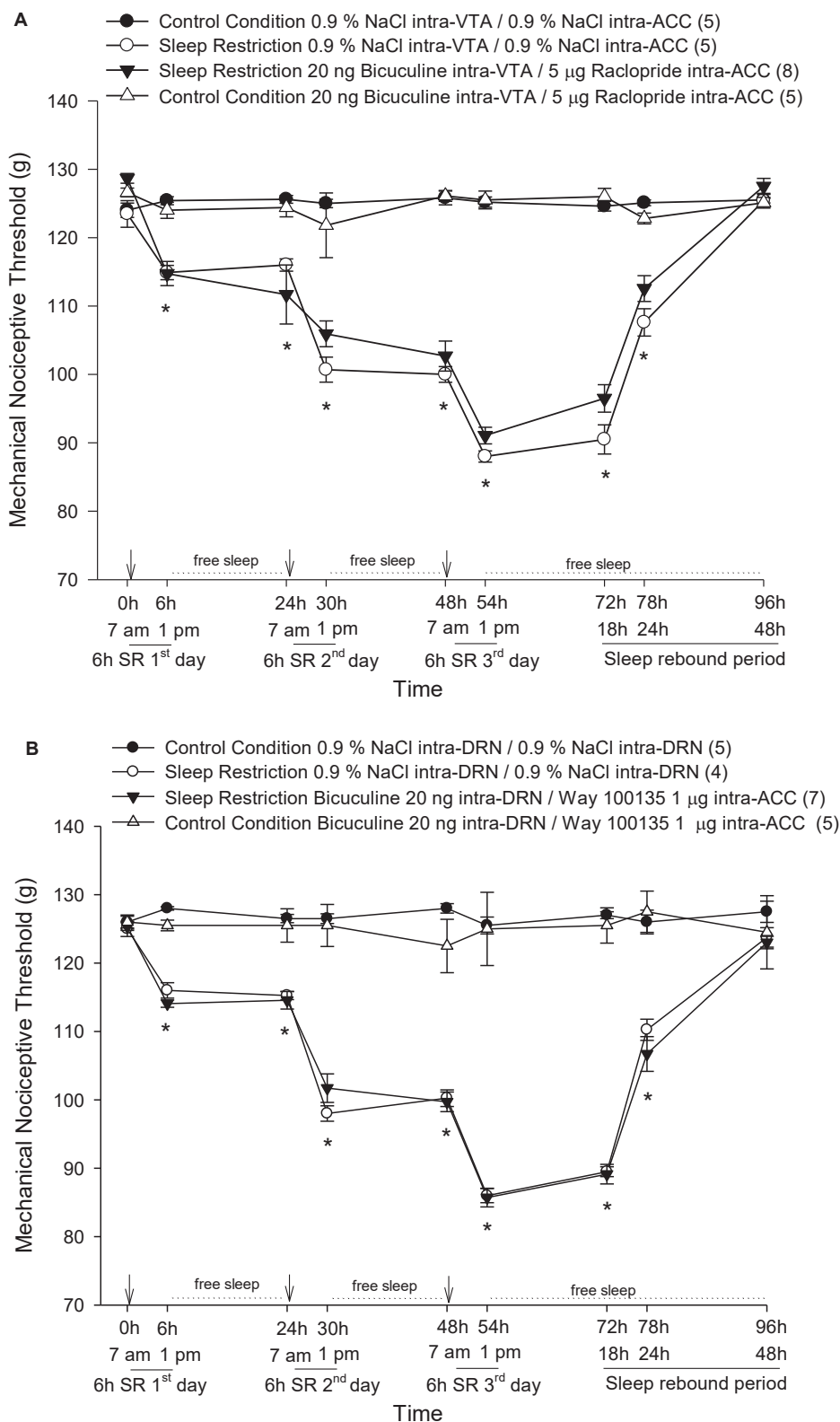
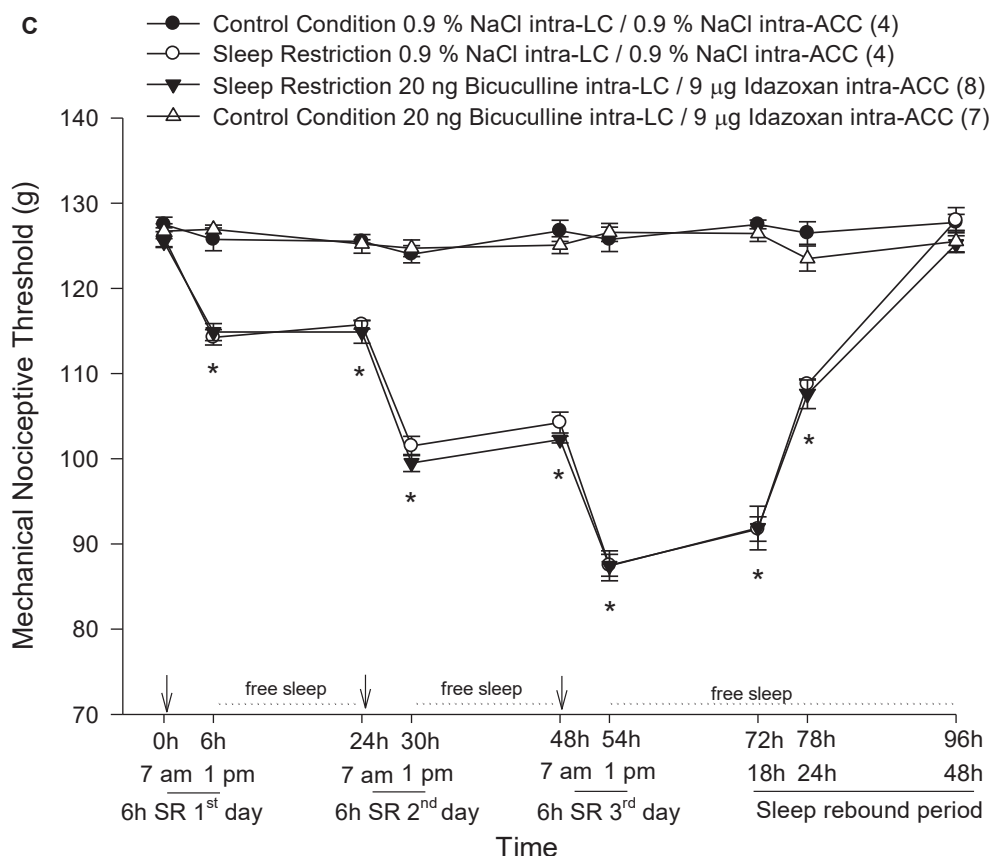


Figure 5 – Blockade of inhibitory monoaminergic receptors located in the ACC restored the pronociceptive effect of sleep restriction that had been prevented by GABAergic inhibition of brainstem monoaminergic nuclei. Sleep restricted animals receiving microinjections of bicuculline (GABA_A antagonist) in the VTA and of raclopride (D₂ antagonist) in the ACC (A); bicuculline in the DRN and Way 100,135 (5-HT_{1A} antagonist) in the ACC (B) or bicuculline in the LC and of idazoxan (selective alpha-2 receptor antagonist) in the ACC (C) behaved like sleep restricted animals receiving vehicle in both regions, that is, the pronociceptive effect of sleep restriction developed normally in these animals. Two way RM ANOVA and Newman-Keuls post hoc test: the symbol, “*” indicates a statistically significant decrease in mechanical nociceptive threshold.



