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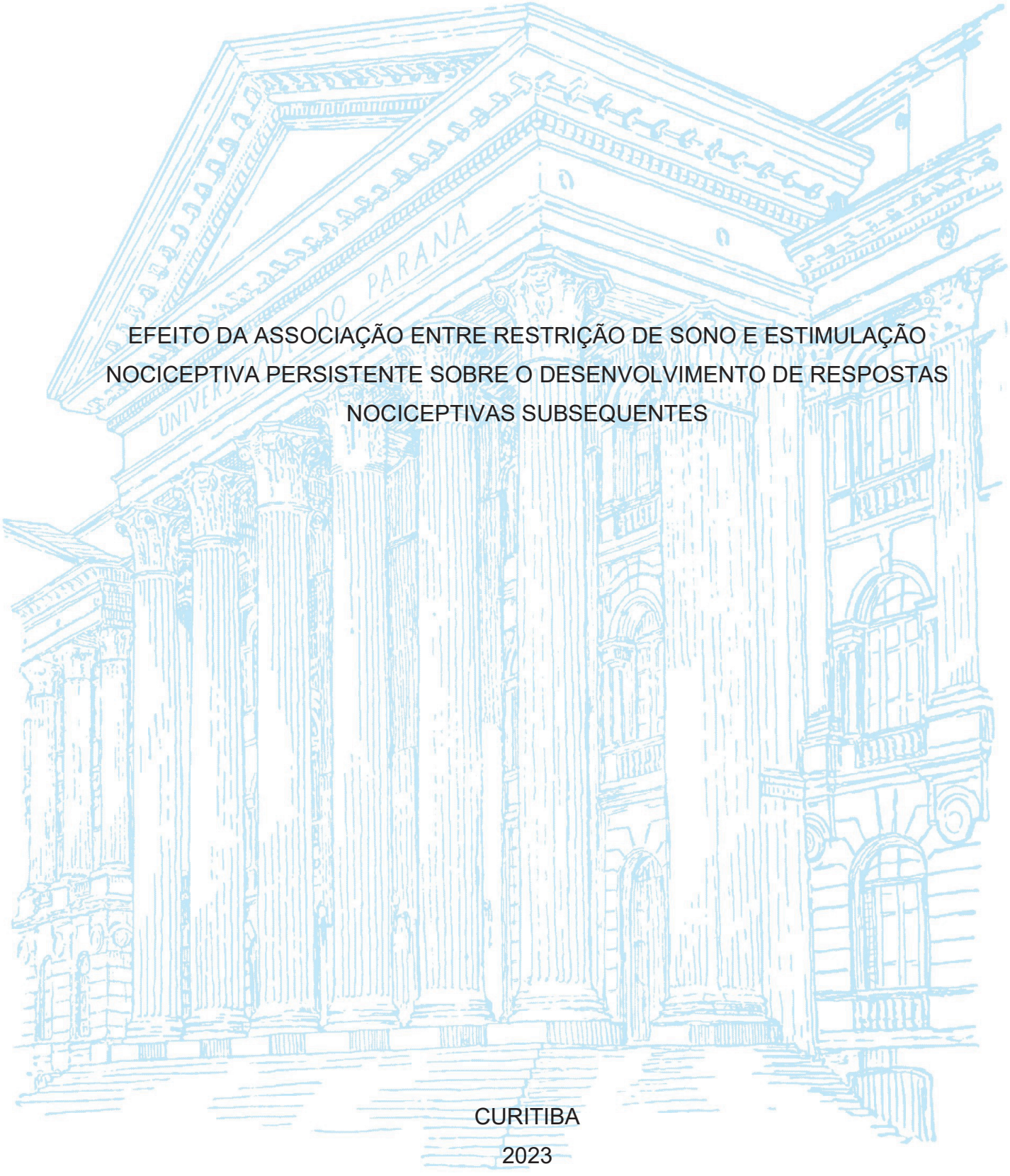
ANA CAROLINA PESCADOR



EFEITO DA ASSOCIAÇÃO ENTRE RESTRIÇÃO DE SONO E ESTIMULAÇÃO NOCICEPTIVA PERSISTENTE SOBRE O DESENVOLVIMENTO DE RESPOSTAS NOCICEPTIVAS SUBSEQUENTES

CURITIBA

2023

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EFEITO DA ASSOCIAÇÃO ENTRE RESTRIÇÃO DE SONO E ESTIMULAÇÃO NOCICEPTIVA PERSISTENTE SOBRE O DESENVOLVIMENTO DE RESPOSTAS NOCICEPTIVAS SUBSEQUENTES

Dissertação apresentada ao Programa de Pós-graduação em Fisiologia, Setor de Ciências Biológicas, Universidade Federal do Paraná, como requisito parcial à obtenção do título de Mestre em Fisiologia.

Orientadora: Profª. Dra. Luana Fischer

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ATA DE SESSÃO PÚBLICA DE DEFESA DE MESTRADO PARA A OBTENÇÃO DO GRAU DE MESTRA EM FISIOLOGIA

No dia vinte e sete de março de dois mil e vinte e tres às 09:00 horas, na sala Auditório do Departamento de Fisiologia (Sala 103), Setor de Ciências Biológicas - Departamento de Fisiologia , foram instaladas as atividades pertinentes ao rito de defesa de dissertação da mestranda **ANA CAROLINA PESCADOR**, intitulada**: Efeito da associação entre restrição de sono e estimulação nociceptiva persistente sobre o desenvolvimento de respostas nociceptivas subsequentes".**, sob orientação da Profa. Dra. LUANA FISCHER. A Banca Examinadora, designada pelo Colegiado do Programa de Pós-Graduação FISIOLOGIA da Universidade Federal do Paraná, foi constituída pelos seguintes Membros: LUANA FISCHER (UNIVERSIDADE FEDERAL DO PARANÁ), MARCO ORESTE FINOCCHIO PAGLIUSI JUNIOR (UNIVERSIDADE DE SÃO PAULO), MAÍRA ASSUNÇÃO BICCA (JOHNS HOPKINS UNIVERSITY - BALTIMORE). A presidência iniciou os ritos definidos pelo Colegiado do Programa e, após exarados os pareceres dos membros do comitê examinador e da respectiva contra argumentação, ocorreu a leitura do parecer final da banca examinadora, que decidiu pela APROVAÇÃO. Este resultado deverá ser homologado pelo Colegiado do programa, mediante o atendimento de todas as indicações e correções solicitadas pela banca dentro dos prazos regimentais definidos pelo programa. A outorga de título de mestra está condicionada ao atendimento de todos os requisitos e prazos determinados no regimento do Programa de Pós-Graduação. Nada mais havendo a tratar a presidência deu por encerrada a sessão, da qual eu, LUANA FISCHER, lavrei a presente ata, que vai assinada por mim e pelos demais membros da Comissão Examinadora.

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Os membros da Banca Examinadora designada pelo Colegiado do Programa de Pós-Graduação FISIOLOGIA da Universidade Federal do Paraná foram convocados para realizar a arguição da dissertação de Mestrado de **ANA CAROLINA PESCADOR** intitulada: **Efeito da associação entre restrição de sono e estimulação nociceptiva persistente sobre o desenvolvimento de respostas nociceptivas subsequentes".**, sob orientação da Profa. Dra. LUANA FISCHER, que após terem inquirido a aluna e realizada a avaliação do trabalho, são de parecer pela sua APROVAÇÃO no rito de defesa.

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“Os que se encantam com a prática sem a ciência são como os timoneiros que entram no navio sem timão nem bússola, nunca tendo certeza do seu destino”

Leonardo da Vinci

RESUMO

A diminuição do tempo de sono facilita a percepção dolorosa e a dor implica em distúrbios de sono. Há uma relação bidirecional entre sono insuficiente e dor. Considerando esta relação, avaliamos a hipótese de que a restrição de sono (RS) atuaria como facilitadora no processo de cronificação da dor no modelo de hiperalgesia crônica induzida por prostaglandina E_2 (PGE_2). Este modelo permite o estudo e manipulação durante a transição da hiperalgesia aguda para crônica, o que o torna ideal para este estudo. A primeira série de experimentos rejeitou a hipótese inicial, demonstrando que a RS não facilita a cronificação da hiperalgesia. Para além, a restrição de sono crônica, paradoxalmente, preveniu o desenvolvimento da hiperalgesia crônica. Alterações na expressão de proteínas relacionadas à sinalização dopaminérgica no núcleo Accumbens forneceram suporte preliminar sobre o papel do sistema dopaminérgico mesolímbico neste efeito paradoxal. Contudo, se a RS não facilita a cronificação da dor, poderia impactar no desenvolvimento futuro da dor? No segundo estudo, animais previamente submetidos à RS associada a injeções de PGE_2 foram mantidos sem intervenções até que o limiar nociceptivo retornasse aos valores basais, quando foram desafiados com uma nova rodada de injeções de PGE_2 ou submetidos a diferentes modelos animais de dor. Surpreendentemente, os animais demonstraram resistência ao subsequente efeito hiperalgésico de diferentes agentes inflamatórios e de lesão neuropática. Em contraste, a resposta a estímulos dolorosos não foi afetada. Nomeamos este fenômeno de “efeito protetor paradoxal da associação prévia entre RS crônica e estimulação nociceptiva sobre o desenvolvimento de hiperalgesia subsequente”. O aumento da função dopaminérgica, por intervenção farmacológica, antes do desafio impediu o efeito protetor, sugerindo que alterações na função dopaminérgica podem ser um dos mecanismos envolvidos neste efeito. Este estudo demonstrou dois efeitos paradoxais da RS, os quais devem ser interpretados com cautela. Qualquer efeito benéfico da RS no processamento da dor não deve ser visto como perspectiva terapêutica, mas sim como uma ferramenta para entender as neuro adaptações que emergem da complexa inter-relação entre diminuição do tempo de sono e cronificação da dor.

Palavras chave: gentle handling; cronificação da dor; sistema mesolímbico.

ABSTRACT

There is a bidirectional relationship between insufficient sleep and pain, that is pain impairs sleep and poor sleep increases pain. Here we hypothesized that sleep restriction (SR) facilitates pain chronification and used the prostaglandin E_2 (PGE_2)-induced chronic hyperalgesia model to test it. This model allows the study of hyperalgesia, a key manifestation of pain, during the transition from its acute to chronic phase. The first series of experiments rejected our hypothesis, showing that SR does not accelerate the transition from acute to chronic hyperalgesia. Furthermore, SR paradoxically prevented the development of chronic hyperalgesia. Changes in the expression of proteins related to dopaminergic signaling and synaptic function in the Nucleus accumbens provided preliminary support for a role of the mesolimbic dopaminergic system in this paradoxical effect. But, if SR does not facilitate pain chronification, could it impact its future development? In a second study, animals previously submitted to SR associated with PGE_2 injections were left to recover baseline nociceptive threshold to be challenged with a new round of PGE_2 injections or subjected to different animals' models of pain. Surprisingly, they showed resistance to the subsequent hyperalgesic effect of several inflammatory agents and a neuropathic injury. In contrast, responses to notably noxious stimulus are unaffected. We called this phenomenon the paradoxical protective effect of long-term SR associated with nociceptive stimulation on the subsequent development of hyperalgesia. The pharmacological increase in dopaminergic function before challenging prevented the protective effect, suggesting that changes in dopaminergic function may be one of the underlying mechanisms. This study demonstrated two paradoxical effects of SR and should be interpreted with caution. Any potential beneficial effect of SR on pain processing should not be seen from a therapeutic perspective, but rather as a tool to understand the neuroadaptations that emerge from the complex interrelationship between decreased sleep time and pain chronification.

Keywords: gentle handling; pain chronification; mesolimbic system.

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LISTA DE ABREVIATURAS E SIGLAS

PGE_2 – prostaglandina E_2

REM – movimento rápido dos olhos, do inglês, “rapid eye moviment”

PAG-RVM – substância cinzenta periaquedutal - bulbo rostro ventral, do inglês, “Periaqueductal Gray – Rostral Ventral Medulla”

NAc – núcleo Accumbens, do inglês, “nucleus Accumbens”

VTA – área tegmental ventral, do inglês, “ventral tegmental area”

RS ou SR – restrição de sono, do inglês, “sleep restriction”

CION – constrição crônica do nervo infraorbital, do inglês, “chronic constriction of the infraorbital nerve”

ACC – córtex cingulado anterior, do inglês, “anterior cingulate cortex”

PFC – córtex pré-frontal, do inglês, “prefrontal cortex”

DAT – transportador de dopamina, do inglês, “dopamine transporter”

DARPP-32 – fosfoproteína regulada por dopamina e AMP-c com peso molecular de 32KDa, do inglês, “dopamine and cyclic-AMP-regulated phosphoprotein of molecular weight 32KDa”

PKA – proteína quinase A, do inglês, “protein kinase A”

cAMP – adenosina 3,5-monofosfato cíclico, do inglês, “cyclic adenosine monophosphate”

PP-1 – proteína fosfatase, do inglês, “protein phosphatase 1”

CREB – proteína de ligação ao elemento de resposta cAMP, do inglês, “cAMP response element-binding protein”

CDK5 – proteína quinase dependente de ciclína, do inglês, “cyclin dependent kinase”

TRPV1 – (receptor de potencial transitório vanilóide tipo 1, do inglês, “transient receptor potential vanilloid 1”)

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1 INTRODUÇÃO

A dor tem função de alerta essencial à vida, pois sinaliza perigo e aciona respostas protetoras apropriadas (JULIUS; BASBAUM, 2001; WOOLF; SALTER, 2000). Contudo, em condições persistentes ou patológicas, a dor perde seu papel biológico e transforma-se em doença (ZEILHOFER, 2005). De fato, a dor crônica é um problema de saúde pública mundial e sua prevalência varia entre 10 e 40% da população (COHEN; VASE; HOOTEN, 2021; DAHLHAMER *et al.*, 2018; FAYAZ *et al.*, 2016; JOHANNES *et al.*, 2010; NAHIN, 2015). As consequências da dor crônica vão além da esfera pessoal, os impactos econômicos são gigantescos. Só nos Estados Unidos, o custo com tratamento e perda de produtividade relacionados à dor no ano de 2010 foi estimado em pelo menos 560 bilhões de dólares (GASKIN; RICHARD, 2012). Ainda, apesar de todo avanço científico das últimas décadas, os mecanismos envolvidos no desenvolvimento e manutenção da dor crônica são amplamente desconhecidos. O entendimento destes mecanismos é essencial ao desenvolvimento de estratégias terapêuticas para prevenir a transição da dor aguda para a crônica e para remediar os estados de dor crônica.

Os distúrbios de sono também são um problema de saúde pública de ordem mundial (MICHAL *et al.*, 2014; STRINGHINI *et al.*, 2015). No Brasil, estima-se que 108 milhões de pessoas sofram com algum distúrbio do sono (HIROTSU *et al.*, 2014). O impacto econômico relacionado a problemas com sono também é alarmante, cerca de 411 bilhões de dólares só nos Estados Unidos (HAFNER *et al.*, 2017). Ainda existe uma clara relação bidirecional entre diminuição do tempo de sono e aumento da sensibilidade à dor (KARAMAN *et al.*, 2014; NICHOLSON; VERMA, 2004; SMITH; HAYTHORNTHWAITE, 2004). Por exemplo, os distúrbios do sono são mais prevalentes em indivíduos com dor crônica (CHEATLE *et al.*, 2016; MORIN *et al.*, 2006; SMITH; HAYTHORNTHWAITE, 2004) e, pelo menos, 50% dos indivíduos com insônia sofrem de dor crônica (TAYLOR *et al.*, 2007).

Embora não haja dúvidas de que a diminuição do tempo de sono aumenta a dor (AFOLALU; RAMLEE; TANG, 2017; LENTZ *et al.*, 1999; SMITH; HAYTHORNTHWAITE, 2004; WEI *et al.*, 2010), os mecanismos são amplamente desconhecidos. Estudos recentes de nosso laboratório têm contribuído para ampliar esse entendimento. Por exemplo, foi demonstrado que a privação de sono REM (movimento rápido dos olhos, do inglês, rapid eye moviment) aumenta a

sensibilidade à dor ao interferir no funcionamento do sistema descendente de modulação da dor PAG-RVM (substância cinzenta periaquedutal - bulbo rostro ventral, do inglês, “Periaqueductal Gray – Rostral Ventral Medulla”), o principal e mais estudado mecanismo endógeno de modulação da dor (TOMIM *et al.*, 2016). Demonstramos também que o efeito pró-nociceptivo da privação de sono REM depende do aumento da atividade adenosinérgica sobre receptores A_{2A} e da diminuição da atividade dopaminérgica sobre receptores D_2 no NAc (núcleo Accumbens, do inglês, “nucleus Accumbens”) (SARDI; TOBALDINI; *et al.*, 2018). Este núcleo faz parte do sistema mesolímbico (HEIMER; HOESEN, 1979), recebe projeções dopaminérgicas da VTA (área tegmental ventral, do inglês, “ventral tegmental area”) e desempenha importante papel na modulação da dor (ALTIER; STEWART, 1998; GEAR; ALEY; LEVINE, 1999; GEAR; LEVINE, 2011) e na regulação do ciclo sono-vigília (OISHI *et al.*, 2017).

O sistema mesolímbico também desempenha importante papel no processo de transição da dor aguda para a crônica e outro estudo do nosso laboratório avançou consideravelmente no entendimento desse papel (VERGARA *et al.*, 2020). Já era conhecido que uma maior conectividade funcional entre o córtex pré-frontal e o NAc parece prever o desenvolvimento da dor crônica em humanos (BALIKI *et al.*, 2012; VACHON-PRESSEAU *et al.*, 2016) e que o aumento da atividade dopaminérgica do NAc facilita a cronificação da dor em ratos (DIAS *et al.*, 2015). Em Vergara et al., 2020 foi demonstrado que a lesão dos neurônios dopaminérgicos da VTA ou o bloqueio farmacológico do sistema kappa opioide no NAc impedem que a dor aguda se torne crônica. No entanto, uma vez que a dor crônica esteja estabelecida, essas manipulações experimentais não afetam sua manutenção (VERGARA *et al.*, 2020). O sistema kappa opioide é conhecido por diminuir a atividade dopaminérgica em estados patológicos, como a adição (MUSCHAMP; CARLEZON, 2013; NIIKURA *et al.*, 2010), e em Vergara et al., 2020 também foi demonstrado que os níveis de dopamina no NAc diminuem a medida que a dor se torna crônica. Portanto, os dados sugerem que a atividade dopaminérgica mesolímbica é essencial para a transição da dor aguda para a crônica, mas essa atividade diminui progressivamente durante o processo de cronificação e, quando a dor se torna crônica, não tem influência sobre sua manutenção.

Com base nos dados de que a diminuição do tempo de sono facilita a percepção dolorosa, este estudo tratará dos efeitos da restrição de sono (RS) sobre o processo

de cronificação da dor. A hipótese inicial era de que a RS atuaria como facilitadora do processo de cronificação da dor. Para testar esta hipótese, foi realizado um estudo preliminar utilizando um modelo de hiperalgesia inflamatória crônica. Neste modelo, são necessários 14 dias de injeção diária de PGE_2 (100 ng, s.c.) para induzir um quadro de hiperalgesia crônica que persiste por pelo menos 30 dias após a interrupção das injeções (FERREIRA; LORENZETTI; DE CAMPOS, 1990; VERGARA *et al.*, 2020). Contudo, o estado de manutenção da hiperalgesia pode ser induzido com apenas 7 dias de injeção diária de PGE_2, desde que associado a manipulações experimentais que facilitam a cronificação da dor (DIAS *et al.*, 2015; VERGARA *et al.*, 2020). Portanto, esperávamos induzir o estado de hiperalgesia crônica em animais submetidos a 7 dias de injeção diária de PGE_2 quando associado a RS por 6 horas diárias. Não foi observado o estado de manutenção da hiperalgesia característico do modelo, contudo, o período de retorno ao limiar nociceptivo basal se estendeu comparado aos controles. Com este resultado em vista, realizamos outro experimento aumentando o período de RS para 14 dias, associado a 7 dias de injeção diária de PGE_2. Novamente, não observamos um efeito facilitador sobre a cronificação e, contra intuitivamente, o período de retorno ao basal foi mais rápido comparado ao experimento anterior de apenas 7 dias de RS. Para mais, após o retorno ao limiar nociceptivo basal, estes animais foram desafiados com uma nova injeção de PGE_2, e os animais previamente submetidos a RS associada a 7 dias de injeção diária de PGE_2 não demonstraram a redução no limiar nociceptivo característica da PGE_2, sendo observado um efeito protetor. Diante destes resultados, este trabalho tem como objetivo ampliar o entendimento sobre o efeito da associação entre RS e estimulação nociceptiva, bem como compilar os dados em formato de artigo científico.