Importantly, the blockade of D₂ (Supplementary fig. 3A – ANOVA with repeated measures – $F_{\text{sleep condition}}(1, 17) = 235.85, p < 0.0001$; $F_{\text{Treatment}}(1, 17) = 0.441, p = 0.51$; $F_{\text{Sleep condition} \times \text{Treatment}}(1, 17) = 2.782, p = 0.11$; $F_{\text{Time}}(8, 136) = 89.099, p < 0.0001$; $F_{\text{Time} \times \text{Sleep condition}}(8, 136) = 100.16, p < 0.0001$; $F_{\text{Time} \times \text{Treatment}}(8, 136) = 1.524, p = 0.15$; $F_{\text{Time} \times \text{Sleep condition} \times \text{Treatment}}(8, 136) = 1.113, p = 0.35$; SNK test $p < 0.05$); alpha-2 (Supplementary fig. 3C – ANOVA with repeated measures – $F_{\text{sleep condition}}(1, 16) = 345.53, p < 0.0001$; $F_{\text{Treatment}}(1, 16) = 0.287, p = 0.59$; $F_{\text{Sleep condition} \times \text{Treatment}}(1, 16) = 5.85, p = 0.027$; $F_{\text{Time}}(8, 128) = 44.274, p < 0.0001$; $F_{\text{Time} \times \text{Sleep condition}}(8, 128) = 43.430, p < 0.0001$; $F_{\text{Time} \times \text{Treatment}}(8, 128) = 1.381, p = 0.21$; $F_{\text{Time} \times \text{Sleep condition} \times \text{Treatment}}(8, 128) = 0.743, p = 0.65$; SNK test $p < 0.05$) or 5-HT_{1A} (Supplementary fig. 3B – ANOVA with repeated measures – $F_{\text{sleep condition}}(1, 16) = 346.31, p < 0.0001$; $F_{\text{Treatment}}(1, 16) = 0.089, p = 0.76$; $F_{\text{Sleep condition} \times \text{Treatment}}(1, 16) = 1.84, p = 0.19$; $F_{\text{Time}}(8, 128) = 96.654, p < 0.0001$; $F_{\text{Time} \times \text{Sleep condition}}(8, 128) = 82.104, p < 0.0001$; $F_{\text{Time} \times \text{Treatment}}(8, 128) = 0.924, p = 0.49$; $F_{\text{Time} \times \text{Sleep condition} \times \text{Treatment}}(8, 128) = 1.389, p = 0.20$; SNK test $p < 0.05$) receptors in the ACC did not affect the pronociceptive effect of sleep restriction by itself. This is important for showing that these antagonists do not induce pronociception by themselves, but are able to restore the effect that bicuculline had blocked (see figure 3 above).

c-Fos expression is increased in the NAc and ACC of sleep restricted animals

Sleep restriction increases c-Fos expression in the NAc (Figure 6 A – One-way ANOVA - $F_{\text{between groups}} (2, 11) = 5.664$, $p = 0.026$, SNK test $p < 0.04$) and ACC (Figure 6 B – One-way ANOVA - $F_{\text{between groups}} (2, 11) = 4.956$, $p = 0.035$, SNK test $p < 0.04$), but not in the VTA (Figure 6 C – One-way ANOVA - $F_{\text{between groups}} (2, 12) = 0.013$ $p = 0.987$); LC (Figure 6 E – One-way ANOVA - $F_{\text{between groups}} (2, 14) = 2.647$ $p = 0.112$) or DRN (Figure 6 D – One-way ANOVA - $F_{\text{between groups}} (2, 13) = 1.929$ $p = 0.191$). After sleep rebound, c-Fos expression was normalized in the NAc and ACC.

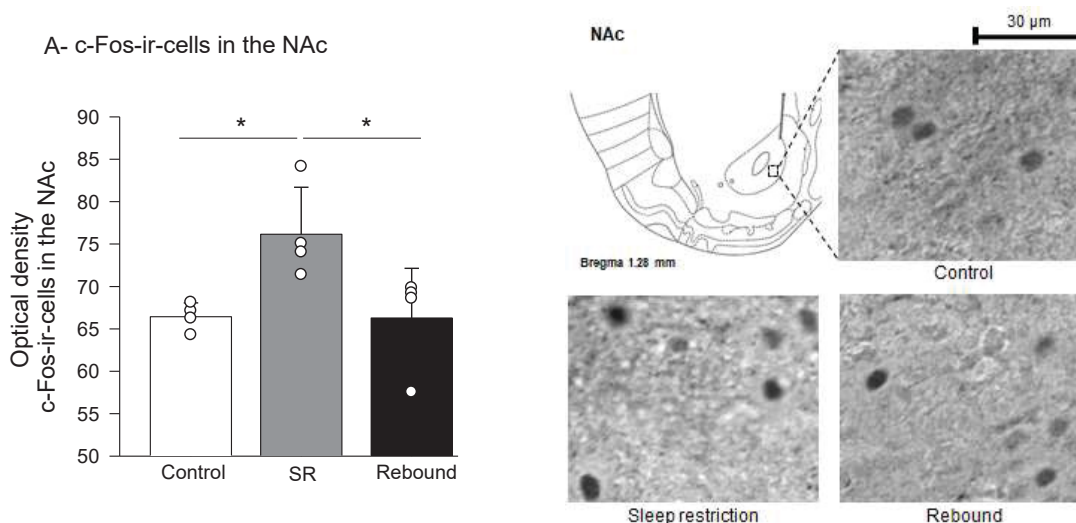


Figure 6 – c-Fos expression is increased in the NAc and ACC of sleep deprived animals. **A**–Sleep restriction increases c-Fos expression in the NAc (A) and ACC (B). After sleep rebound, c-Fos expression was normalized both in the NAc and ACC. Sleep restriction and sleep rebound did not significantly change c-Fos expression in the VTA (C); DRN (D) and LC (E). One-way ANOVA and Newman-Keuls post hoc test: the symbol “*” statistically significant differences are indicated by the symbol “*” Right panels shown representative figures of c-Fos expression, schematic drafts were adapted from the atlas by Paxinos and Watson (2007). Numbers next to the brain diagram represent the atlas frontal coordinates in millimeters from bregma.