2 OBJETIVOS

2.1 OBJETIVO GERAL

Os objetivos centrais foram pautados em três pilares: (1) avaliar o efeito da restrição de sono no processo de cronificação da dor; (2) determinar se o efeito protetor, induzido pela associação prévia entre restrição de sono e estimulação nociceptiva, é observado em diferentes modelos animais de dor e de hiperalgesia; (3) verificar se o aumento da atividade dopaminérgica mesolímbica durante o período de retorno ao limiar basal afeta o efeito protetor.

2.2 OBJETIVOS ESPECÍFICOS

- *1.1* Avaliar se 14 dias de restrição de sono impede a cronificação da resposta hiperalgésica que é classicamente induzida por 14 injeções diárias de PGE_2;
- *1.2* Investigar se a restrição de sono e o processo de cronificação da dor (induzido por injeções diárias de PGE_2), assim como a associação entre eles, altera de forma antagônica a atividade dopaminérgica mesolímbica, através da quantificação de proteínas recrutadas pela atividade dopaminérgica;
- *1.3* Avaliar se um antipsicótico, droga que reduz a atividade dopaminérgica, produz efeito semelhante à restrição de sono, quando associada à estimulação nociceptiva;

- *2.1* Avaliar se o efeito protetor previamente induzido pela associação entre restrição de sono e injeções diárias de PGE_2 também é observado em:
    - *2.1.1* Modelo de dor inflamatória aguda induzida por formalina;
    - *2.1.2* Modelo de hiperalgesia inflamatória aguda induzida por carragenina;
    - *2.1.3* Modelo de dor e hiperalgesia inflamatória aguda induzida por capsaicina;
    - *2.1.4* Modelo de hiperalgesia de origem neuropática induzida por constrição do nervo infraorbital (CION);

- *3.1* Avaliar se o aumento dos níveis sistêmicos de dopamina, durante o período de retorno ao limiar nociceptivo basal, previne o efeito protetor;
- *3.2* Avaliar se o aumento da disponibilidade sináptica de dopamina no núcleo Accumbens, durante o período de retorno ao limiar nociceptivo basal, previne o efeito protetor.

3 REVISÃO BIBLIOGRÁFICA

3.1 DOR E DOR CRÔNICA

A dor tem função de alerta essencial para a manutenção da vida, pois, embora possua um caráter emocional desagradável, serve como sinalizador para direcionar o comportamento a fim de cessar o estímulo causador da dor, comumente associado a uma lesão tecidual que gera risco à vida (JULIUS; BASBAUM, 2001; RAJA *et al.*, 2020; WOOLF; SALTER, 2000). Em situação não patológica de dor, dor nociceptiva, é necessário que haja um estímulo físico ou químico de alta intensidade, e que este, por meio de mecanismos de transdução de sinal, induza a deflagração do potencial de ação no nociceptor (FIELDS, 2004; JULIUS; BASBAUM, 2001; PEIRS; SEAL, 2016). Os nociceptores, através da via nociceptiva, transmitem a informação associada ao estímulo doloroso até o encéfalo, onde a dor, em toda a sua complexa experiência, é percebida (JULIUS; BASBAUM, 2001; PEIRS; SEAL, 2016) (Figura A).

A complexidade da experiência dolorosa é resultado da integração de projeções para diferentes áreas, o que permite a percepção multidimensional da dor, com características emocionais, discriminativas e motoras (APKARIAN, 2008; HUNT; MANTYH, 2001; PEIRS; SEAL, 2016). Ainda, a fragmentação da via nociceptiva em sinapses possibilita o recrutamento de múltiplos sítios de modulação em neurônios de diferentes ordens (HEINRICHER *et al.*, 2009) a depender do contexto em que o organismo se encontra (OSSIPOV; MORIMURA; PORRECA, 2014). A modulação da experiência dolorosa pode resultar na ampliação ou redução das entradas nociceptivas (NAVRATILOVA; PORRECA, 2014; VANEGAS; SCHAIBLE, 2004; YOU *et al.*, 2010).

Em resumo, a relação entre ativação de nociceptores e dor não é linear e a via nociceptiva é constantemente modulada a depender do conjunto de estímulos percebidos pelo organismo (HEINRICHER *et al.*, 2009; NAVRATILOVA; PORRECA, 2014; YOU *et al.*, 2010). Por exemplo: em situações de medo e estresse intensos, ocorre a diminuição da responsividade ao estímulo nociceptivo (hipoalgesia) (HEINRICHER et al., 2009), já na inflamação, ocorre uma redução do limiar nociceptivo (alodinia) e um aumento da responsividade ao estímulo nociceptivo (hiperalgesia) (HEINRICHER *et al.*, 2009; WOOLF, 2011). Contudo, em alguns

indivíduos e por razões ainda não totalmente conhecidas, podem ocorrer alterações plásticas na via nociceptiva de caráter não adaptativo (NAVRATILOVA; PORRECA, 2014). Alterações plásticas estas que levam a um desbalanço entre facilitação e inibição da nocicepção, podendo ainda resultar em um quadro patológico de dor, comumente descrito como dor crônica (NAVRATILOVA; PORRECA, 2014). Em uma situação de dor patológica, a dor pode estar presente mesmo na ausência de um estímulo nociceptivo (NAVRATILOVA; PORRECA, 2014; ZEILHOFER, 2005), assim, a dor perde o seu caráter protetivo e se transforma em doença.

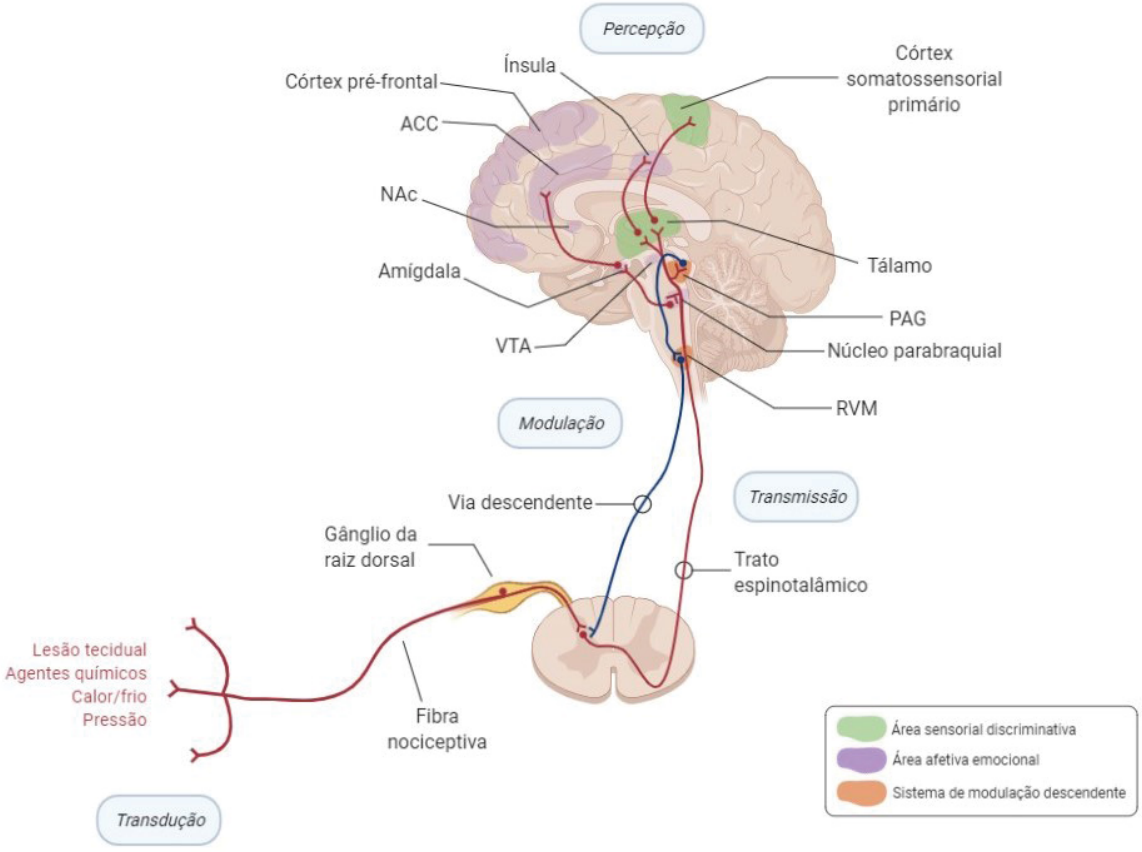

Figura A: Esquema representativo da via nociceptiva e regiões associadas ao processamento do estímulo doloroso e geração da experiência dolorosa. Em situação não patológica, a percepção da dor se inicia com a transdução de um estímulo mecânico, térmico ou químico de alta intensidade em potencial de ação. Este primeiro evento ocorre nos nociceptores, neurônios sensoriais primários que, através de receptores presentes nas terminações nervosas livres, são capazes de transdução de sinal e codificação de estímulos nocivos em potencial de ação (FIELDS, H., 2004; JULIUS; BASBAUM, 2001). Após a codificação, a informação é transmitida, por meio de potenciais de ação, ao longo da via nociceptiva, sendo no corno dorsal da medula espinhal onde se localiza a primeira sinapse da via. A partir da primeira sinapse, a maioria das fibras

nociceptivas secundárias ascendem (1) até o tálamo, através dos tratos espinotalâmico lateral e medial (HODGE; APKARIAN, 1990) ou (2) até o núcleos da formação reticular, através do trato espinoparabraquial (BURITOVA; BESSON; BERNARD, 1998; HUNT; MANTYH, 2001). A partir do tálamo e dos núcleos da formação reticular (principalmente o núcleo parabraquial e a PAG) ocorrem projeções para regiões relacionadas à transformação do estímulo nociceptivo na experiência de "dor" (HUNT; MANTYH, 2001; FIELDS, 2004). Projeções para o córtex somatossensorial e para a ínsula estão mais relacionadas ao aspecto discriminativo somático da dor, que representa a localização, qualidade e duração do estímulo (HUNT; MANTYH, 2001; APKARIAN, 2008). Projeções para regiões como: amigdala, hipotálamo, ACC (córtex cingulado anterior, do inglês, "anterior cingulate cortex"), PFC (córtex pré-frontal, do inglês, "prefrontal cortex"), VTA, NAc e, novamente, a ínsula, são responsáveis pela dimensão afetiva da dor (HUNT; MANTYH, 2001), motivação de escape, planejamento para cessar o dano e aprendizagem/reforço negativo relacionado ao dano (APKARIAN *et al.*, 2005; BALIKI *et al.*, 2010). Existem ainda regiões responsáveis pela modulação das entradas nociceptivas e o sistema descendente é o mais conhecido deles, possuindo como figura central o circuito PAG-RVM. Múltiplas aferências, periféricas e encefálicas, convergem para a PAG e eferências, a partir do RVM, modulam a transmissão nociceptiva a nível medular (FIELDS, H., 2004; FIELDS; HEINRICHER; MASON, 1991; REYNOLDS, 1969). Por conta de uma arquitetura com duas subpopulações de neurônios antagônicos, "ON-cells" e "OFF-cells", o RVM possui a capacidade dinâmica de integrar as aferências e balancear eferências que facilitem e que inibem a nocicepção (HEINRICHER et al., 2009). As "OFF-cells" são responsáveis pelas projeções antinociceptivas, enquanto as "ON-cells" são responsáveis pelas projeções pró-nociceptivas (HEINRICHER et al., 2009).

A dor crônica é um dos maiores problemas de saúde pública do mundo, com estimativa de prevalência entre 10-40% da população (COHEN; VASE; HOOTEN, 2021; DAHLHAMER *et al.*, 2018; FAYAZ *et al.*, 2016; JOHANNES *et al.*, 2010; NAHIN, 2015). Além de afetar diretamente a qualidade de vida de milhões de pessoas, está associada a um gigantesco impacto econômico (GASKIN; RICHARD, 2012; PORRECA; OSSIPOV; GEBHART, 2002). Problemas também são observados no tratamento, pois a taxa de sucesso é de cerca de apenas 30% (BORSOOK; BECERRA; HARGREAVES, 2011).

Dentre as características observadas na dor crônica, e que correspondem a uma percepção dolorosa alterada, estão: dor espontânea, hiperalgesia e alodinia (APKARIAN, 2008). A dor espontânea é caracterizada por ativação da fibra nociceptiva na ausência de um estímulo nociceptivo (BENNETT, 2012) e, como explorado nos parágrafos anteriores, a hiperalgesia e alodinia são características

observadas quando há redução no limiar nociceptivo (MERSKEY *et al.*, 1994). As alterações descritas culminam na amplificação da percepção dolorosa e se relacionam com a diminuição na qualidade de vida observada no indivíduo com dor crônica, isto porque a constante demanda por cessar a dor suprime outras emoções, levando a um estado de anedonia (COHEN; VASE; HOOTEN, 2021; NAVRATILOVA; PORRECA, 2014)

A transição da dor aguda para dor crônica se desenvolve a partir de um processo, a cronificação da dor. A persistência de entradas nociceptivas e/ou a sensibilização de nociceptores inicia uma cascata de alterações na via nociceptiva à nível periférico (ELMAN; BORSOOK, 2016; MIRANDA *et al.*, 2015; NAVRATILOVA; PORRECA, 2014; PORRECA; OSSIPOV; GEBHART, 2002), que podem ou não culminar na dor crônica. Neste contexto, certas características que influenciam a via nociceptiva ou a percepção dolorosa podem facilitar a cronificação, como medicações, estressores e a responsividade do sistema imunológico (BORSOOK *et al.*, 2018; DENK; MCMAHON; TRACEY, 2014; YARNITSKY *et al.*, 2008).

Durante o processo de cronificação da dor, alterações à nível periférico influenciam a transmissão nociceptiva a nível medular, aumentando a eficiência das aferências nociceptivas para centros superiores (LUO; KUNER; KUNER, 2014). Além disso, também são observadas alterações na modulação descendente da dor, aumentando as eferências que facilitam a transmissão da informação nociceptiva e reduzindo as eferências inibitórias (MIRANDA *et al.*, 2015; OSSIPOV; MORIMURA; PORRECA, 2014; PORRECA; OSSIPOV; GEBHART, 2002).

Há também uma importante influência de sistemas encefálicos na cronificação (PORRECA; OSSIPOV; GEBHART, 2002). Isto porque durante o processo de cronificação da dor ocorrem alterações em áreas como a amígdala (NEUGEBAUER *et al.*, 2004), o PFC e o NAc (APKARIAN, 2008; BALIKI *et al.*, 2012), alterando o processamento encefálico da nocicepção, principalmente no aspecto afetivo-emocional. Estas regiões não são exclusivas ao processamento nociceptivo, possuem também funções relacionadas à aprendizagem, adição e mecanismos de recompensa (APKARIAN, 2008). O que reflete em diversas hipóteses que relacionam mecanismos de adição e de aprendizagem com a cronificação da dor (APKARIAN, 2008; ELMAN; BORSOOK, 2016).

Em última instância, as mudanças plásticas ocorridas na cronificação resultam na manutenção da dor crônica. Neste estágio há evidentes alterações nos

circuitos neuronais recrutados para o processamento da dor (BORSOOK *et al.*, 2018) gerando um estado de dor constante, além de comorbidades como depressão, ansiedade, prejuízos cognitivos e distúrbios de sono (BAIR *et al.*, 2008; GUREJE, 2008; OSSIPOV; MORIMURA; PORRECA, 2014; TAYLOR *et al.*, 2016).

3.2 MODELO DE HIPERALGESIA INFLAMATÓRIA CRÔNICA

O processamento nociceptivo alterado na dor crônica pode ter diferentes origens, sendo, de maneira simplista, dividido em (1) dor crônica de origem inflamatória (resultado da ativação crônica do sistema imunológico) ou (2) dor crônica de origem neuropática (resultado de lesão neural) (APKARIAN, 2008). Além das diferentes origens, características individuais também dificultam o entendimento sobre o processo de cronificação da dor (APKARIAN, 2008). Contudo, estudos em diferentes modelos enriquecem os conhecimentos sobre os mecanismos envolvidos e são de extrema importância para a busca de melhores terapias para a dor crônica.

Considerando a dor crônica de origem inflamatória, Ferreira *et al*., 1990, desenvolveram um protocolo que utiliza o potencial de sensibilização da PGE_2 para induzir um estado crônico de hiperalgesia. Existem duas fases bem definidas neste modelo: a fase de indução e a fase de manutenção. A fase de indução é definida como o período em que o animal é submetido a 14 dias de injeção diária de PGE_2 na pata traseira. A fase de manutenção é definida como o período em que mesmo na ausência de estimulação pró-nociceptiva ocorre a manutenção da diminuição do limiar nociceptivo por, pelo menos, 30 dias (FERREIRA; LORENZETTI; DE CAMPOS, 1990).

A principal vantagem deste modelo é o extenso período de cronificação da dor (período de indução), o que possibilita avaliar mais facilmente as intervenções experimentais capazes de facilitar a cronificação. Já foi demonstrado que em intervenções que facilitam a cronificação apenas 7 dias de injeção diária são necessários para induzir a fase de manutenção (DIAS *et al.*, 2015; PIARDI *et al.*, 2020; VERGARA *et al.*, 2020).

3.3 SONO E DISTÚRBIOS DE SONO

O sono é componente vital para a manutenção da homeostase do organismo, visto que este influencia em diversos aspectos cognitivos e metabólicos (BRINGMANN, 2019; BROWN *et al.*, 2012; NASCIMENTO *et al.*, 2007). Já a vigília detém considerável vantagem evolutiva, pois permite que o organismo interaja com o ambiente (BRINGMANN, 2019; BROWN *et al.*, 2012). Estes estados, sono e vigília, são classificados de acordo com alterações na atividade elétrica cortical e se alternam durante um período de 24h, característica de um ritmo circadiano (BROWN *et al.*, 2012; BUZSÁKI; DRAGUHN, 2004).

O padrão de atividade elétrica cortical é gerado por diversos circuitos neurais que se inter-relacionam (OH *et al.*, 2019). Durante a vigília há ativação dos núcleos promotores da vigília: Locus Coeruleus, Núcleo da Rafe, VTA, Núcleo túbero mamilar e prosencéfalo (SCAMMELL; ARRIGONI; LIPTON, 2017), que, quando ativados, mandam eferências para regiões como o hipotálamo, o tálamo e o córtex, e possibilitam a manutenção do padrão de ativação característico da vigília (SCAMMELL; ARRIGONI; LIPTON, 2017). Durante o sono ocorre a inibição dos núcleos promotores da vigília, via eferências de neurônios GABAérgicos localizados no núcleo pré-óptico do hipotálamo e via moléculas inibitórias produzidas nos núcleos promotores da vigília (BROWN *et al*., 2012). Estas moléculas inibitórias são figura central do fator homeostático que regula o ciclo sono-vigília, estas acumulam ao longo da vigília, influenciam os circuitos neurais relacionados à indução do sono e, ao longo do período de sono, diminuem sua concentração (OISHI *et al.,* 2017). Dentre as moléculas, a adenosina possui figura central, mas, oxido nítrico, prostaglandinas e outras citocinas também estão relacionadas (BROWN *et al.,* 2012). Observa-se a necessidade do sono para a variação cíclica dos fatores homeostáticos, pois estes necessitam de mecanismos que ocorrem no sono para diminuir sua concentração tecidual (OISHI *et al.,* 2017), se o sono não ocorre há necessidade do período de rebote/recuperação de sono.

É importante salientar que as áreas que induzem os estados de sono e de vigília são comuns a outros processamentos neurais, possibilitando a influência de alterações cognitivas, emocionais, sensoriais e metabólicas no ciclo sono-vigília e o contrário também ocorre (OISHI *et al.*, 2017; SAPER; CANO; SCAMMELL, 2005; SCAMMELL; ARRIGONI; LIPTON, 2017).

A sociedade moderna tornou natural a redução do tempo de sono (CHHANGANI *et al.*, 2009; MATSUMOTO; CHIN, 2019), e a prevalência de

distúrbios de sono e qualidade de sono reduzida escalona de forma alarmante (HIROTSU *et al.*, 2014; MATSUMOTO; CHIN, 2019; MORAES *et al.*, 2013; OHAYON, 2011). Observa-se uma redução de aproximadamente uma hora e meia de sono por noite, quando comparado ao início do século passado (CHHANGANI *et al.*, 2009; WALSLEBEN *et al.*, 2004), possivelmente reflexo da evolução tecnológica, econômica e social. Tratando de distúrbios de sono, estima-se uma prevalência entre 20-60% na população em geral (BHASKAR; HEMAVATHY; PRASAD, 2016; HIROTSU *et al.*, 2014; HOSSAIN; SHAPIRO, 2002; MICHAL *et al.*, 2014; OHAYON, 2011). As consequências da baixa qualidade de sono são observadas a nível individual e coletivo (CHATTU *et al.*, 2018; RAMAR *et al.*, 2021; VETTER, 2018), ainda com o dado alarmante sobre o impacto econômico de cerca de 411 bilhões de dólares só nos Estados Unidos (HAFNER *et al.*, 2017).

A diminuição no tempo e/ou qualidade de sono leva a consequências na homeostase encefálica que resultam em alterações em diversos circuitos neurais (BROWN *et al.*, 2012; PALAGINI *et al.*, 2022). Um mecanismo evolutivo para reduzir os prejuízos relacionados à diminuição no tempo de sono leva à inibição dos mecanismos da vigília, propiciando assim o período de sono rebote (BROWN *et al.*, 2012). Como discutido, os mecanismos relacionados à indução e manutenção da vigília são comuns a diversos processos neurais, o que leva a alterações comportamentais e fisiológicas relacionadas à plasticidade ocasionada pela restrição de sono (KILLGORE; BALKING; WESENSTEN, 2006; KNUTSON *et al.*, 2007; MULLINGTON *et al.*, 2009; SPIEGEL; LEPROULT; VAN CAUTER, 1999). Como exemplos temos: memorização deficiente (SANTOS *et al.*, 2016), alterações na tomada de decisão (ORZEŁ-GRYGLEWSKA, 2010), aumento da sensibilidade dolorosa (SARDI; LAZZARIM; *et al.*, 2018; SARDI; TOBALDINI; *et al.*, 2018) e ainda relação com patologias como: hipertensão (ALTMAN *et al.*, 2012; KIM *et al.*, 2013), obesidade (HART *et al.*, 2013) e depressão (VOLKOW *et al.*, 2008).

3.4 EFEITO PRÓ-NOCICEPTIVO DA DIMINUIÇÃO DO TEMPO DE SONO

Diversos estudos epidemiológicos já observaram uma relação bidirecional entre dor e sono (Tabela A); a dor altera o sono e distúrbios do sono alteram a percepção dolorosa (AFOLALU; RAMLEE; TANG, 2017; CHEATLE *et al.*, 2016; SMITH; HAYTHORNTHWAITE, 2004; VANINI, 2016). A prevalência de distúrbios de

sono em indivíduos com dor crônica está entre 50-80% (CHEATLE *et al.*, 2016; SUN *et al.*, 2021; ZHANG; SHEN; LIU, 2020), enquanto aproximadamente 50% de indivíduos com distúrbios de sono sofrem de dor crônica (TAYLOR *et al.*, 2007). A baixa qualidade de sono possui relação direta com a magnitude e duração com que a dor é percebida, aumentando ambos, tanto em indivíduos com dor crônica quanto em modelos experimentais que induzem dor e pró-nocicepção (AFFLECK *et al.*, 1996; HAMBRECHT-WIEDBUSCH *et al.*, 2017; HUANG *et al.*, 2014; JENNUM *et al.*, 2013; LI *et al.*, 2019; MORPHY *et al.*, 2007; O'BRIEN *et al.*, 2011; PAGE; OPP; KOZACHIK, 2014; STONE *et al.*, 1997; SUTTON; OPP, 2014; TANG *et al.*, 2012; TOMIM *et al.*, 2016; VANINI, 2016; XUE *et al.*, 2018). Ainda, diversos estudos longitudinais observaram uma maior incidência de dor crônica em pessoas com distúrbios de sono de base (AFOLALU; RAMLEE; TANG, 2017; AGMON; ARMON, 2014; ANDERSEN *et al.*, 2018; CHOUCHOU *et al.*, 2014; CREMEANS-SMITH *et al.*, 2006; GENERAAL *et al.*, 2017; GUPTA *et al.*, 2007; JONES *et al.*, 2009; LINDELL; GRIMBY-EKMAN, 2022). Por fim, já foi demonstrado em diferentes modelos experimentais que a diminuição do tempo de sono *per se* (sem dor associada) reduz o limiar nociceptivo (HAACK; MULLINGTON, 2005; SARDI; LAZZARIM; *et al.*, 2018; SARDI; TOBALDINI; *et al.*, 2018; SCHUH-HOFER *et al.*, 2013; TIEDE *et al.*, 2010; WODARSKI *et al.*, 2014)

Apesar de todos estes dados experimentais e observacionais, os mecanismos envolvidos na relação entre dor e sono não são completamente compreendidos. A maioria dos achados se concentram em alterações supraespinhais geradas pela diminuição no tempo de sono que resultam em pró-nocicepção, e serão detalhados a seguir.

Tabela A - Compilação de artigos que retratam as relações entre dor e sono

Referência	Tipo de estudo	Observações	Conclusões gerais
Haack e Mullington, 2005	Experimental (humanos)	12 dias de RS (4h de sono por dia) em indivíduos saudáveis = ↑ dor no corpo generalizada, dor nas costas e irritação no estômago	A diminuição do tempo de sono *per se* reduz o limiar nociceptivo
Wodarski *et al*., 2014	Experimental (ratos)	8h de perturbação do sono = ↓ limiar nociceptivo mecânico e térmico	
Schuh-Hofer *et al*., 2013	Experimental (humanos)	Uma noite de privação de sono = ↓ limiar nociceptivo mecânico e térmico e ↑ ansiedade	
Tiede et al., 2010	Experimental (humanos)	Uma noite de RS (4h de sono) = ↓ do limiar nociceptivo	
Sardi; Tobaldini *et al*., 2018	Experimental (ratos)	3 dias de PS-REM = ↓ do limiar nociceptivo mecânico	
Sardi; Lazzarim *et al*., 2018	Experimental (ratos)	30 dias de RS (6h de RS por dia) = ↓ do limiar nociceptivo mecânico	
Huang *et al*., 2014	Experimental (ratos)	3 dias de PS pós CCI = ↑ a magnitude da hiperalgesia induzida por CCI	Efeito sinérgico, a diminuição do tempo de sono aumenta a magnitude e/ou duração da dor/hiperalgesia
Kozachik *et al*., 2014	Experimental (ratos)	6h de RS por dia concomitante com quimioterápico = ↑ a hipersensibilidade mecânica induzida por quimioterápico	
Vanini, 2016	Experimental (ratos)	9h de PS = ↑ magnitude da alodinia induzida por formalina 5%	
Hambrecht-Wiedbusch *et al*., 2017	Experimental (ratos)	6h de restrição de sono previamente à incisão na pata = ↑ a hipersensibilidade mecânica pós operatória e o tempo de recuperação da dor pós cirúrgica	
Sutton e Opp, 2014	Experimental (camundongo)	12h de perturbação do sono por dia durante 5 dias e injeção de salina acidificada no musculo = ↑ a hipersensibilidade mecânica induzida por injeção de salina acidificada	
Xue *et al.*, 2018	Experimental (ratos)	Privação de sono por 24h pré incisão plantar = ↑ a magnitude e prolonga a hipersensibilidade pós operatória	
Li *et al*., 2019	Experimental (ratos)	6h de RS-REM por dia por 3 dias pré incisão plantar = prolonga a hiperalgesia pós cirúrgica	
Tomim *et al*., 2016	Experimental (ratos)	24h ou 48h de RS-REM = ↑ a nocicepção induzida por formalina e ↓ o limar nociceptivo mecânico	
Morphy *et al.*, 2007	Observacional (humanos)	Insônia = ↑ incidência de dor, ansiedade e depressão em pacientes com dor no corpo generalizada	Efeito sinérgico, distúrbios de sono aumentam a incidência e magnitude de dor em pacientes com dor crônica
Jennum *et al*., 2013	Observacional (humanos)	Narcolepsia = ↑ incidência de dor em pacientes com dor musculoesquelética	
Tang *et al*., 2012	Observacional (humanos)	Baixa qualidade de sono = ↑ incidência de dor no dia seguinte em pacientes com dor crônica	
Affleck *et al*., 1996	Observacional (humanos)	Baixa qualidade de sono = ↑ incidência de dor no dia seguinte em pacientes com fibromialgia; o contrário também ocorre	
Stone *et al.*, 1997	Observacional (humanos)	Baixa qualidade de sono = ↑ dor e fadiga em pacientes com artrite reumatoide	
O'Brien *et al*., 2011	Observacional (humanos)	Baixa qualidade de sono = ↑ dor; o contrário também ocorre	
Sun *et al*., 2021	Observacional (humanos)	A prevalência de distúrbios de sono em pacientes com dor crônica é de aprox. 73%	Distúrbios de sono estão associados a uma maior prevalência de dor crônica; o contrário também é valido
Cheatle *et al*., 2016	Revisão	Pacientes com dor crônica = ↑ prevalência de distúrbios de sono	
Zhang *et al*., 2020	Observacional (humanos)	Pacientes com artrite reumatoide = ↑ prevalência de distúrbios de sono	
Taylor *et al*., 2007	Observacional (humanos)	Insônia = ↑ prevalência de dor crônica	

Gupta *et al.*, 2007	Observacional (humanos)	Insônia = ↑ incidência de dor crônica	Distúrbios de sono de base aumentam a incidência de dor crônica no futuro
Jones *et al*., 2009	Observacional (humanos)	Distúrbios de sono = ↑ incidência de dor	
Agmon e Armon, 2014	Observacional (humanos)	Insônia = ↑ incidência de dor nas costas	
Chouchou *et al*., 2014	Revisão	Problemas de sono = ↑ incidência de dor pós operatória	
Afolalu *et al*., 2018	Revisão	Baixa qualidade de sono = ↑ incidência de dor crônica	
Cremeans-Smith *et al*., 2006	Observacional (humanos)	Distúrbios de sono = prediz uma recuperação mais lenta pós cirurgia do joelho	
Generaal *et al*., 2017	Observacional (humanos)	Insônia = ↑ incidência de dor crônica	
Lindell e Grimby-Ekman, 2022	Observacional (humanos)	Baixa qualidade de sono = ↑ incidência de dor crônica	
Andersen *et al.*, 2018	Revisão	Insônia = ↑ a incidência de dor crônica ou piora a condição dolorosa já existente	

RS = restrição de sono; PS = privação de sono; CCI = constrição do nervo ciático; REM = movimento rápido dos olhos

Como tratado no tópico sobre sono, a adenosina possui importante função na homeostase do ciclo sono/vigília, e esta parece ter influência também sobre o efeito pró-nociceptivo da diminuição do tempo de sono. Em Alexandre *et al*., 2017 foi demonstrado que a administração por via oral de cafeína, um antagonista não seletivo de receptores adenosinérgicos, reverteu a hiperalgesia causada por privação de sono total. A cafeína também é capaz de prevenir a hiperalgesia pós cirúrgica induzida por restrição de sono (HAMBRECHT-WIEDBUSCH *et al.*, 2017). Já em Sardi; Tobaldini *et al*. 2018 observou-se que a administração intra-NAc de antagonista de receptor A_{2A} preveniu a hiperalgesia induzida por privação de sono REM. Este atesta que o aumento da atividade de receptores A_{2A} no NAc é fundamental para o efeito da privação de sono REM. O aumento da atividade de receptores A_{2A} no núcleo pré-optico também parece ser necessário, visto que a administração local de antagonista A_{2A} bloqueou a hiperalgesia pós cirúrgica induzida por restrição de sono (HAMBRECHT-WIEDBUSCH *et al.*, 2017). Com isso, até o momento de finalização deste trabalho, é possível concluir que a ativação de receptores adenosinérgicos do tipo A_{2A} parece ser fundamental para o aumento da sensibilidade à dor induzido por diminuição no período de sono. De fato, a adenosina acumula em situações de diminuição no tempo de sono (IAN SCHMITT *et al.*, 2012; KALINCHUK *et al.*, 2011; LEENAARS *et al.*, 2018; PORKKA-HEISKANEN; STRECKER; MCCARLEY, 2000) e isso pode estar relacionado com o aumento da atividade do sistema adenosinérgico.

O aumento da atividade do sistema adenosinérgico resulta em alterações intracelulares complexas, podendo contemplar: modificação de canais iônicos; alterações em segundos mensageiros e alterações na liberação de neurotransmissores (WILSON; MUSTAFA, 2009). O sistema dopaminérgico é um dos alvos de modulação. Sabe-se que uma maior ativação de receptores adenosinérgicos do tipo A_{2A} leva à internalização (FUXE *et al.*, 2002; HILLION *et al.*, 2002) e diminuição da afinidade (FERRE *et al.*, 1991) de receptores dopaminérgicos do tipo D_2. Estes co-expressos principalmente em neurônios GABAérgicos do estriado (MOINE *et al.*, 1997). Com uma menor sinalização dopaminérgica por receptores inibitórios do tipo D_2, os neurônios GABAérgicos estão mais ativados, resultando em uma redução da atividade motora e do sistema de recompensa, o que vai ao encontro com a hipótese de que a diminuição no tempo de sono está associada à hipofunção dopaminérgica mesolímbica (VOLKOW *et al.*, 2012).

Estas alterações no sistema dopaminérgico ainda se relacionam com achados sobre a influência deste no efeito pró-nociceptivo da diminuição do tempo de sono. Já foi demonstrado, por nosso laboratório, que a administração de agonista de receptores D_2 no NAc bloqueia o efeito pró-nociceptivo induzido pela privação de sono REM (SARDI; TOBALDINI; *et al.*, 2018). Este resultado vai ao 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 ainda fortalecido com o dado de que tanto a administração de modafinil, droga que aumenta, principalmente, a disponibilidade de dopamina, quanto a administração de L-DOPA normalizam o aumento da sensibilidade dolorosa induzida por privação de sono, e o mesmo não ocorre com administração de ibuprofeno ou morfina (ALEXANDRE *et al.*, 2017; SKINNER *et al.*, 2011).

Se tratando de processamento cortical alterado, já foi observada uma menor atividade no ACC e na ínsula após estímulo doloroso associado à restrição de sono (KRAUSE *et al.*, 2019; TIEDE *et al.*, 2010), o que configura possível prejuízo na percepção afetiva da dor, motivação de escape e reforço negativo relacionado ao dano (APKARIAN *et al.*, 2005; BALIKI *et al.*, 2010; HUNT; MANTYH, 2001). Um aumento na atividade do córtex somatossensorial primário também já foi demonstrado após experimentos com restrição de sono em humanos, utilizando ressonância magnética funcional (KRAUSE *et al.*, 2019). No mesmo estudo ainda foi

evidenciada uma correlação positiva entre reatividade do córtex somatossensorial e diminuição do limiar nociceptivo (KRAUSE *et al.*, 2019).

O principal sistema de modulação descendente da dor, PAG-RVM, também está alterado. Há uma diminuição das eferências inibitórias e um aumento das eferências que facilitam a dor (TOMIM *et al.*, 2016). Em Tomim et al., 2016 também foi demonstrado que, após privação de sono REM, a expressão de c-fos no RVM aumenta, possivelmente resultado de uma maior ativação, e, em Sardi; Lazzarim et al., 2018, foi demonstrado que uma lesão na PAG impede o efeito pró-nociceptivo da restrição de sono total. É interessante salientar, ainda, que a PAG é o local onde convergem as informações encefálicas vindas de núcleos superiores, como NAc e ACC, e estes influenciam no processamento e na saída que facilita a nocicepção (GEAR; ALEY; LEVINE, 1999; ZHANG *et al.*, 2013). O sistema opioidérgico também é alterado em função da diminuição do tempo de sono, levando a uma desregulação e diminuição da eficácia de medicamentos opioides (NASCIMENTO *et al.*, 2007; SKINNER *et al.*, 2011; TOMIM *et al.*, 2016). Levando em conta que pacientes com dor crônica também possuem alterações nos receptores opioides, como diminuição do potencial de ligação (HARRIS *et al.*, 2007), que também implicam na diminuição da eficácia de tratamento, a associação entre diminuição do tempo de sono e dor crônica pode intensificar estas alterações funcionais. De fato, um estudo recente demonstrou que a baixa qualidade de sono antes de cirurgia do quadril prediz maior tratamento com opioides após a cirurgia (BJURSTRÖM *et al.*, 2021), possivelmente devido à baixa eficácia do tratamento opioide em indivíduos com prejuízo de sono.

A resposta inflamatória está diretamente relacionada com o aumento da percepção dolorosa (MCMAHON; BEVAN, 2005) e também influencia nos processos de cronificação da dor (DIAS *et al.*, 2015; VERGARA *et al.*, 2020). Neste contexto, já foi demonstrado que a diminuição no tempo de sono altera os marcadores inflamatórios e leva a prevalência do estado pró-inflamatório (HAACK *et al.*, 2009, 2020; HAACK; SANCHEZ; MULLINGTON, 2007). Por exemplo, já foi demonstrado que a diminuição do tempo em 50% durante 10 dias induz aumento nos níveis séricos de IL-6 (interleucina 6) (HAACK; SANCHEZ; MULLINGTON, 2007) e que 88 horas de privação de sono induz um aumento nos metabólitos de PGE_2 na urina (HAACK *et al.*, 2009).

Em resumo, a diminuição no tempo de sono impacta a percepção da dor e, isso ocorre por conta de alterações em diferentes segmentos da via nociceptiva que,

em última instância, facilitam a percepção dolorosa. Contudo, não há estudos avaliando o impacto da restrição de sono durante o processo de cronificação da dor, o que, utilizando o modelo de dor crônica inflamatória induzida por PGE_2, será investigado neste trabalho.

4 ARTIGO CIENTÍFICO

Paradoxical effect of long-term insufficient sleep on pain chronification and on the subsequent development of acute and chronic pain

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Original Article

Introduction

Pain conditions and sleep disorders are public health problems worldwide (CHATTU *et al.*, 2018; COHEN; VASE; HOOTEN, 2021) and there is a well-recognized bidirectional relationship between them. That is, pain impairs sleep and poor sleep increases pain (AFOLALU; RAMLEE; TANG, 2017; ANDERSEN *et al.*, 2018; KRAUSE *et al.*, 2019). With a prevalence reaching up to 40% of the general population (COHEN; VASE; HOOTEN, 2021; DAHLHAMER *et al.*, 2018), the personal and socioeconomic costs of chronic pain are among the highest for any disease (GASKIN; RICHARD, 2012). The understanding of how chronic pain development and maintenance are affected by negative changes in sleep seems to be particularly relevant in a time where sleep is constantly degraded or voluntarily restricted (MATSUMOTO; CHIN, 2019).