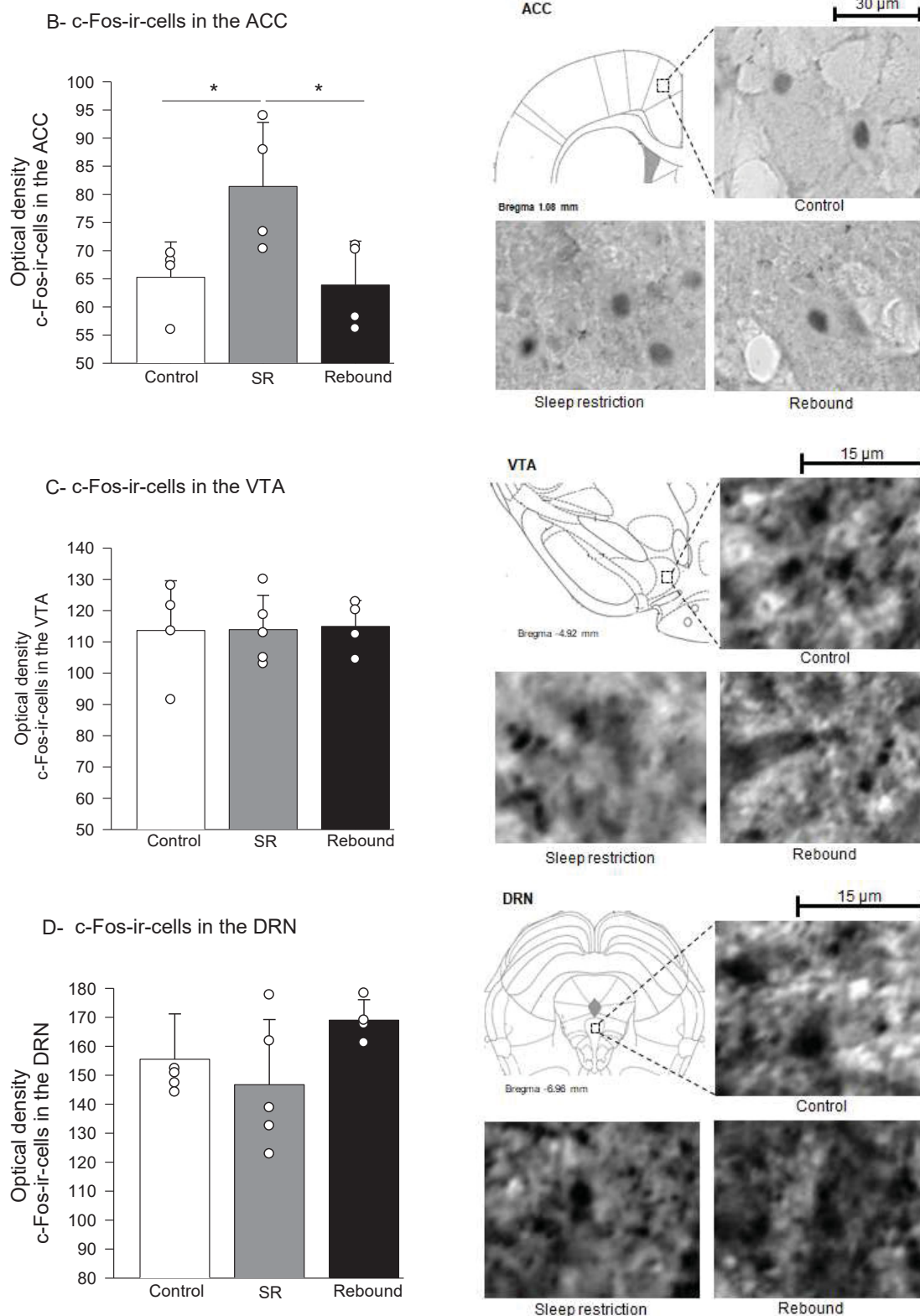


Figure 6 – c-Fos expression is increased in the NAc and ACC of sleep deprived animals. **B**-Sleep restriction increases c-Fos expression in the ACC, but not in the VTA (**C**) and DRN (**D**). After sleep rebound, c-Fos expression was normalized in the ACC. One-way ANOVA and Newman-Keuls post hoc test: the symbol, “*” indicates a statistically significant increase in c-Fos expression in the ACC compared sleep restriction with control and rebound groups. B, C and D- right, representative figures of c-Fos expression. The representative image were adapted from the atlas by Paxinos and Watson (2007). Numbers next to the brain diagrams represent the atlas frontal coordinates in millimeters from bregma.

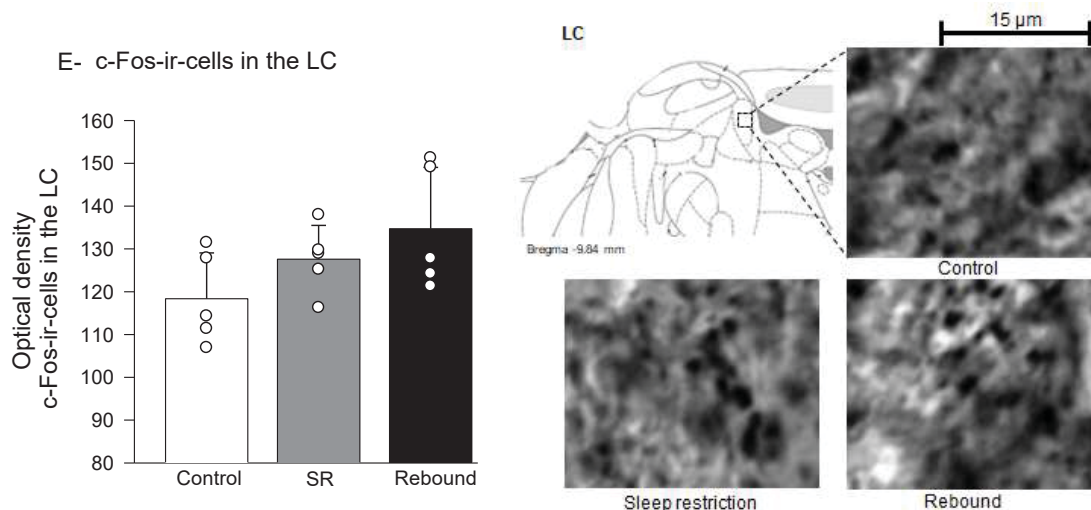


Figure 6 – c-Fos expression is increased in the NAc and ACC of sleep deprived animals. E-Sleep restriction did not change the expression of c-Fos in the LC. E- right, representative figure of c-Fos expression. The representative image were adapted from the atlas by Paxinos and Watson (2007). Numbers next to the brain diagram represent the atlas frontal coordinates in millimeters from bregma.

Levels of fecal glucocorticoid metabolites did not significantly increase in sleep-restricted animals

The fluctuation of fecal glucocorticoid levels throughout the experiment is similar between sleep-restricted animals and controls animals (Figure 7 – Two-way ANOVA with repeated measures – $F_{\text{sleep condition}}(1, 11) = 0.319, p = 0.58$; $F_{\text{time}}(6, 66) = 5.225, p = 0.0001$; $F_{\text{time} \times \text{sleep condition}}(6, 66) = 0.882, p = 0.51$), suggesting that sleep restriction did not induce a significant stress response.

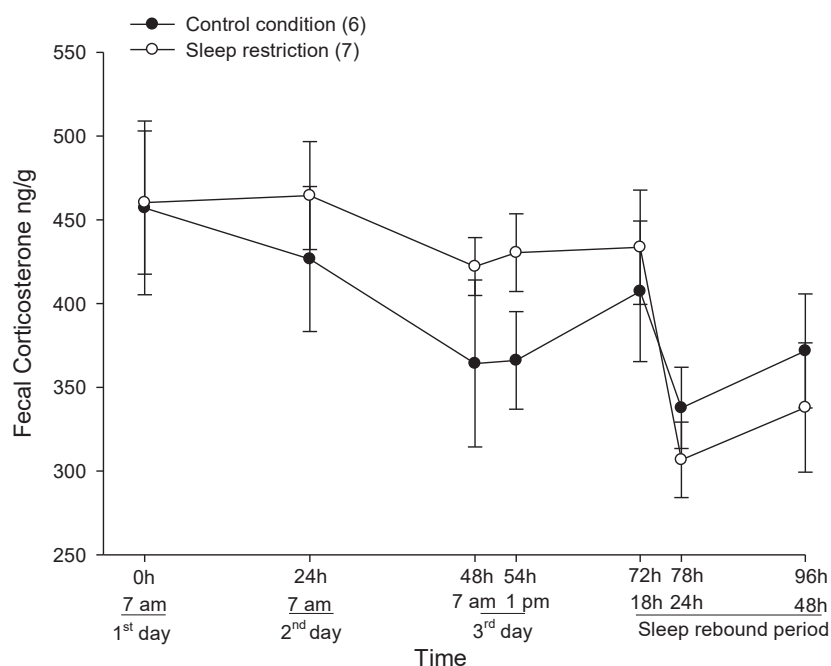


Figure 7 – Fecal glucocorticoid metabolites levels did not significantly increase in sleep-restricted animals. Two-way ANOVA with repeated measures: corticosterone levels do not differ statistically throughout the experiment.

Discussion

The findings of this study demonstrated that for sleep restriction to increase pain sensitivity it is necessary the activation of A_{2A} receptors in the NAc and GABA_A receptors in the VTA, LC or DRN. Activation of dopamine D₂, norepinephrine alpha-2 or serotonin 5-HT_{1A} receptors in the ACC prevented the pronociceptive effect of sleep restriction, while their inhibition restored this effect in animals under GABAergic blockade of the VTA, LC or DRN. Sleep restriction increased c-Fos expression in the NAc and ACC, but not in the VTA; LC or DRN.