There is full consensus that sleep loss increases pain sensitivity in humans (AFFLECK *et al.*, 1996; HAACK; SANCHEZ; MULLINGTON, 2007; JENNUM *et al.*, 2013; MORPHY *et al.*, 2007; O'BRIEN *et al.*, 2011; ØDEGÅRD *et al.*, 2015; SIMPSON *et al.*, 2018; STONE *et al.*, 1997; TANG *et al.*, 2012) and animals (ALEXANDRE *et al.*, 2017; HAMBRECHT-WIEDBUSCH *et al.*, 2017; HUANG *et al.*, 2014; LI *et al.*, 2019; PAGE; OPP; KOZACHIK, 2014; SARDI; LAZZARIM; *et al.*, 2018; SARDI; TOBALDINI; *et al.*, 2018; SUTTON; OPP, 2014; TOMIM *et al.*, 2016; VANINI, 2016; XUE *et al.*, 2018) and some longitudinal studies have indicated that self-reported sleep problems is a risk factor for developing a future pain condition (GUPTA *et al.*, 2007; MCBETH; LACEY; WILKIE, 2014; MUNDAL *et al.*, 2014). However, a causal relationship between sleep loss and increased risk of developing chronic pain remains to be determined. In this regard, animal studies can be interesting, as they allow strict control of the variables and broad possibilities of intervention required to elucidate the underlying mechanisms.

Therefore, the first aim of this study was to test the hypothesis that long-term insufficient sleep facilitates the transition from acute to chronic pain. We modeled chronic pain in animals through the PGE_2-induced chronic hyperalgesia model (DIAS *et al.*, 2015; FERREIRA; LORENZETTI; DE CAMPOS, 1990; VERGARA *et al.*, 2020). This model allows the study of hyperalgesia, a key manifestation of pain, not

only in its acute or chronic phase, but also and specifically in the transition between them. Surprisingly, not only was our hypothesis rejected, but also the findings ran precisely in the opposite direction. This is unexpected because we (SARDI; LAZZARIM; *et al.*, 2018; SARDI; TOBALDINI; *et al.*, 2018; TOMIM *et al.*, 2016) and others (ALEXANDRE *et al.*, 2017; HAACK; MULLINGTON, 2005; SCHUH-HOFER *et al.*, 2013; TIEDE *et al.*, 2010; WODARSKI *et al.*, 2014) have consistently demonstrated the pronociceptive effect of sleep restriction in several experimental approaches.

If SR does not facilitate pain chronification, could it impact its future development? It has been demonstrated that the remarkable plasticity of the nociceptive system in response to previous activity history is implicated in the development of chronic pain (BALIKI; APKARIAN, 2015; BORSOOK *et al.*, 2016; CHEN; HEINRICHER, 2019; KUNER; KUNER, 2021; LATREMOLIERE; WOOLF, 2009; LUO; KUNER; KUNER, 2014; PACE *et al.*, 2018; WOOLF; SALTER, 2000). Therefore, in a second study we investigated whether long-term sleep restriction associated with nociceptive stimulation could affect the subsequent development of acute and chronic pain. The results were no less surprising!

Materials and Methods

Animals

The experiments were performed in male Wistar rats (270-330g), randomly housed in groups of four in polypropylene cage lined with shavings and provided with *ad libitum* access to food and water. The animals were placed in a temperature- (22°C ± 2) and light- (12-h/12-h light/dark cycle) controlled room. All experimental procedures and protocols were approved by Commitee on Animal Research of the Federal University of Parana, Brazil (approval ID #1379).

Drugs

Prostaglandin E_2 (100 ng / 30 µL) was diluted in 0.9% NaCl (DIAS *et al.*, 2015; FERREIRA; LORENZETTI; DE CAMPOS, 1990; VERGARA *et al.*, 2020); Carrageenan-λ (100 µg / 30 uL) was dissolved in 0.9% NaCl, freshly prepared (BONET *et al.*, 2013); Capsaicin (1.6 µg / 20 µL) was initially dissolved in Tween-80 (50%) and etanol (50%) to a concentration of 50 µg/µL and then diluted in 0.9% NaCl (SANTOS; CALIXTO, 1997; TOBALDINI *et al.*, 2019); Formalin (50 µL) (an aqueous solution of 37% of formaldehyde) was diluted in 0.9% NaCl to a concentration of 0.5% (TJØLSEN *et al.*, 1992); GBR12909, 1-(2-[bis(4-fluorophenyl) methoxy]ethyl)-4-(3-phenylpropyl) piperazine dihydrochloride, a dopamine reuptake inhibitor, was diluted in 0.9% NaCl to a concentration of 0.5 nmol / 0.25 µL (DIAS *et al.*, 2015); All drugs above were obtained from Sigma-Aldrich (St. Louis, MO, USA); Prolopa® (levodopa and benserazide hydrochloride) (37.5 mg / kg) was dissolved in 0.9% NaCl and obtained from Hoffman-LaRoche, Brazil (PADOVAN-NETO *et al.*, 2015). Haldol® (haloperidol) (1 mg/kg and 0.1 mg/kg) was diluted in 0.9% NaCl and obtained from Janssen-Cilag, Brazil (WILSON *et al.*, 2015; ZHANG; LI, 2015).

Partial restriction of total sleep (SR): The enriched gentle handling method

Sleep restriction was achieved using the enriched gentle handling method (ALEXANDRE *et al.*, 2017; SARDI; LAZZARIM; *et al.*, 2018). The method consists in keeping the animals awake using gentle stimuli, such as the introduction of new objects in their cage, touch them with a soft bristle brush and move the cage gently. During the sleep restriction period, the animals were in their habitual cages with water and food *ad libitum*. Control groups were kept in the same condition, but were

free to sleep. Sleep restriction sessions lasted 6 hours a day (from 7:00 am to 1:00 pm) for 7 or 14 days, depending on the experiment.

Pain models

Prostaglandin E_2-induced chronic hyperalgesia

In this chronic pain model, 14 daily subcutaneous injections of PGE_2 into the rat's hind paw induces a chronic decrease in the mechanical nociceptive threshold, defined as hyperalgesia, that persists for at least 30 days after the discontinuation of the injections (FERREIRA; LORENZETTI; DE CAMPOS, 1990).

There are two well-defined phases in this model: the induction phase and the maintenance phase. The induction phase is defined as the 14-day period of daily PGE_2 injections, during which PGE_2 (100 ng / 30 µL) was injected into the dorsal surface of the rat's hind paw, always after the measurement of the mechanical nociceptive threshold (described below). The maintenance phase is defined as the long-lasting period after the discontinuation of the PGE_2 injections, during which the mechanical nociceptive threshold remains low, even in the absence of additional interventions.

Carageenan-induced acute inflammatory hyperalgesia

The injection of Carageenan induces a well-recognized inflammatory response resulting in hyperalgesia and is classically used as a model of acute inflammatory pain (BONET *et al.*, 2013; FERREIRA; LORENZETTI; POOLE, 1993). A single subcutaneous injection of carrageenan (100 µg / 30 µL) was performed in the dorsal surface of hind paw. The mechanical nociceptive threshold was measured 3 hours after the injection (described below), when the resultant hyperalgesia reaches its peak (BONET *et al.*, 2013).

Formalin-induced nociception

The injection of formalin in different body regions induces overt nociception and has been largely used as a model of acute inflammatory pain (TJØLSEN *et al.*, 1992; TOBALDINI *et al.*, 2019). A single subcutaneous injection of formalin 0.5 % (50 µL) was performed in the dorsal surface of hind paw. The overt nociception was quantified by counting the number of hind paw flinches (described below) during 60 min.

Capsaicin-induced acute nociception and hyperalgesia

Capsaicin is an agonist of the transient receptor potential vanilloid 1 (TRPV1). When injected in the subcutaneous tissue, it induces short-term overt nociception (5 min) followed by hyperalgesia (RO; LEE; ZHANG, 2009). A single subcutaneous injection of capsaicin (1.6 µg / 20 µL) (SANTOS; CALIXTO, 1997) was performed in the dorsal surface of hind paw and the overt nociception (described below) and hyperalgesia were evaluated up to 3 h later.

Chronic constriction of the infraorbital nerve-induced neuropathic pain

The chronic constriction of the infraorbital nerve (CION) has been largely used as a model of trigeminal neuropathic pain (CHICHORRO *et al.*, 2006; GAMBETA *et al.*, 2018). Under deep anesthesia induced by intraperitoneal injection of xylazine (10 mg / kg) and ketamine (60 mg / kg) an incision was made beneath the right eye, about 3 mm caudal to the mystacial pads. After the dissection of superficial muscles, masseter and superior lip elevator, the infraorbital nerve was exposed and two silk 4-0 ligatures were tied loosely around the nerve with 2 mm apart. The wound was closed with silk 4-0 sutures. Sham operated animals underwent the same procedure, but no ligatures were performed. Neuropathic sensory changes were assessed by measuring latency to escape from a heat source (described below).

Behavioral tests

Behavioral tests were performed during the light phase - between 7 a.m. and 12 a.m - in a quiet room maintained at 22 °C. Before the experiments, each animal was manipulated for 7 days to be habituated to the experimental manipulation.

The Randall-Selitto mechanical nociceptive threshold test: The nociceptive mechanical paw-withdrawal threshold (RANDALL; SELITTO, 1957) was used as a measure of nociception. The animals were previously habituated to the testing conditions. In this test, increasing pressure is applied to the dorsal surface of the rat's hind paw, the mechanical nociceptive threshold is defined as the force (mean of three readings) in grams at which the rat withdrew its paw. Data were expressed as mean ± SEM of mechanical nociceptive threshold.

Overt nociception: Formalin- or capsaicin-induced nociception was assessed by quantifying the number of hind paw flinches. Animals were previously habituated to the test boxes (30 × 30 × 30 cm mirrored-wood chamber with a glass at the front side) and immediately after the hind paw injection of formalin or capsaicin the number of hind paw flinches was quantified during 60 minutes or 5 minutes, respectively.

Thermal allodynia: Thermal sensory changes induced by infraorbital nerve constriction was assessed by measuring latency to escape from a heat source, as previously described (GAMBETA *et al.*, 2018). Briefly, the animal was gently held by a trained experimenter and a heat source was presented 1 cm from the right mystacial pads. The nociceptive response latency, as either head withdrawal or vigorous flicking of the snout, was record in seconds and used as a measure of the thermal nociceptive threshold. To prevent tissue damage, a cut-off of 20s was established.

Spontaneous locomotion: the Open Field test

The Open Field test was used to evaluate the locomotor activity and exploratory behavior determining if experimental manipulations affected animals motor behavior. Animals were individually placed at the center of a circular arena (60 cm of diameter), divided into 12 squares (Insight®, Ribeirao Preto, SP, BR). The number of crossed squares was quantified for 5 min and used as a measure of the exploratory behavior.

Stereotaxic surgery and drug infusion

Under deep anesthesia induced by intraperitoneal injection of xylazine (10 mg / kg) and ketamine (60 mg / kg) the animals were placed in a stereotaxic apparatus. Once positioned the skull was exposed and a small hole was made to implant a 25-gauge stainless steel cannula, bilaterally. The cannulas were fixed with acrylic resin and two screws, to prevent them to move. Their lower extremity was positioned 2 mm above the NAc core, the site of interest. The coordinates from bregma were, in the rostro-caudal direction were +1.30, dorso-ventral -6.20 mm and latero-lateral ±1.80 mm (PAXINOS; WATSON, 2007; SARDI; LAZZARIM; *et al.*, 2018; SARDI; TOBALDINI; *et al.*, 2018). After surgery, animals received dipyrone (50 mg/kg) and gentamicin (0.5 mg/kg) and the experiments were conducted 5-7 days later.

At the day of the experiment, the injection volume of 0.25 µL was slowly delivered in the NAc by a mechanical infusion pump (model KDS-100 KD Scientific Holliston, MA, USA). A cannula connected to a PE-10 polyethylene tube and a 5µL Hamilton syringe was introduced in the implanted guide cannula to enable the drug delivery. After injection, the cannula remained for a while in the site to prevent retrograde flow.

To evaluate the correct site of injection, at the end of the experiment, the animals were transcardially perfused with 0.9% NaCl under general anesthesia. Evans Blue dye (1%, 0.3 µL) was delivered through the implanted guide cannula and 50 µm coronal sections were performed to assess the location of the dye marking (PAXINOS; WATSON, 2007). Only animals with Evans Blue dye marking restricted to NAc were included in analysis.

Western Blot

Western blotting was performed to determine the relative state of phosphorylation of DARPP-32 (dopamine and adenosine 3′, 5′-monophosphate-regulated phospho-protein, Mr 32 kDa) and the expression of DAT (dopamine transporter) in the Nac. The rats were killed by decapitation then Nac were freshly collected, frozen in liquid nitrogen and stored at -80 °C. For analysis, the samples were first homogenized in ice-cold lysis buffer (150mM NaCl, 50mM Tris-HCl, 1% TX-100) containing protease inhibitor (Protease Inhibitor Cocktail powder, P2714, Sigma- Aldrich, EUA) and phosphatase inhibitor (Halt Phosphatase Inhibitor Cocktail, 78427, Thermo Fisher, EUA) and then centrifuged at 4 °C, 14,000 rpm for 20 min. The supernatant was extracted, and total protein was quantified using Bradford reagent. The proteins were denatured in a water bath for 10 min at 95 °C in sample buffer (5x; 1,6% SDS; 4% 2-Mercaptoethanol; 8% glicerol; 0,0016% bromophenol blue; 0,125M tris-HCl). Equal amounts (20 ug) of total protein extracts were separated by electrophoresis in 12% polyacrylamide (SDS-page) and transferred to a PVDF membrane. The membranes were blocked with 2% BSA (bovine serum albumin) for 1h30min and incubated overnight at 4 °C with anti-phospho-thr75-DARPP-32 (1:1000) (#2301s, Cell Signaling; rabbit), anti-phospho-thr34-DARPP-32 (1:1000) (#12438s, Cell Signaling; rabbit), anti-dopamine transporter (1:1000) (DAT, #22524-1-ap, Proteintech; rabbit) or anti-β-actin antibody (1:10000, #AM4302, Thermo; mouse). After TBST washes, the membranes were incubated with the

appropriate secondary antibody for 1h. The bands were revealed by chemiluminescence ECL system (SuperSignal West pico, 34580, Thermo, EUA) in a photodocumenter. The quantification was performed by optical density using ImageJ 1.37c (Public Domain) image analysis software and data was normalized using β-actin optical density.

Statistical Analysis

Nociceptive behavioral data were expressed as mean + SEM, evaluated by descriptive and inferential statistics. All data were evaluated for normality (Shapiro-Wilk test) and homoscedasticity (Bartlett test). Then, the data were analyzed in two ways: (1) repeated measures analysis of variance of (ANOVA) with one within subject factor (time) and two or three between subject's factors (depending on the experiment) or (2) analysis of variance (ANOVA) with different between subject's factors (depending on the purpose of the experiment). In all cases, the follow-up test used was the Tukef-HSD. The significance level adopted was $p < 0.05$. SigmaPlot software (Systat Software, San Jose, California, USA) was used for graphing and R Software (R core Team, 2022) for data analysis.

Results

First study: Does Sleep Restriction facilitate pain chronification?

1.1- Chronic pain model

To characterize chronic hyperalgesia, PGE_2 was daily injected into the dorsal surface of the rat's hind paw for 14 days (induction phase, see methods for details regarding the model). Daily PGE_2 injections significantly decreased the mechanical nociceptive threshold in the injected paw (Fig. 1, repeated-measures ANOVA followed by Tukey test, $F_{\text{paw injection}}(1, 1) = 999.787$, $p < 0.0001$; $F_{\text{time}}(1, 15) = 32.397$, $p < 0.0001$; $F_{\text{paw injection x time}}(1, 14) = 36.733$, $p < 0.0001$). Specifically, three hours after the first injection, the mechanical nociceptive threshold was significantly decreased, characterizing PGE_2-induced acute inflammatory hyperalgesia (Fig. 1). This acute hyperalgesic state was completely resolved 24h later, when a second injection was performed. During this induction phase, the mechanical nociceptive threshold was always assessed before the subsequent daily injection and, therefore, reflects the cumulative effect of previous injections. The mechanical nociceptive threshold did not return to the baseline values after the second injection, indicating that the chronification process is developing (Fig. 1, shaded area).

After 14 days of daily PGE_2 injections, the maintenance phase began, and no additional interventions were performed. However, the hyperalgesic state lasts for weeks after the injections were discontinued, characterizing PGE_2-induced chronic inflammatory hyperalgesia (Fig. 1, shade free area).

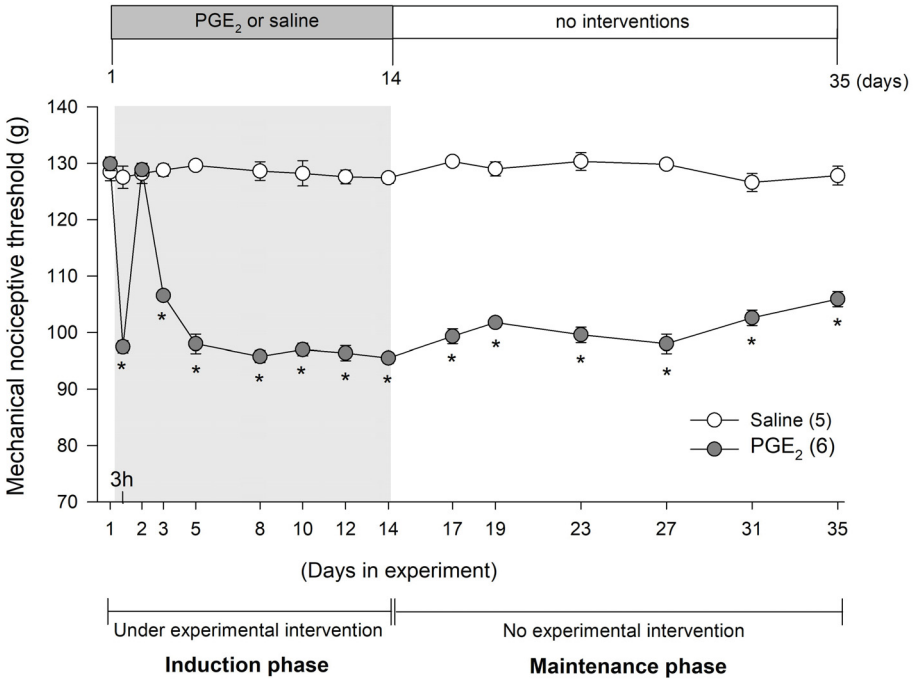

Fig. 1 – PGE_2-induced acute and chronic hyperalgesia. The experiment was performed in two phases. During the induction phase, 14 days of daily injections of PGE_2 or saline were performed into the hind paw (shaded area); during the maintenance phase, injections were discontinued, and no additional interventions were performed. The mechanical nociceptive threshold was always evaluated before each daily injection, except for the measurement performed 3 hours after the first injection. *Induction phase*: The first PGE_2 injection induced acute hyperalgesia assessed 3h later, and subsequent daily injections induced a persistent hyperalgesic state along the induction phase. *Maintenance phase*: A chronic hyperalgesic state lasted for at least 21 days after the injections were discontinued. RM ANOVA with one between-subjects factor (paw injection) followed by the Tukey post hoc test ($p < 0.05$). The symbol "*" indicates a statistically significant decrease in mechanical nociceptive threshold. In this and subsequent figures data are expressed as mean ± S.E.M and numbers in parenthesis indicate the number of animals in each group.

1.2- Association of sleep restriction (SR) and daily PGE_2 injection for 7 days.

This chronic pain model has the great advantage of allowing the determination of whether any concomitant intervention accelerates or hinders the transition from acute to chronic pain. During the induction phase, the chronic hyperalgesic state is developing and it is well established that the association of interventions that facilitate the chronification of pain allows the development of the chronic hyperalgesic state after only 7 (not 14) days (DIAS *et al.*, 2015; FERREIRA; LORENZETTI; DE CAMPOS, 1990; PIARDI *et al.*, 2020; VERGARA *et al.*, 2020).

Therefore, the first step was to test whether the association of daily PGE_2 injections and SR (6 hours a day, see methods) for 7 days would result in chronic hyperalgesia. During the induction phase, both SR and daily PGE_2 injections, in association or alone, decreased the mechanical nociceptive threshold, however, the nociceptive threshold progressively returned to baseline values throughout the maintenance phase (Fig. 2A, repeated-measures ANOVA followed by Tukey test, $F_{\text{sleep condition}}(1, 1) = 89.886$, $p < 0.0001$; $F_{\text{paw injection}}(1, 1) = 173.539$, $p < 0.0001$; $F_{\text{time}}(1, 14) = 93.221$, $p < 0.0001$; $F_{\text{sleep condition x paw injection}}(1, 1) = 128.668$, $p < 0.0001$; $F_{\text{time x sleep condition}}(1, 14) = 21.469$, $p < 0.0001$); $F_{\text{time x paw injection}}(1, 9) = 34.391$, $p < 0.0001$); $F_{\text{sleep condition x paw injection x time}}(1, 7) = 20.331$, $p < 0.0001$). Although the association of SR with daily PGE_2 injections for 7 days delayed recovery, baseline values were reached 15 days later, indicating that the chronic hyperalgesic state did not develop (Fig. 2A).

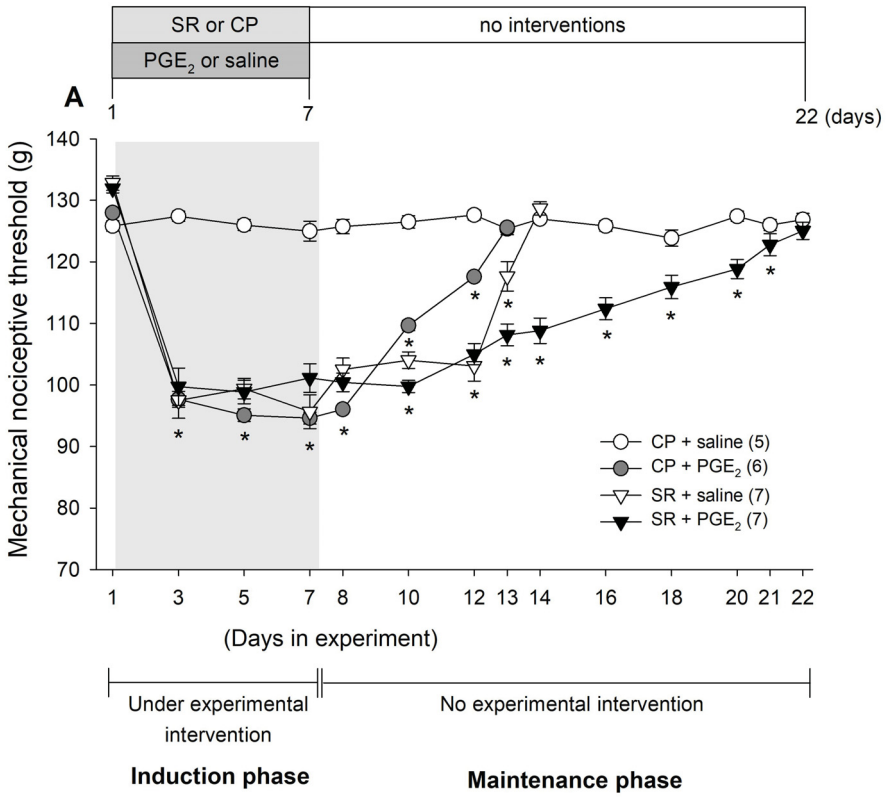

Fig. 2A – The nociceptive response induced by 7 days of SR associated with daily PGE_2 injection. During the induction phase, 7 days of daily PGE_2 injections were associated with 6h a day of SR (see methods for details regarding SR); during the maintenance phase, injections and SR were stopped and no additional interventions were performed. The experiment was interrupted when each group

returned to baseline nociceptive threshold. The association of SR with PGE_2 injections resulted in longer-lasting hyperalgesia, but not in chronic hyperalgesia. RM ANOVA with two between-subjects factors (sleep condition and paw injection) followed by the Tukey post hoc test ($p < 0.05$). The symbol "*" indicates a statistically significant decrease in mechanical nociceptive threshold. SR = sleep restriction; CP = control procedure.

1.3- Association of 14 days of SR and 7 days of daily PGE_2 injections

Since 7 days of SR did not facilitate the transition from acute to chronic pain, we evaluated the ability of 14 days of SR to do so. The animals underwent 14 days of SR (6 hours a day) and in the last 7 days they also received a daily injection of PGE_2.

During the induction phase, the mechanical nociceptive threshold was decreased by SR and daily PGE_2 injections combined or alone; during the maintenance phase, the nociceptive threshold progressively returned to baseline values (Fig. 2B, repeated-measures ANOVA followed by Tukey test, $F_{\text{sleep condition}}(1, 1) = 718.651$, $p < 0.0001$; $F_{\text{paw injection}}(1, 1) = 141.622$, $p < 0.0001$; $F_{\text{time}}(1, 14) = 270.098$, $p < 0.0001$; $F_{\text{sleep condition x paw injection}}(1, 1) = 36.598$, $p < 0.0001$; $F_{\text{time x sleep condition}}(1, 14) = 113.449$, $p < 0.0001$); $F_{\text{time x paw injection}}(1, 12) = 83.831$, $p < 0.0001$); $F_{\text{sleep condition x paw injection x time}}(1, 10) = 48.978$, $p < 0.001$). The association of SR with daily PGE_2 injections extended the hyperalgesia, but baseline values were reached 10 days later, indicating that the chronic hyperalgesic state did not develop.

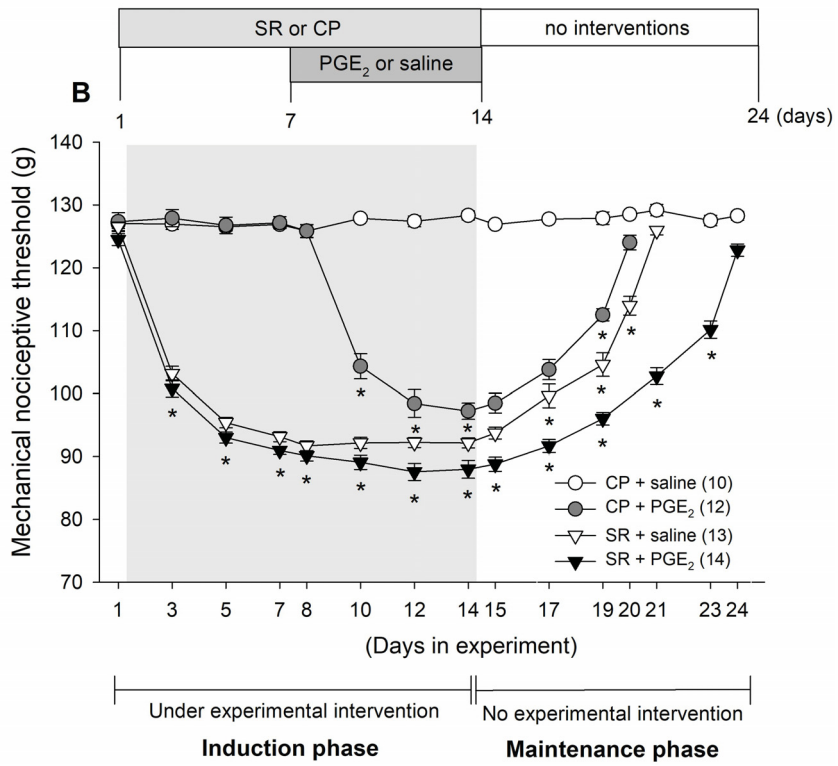

Fig. 2B – The nociceptive response induced by 14 days of SR associated with 7 days of daily PGE_2 injection. During the induction phase, 14 days of SR were associated with 7 days of daily injections of PGE_2 or saline; during the maintenance phase, injections and SR were stopped and no additional interventions were performed. The experiment was interrupted when each group returned to baseline nociceptive threshold. The association of SR with PGE_2 injections resulted in longer-lasting hyperalgesia, but not in chronic hyperalgesia. RM ANOVA with two between-subjects factors (sleep condition and paw injection) followed by the Tukey post hoc test ($p < 0.05$). The symbol "*" indicates a statistically significant decrease in mechanical nociceptive threshold. SR = sleep restriction; CP = control procedure.

1.4- Association of SR and daily PGE_2 injection for 14 days.

Since SR did not facilitate pain chronification in previous experiments, we sought to test its effect on the development of chronic pain in the standard model. So, SR was performed in association with daily PGE_2 injections for 14 days. Surprisingly, this association prevented the development of chronic hyperalgesia: animals submitted only to 14 days of daily PGE_2 injections entered the maintenance phase as expected, remaining in chronic hyperalgesia for more than 30 days, while those who also underwent SR (in association with PGE_2 injections) returned to baseline values after 10 days (Fig. 2C, repeated-measures ANOVA followed by Tukey test, $F_{\text{sleep condition}}(1, 1) = 293.990$, $p < 0.0001$; $F_{\text{paw injection}}(1, 1) = 672.497$, $p < 0.0001$; $F_{\text{time}}(1, 18) = 157.103$, $p < 0.0001$; $F_{\text{sleep condition x paw injection}}(1, 1) =$

501.127, p < 0.0001; $F_{\text{time x sleep condition}}$ (1, 11) = 69.301, p < 0.0001); $F_{\text{time x paw injection}}$ (1, 18) = 42.553, p < 0.0001; $F_{\text{sleep condition x paw injection x time}}$ (1, 9) = 22.584, p < 0.0001). From a literature standpoint, these data are completely unexpected. Pragmatically, they support that long-term (14 days) SR protect animals from developing chronic pain, at least in this animal model.

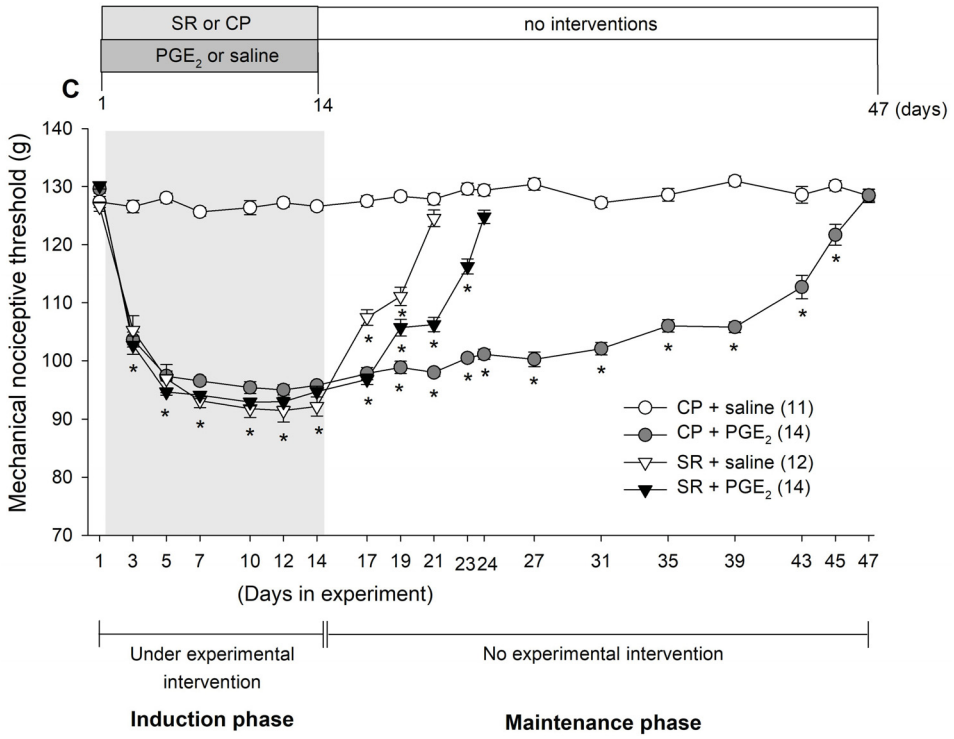

Fig. 2C – The nociceptive response induced by 14 days of SR associated with PGE_2 injections. During the induction phase, 14 days of SR were associated with daily injections of PGE_2 or saline; during the maintenance, no interventions were performed. The experiment was interrupted when the nociceptive threshold for each group returned to baseline value. After 14 days of daily injections of PGE_2, animals developed chronic hyperalgesia, as expected. However, the association of SR with PGE_2 injections prevented chronic hyperalgesia development; the nociceptive threshold returned to baseline values 10 days after injections were discontinued. RM ANOVA with two between-subjects factors (sleep condition and paw injection) followed by the Tukey post hoc test (p <0.05). The symbol "*" indicates a statistically significant decrease in mechanical nociceptive threshold. SR = sleep restriction; CP = control procedure.

1.5- The paradoxical protective effect induced by long-term SR in chronic pain development.

In order to provide an overview of the effect of SR on PGE_2-induced hyperalgesia over time, we compared the area above the curve of the maintenance phase among the key groups of Figure 2. As it can be seen in Fig. 3, the association of 7 days of SR with daily PGE_2 injection induced longer-lasting hyperalgesia than that induced by each of them alone (Fig. 3, ANOVA followed by Tukey test, $F_{treatment}$ (6, 92) = 8545.5, p > 0.0001). However, when SR is increased to 14 days this effect disappears and an apparent recovery-accelerating effect was detected (Fig. 3). Surprisingly, the association of 14 days of SR dramatically decreased the duration of hyperalgesia induced by 14 days of daily injections of PGE_2 (Fig. 3).

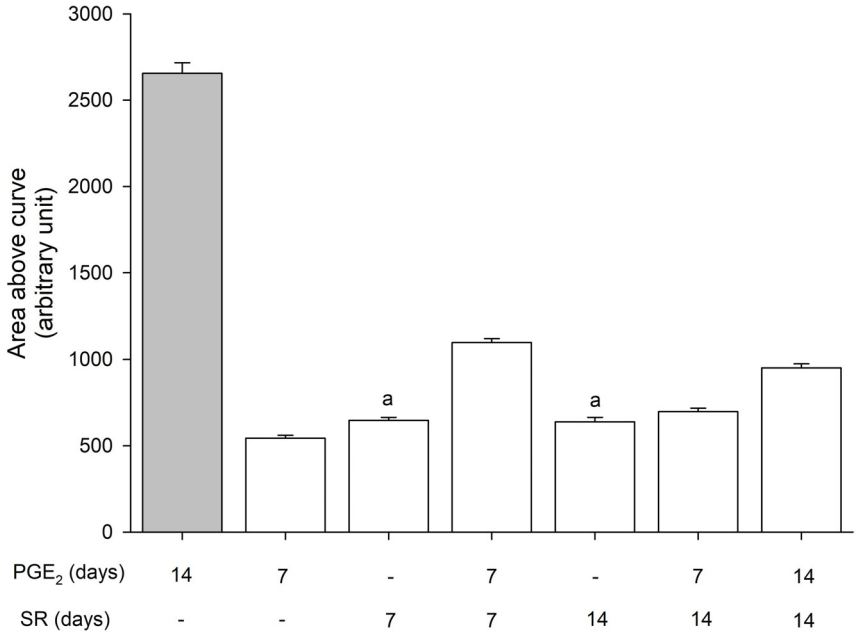

Fig. 3 – The effect of SR on PGE_2-induced hyperalgesia over time: 7 but not 14 days of SR increased the area above curve after 7 days of daily PGE_2 injection; while 14 days of SR decreased the area above curve after 14 days of daily PGE_2 injection. Data represent the area above curve of the maintenance phase. One way ANOVA followed by the Tukey post hoc test ($p < 0.05$). All groups differ from each other, except those indicated by the letter “a”. SR = sleep restriction.

1.6- Effects of SR and daily PGE_2 injections on the expression of DAT and DARPP-32 in the Nucleus Accumbens

The data presented so far are surprising and counterintuitive, which prompted us to look for some mechanism that could begin to explain them. Previous data from

our lab supports that (1) SR increases pain sensitivity by decreasing mesolimbic dopaminergic function (SARDI; TOBALDINI; *et al.*, 2018), while (2) mesolimbic dopaminergic function is required for PGE_2-induced chronic hyperalgesia (VERGARA *et al.*, 2020). Therefore, we chose to focus on the expression of proteins related to dopaminergic signaling and synaptic function in the Nucleus Accumbens. Our choice fell on DAT (the dopamine reuptake transporter) which controls the extracellular dopamine clearance (VAUGHAN; FOSTER, 2013) and DARPP-32 (dopamine and adenosine 3′, 5′-monophosphate-regulated phospho-protein, Mr 32 kDa) a bifunctional signal transduction molecule with antagonistic functions when it is phosphorylated at threonine 34 or 75 (GIRAULT; NAIRN, 2021).

Animals underwent 14 days of SR or control condition associated with daily injections of PGE_2 or saline. At the end of this 14-day period, the levels of DAT were significantly increased by PGE_2 injections, independent on sleep condition and significantly decreased by SR, independent on hind paw injections (Fig. 4A, two-way ANOVA, $F_{\text{sleep condition}}(1, 28) = 6.84$, $p = 0.014$; $F_{\text{paw injection}}(1, 28) = 4.78$, $p = 0.037$; $F_{\text{sleep condition x paw injection}}(1, 28) = 0.0003$, $p = 0.985$). The levels of DARPP-32 phosphorylation at threonine 34 (P-Thr34-DARPP-32) in the NAc did not differ among treatment groups (Fig. 4B, two-way ANOVA, $F_{\text{sleep condition}}(1, 35) = 0.31$, $p = 0.57$; $F_{\text{paw injection}}(1, 35) = 2.95$, $p = 0.094$; $F_{\text{sleep condition x paw injection}}(1, 35) = 0.69$, $p = 0.41$), while the levels of DARPP-32 phosphorylation at threonine 75 (P-Thr75-DARPP-32) were significantly increased by PGE_2 injections, independent on sleep condition (Fig. 4C, two-way ANOVA, $F_{\text{sleep condition}}(1, 35) = 0.09$, $p = 0.75$; $F_{\text{paw injection}}(1, 35) = 13.53$, $p < 0.001$; $F_{\text{sleep condition x paw injection}}(1, 35) = 0.76$, $p = 0.38$).