NAc has long been implicated in controlling both sleep-wake cycle (Lazarus et al., 2013, Oishi and Lazarus, 2017) and pain (Harris and Peng, 2020), but recently, we have demonstrated that the NAc may also be the link between sleep impairment and increased pain sensitivity. For example, NAc integrity's is essential for sleep deprivation to increase pain sensitivity (Sardi et al., 2018a, Sardi et al., 2018b). The understanding of how NAc mediates the pronociceptive effect of sleep restriction may lie, in part, in understanding how it affects sleep-wake cycle. Briefly, during prolonged wakefulness, adenosine accumulates in the basal forebrain and recent evidence support NAc as a key site for adenosine to induce sleep (Lazarus et al., 2011, Oishi and Lazarus, 2017, Oishi et al., 2017b, Lazarus et al., 2019). A subset of NAc projection neurons, which express adenosine A_{2A} and dopamine D₂ receptors, promote sleep (Qiu et al., 2010, Oishi et al., 2017b) and their activity is regulated by a balance between dopamine (which inhibits their activity and induce wakefulness) and adenosine (Lazarus et al., 2011, Lazarus et al., 2012). We have previously demonstrated that this balance between A_{2A} and D₂ receptor activity in the NAc underlies the pronociceptive effect of REM sleep deprivation (Sardi et al., 2018b). The present study extends these findings by showing for the first time that the blockade of A_{2A} receptors in the NAc prevents the pronociceptive effect of persistent partial restriction of total sleep (Fig. 2). Therefore, the role of NAc adenosine A_{2A} receptors in the increased pain sensitivity that results from sleep impairment is not limited to REM sleep deprivation. All together, these data suggest that prolonged wakefulness increases adenosine activity on NAc A_{2A} receptors to increase sleep pressure (Lazarus et al., 2011, Lazarus et al., 2012, Lazarus et al., 2013, Oishi and Lazarus, 2017, Oishi et al., 2017b, Lazarus et al., 2019) and pain sensitivity ((Sardi et al., 2018b) Fig. 2).

The in vivo activity of NAc neurons across the sleep/wake stages and during prolonged wakefulness has not been determined, but this study demonstrated that six hours a day of total sleep deprivation for three days significantly increases c-Fos expression in the NAc, which was normalized after sleep rebound (Fig. 6A). c-Fos is a general marker of neuronal activation and

its increased expression suggests that sleep restriction increases NAc activity. We have previously demonstrated that chronic sleep restriction increases c-Fos expression in the NAc (Sardi et al., 2018a), the present findings demonstrated that just three days of sleep restriction are enough to induce this increase. The mechanism responsible for increasing NAc activity may be the activation of A_{2A} receptors by adenosine. It was previously showed that the subarachnoid infusion of an A_{2A} agonist increases sleep and the expression of c-Fos in the NAc (Scammell et al., 2001) and chemogenetic or optogenetic activation of NAc A_{2A} expressing-neurons induces sleep, whereas their inhibition prevents sleep (Oishi et al., 2017b). Therefore, by increasing adenosine levels, prolonged wakefulness results in an A_{2A} dependent activation of NAc projection neurons to induce sleep (Oishi and Lazarus, 2017, Oishi et al., 2017b, Zhou et al., 2019). Future studies using selective optogenetic or pharmacogenetic manipulations will determine if these A_{2A}-expressing projection neurons also mediate the pronociceptive effect of sleep restriction.

NAc is reciprocally connected with many nuclei in the forebrain, diencephalon, and brainstem (Matsuzaki et al., 1993, Delfs et al., 1998, Ikemoto, 2007, Zhang et al., 2013, Beliveau et al., 2015). Of special interest for pain modulation are the monoaminergic nuclei of the brainstem: VTA, LC and DRN, which receive GABAergic projections from the NAc (Zhang et al., 2013). This study demonstrated for the first time that these nuclei are implicated in the increased sensitivity to pain resulting from sleep deficiency. The evidence is that the blockade of GABA_A receptors located in the VTA (Fig. 3A), DRN (Fig. 3B) or LC (Fig. 3C) prevented the pronociceptive effect of sleep restriction. In contrast, the blockade of GABA_A receptors in the lateral hypothalamus slightly increased this effect (Fig. 3D). These findings suggest that for sleep impairment to increase pain sensitivity, the brainstem monoaminergic nuclei should be inhibited. An important source of GABAergic inhibition for these nuclei is the NAc (Kreitzer, 2009, Lazarus et al., 2012, Harris and Peng, 2020) and our data from c-Fos expression indicate that sleep restriction increases neural activity in the NAc, but not in the VTA, LC and DRN (Fig 6). Future studies are needed to determine whether the increased NAc activity during sleep restriction enhances inhibition of these nuclei to increase pain sensitivity.

The monoaminergic systems have a broad modulatory role in pain processing (Bannister and Dickenson, 2016). For this reason, we tested the ability of the GABAergic blockade of monoaminergic nuclei in decreasing the nociceptive response elicited by carrageenan, a classical animal pain model. Similar to what was observed in sleep deprived animals, the blockade of GABA_A receptors located both in the VTA (Supplementary Fig. 1A), LC (Supplementary Fig. 1C) or DRN (Supplementary Fig. 1B) inhibited the hyperalgesic response

induced by carrageenan, but this inhibition was transient and lasted no longer than two hours. After the expected end of bicuculline's effect, hyperalgesia resumes its normal course, which contrasts with sleep deprived animals in which the hyperalgesic response was completely prevented.

Pre-frontal and adjacent cortical regions are among the areas most densely innervated by monoaminergic projections from the brainstem (Chandler et al., 2013). The ACC stands out as the cortical region with probably the most prominent role in pain processing (Xiao et al., 2021) and it is innervated by the three monoaminergic nuclei we are studying (Chandler et al., 2013). We have previously demonstrated that the integrity of the ACC is essential for sleep restriction to increase pain sensitivity (Sardi et al., 2022a; unpublished data), but whether monoaminergic receptors contribute to the role of ACC in the interplay between sleep and pain had never been studied. In this study we focus on dopamine D₂ and norepinephrine alpha-2 receptors because of their consistent role in pain modulation (Ortiz et al., 2007, Pertovaara, 2013, Sardi et al., 2018b, Wang et al., 2021), the 5-HT_{1A} receptor was another choice because of its key role in regulating serotonergic function (Popova and Naumenko, 2013). First, we tested the ability of each receptor agonist, when injected into the ACC, to decrease the pronociceptive effect of sleep restriction. The findings showed that the pharmacological activation of D₂ (Fig. 4A), alpha-2 (Fig. 4C) or 5-HT_{1A} (Fig. 4B) receptors located in the ACC prevents sleep restriction from increasing pain sensitivity, with no effect in the nociceptive response of control (not sleep restricted) animals. This is the first suggestion for a role of ACC alpha-2 and 5-HT_{1A} receptors in the pronociceptive effect of sleep restriction, while we have previously demonstrated that the activation of ACC D₂, but not D₁, receptors prevented this effect (Sardi et al., 2022a; unpublished data).

The most relevant source of monoamines for ACC are precisely the VTA, LC and DRN that our findings indicate should be inhibited for sleep restriction to increase pain sensitivity (Fig. 3). The inhibition of these nuclei in sleep-restricted animals may result in a decrease of the monoaminergic activity in the ACC. Therefore, we tested the effect of the blockade of monoaminergic receptors in the ACC of animals in which the GABA_A receptors were blocked in the brainstem nuclei. The concomitant blockade of GABA_A receptors in the VTA and D₂ receptors in the ACC allowed the pronociceptive effect to manifest itself without any change in its normal course (Fig. 5A). Similar result was obtained with the concomitant blockade of GABA_A receptors in the LC and alpha-2 receptors in the ACC (Fig. 5C) or GABA_A receptors in the DRN and 5-HT_{1A} receptors in the ACC (Fig. 5B). Taken together, the data so far discussed demonstrated that the blockade of GABA_A receptors in both VTA, LC or DRN

prevents the pronociceptive effect of sleep restriction (Fig. 3), but a concomitant blockade of the ACC D₂, alpha-2 or 5-HT_{1A} receptors, respectively, rescues this effect, allowing it to develop normally (Fig. 5). This is consistent with the idea that sleep restriction increases GABAergic inhibition of VTA, LC and DRN decreasing their monoaminergic influence over the ACC to increase pain sensitivity. Data obtained with monoaminergic agonists in the ACC provide additional support to this idea.