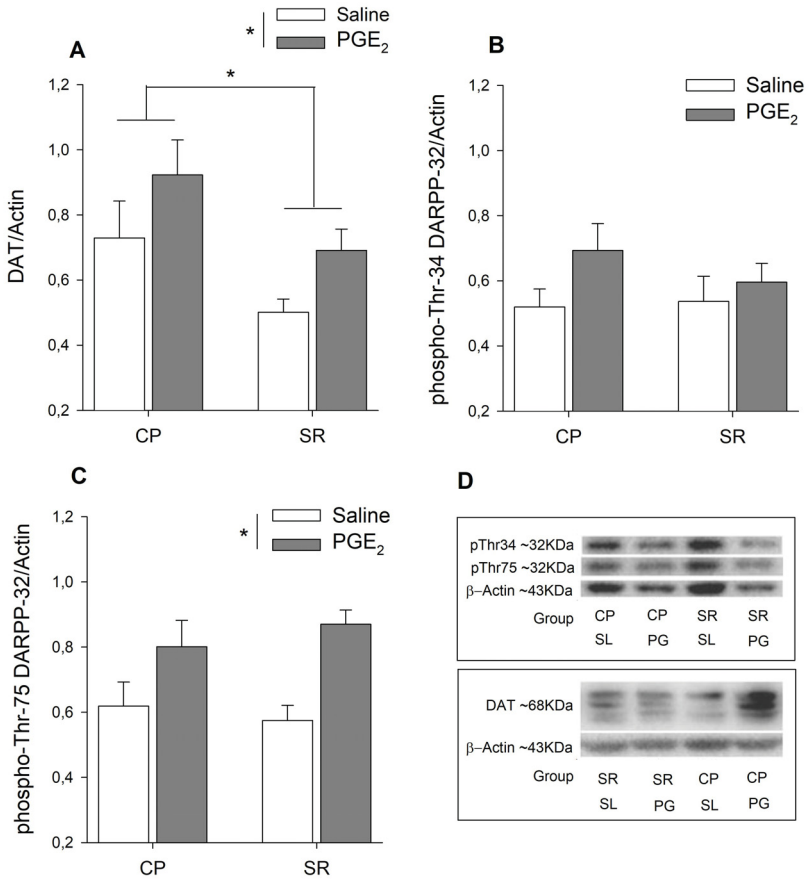

Fig. 4 – Effect of SR associated with PGE_2 injections on the relative levels of DARPP-32 phosphorylated at threonine 34 or 75 and DAT in Nucleus Accumbens. Samples were collected at the end of a 14-day period of SR or control condition associated with daily injections of PGE_2 or saline. Protein levels were normalized to actin. The levels of DAT were significantly increased by 14 days of daily PGE_2 and decreased by 14 days of SR (A). No significant change was observed in phosphor-Thr34 DARPP-32 (B). 14 days of daily PGE_2 injections significantly increased the levels of phosphor-Thr75 DARPP-32 independent on the sleep condition (C). ANOVA with two between-subjects factors (sleep condition and paw injection). The symbol "*" indicates significative difference ($p<0,05$) within factors. (D) Representative figures of western blot assay. 7-10 animals per group. SR = sleep restriction; CP = control procedure; SL = saline; PG = PGE_2; P-Thr34-DARPP-32 = DARPP-32 phosphorylation at threonine 34; DARPP-32 phosphorylation at threonine 75 (P-Thr75-DARPP-32)

Second study: What is the effect of an earlier period of SR associated with nociceptive stimulation on the subsequent development of acute and chronic pain?

Surprised and intrigued by the previous results, we decided to investigate whether an earlier history of SR associated with nociceptive stimulation would impact the subsequent development of acute and chronic pain.

To answer this question, we performed SR in association with daily PGE_2 injections, just as we did in the first study, then let the animals recover until they reached the baseline nociceptive threshold to test them again in different animal models of pain.

2.1 - 14 but not 7 days of SR associated with daily PGE_2 injection prevent the subsequent development of acute and chronic pain

After returning to baseline nociceptive threshold, animals who had undergone 7 days of SR associated with daily injections of PGE_2 (and their matched controls) were challenged with new PGE_2 injections. Regardless of previous treatment, PGE_2-induced acute hyperalgesia was similar among the groups, that is, the nociceptive threshold was significantly decreased 3h after injection, but returned to baseline values within 24h, when a second injection was performed. The nociceptive threshold decreased again after the second injection and did not return to baseline values within the next 24h (Fig. 5A, repeated-measures ANOVA followed by Tukey test, $F_{\text{sleep condition}}(1, 1) = 55.761$, $p < 0.0001$; $F_{\text{paw injection}}(1, 1) = 0.058$, $p = 0.812$; $F_{\text{time}}(1, 4) = 384.13$, $p < 0.0001$; $F_{\text{sleep condition x paw injection}}(1, 1) = 0.369$, $p = 0.55$; $F_{\text{sleep condition x time}}(1, 4) = 2.11$, $p = 0.087$; $F_{\text{paw injection x time}}(1, 4) = 3.29$, $p = 0.014$; $F_{\text{sleep condition x paw injection x time}}(1, 4) = 6.74$, $p < 0.0001$), indicating that the chronification process is developing (see Fig. 1).

After returning to baseline nociceptive threshold, animals who had undergone 14 days of SR associated with 7 days of daily injections of PGE_2 were challenged with new PGE_2 injections. While animals in the control groups (which underwent only SR or daily PGE_2 injections) developed hyperalgesia as expected, those previously submitted to 14 days of SR associated with 7 days of daily PGE_2 injections were resistant to the acute and chronic hyperalgesic effect of this new round of daily PGE_2 injections (Fig. 5B, repeated-measures ANOVA followed by Tukey test, $F_{\text{sleep condition}}$

$(1, 1) = 155.27$, $p < 0.0001$; $F_{\text{paw injection}}$ $(1, 1) = 45.106$, $p < 0.0001$; F_{time} $(1, 6) = 136.68$, $p < 0.0001$; $F_{\text{sleep condition x paw injection}}$ $(1, 1) = 199.43$, $p < 0.0001$; $F_{\text{sleep condition x time}}$ $(1, 6) = 8.84$, $p < 0.0001$; $F_{\text{paw injection x time}}$ $(1, 6) = 12.97$, $p < 0.0001$; $F_{\text{sleep condition x paw injection x time}}$ $(1, 6) = 11.02$, $p < 0.0001$). We repeated the daily injections for 14 days to compare the development of the chronic hyperalgesic effect among the groups. Surprisingly, in animals previously submitted to 14 days of SR associated with daily PGE_2 injections, the nociceptive threshold decreased only moderately and only after the tenth injection. Noteworthy, animals previously submitted to 14 days of daily PGE_2 injections (without SR) presented an exacerbated response to this subsequent round of daily PGE_2 injections.

After returning to baseline nociceptive threshold, animals who had undergone 14 days of SR associated with daily injections of PGE_2 were challenged with new PGE_2 injections. As expected, animals in the control groups (which were submitted only to SR or to daily PGE_2 injections) developed acute hyperalgesia and entered the chronification process (evidenced by the absence of a return to baseline after the second injection). In contrast, those previously submitted to 14 days of SR associated with daily injections of PGE_2 showed resistance to the hyperalgesic effect of PGE_2 (Fig. 5C, repeated-measures ANOVA followed by Tukey test, $F_{\text{sleep condition}}$ $(1, 1) = 30.48$, $p < 0.0001$; $F_{\text{paw injection}}$ $(1, 1) = 187.59$, $p < 0.0001$; F_{time} $(1, 4) = 455.6$, $p < 0.0001$; $F_{\text{sleep condition x paw injection}}$ $(1, 1) = 161.92$, $p < 0.0001$); $F_{\text{sleep condition x time}}$ $(1, 4) = 31.26$, $p < 0.0001$; $F_{\text{paw injection x time}}$ $(1, 4) = 72.91$, $p < 0.0001$; $F_{\text{sleep condition x paw injection x time}}$ $(1, 4) = 33.38$, $p < 0.0001$).

Taken together findings in figure 5 support the idea that long-term SR (14 but not 7 days) associated with persistent nociceptive stimulation (daily PGE_2 injections) may protect against the subsequent development of acute and chronic pain.

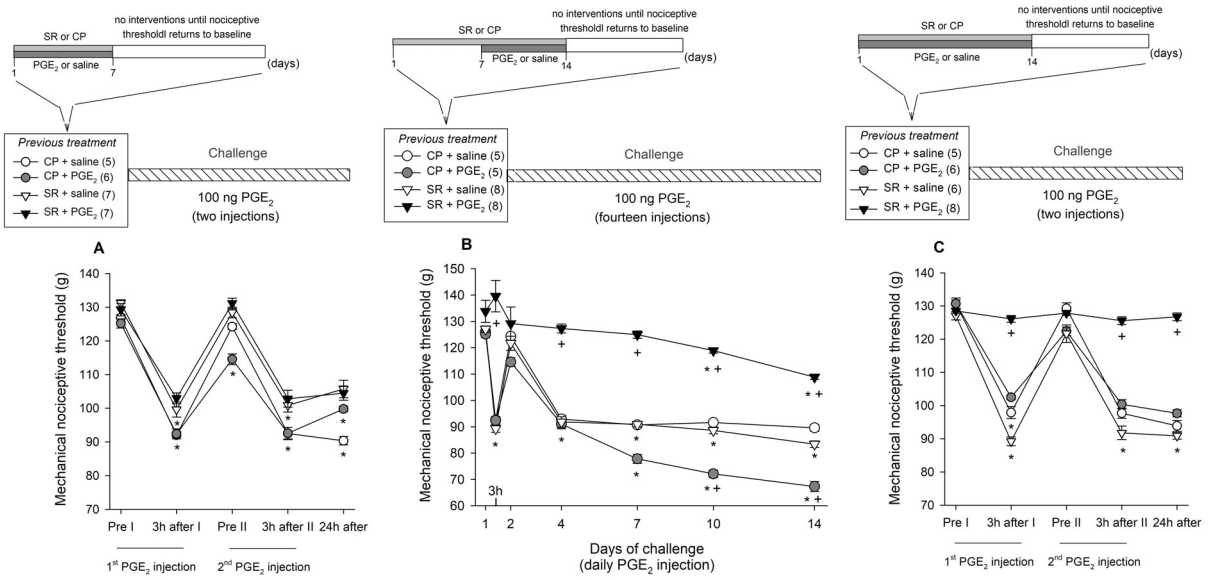

Fig. 5 – Effect of SR associated with daily PGE_2 injections at three different protocols on the subsequent development of acute and chronic hyperalgesia. SR and PGE_2 injections were combined according to the three different protocols drawn at the top of each figure. Then the animals were left to recover and as soon as each group returned to baseline nociceptive threshold a new round of PGE_2 injections was started (challenge). The nociceptive response induced by this new round of PGE_2 injections is plotted in the bottom panel of each figure. After a previous exposition to 7 days of SR associated with daily PGE_2 injections, the mechanical nociceptive threshold decreased similarly in all groups (A). Animals previously submitted to 14 days of SR associated with 7 (B) or 14 (C) days of daily PGE_2 injections became resistant to the hyperalgesic effect of the new PGE_2 injections. RM ANOVA with two between-subjects factors (sleep condition and paw injection) followed by the Tukey post hoc test ($p < 0.05$). The symbol "*" indicates a statistically significant decrease in mechanical nociceptive threshold. The symbol "+" indicates significant difference compared to all other groups at the same time point. SR = sleep restriction; CP = control procedure; Pre = pre injection; 3h after = 3h after injection; 24h after = 24h after injection.

2.2 - A role for the dopamine system in the paradoxical protective effect of long-term SR associated with nociceptive stimulation on the subsequent development of pain

Once again, our data are surprising and counterintuitive, and we asked whether manipulating dopaminergic function could affect what we are going to call "the paradoxical protective effect of long-term SR associated with nociceptive stimulation on the subsequent development of pain".

At the end of 14 days of SR associated with 7 days of daily injections of PGE_2 the animals were allowed to recover until returning to baseline nociceptive threshold. During this recovery phase, the animals received one of two different treatments aimed at increasing dopamine availability (treatment performed between days 16 to 19; see below); then they were challenged with new PGE_2 injections.

The oral administration of Prolopa® (L-DOPA plus benserazide hydrochloride 37.5 mg / kg); once a day, from day 16 to 19, Fig. 6A: repeated-measures ANOVA followed by Tukey test, $F_{\text{sleep condition}}(1, 1) = 0.733$, $p = 0.395$; $F_{\text{paw injection}}(1, 1) = 43.59$, $p < 0.0001$; $F_{\text{oral drug}}(1, 1) = 275.91$, $p < 0.0001$; $F_{\text{time}}(1, 4) = 265.49$, $p < 0.0001$; $F_{\text{sleep condition x paw injection}}(1, 1) = 0.179$, $p = 0.67$; $F_{\text{sleep condition x time}}(1, 4) = 1.45$, $p = 0.218$; $F_{\text{paw injection x time}}(1, 4) = 106.57$, $p < 0.0001$; $F_{\text{sleep condition x paw injection x oral drug x time}}(1, 8) = 78.83$, $p < 0.0001$), or the microinjection of GBR (a dopamine reuptake inhibitor, 0.5 nmol / 0.25µL), once a day, from day 16 to 19, Fig. 6B: repeated-measures ANOVA followed by Tukey test, $F_{\text{sleep condition}}(1, 1) = 0.007$, $p = 0.92$; $F_{\text{paw injection}}(1, 1) = 36.01$, $p < 0.0001$; $F_{\text{intra-NAc drug}}(1, 1) = 361.32$, $p < 0.0001$; $F_{\text{time}}(1, 4) = 285.64$, $p < 0.0001$; $F_{\text{sleep condition x paw injection}}(1, 1) = 1.34$, $p = 0.24$; $F_{\text{sleep condition x time}}(1, 4) = 2.21$, $p = 0.071$; $F_{\text{paw injection x time}}(1, 4) = 127.70$, $p < 0.0001$; $F_{\text{sleep condition x paw injection x intra-NAc drug x time}}(1, 8) = 75.79$, $p < 0.0001$) into the Nucleus Accumbens (NAc) restored the nociceptive phenotype, allowing animals to develop the acute and chronic hyperalgesic responses to the new round of PGE_2 injections.

Together, these findings demonstrate that increasing dopamine levels, either systemically (Prolopa® p.o.) or at the mesolimbic system (GBR intra-NAc), restores the nociceptive phenotype, preventing the paradoxical protective effect of SR associated with nociceptive stimulation on the subsequent development of pain. Therefore, whatever the mechanism underlying this effect, it appears to depend on the dopaminergic system.

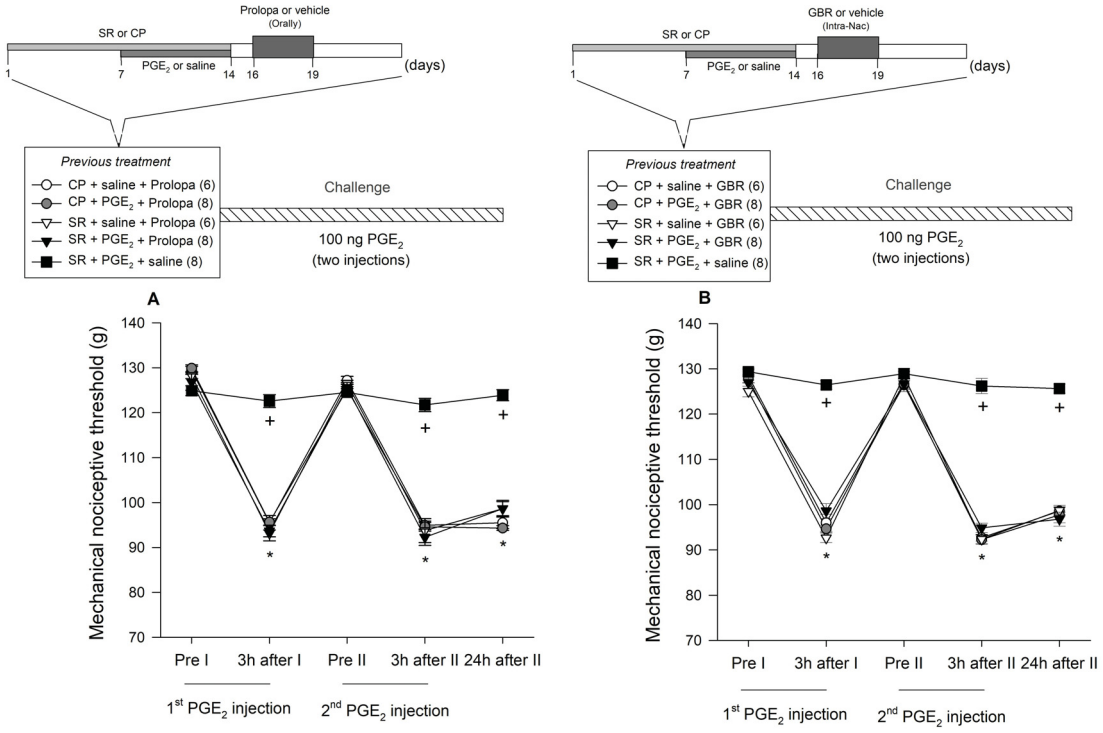

Fig. 6 – Effect of increasing dopamine availability on the protective effect of SR associated with nociceptive stimulation on the subsequent development of pain. Animals were previously submitted to 14 days of SR associated with 7 days of daily injections of PGE_2, then they were left to recover and between days 16 to 19 received Prolopa® (37.5 mg / kg p.o.) or GBR (0.5 nmol / 0.25 µL, intra-Nac), according to the drawn at the top of each figure. As soon as each group returned to baseline nociceptive threshold, a new round of PGE_2 injections was started (challenge). The nociceptive response induced by new PGE_2 injections is plotted in the bottom panel of each figure. The administration of Prolopa® (A) or GBR (B) normalized the hyperalgesic response induced by PGE_2 in animals previously submitted to SR associated with daily PGE_2 injections. RM ANOVA with three between-subjects factors (sleep condition, paw injection and oral or intra-NAc treatment) followed by the Tukey post hoc test ($p < 0.05$). The symbol "*" indicates a statistically significant decrease in mechanical nociceptive threshold. The symbol "+" indicates significant difference compared to all other groups at the same time point. SR = sleep restriction; CP = control procedure; Pre = pre injection; 3h after = 3h after injection; 24h after = 24h after injection.

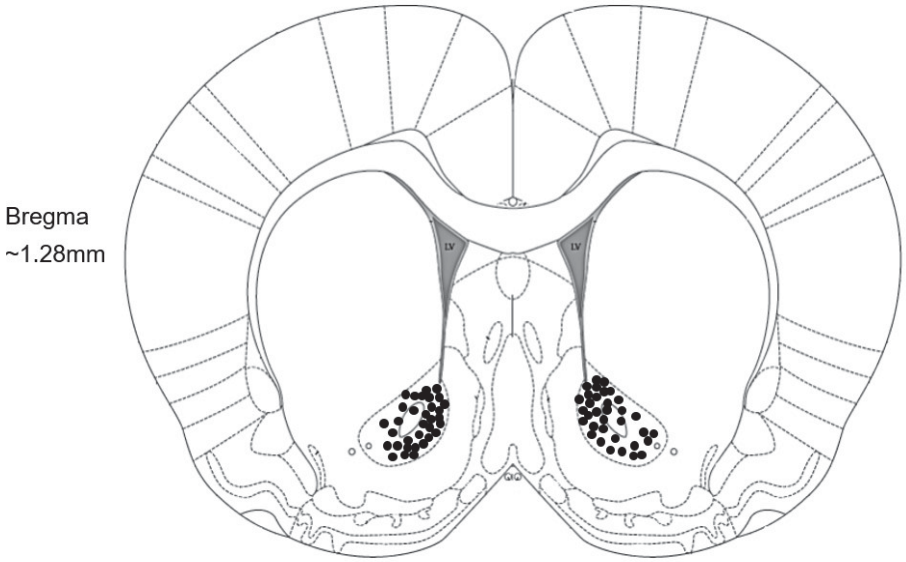

Fig. 6C – NAc microinjection sites. Reconstruction adapted from Paxinos and Watson (2007). The dots represent the microinjection sites on a cross-section through the rats NAc.

Although with some discrepancies (see discussion), data from the literature support that the decrease in sleep time decreases dopaminergic D_2 activity. Therefore, we sought a pharmacological strategy to suppress D_2 activity and test whether it somehow replicates the effects of SR on the nociceptive response. We chose to use Haldol® (haloperidol, a typical antipsychotic) because it antagonizes the D_2 receptor and is in current clinical use. Haldol® (0.1 or 1mg/kg) was orally administered, once a day for 14 days, concomitantly with daily PGE_2 injections. The experiment was divided in phases A and B; the group who developed chronic hyperalgesia (PGE_2 / vehicle) was excluded from phase B. Phase A evaluated Haldol's ability to prevent pain chronification; while phase B, its ability to protect individuals from developing subsequent pain. Therefore, a division similar to the one we used with SR (see figure 2 and 5).