The *in vivo* activity of ACC neurons during prolonged wakefulness has not been determined, but our findings demonstrated for the first time that c-Fos expression is increased in the ACC after sleep restriction and is normalized after sleep rebound (Fig 6B). This indicates that sleep restriction increases neural activity in the ACC, which is in line with other indirect data suggesting that ACC activity increases with sleep restriction (Deurveilher et al., 2013, Park et al., 2020) and sustained wakefulness in animals (Bertini et al., 2002, Wirtshafter, 2005, Gompf et al., 2010) and humans (Habeck et al., 2004, Choo et al., 2005, Poudel et al., 2013, Elvsashagen et al., 2019). Neural activity of the ACC is consistently associated with increased pain and top-down pain facilitation (Xiao et al., 2021). The increased neural activity in the ACC is also consistent with decreased monoaminergic neurotransmission, since evidence support that both dopamine, norepinephrine and 5-HT inhibit neural activity of the ACC (Takeda et al., 2009, Darvish-Ghane et al., 2016, Godlewska et al., 2016). Future studies should determine whether decrease in monoaminergic function increases neural activity of the ACC to increase pain sensitivity under sleep-restriction conditions

Despite growing interest, the role of monoamines in the interplay between sleep impairment and pain is still poorly understood. This study opens up new avenues for the study of the mechanisms by which sleep impairment increases pain sensitivity. For example, the present findings demonstrated that for sleep restriction to increase pain sensitivity it is necessary the activation of A_{2A} receptors in the NAc and GABA_A receptors in the VTA, LC and DRN. Whether NAc A_{2A} expressing neurons inhibit these brainstem nuclei to increase pain sensitivity in sleep deprived subjects is a possibility to be explored. The blockade of dopamine D₂, norepinephrine alpha-2 or serotonin 5-HT_{1A} receptors in the ACC rescued the pronociceptive effect that had been blocked by GABAergic blockade of the VTA, LC or DRN. Whether the inhibition of the brainstem nuclei decreases monoaminergic influence in the ACC to increase pain sensitivity in sleep deprived subjects is another possibility to be explored. A decrease in monoaminergic function may help to explain the increase in c-Fos expression in the ACC, since monoamines play a mainly inhibitory role in the ACC (Takeda et al., 2009, Darvish-Ghane et al., 2016, Godlewska et al., 2016). Whether a decrease in monoaminergic function disinhibits

ACC neural activity to increase pain sensitivity in sleep deprived subjects is a further possibility to be explored.

Sleep restriction and persistent pain conditions are public health problems worldwide and the understanding of the mechanisms by which sleep impairment increases pain sensitivity is essential to encourage the development of new drugs and behavioral interventions that would impact pain prevention and management. This study contributes to advance our knowledge on the interplay between sleep impairment and pain, extending our previous studies showing that the integrity of NAc (Sardi et al., 2018a, Sardi et al., 2018b) and ACC (Sardi et al., 2022a; unpublished data) is essential for sleep restriction to increase pain sensitivity. However, maybe the greatest contribution of this study is that it has proposed some mechanisms to be tested under conditions that allow massive investment in science and technology.

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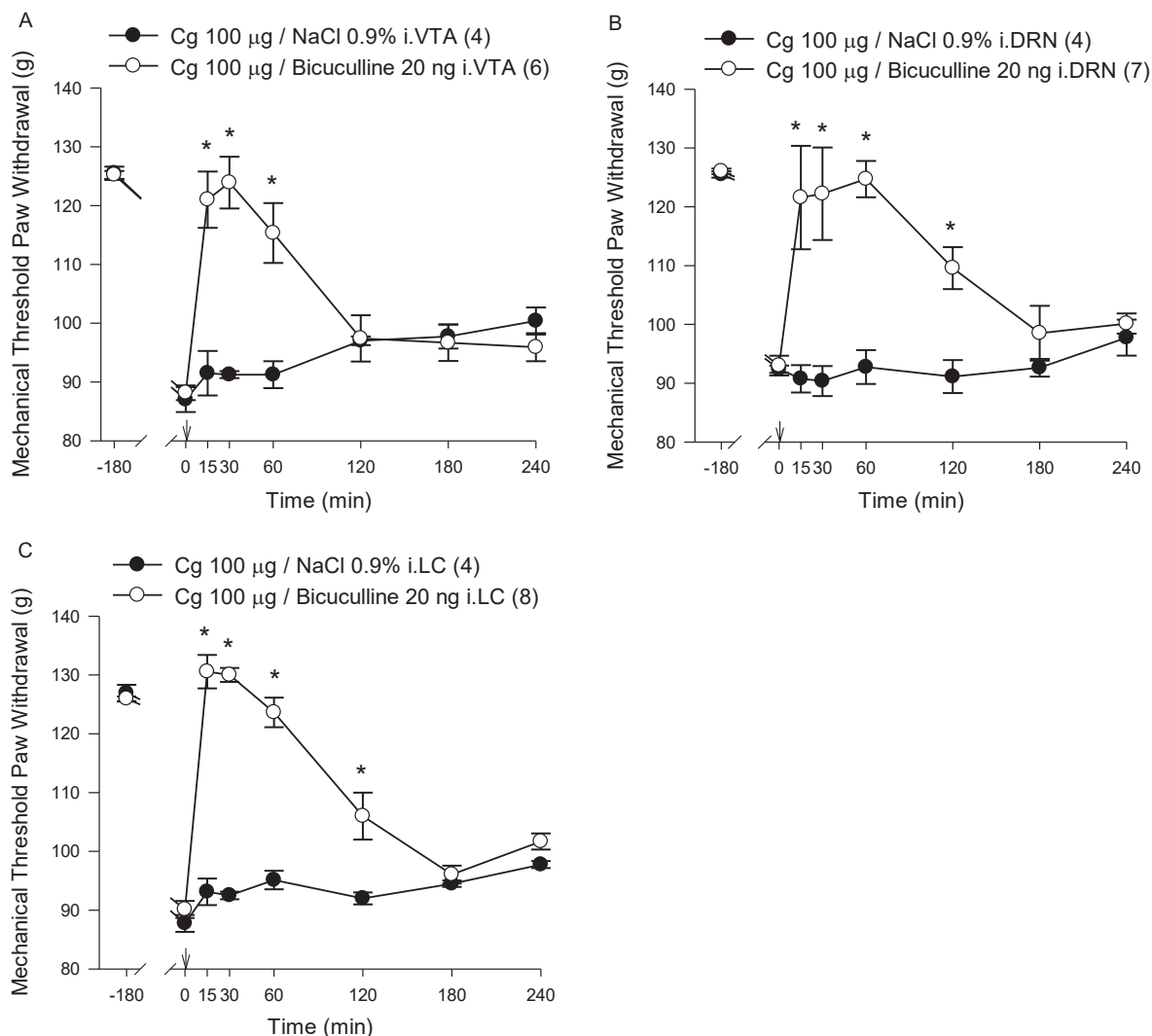
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Supplementary material