In phase A of the experiment, Haldol® at the lower dose (0.1mg/kg) did not significantly affect PGE_2-induced hyperalgesia throughout the induction phase but prevented its chronification during the maintenance phase (Fig. 7A, repeated-measures ANOVA followed by Tukey test, $F_{\text{paw injection}}(1, 1) = 103.68$, $p < 0.0001$; $F_{\text{oral drug}}(1, 2) = 15.87$, $p < 0.0001$; $F_{\text{time}}(1, 12) = 25.83$, $p < 0.0001$; $F_{\text{oral drug x paw injection}}(1, 1) = 19.89$, $p < 0.0001$; $F_{\text{oral drug x time}}(1, 24) = 6.59$, $p < 0.0001$; $F_{\text{paw injection x time}}(1, 12) = 13.66$, $p < 0.0001$; $F_{\text{paw injection x oral drug x time}}(1, 12) = 5.45$, $p < 0.0001$). Haldol®

at the higher dose (1 mg/kg) decreased the effect of PGE_2; however, it also significantly decreased locomotor activity (Table 1), which may affect the paw withdrawal test itself.

At the end of the 14-day period of daily administration of Haldol® and PGE_2, the animals were allowed to recover, until returning to baseline nociceptive threshold, when they were challenged with a new round of 14 daily PGE_2 injections, in phase B of the experiment. Animals who had received Haldol® at the higher dose (1mg/kg) - but not at the lower dose - associated with daily PGE_2 injection showed resistance to the acute and chronic hyperalgesic effect of PGE_2 in phase B (Fig. 7B, repeated-measures ANOVA followed by Tukey test, $F_{treatment}$ (3, 20) = 6.30, $p < 0.01$; F_{time} (6, 120) = 70.93, $p < 0.0001$; $F_{treatment \times time}$ (18, 120) = 8.44, $p < 0.0001$). In contrast, animals in the control groups (which received only Haldol® or only daily PGE_2 injections in the first phase) developed acute and chronic hyperalgesia, as expected. This finding supports that antagonism at the D2-like dopamine receptor prevents pain chronification and protects from subsequent pain development.

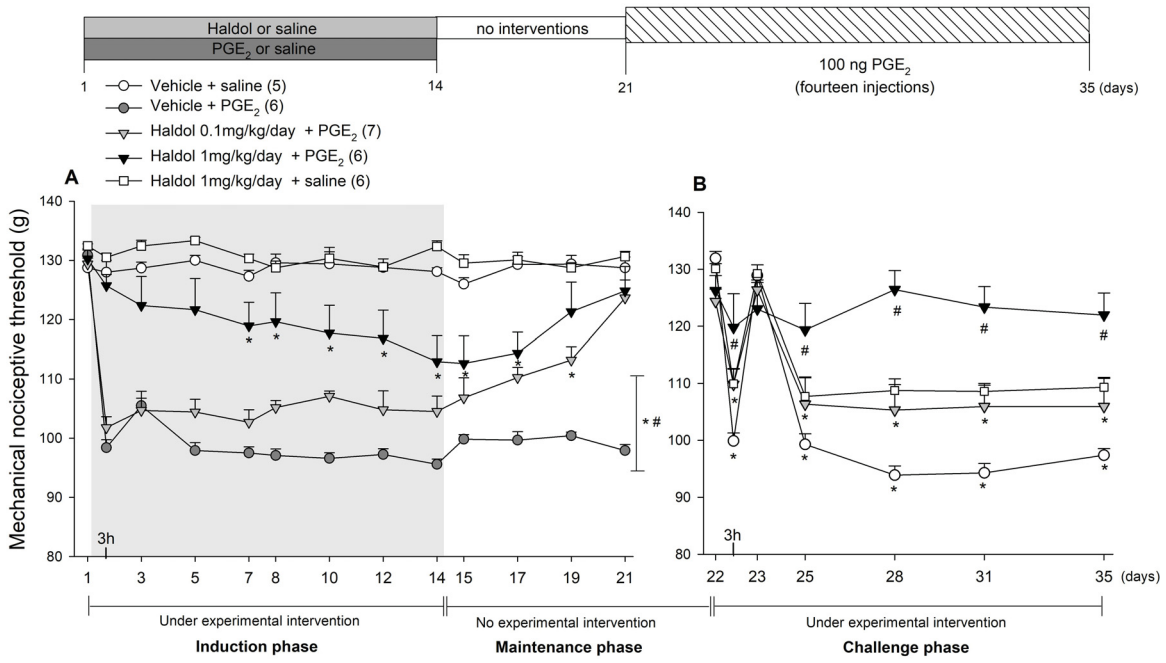

Fig. 7 – Effect of antagonizing D_2-like dopamine receptor in the transition from acute to chronic hyperalgesia and in the subsequent development of pain. The experiment was performed in two phases. First, animals were submitted to a 14-day period of daily administration of Haldol® / vehicle and PGE_2 / saline. Then they were left to recover and those who returned to baseline nociceptive threshold (did not develop chronic hyperalgesia) were included in the second phase, when a new

round of PGE_2 injections was started (challenge). (A) Daily injections of PGE_2 significantly decreased the mechanical nociceptive threshold, but this decrease was milder in animals receiving the higher dose of Haldol®. In contrast to animals receiving vehicle, those receiving Haldol® did not develop chronic hyperalgesia. RM ANOVA with two between-subjects factors (oral treatment and paw injection) followed by the Tukey post hoc test ($p < 0.05$). As soon as each group returned to baseline nociceptive threshold a new round of PGE_2 injections was started (challenge). (B) The nociceptive response induced by this new round of PGE_2 injections was similar among groups, except in animals previously submitted to the higher dose of Haldol® and PGE_2, which became resistant to the acute and chronic hyperalgesic effect of PGE_2. RM ANOVA with one between-subjects factors (treatment) followed by the Tukey post hoc test ($p < 0.05$). The symbol "*" indicates a statistically significant decrease compared to baseline values. The symbol "#" indicates statistically significant difference compared to the vehicle / saline group at the same time point.

2.3 - The paradoxical protective effect of long-term SR associated with nociceptive stimulation on the subsequent development of pain in other animal models.

A natural question that arises from the surprising data we are presenting is whether this protective effect is restricted to PGE_2-induced hyperalgesia or if it extends to other pain models. To answer this question, animals who had undergone 14 days of SR associated with 7 days of daily injections of PGE_2 were allowed to recover and then were tested in different animal models of pain.

The injection of formalin in different body regions induces overt nociception and has been largely used as a model of acute inflammatory pain. After returning to baseline nociceptive threshold, animals who had undergone 14 days of SR associated with 7 days of daily injections of PGE_2 received a subcutaneous injection of formalin (50 µL 0.5%). The nociceptive response induced by formalin in these animals was similar to that induced in the control group (Fig. 8A, t-test, $T = 0.769$, $df = 7.63$, $p = 0.465$). This finding demonstrated that a previous history of long-term SR associated with nociceptive stimulation does not affect the subsequent nociceptive response in the formalin model.

The injection of Carrageenan induces a well-recognized inflammatory response resulting in hyperalgesia and is classically used as a model of acute inflammatory hyperalgesia. After returning to baseline nociceptive threshold, animals who had undergone 14 days of SR associated with 7 days of daily injections of PGE_2

received a subcutaneous injection of Carrageenan (100 µg). These animals were resistant to carrageenan-induced hyperalgesia, in contrast to their matched controls, who developed the characteristic hyperalgesic response (Fig. 8B, repeated-measures ANOVA followed by Tukey test, $F_{\text{sleep condition}}(1, 1) = 68.455$, $p < 0.0001$; $F_{\text{paw injection}}(1, 1) = 99.304$, $p < 0.0001$; $F_{\text{time}}(1, 1) = 2257.87$, $p < 0.0001$; $F_{\text{sleep condition x paw injection}}(1, 1) = 118.71$, $p < 0.0001$; $F_{\text{paw injection x time}}(1, 1) = 257.60$, $p < 0.0001$; $F_{\text{sleep condition x time}}(1, 1) = 196.88$, $p < 0.0001$; $F_{\text{sleep condition x pa injection x time}} = 201.35$, $p < 0.0001$). This finding suggests that a previous history of long-term sleep restriction associated with nociceptive stimulation may protect animals from developing subsequent hyperalgesia in the carrageenan model.

Capsaicin is an agonist of the transient receptor potential vanilloid 1 (TRPV1). When injected in the subcutaneous tissue, it induces short-term overt nociception followed by hyperalgesia. After returning to baseline nociceptive threshold, animals who had undergone 14 days of SR associated with 7 days of daily injections of PGE_2 received a subcutaneous injection of capsaicin (1.6 µg). These animals developed overt nociception similar to control groups (Fig. 9A, ANOVA followed by Tukey test, $F_{\text{sleep condition}}(1, 33) = 3.29$, $p = 0.07$; $F_{\text{paw injection}}(1, 33) = 0.17$, $p = 0.67$; $F_{\text{sleep condition x paw injection}}(1, 33) = 1.34$, $p = 0.25$), but were resistant to capsaicin-induced hyperalgesia, in contrast to their matched controls (Fig. 9B, repeated-measures ANOVA followed by Tukey test, $F_{\text{sleep condition}}(1, 1) = 18.51$, $p = 0.0001$; $F_{\text{paw injection}}(1, 1) = 10.79$, $p = 0.002$; $F_{\text{time}}(1, 4) = 135.54$, $p < 0.0001$; $F_{\text{sleep condition x paw injection}}(1, 1) = 135.54$, $p < 0.0001$; $F_{\text{paw injection x time}}(1, 4) = 19.28$, $p = 0.0001$; $F_{\text{sleep condition x time}}(1, 4) = 17.37$, $p < 0.0001$; $F_{\text{sleep condition x paw injection x time}} = 13.15$, $p < 0.0001$). This finding suggests that a previous history of long-term sleep restriction associated with nociceptive stimulation does not affect capsaicin-induced nociception but may protect against the subsequent development of capsaicin-induced hyperalgesia.

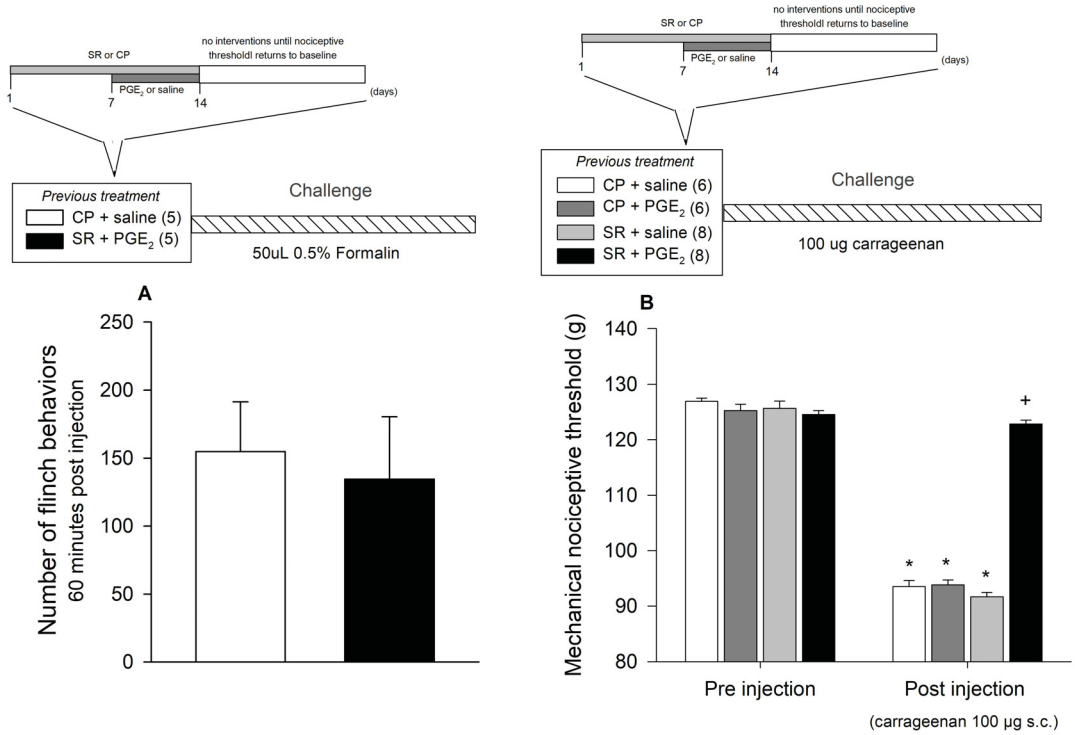

Fig. 8 – Effect of long-term SR associated with nociceptive stimulation on the subsequent nociceptive response induced by formalin or carrageenan. SR and PGE_2 injections were combined according to protocol drawn at the top of each figure. Challenge (injection of formalin or carrageenan) was performed a day after each group returned to baseline nociceptive threshold. The injection of formalin (50 µL 0.5%) into the hind paw induced similar nociceptive behavior in control animals and in those previously submitted to SR associated with daily PGE_2 (A). T test p = 0.46. The injection of carrageenan (100 ng) into the hind paw induced hyperalgesia in control groups but not in that previously submitted to SR associated with daily PGE_2 (B). RM ANOVA with two between-subjects factors (sleep condition and paw injection) followed by the Tukey post hoc test ($p < 0.05$). The symbol "*" indicates significant decrease compared to baseline values. The symbol "+" indicates significant difference compared to all other groups at the same time point. SR = sleep restriction; CP = control procedure.

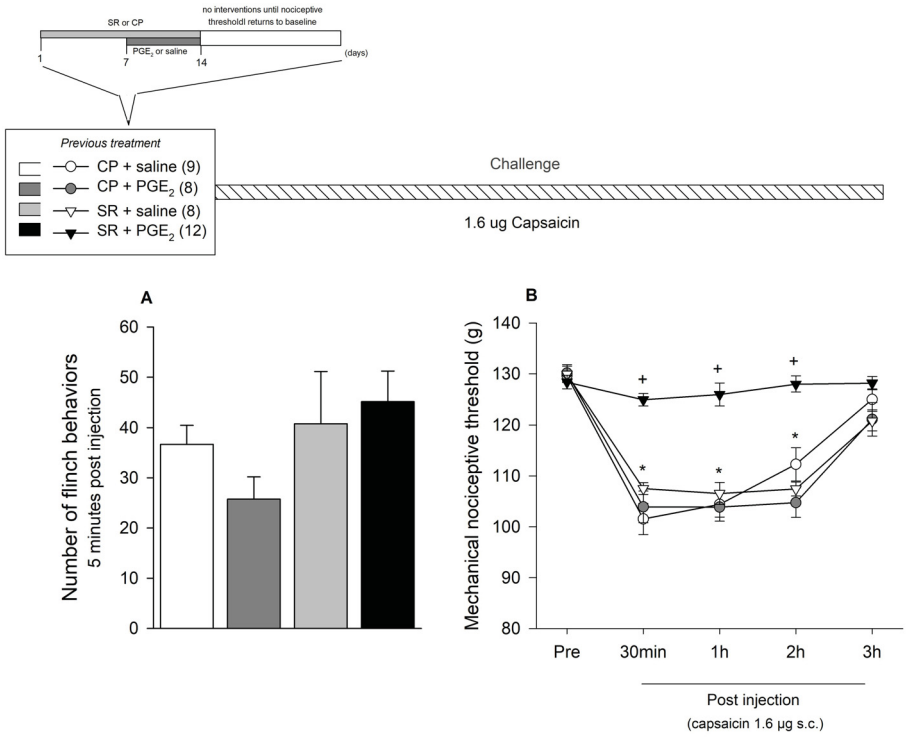

Fig. 9 – Effect of long-term SR associated with nociceptive stimulation on the subsequent nociceptive response induced by capsaicin. SR and PGE_2 injections were combined according to the protocol drawn at the top the figure. Challenge (injection of capsaicin, 1.6 µg, 50 µL) was performed a day after each group returned to baseline nociceptive threshold. Capsaicin induced similar flinch behavior in control animals and in those previously submitted to SR associated with daily PGE_2 (A). ANOVA, with two between-subjects factors (sleep condition and paw injection). Capsaicin induced hyperalgesia in control groups but not in that previously submitted to SR associated with daily PGE_2 (B). RM ANOVA, with two between-subjects factors (sleep condition and paw injection) followed by the Tukey post hoc test ($p < 0.05$). The symbol "*" indicates significant decrease compared to baseline values. The symbol "+" indicates significant difference compared to all other groups at the same time point. SR = sleep restriction; CP = control procedure.

The models used so far in this study are inflammatory pain models with the paw as the source of nociceptive input. The chronic constriction of the infraorbital nerve (CION) has been largely used as a model of trigeminal neuropathic pain. Such characteristics, that is, being a model of neuropathic pain in the facial region, make it a particularly attractive model to test the protective effect we are studying.

Animals that underwent 14 days of SR associated with 7 days of daily injections of PGE_2 in the hind paw (and their matched controls) were tested for orofacial thermal withdrawal latency before experiment and at day 14; CION was

performed at day 18 and the development of thermal neuropathic allodynia was assessed 2 and 5 days later.

Thermal withdrawal latency in the orofacial region (see methods for additional details) was significantly decreased by SR, but not by daily PGE_2 injections in the hind paw (Fig. 10A, two-way Repeated Measurements ANOVA, $F_{\text{sleep condition}}(1, 35) = 18.05$, $p > 0.001$; $F_{\text{paw injection}}(1, 35) = 0.21$, $p = 0.64$; $F_{\text{time}}(1, 35) = 112.09$, $p < 0.0001$; $F_{\text{sleep condition x paw injection}}(1, 35) = 0.21$, $p = 0.65$; $F_{\text{sleep condition x time}}(1, 35) = 43.75$, $p < 0.0001$; $F_{\text{paw injection x time}}(1, 35) = 0.06$, $p = 0.81$; $F_{\text{sleep condition x paw injection x time}}(1, 35) = 0.16$, $p = 0.69$).

CION significantly decreased orofacial thermal withdrawal latency, characterizing neuropathic allodynia, except in animals previously submitted to 14 days of SR associated with 7 days of daily injections of PGE_2 in the hind paw, in which withdrawal latency did not differ from the sham groups (Fig. 10B three-way Repeated Measurements ANOVA, $F_{\text{sleep condition}}(1, 1) = 0.36$, $p = 0.55$; $F_{\text{paw injection}}(1, 1) = 4.86$, $p < 0.05$; $F_{\text{surgery}}(1, 1) = 16.94$, $p < 0.001$; $F_{\text{time}}(1, 1) = 0.29$, $p = 0.59$; $F_{\text{sleep condition x paw injection}}(1, 1) = 3.40$, $p = 0.07$; $F_{\text{sleep condition x surgery}}(1, 1) = 55.74$, $p = 0.11$; $F_{\text{sleep condition x time}}(1, 1) = 1.64$, $p = 0.21$; $F_{\text{paw injection x time}}(1, 1) = 0.71$, $p = 0.41$; $F_{\text{surgery x time}}(1, 1) = 0.22$, $p = 0.64$; $F_{\text{sleep condition x paw injection x surgery x time}}(1, 3) = 2.74$, $p = 0.07$). This finding suggests that a previous history of long-term SR associated with nociceptive stimulation may protect against the subsequent development of trigeminal neuropathic pain.