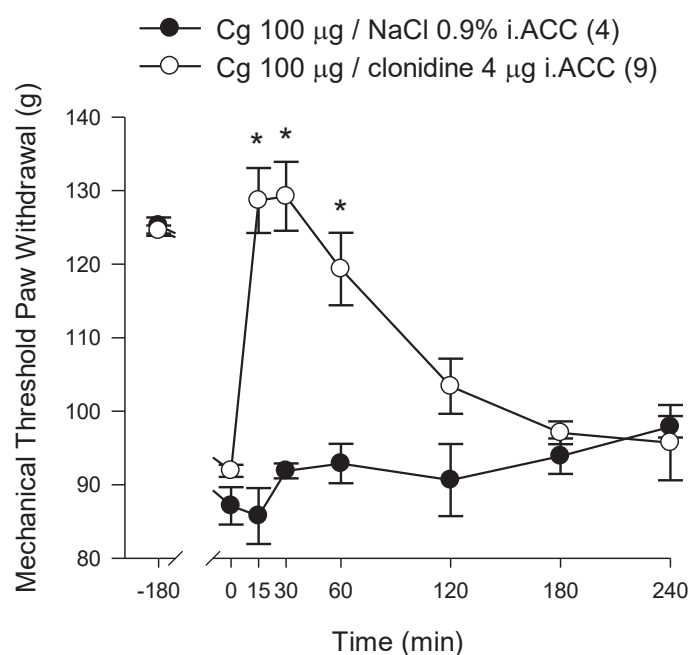
Supplementary figures

Supplementary figure 1



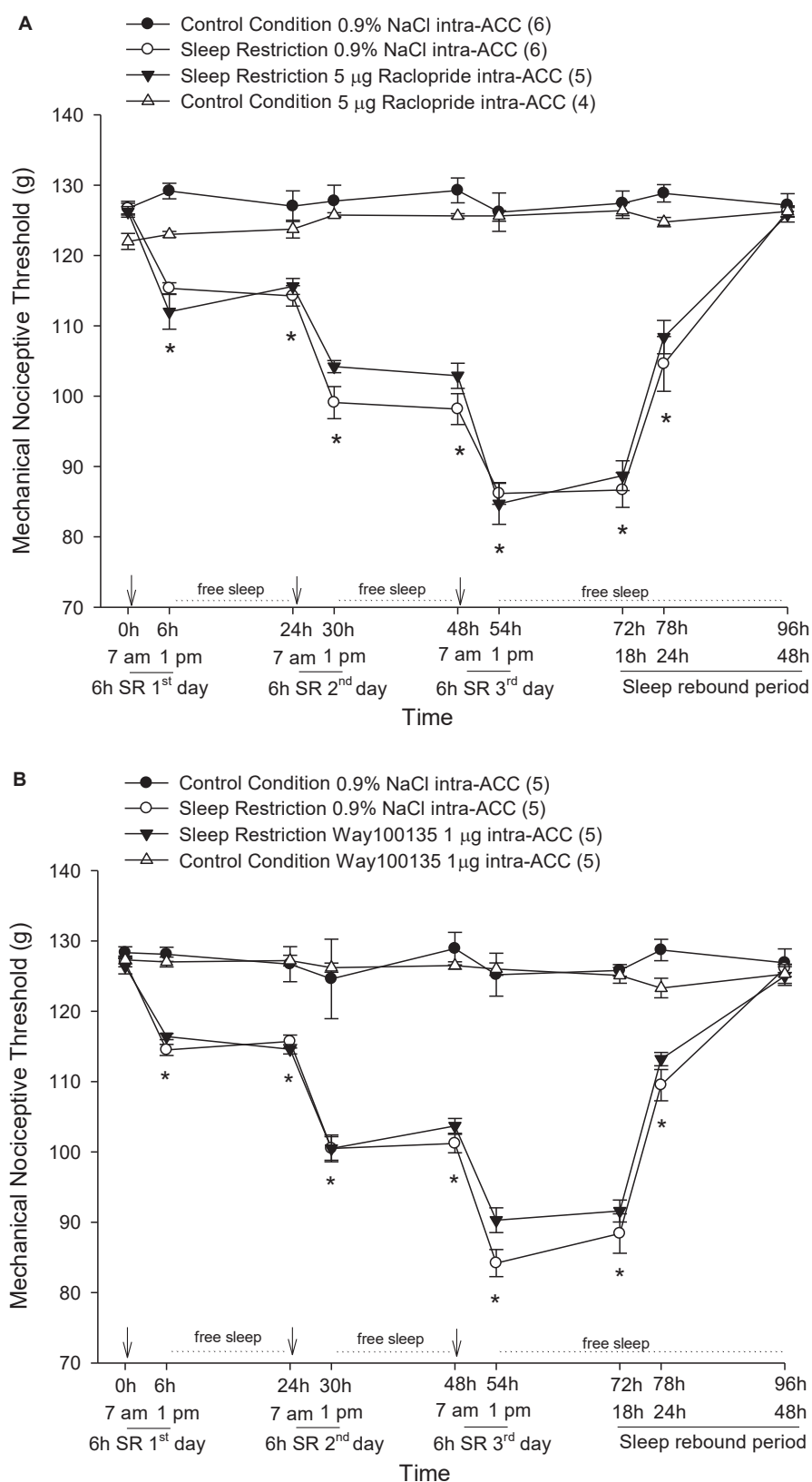
Supplementary Figure 1 – The GABAergic blockade of brainstem monoaminergic nuclei transiently decreases carrageenan-induced hyperalgesia. A single microinjection of the selective GABA_A receptor antagonist, bicuculline, into the VTA (A); DRN (B) or LC (C) transiently inhibited carrageenan-induced hyperalgesia. Two way RM ANOVA and Newman-Keuls post hoc test: the symbol, “*” indicates a statistically significant increase in mechanical nociceptive threshold with bicuculline microinjection. Cg = carrageenan.

Supplementary figure 2

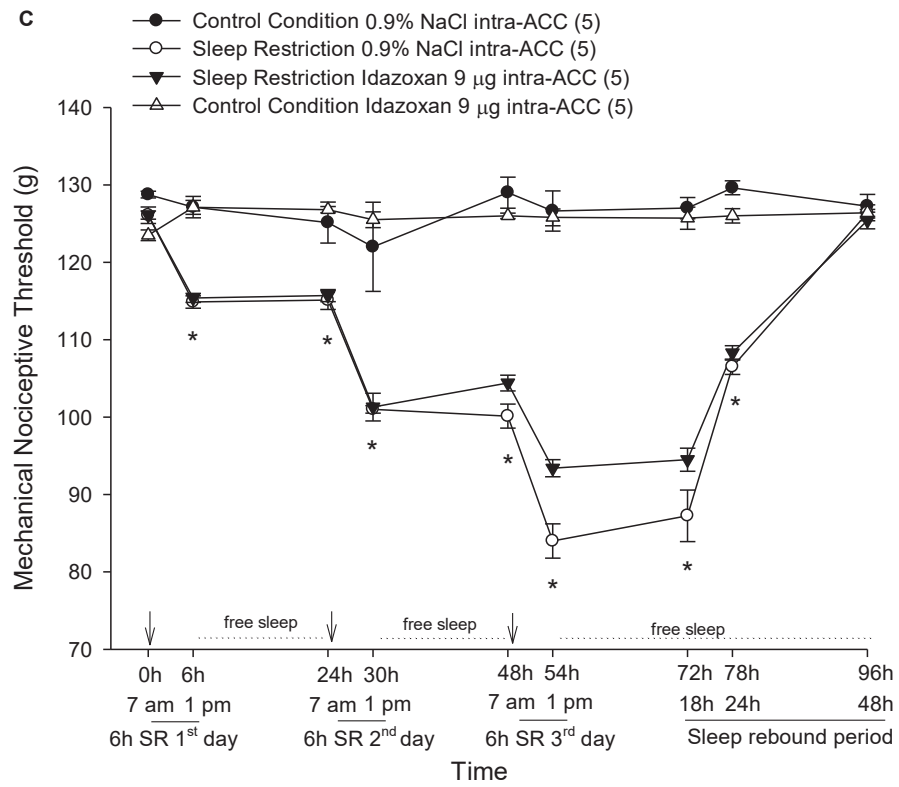


Supplementary Figure 2 – The activation of noradrenergic alpha-2 receptor in the ACC transiently decreases carrageenan-induced hyperalgesia. The microinjection of clonidine in the ACC induced a transient analgesic effect for about two hours. Two way RM ANOVA and Newman-Keuls post hoc test: the symbol, “*” indicates a statistically significant increase in mechanical nociceptive threshold with clonidine microinjection. Cg = carrageenan.

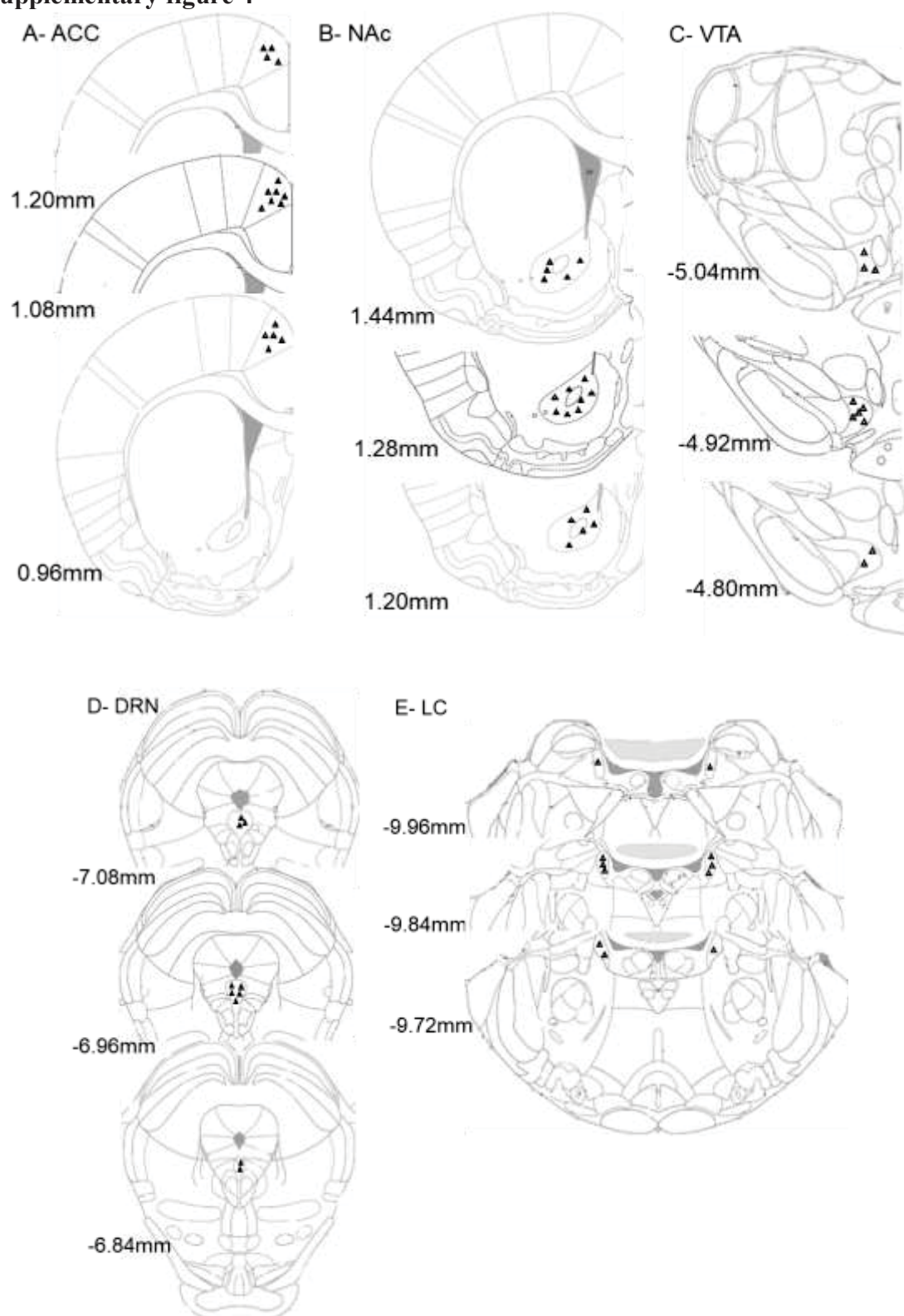
Supplementary figure 3



Supplementary Figure 3 – The blockade of inhibitory monoaminergic receptors located in the ACC did not affect the pronociceptive effect of sleep restriction by itself. The microinjection of the D₂ (raclopride, **A**); 5-HT_{1A} (way100135, **B**) or alpha-2 (idazoxan, **C**) receptor agonist in the ACC did not affect the pronociceptive effect of sleep restriction by itself. Two way RM ANOVA and Newman-Keuls post hoc test: the symbol, “*” indicates a statistically significant decrease in mechanical nociceptive threshold.



Supplementary figure 4



Supplementary Figure 4 - Representative figures of microinjections. **A-** ACC, **B-** NAc, **C-** VTA, **D-** DRN and **E-** LC the triangles represent the microinjection sites. The images were adapted from the atlas by Paxinos and Watson (2007). Numbers next to the brain diagrams represent the atlas frontal coordinates in millimeters to the bregma.

CONSIDERAÇÕES FINAIS

Este trabalho juntamente com os trabalhos anteriores do grupo, contribui consideravelmente para o entendimento de como a diminuição do tempo de sono aumenta a sensibilidade à dor. Os dados anteriores do nosso grupo demonstram que a privação de sono REM impacta o sistema descendente de modulação da dor, aumentando a facilitação e diminuindo a inibição da dor (Tomim et al., 2016); além disso, o aumento da atividade adenosinérgica e diminuição da atividade dopaminérgica no NAc, também medeiam o efeito pró-nociceptivo da privação de sono REM (Sardi et al., 2018b). Neste trabalho, apresentamos dados que reforçam esses achados e adicionamos mais peças ao quebra-cabeça.

No primeiro capítulo, demonstramos que o estado hiperalgésico induzido pela privação de sono REM é totalmente suprimido pela ativação de mecanismos endógenos de modulação da dor desencadeados pela acupuntura, estresse ou estimulação nociva. Sendo assim, a privação de sono impacta a modulação endógena da dor, no entanto, não se sobrepõe a ela, ou seja, não impede que seja ativada.

Em seguida, no segundo capítulo, investigamos se a PAG – que compõe o sistema descendente de modulação da dor – e o NAc também são necessários para o aumento da sensibilidade à dor induzida por restrição crônica de sono, visto que para o efeito pró-nociceptivo da privação de sono REM são essenciais (Tomim et al., 2016, Sardi et al., 2018b). Demonstramos que sim, a PAG e o NAc são essenciais para o desenvolvimento do efeito pró-nociceptivo da restrição crônica de sono, pois tal efeito foi prevenido por lesão excitotóxica de ambas as áreas.

No terceiro capítulo, baseado em nossos dados anteriores que suportam o envolvimento do NAc no efeito pró-nociceptivo da diminuição do tempo de sono (Sardi et al., 2018a, Sardi et al., 2018b), e em dados da literatura, que suportam o envolvimento do NAc e do ACC na modulação do ciclo sono-vigília (Lazarus et al., 2011, Lazarus et al., 2012, Lazarus et al., 2013, Oishi et al., 2017a, Oishi et al., 2017b, Scammell et al., 2017, Zhou et al., 2019) e na modulação dor (Gear and Levine, 2011, Yamashita et al., 2014, Tobaldini et al., 2019, Sun et al., 2020, Vergara et al., 2020, Xiao et al., 2021), e sabendo que o ACC se conecta ao sistema descendente (Calejesan et al., 2000, Zhang et al., 2005, Peyron et al., 2007, Ikeda et al., 2014, Bliss et al., 2016), investigamos a importância do ACC, e os mecanismos dopaminérgicos no ACC e NAc envolvidos no efeito pró-nociceptivo da restrição de sono. Nossos achados sugerem que uma

diminuição na neurotransmissão dopaminérgica no ACC e NAc contribui para o efeito pró-nociceptivo da restrição do sono.

No quarto capítulo, devido ao importante envolvimento dos núcleos monoaminérgicos que sabidamente projetam ao ACC (Lopez-Avila et al., 2004, Chandler et al., 2013, Scammell et al., 2017) na modulação da dor (Wang and Nakai, 1994, Baliki and Apkarian, 2015, Bannister and Dickenson, 2016, Bravo et al., 2016, Haack et al., 2020) e ciclo sono-vigília (Oishi and Lazarus, 2017, Scammell et al., 2017, Haack et al., 2020), investigamos o papel dos núcleos monoaminérgicos do tronco cerebral no efeito pró-nociceptivo da restrição de sono. Os nossos dados sustentam que o efeito pró-nociceptivo da restrição de sono depende do aumento da atividade GABAérgica nos núcleos monoaminérgicos VTA, DRN e LC e, também depende da diminuição da atividade monoaminérgica inibitória no ACC.

Portanto, a partir do conjunto de dados até aqui exposto, sugerimos um mecanismo pelo qual a redução do tempo de sono induz efeito pró-nociceptivo. Propomos ser possível que, o aumento da atividade eferente do NAc pela restrição de sono (Figura 1, #1) diminua as projeções monoaminérgicas para o ACC (Figura 1, #2) resultando em aumento da facilitação descendente (Figura 1, #3).

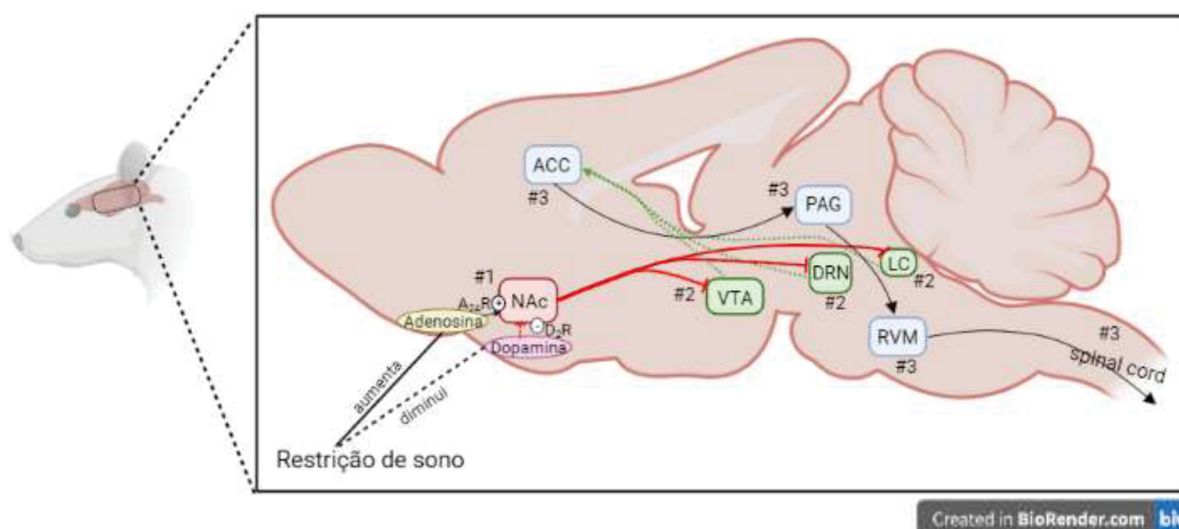


Figura 1 - Representação esquemática do mecanismo que propomos estar envolvido no efeito pró-nociceptivo induzido pela diminuição do tempo de sono. A restrição de sono aumenta a atividade adenosinérgica nos receptores de adenosina A_{2A} ($A_{2A}R$) e diminui a dopaminérgica nos receptores de dopamina D_2 (D_2R) no NAc, o que aumenta a atividade eferente do NAc (#1), que pode estar diminuindo a atividade de núcleos importantes para a modulação da dor e do ciclo sono-vigília, entre eles a VTA, o DRN e o LC. Portanto, as projeções de tais núcleos ao ACC estariam diminuídas (#2), o que poderia alterar o controle cortical sobre o sistema descendente PAG-RVM, levando ao aumento da atividade descendente facilitatória no corno dorsal da medula espinhal (#3). Linhas contínuas vermelhas: aumento da neurotransmissão GABAérgica; Setas pontilhadas verdes: diminuição da neurotransmissão monoaminérgica inibitória, favorece a excitação cortical; Setas pretas: aumento da atividade para facilitação descendente. NAc: núcleo accumbens, do inglês *nucleus accumbens*; VTA: área tegmentar ventral, do inglês *ventral tegmental area*; DRN: núcleo dorsal da rafe, do inglês *dorsal raphe nucleus*; LC: locus coeruleus; ACC: córtex cingulado anterior, do inglês *anterior cingulate cortex*; PAG-RVM: substância cinzenta periaquedutal – bulbo rostro ventral, do inglês *periaqueductal grey matter – rostral ventral medula*. Figura criada com a ferramenta gratuita BioRender.com.

Concluindo, mais estudos com tecnologias que não tivemos acesso devem ser realizados para nos ajudar a montar o quebra-cabeça dos mecanismos envolvidos no aumento da sensibilidade a dor induzida pela redução do tempo de sono. Até o momento, com as peças que temos, podemos dizer que a privação de sono aumenta a facilitação descendente da dor (Tomim et al., 2016, Xue et al., 2018), no entanto, não impede a ativação do sistema endógeno de modulação da dor por mecanismos que o ativam, tais como a acupuntura, o estresse e a estimulação nociva. Podemos dizer também que o efeito pró-nociceptivo induzido pela redução do tempo de sono depende da integridade do NAc, da PAG (Sardi et al., 2018a, Sardi et al., 2018b) e do ACC. Além disso, o efeito pró-nociceptivo induzido por restrição de sono parece depender de uma redução da atividade dopaminérgica no NAc e no ACC, sugerimos também que dependa do aumento da atividade GABAérgica na VTA, DRN e LC e, em adição, pode depender de uma diminuição da atividade monoaminérgica inibitória no ACC. Desta maneira, os achados deste trabalho contribuem para o conhecimento adequado dos mecanismos pelos quais o comprometimento do sono aumenta a dor e isso é essencial para incentivar o desenvolvimento de novos medicamentos e intervenções comportamentais que possam impactar a prevenção e o manejo da dor.

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ANEXOS

Anexo 1 – Certificado da Comissão de Ética nº 1126



Ministério da Educação
UNIVERSIDADE FEDERAL DO PARANÁ
Setor de Ciências Biológicas
Comissão de Ética no Uso de Animais
(CEUA)



Nº 1126

CERTIFICADO

A Comissão de Ética no Uso de Animais do Setor de Ciências Biológicas da Universidade Federal do Paraná (CEUA/BIO – UFPR), instituída pela Resolução Nº 86/11 do Conselho de Ensino Pesquisa e Extensão (CEPE), de 22 de dezembro de 2011, **CERTIFICA** que os procedimentos utilizando animais no projeto de pesquisa abaixo especificado estão de acordo com a Diretriz Brasileira para o Cuidado e a Utilização de Animais para fins Científicos e Didáticos (DBCA) estabelecidas pelo Conselho Nacional de Controle de Experimentação Animal (CONCEA) e com as normas internacionais para a experimentação animal.

STATEMENT

The Ethics Committee for Animal Use from the Biological Sciences Section of the Federal University of Paraná (CEUA/BIO – UFPR), established by the Resolution Nº 86/11 of the Teaching Research and Extension Council (CEPE) on December 22nd 2011, **CERTIFIES** that the procedures using animals in the research project specified below are in agreement with the Brazilian Guidelines for Care and Use of Animals for Scientific and Teaching purposes established by the National Council for Control of Animal Experimentation (CONCEA) and with the international guidelines for animal experimentation.

PROCESSO/PROCESS: 23075.213783/2017-78

APROVADO/APPROVAL: 12/12/2017 – R.O. 11/2017

TÍTULO: Efeito pró-nociceptivo da privação de sono em ratos: Mecanismos estriado-reticulocorticais.

TITLE: The pronociceptive effect of sleep deprivation in rats: Striato-reticulo-cortical mechanisms.

AUTORES/AUTHORS: Luana Fischer, Natalia Fantin Sardi.

DEPARTAMENTO/DEPARTMENT: Fisiologia

Prof. D.a. Katya Naliwaiko
Coordenadora da CEUA