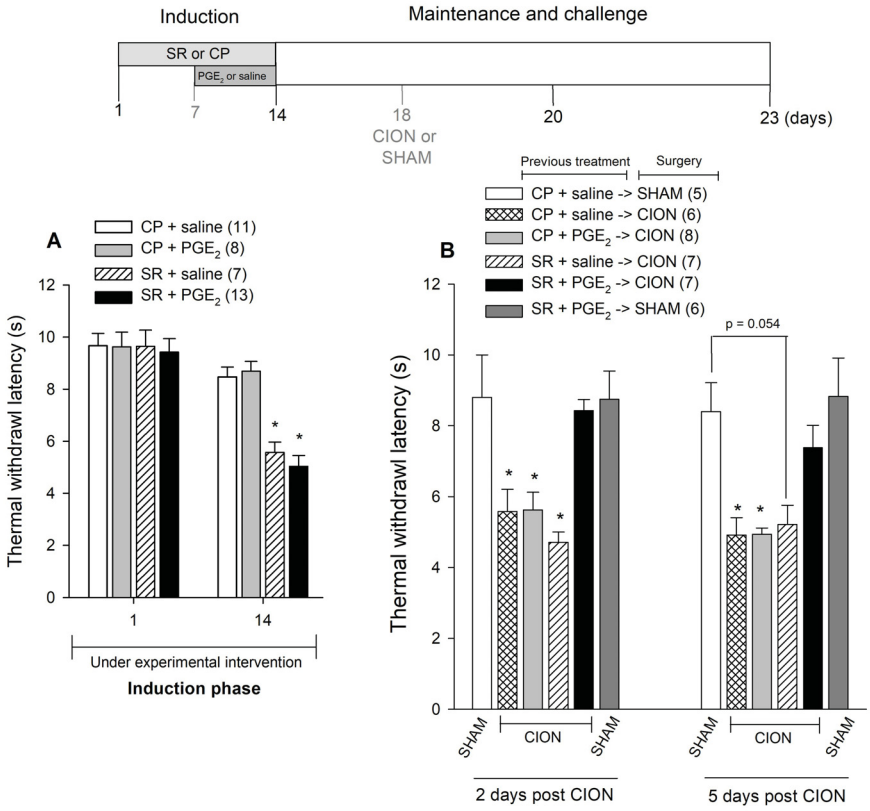

Fig. 10 – Effect of long-term SR associated with nociceptive stimulation on the subsequent development of trigeminal neuropathic allodynia. SR and PGE_2 injections were combined according to the protocol drawn at the top the figure. CION was performed at day 18 and the development of thermal neuropathic allodynia was assessed at days 20 and 23. At the end of 14 days of SR associated with 7 days of daily PGE_2 injections, thermal withdrawal latency in the orofacial region was decreased in SR groups (A). RM ANOVA with two between-subjects factors (sleep condition, paw injection) followed by the Tukey post hoc test ($p < 0.05$). The symbol "*" indicates statistically significant difference compared to CP + saline. After CION all groups developed orofacial thermal allodynia, characterized by the decrease in thermal withdrawal latency, except the group previously submitted to SR and PGE_2 injections (B). RM ANOVA with three between-subjects factors (sleep condition, paw injection and CION) followed by the Tukey post hoc test ($p < 0.05$). The symbol "*" indicates statistically significant difference compared to CP + saline (SHAM). SR = sleep restriction; CP = control procedure; CION = chronic constriction of the infraorbital nerve.

2.4 - The effect of the different experimental protocols on motor behavior.

Spontaneous locomotion in the open field arena was assessed in all groups at the end of SR / daily injections period or Haldol® treatment (groups that received Prolopa® or GBR were submitted to an additional evaluation at the end of this treatment). Except for Haldol® treatment, none of the interventions significantly affected the spontaneous locomotion.

Table 1

Effect of experimental manipulations on locomotor activity

Experimental manipulations	Mean ± SEM	p value	F value
14 days of saline s.c.	104.60 ± 8.75	0.86	n/a
14 days of PGE_2 s.c.	89.33 ± 8.89		
7 days of CP + saline s.c.	64.20 ± 6.63	0.87	0.28
7 days of CP + PGE_2 s.c.	69.33 ± 6.12		
7 days of SR + saline s.c.	69.86 ± 8.72		
7 days of SR + PGE_2 s.c.	72.57 ± 6.02		
14 days of CP + 7 final days saline s.c.	69.80 ± 10.51	0.23	1.51
14 days of CP + 7 final days of PGE_2 s.c.	80.40 ± 16,77		
14 days of SR + 7 final days of saline s.c.	72.00 ± 8.22		
14 days of SR + 7 final days of PGE_2 s.c.	68.50 ± 9.71		
14 days of CP + saline s.c.	84.40 ± 2.99	0.36	0.86
14 days of CP + PGE_2 s.c.	76.87 ± 4.79		
14 days of SR + saline s.c.	78.50 ± 5.72		
14 days of SR + PGE_2 s.c.	70.00 ± 7.81		
CP + saline s.c. + Prolopa p.o. (day 14)	74.00 ± 10.98	0.20	1.67
CP + saline s.c. + Prolopa p.o. (day 19)	73.83 ± 15.45		
CP + PGE_2 s.c. + Prolopa p.o. (day 14)	74.12 ± 7.86		
CP + PGE_2 s.c. + Prolopa p.o. (day 19)	78.87 ± 7.62		
SR + PGE_2 s.c. + Prolopa p.o. (day 14)	71.37 ± 7.80		
SR + PGE_2 s.c. + Prolopa p.o. (day 19)	51.75 ± 5.53		
SR + PGE_2 s.c. + saline p.o. (day 14)	76.00 ± 7.88		
SR + PGE_2 s.c. + saline p.o. (day 19)	68.75 ± 8.40		
CP + saline s.c. + GBR intra-NAc (day 14)	70.00 ± 2.70	0.19	1.73
CP + saline s.c. + GBR intra-NAc (day 19)	48.40 ± 12.16		
CP + PGE_2 s.c. + GBR intra-NAc (day 14)	79.00 ± 9.23		
CP + PGE_2 s.c. + GBR intra-NAc (day 19)	51.67 ± 14.10		
SR + PGE_2 s.c. + GBR intra-NAc (day 14)	77.87 ± 7.02		
SR + PGE_2 s.c. + GBR intra-NAc. (day 19)	103.75 ± 18.73		
SR + PGE_2 s.c. + saline intra-NAc (day 14)	75.58 ± 9.01		
SR + PGE_2 s.c. + saline intra-Nac (day 19)	84.57 ± 15.54		

Saline p.o. + saline s.c.	102.60 ± 9.75		
Saline p.o. + PGE_2 s.c.	91.33 ± 6.23		
Haldol 1mg/kg/day p.o. + PGE_2 s.c.	16.50* ± 3.44	p<0.001	49.87
Haldol 1mg/kg/day p.o. +saline s.c.	20.33* ± 2.75		
Haldol 0.1mg/kg/day p.o. + PGE_2 s.c.	49.33* ± 6.96		

Tab. 1 – Spontaneous locomotion. T test, ANOVA or RM ANOVA were performed. The symbol "*" indicates significant difference compared to control group. The administration of Haldol® for 14 days was the only intervention that affected spontaneous locomotion on the open field, leading to its significant reduction. CP = control procedure; SR = sleep restriction; s.c.= subcutaneous injection; p.o. = oral administration. SEM = standard error of the mean.

Discussion

Sleep disorders are highly comorbid with pain conditions (AFOLALU; RAMLEE; TANG, 2017). Our current understanding of the pain-sleep binomial supports a bidirectional relationship: pain can disrupt sleep, and short or disturbed sleep lowers pain thresholds and aggravates clinical pain (AFOLALU; RAMLEE; TANG, 2017; ANDERSEN et al., 2018; KRAUSE et al., 2019). However, the current findings demonstrate two paradoxical effects of sleep restriction (SR) in pain processing. In the first study, we hypothesized that SR would facilitate the transition from acute to chronic pain. Not only this did not happen, but surprisingly long-term SR (6 hours a day of SR for 14 days, but not for 7 days) prevented pain chronification. In the second study, we investigated whether the previous history of long-term SR associated with nociceptive stimulation would facilitate the subsequent development of acute and chronic pain. It did not facilitate, on the contrary, it paradoxically prevented later pain development in several animal models. Additional findings suggested a role of mesolimbic dopaminergic system in the underlying mechanisms. Although consistent, these data contradict the current understanding of the sleep-pain relationship and should be interpreted with caution. We do not believe in and will not advocate any beneficial effect of sleep restriction on pain. But we confirmed all data, and we are sure they are replicable, at least under the same conditions. Therefore, our discussion will be limited to try to understand the phenomenon and how it emerges from the complex interrelationship between decreased sleep time and pain chronification.

First study: The paradoxical protective effect of SR on chronic pain development

Daily injections of PGE_2 have been used to induce chronic hyperalgesia in rodents for more than three decades (DIAS *et al.*, 2015; FERREIRA; LORENZETTI; DE CAMPOS, 1990; MIRANDA *et al.*, 2015; VERGARA *et al.*, 2020). As shown in figure 1, 14 days of daily injections of PGE_2 into the rat´s hind paw induce a chronic hyperalgesic state that lasts for weeks after the injections were discontinued. When PGE_2 injections are associated with interventions that facilitate pain chronification, the chronic hyperalgesic state emerges after 7 days of daily injections (DIAS *et al.*, 2015; VERGARA *et al.*, 2020). This was what we expected to see when we associated 7 days of daily injections of PGE_2 with 7 (Fig. 2A) or 14 (Fig. 2B) days of

SR. The rapid return to baseline nociceptive threshold supports that SR does not facilitate the transition from acute to chronic pain. Certainly unexpected, but still plausible, since SR could increase pain, as unequivocally demonstrated in literature, without necessarily increasing the risk of developing chronic pain. But when we associate SR with the 14 days of daily injections of PGE_2, the expected development of chronic hyperalgesia was prevented (Fig. 2C)! The protective effect of long-term SR (6 hours a day for 14 days) against the development of chronic pain is clear when comparing the area above the curve from the moment the interventions are discontinued (maintenance phase) until the return to baseline nociceptive threshold (Fig. 3). The area above the curve resultant from 7 days of daily injections of PGE_2 was increased by 7, but not by 14 days of SR. This finding demonstrates that short-term (7 days) SR potentiates the pronociceptive effect of PGE_2, however this potentiation degrades as SR period becomes longer. Remarkably, the area above the curve resulting from 14 days of daily injections of PGE_2 was dramatically decreased by its association with 14 days of SR. The comparison of the first and last bars in figure 3 gives the dimension of the impact of long-term SR on the duration of the hyperalgesia induced by 14 days of daily injections of PGE_2. Undoubtedly, 14 days of SR prevents the transition from acute to chronic hyperalgesia.

How to explain that SR protects against the development of chronic pain? Unfortunately, we do not have that answer, but we would like to propose a line of reasoning. Recently, the mesolimbic dopaminergic system has achieved prominence in pain and sleep research (HAACK *et al.*, 2020; KOURBANOVA; ALEXANDRE; LATREMOLIERE, 2022). In that system, the midbrain VTA send dopaminergic projections to the NAc and other subcortical and cortical structures to influence a variety of functions ranging from motivation to sleep-wake cycle and pain (YANG *et al.*, 2020). Current studies have focused on mesolimbic neuroadaptations to understand the mechanisms underlying sleep disorders (ALEXANDRE *et al.*, 2017; EACRET *et al.*, 2022; HAACK *et al.*, 2020; HANLON *et al.*, 2010; KRAUSE *et al.*, 2017, 2019; ROODSARI *et al.*, 2022; SARDI; TOBALDINI; *et al.*, 2018; SKINNER *et al.*, 2011; TOMASI; WANG; VOLKOW, 2016; UGALDE-MUÑIZ *et al.*, 2022; VOLKOW *et al.*, 2012; WIERS *et al.*, 2016; ZANT *et al.*, 2011), chronic pain development (CHANG *et al.*, 2014; CHEN *et al.*, 2022; DIAS *et al.*, 2015; ELMAN; BORSOOK, 2016; REN *et al.*, 2015; SAGHEDDU *et al.*, 2015; SCHWARTZ *et al.*, 2014; TAYLOR *et al.*, 2016; VERGARA *et al.*, 2020; ZHANG *et al.*, 2017) and their

relationship (ALEXANDRE *et al.*, 2017; SARDI; LAZZARIM; *et al.*, 2018; SARDI; TOBALDINI; *et al.*, 2018; SEMINOWICZ *et al.*, 2019). We will revisit recent literature, part of it from our lab, to support that the decrease in sleep time and the transition from acute to chronic pain result in opponent neuroadaptations in the mesolimbic system. A resultant antagonist dopaminergic state could help to explain why the transition from acute to chronic pain was prevented in sleep restricted animals. Our data regarding the changes in NAc expression of DARPP-32 and DAT will be discussed, in terms of their significance for dopaminergic signaling and synaptic function, as indirect and preliminary support for some of the ideas we are proposing.

Mesolimbic dopaminergic system and pain

Literature converges to support that the transition from acute to chronic pain depends on biphasic changes in mesolimbic dopaminergic function. That is, (1) acute pain increases dopaminergic signaling (BALIKI *et al.*, 2010; ELMAN; BORSOOK, 2016; MORIYA *et al.*, 2018; NAVRATILOVA; PORRECA, 2014; NEES; BECKER, 2017) and (2) this increased dopaminergic signaling contribute to the transition from acute to chronic pain (CHANG *et al.*, 2014; DIAS *et al.*, 2015; ELMAN; BORSOOK, 2016; SAGHEDDU *et al.*, 2015; VERGARA *et al.*, 2020; ZHANG *et al.*, 2017), but once established (3), chronic pain is characterized by a hypodopaminergic state (CHANG *et al.*, 2014; CHEN *et al.*, 2022; REN *et al.*, 2015; SCHWARTZ *et al.*, 2014; TAYLOR *et al.*, 2016; VERGARA *et al.*, 2020; WOOD *et al.*, 2007), which may underlie the decreased motivation in chronic pain patients (DENK; MCMAHON; TRACEY, 2014; MARGOLIS; KARKHANIS, 2019; SEIXAS; PALACE; TRACEY, 2016; SERAFINI; PRYCE; ZACHARIOU, 2020). Importantly, we have demonstrated, specifically in this model of PGE_2-induced chronic hyperalgesia, that the dopaminergic mesolimbic system drives the transition from acute to chronic pain, but not affect the maintenance of chronic pain (VERGARA *et al.*, 2020). These findings fits well with recent human studies showing that the functional connectivity between mesolimbic and prefrontal areas is increased during the transition from acute to chronic pain (VACHON-PRESSEAU *et al.*, 2016), but is decreased in chronic pain patients (YU *et al.*, 2020).

Mesolimbic dopaminergic system and decreased sleep time.

The impact of decreased sleep time on dopaminergic function is under intense debate. Although contradictory data have been reported (ANDERSEN *et al.*, 2005; BERRO *et al.*, 2014; ZANT *et al.*, 2011), most of the recent findings agree that

negative changes in sleep are associated with a decrease in mesolimbic dopaminergic function (ALEXANDRE *et al.*, 2017; EACRET *et al.*, 2022; FIFEL; MEIJER; DEBOER, 2018; HAACK *et al.*, 2020; HANLON *et al.*, 2010; KRAUSE *et al.*, 2017, 2019; ROODSARI *et al.*, 2022; SARDI; TOBALDINI; *et al.*, 2018; SKINNER *et al.*, 2011; TOMASI; WANG; VOLKOW, 2016; UGALDE-MUÑIZ *et al.*, 2022; VOLKOW *et al.*, 2012; WIERS *et al.*, 2016). For example, sleep deprivation decreases neuronal activity in the VTA (FIFEL; MEIJER; DEBOER, 2018), downregulates dopamine D_2 receptors in the striatum (VOLKOW *et al.*, 2012; WIERS *et al.*, 2016) and induces behavioral alterations compatible with hypodopaminergic states, as reduced motivation and cognitive impairments (EACRET *et al.*, 2022; HANLON *et al.*, 2010; KRAUSE *et al.*, 2017; ROODSARI *et al.*, 2022; TOMASI; WANG; VOLKOW, 2016; UGALDE-MUÑIZ *et al.*, 2022). Indeed, strategies to increase dopaminergic signaling reverse some of the effects resultant from decreased sleep time (HANLON *et al.*, 2010), including the enhanced pain sensitivity in sleep-deprived animals, as we (SARDI; TOBALDINI; *et al.*, 2018) and others (ALEXANDRE *et al.*, 2017; SKINNER *et al.*, 2011; UGALDE-MUÑIZ *et al.*, 2022) have demonstrated.

Opponent neuroadaptations

Taken together the findings exposed in the previous paragraphs provide a possible explanation for understanding the neural and mechanistic linkages between decreased sleep and chronic pain development. This mechanistic linkage is the dopaminergic mesolimbic system, which may undergo opponent neuroplastic changes during SR and during the transition from acute to chronic pain. That is, if SR decreases the mesolimbic dopaminergic function (ALEXANDRE *et al.*, 2017; EACRET *et al.*, 2022; HAACK *et al.*, 2020; HANLON *et al.*, 2010; KRAUSE *et al.*, 2017, 2019; ROODSARI *et al.*, 2022; SARDI; TOBALDINI; *et al.*, 2018; SKINNER *et al.*, 2011; TOMASI; WANG; VOLKOW, 2016; UGALDE-MUÑIZ *et al.*, 2022; VOLKOW *et al.*, 2012; WIERS *et al.*, 2016), necessary for the transition from acute to chronic pain (APKARIAN, 2008; CHANG *et al.*, 2014; DIAS *et al.*, 2015; LI *et al.*, 2020; MANSOUR *et al.*, 2014; VERGARA *et al.*, 2020), then SR could theoretically prevent this transition.

Indirect evidence on dopaminergic signaling and synaptic function.

Whether opponent mesolimbic neuroadaptations explain why SR prevents chronic pain development remains to be proven in future studies. However, our data

for NAc changes in DAT and DARPP-32 expression may provide an indirect support. The synaptic availability of dopamine is mainly regulated by its reuptake transporter (DAT), which expression and clearance efficiency are regulated following homeostatic demands (VAUGHAN; FOSTER, 2013). The increase in dopaminergic transmission commonly results in increased DAT expression and clearance efficiency (ALONSO *et al.*, 2021; MASH *et al.*, 2002; RAMAMOORTHY; SHIPPENBERG; JAYANTHI, 2011; ROODSARI *et al.*, 2022); while a decrease in dopaminergic transmission commonly results in the opposite effect (AFONSO-ORAMAS *et al.*, 2010; BA; MARTIN, 2014; PALERMO *et al.*, 2020, 2021). Therefore, the decrease in NAc DAT expression induced by SR (Fig. 4A) may result from a homeostatic compensatory mechanism to increase dopamine synaptic availability and reverse the decreased dopaminergic function resulting from SR. In accordance with this idea, it has been demonstrated that DAT is downregulated in hypodopaminergic states, such as Parkinson's disease (BA; MARTIN, 2014; PALERMO *et al.*, 2020, 2021; PALERMO; CERAVOLO, 2019) as well as in patients with sleep disorders (HUANG *et al.*, 2020; MIYAMOTO *et al.*, 2020).

In contrast to SR, persistent nociceptive stimulation through 14 days of daily PGE_2 injections increased the expression of DAT (Fig. 4A) and P-Thr75-DARPP-32 (phosphorylation at threonine 75) (Fig. 4C), both of which may result from the increase in dopaminergic transmission necessary for the transition from acute to chronic pain. DARPP-32 is a bifunctional dopamine downstream transduction molecule. The initial increase in dopamine neurotransmission results in a protein kinase A (PKA)-mediated increase in P-Thr34-DARPP-32 (phosphorylation at threonine 34), which amplifies dopamine signaling by inhibiting protein phosphatase 1 (NISHI *et al.*, 2000; WALAAS *et al.*, 2011). However, sustained dopamine action increases P-Thr75-DARPP-32, which inhibits PKA, working as a homeostatic brake to the prolonged effects of dopamine neurotransmission (BENAVIDES; BIBB, 2004; BORGKVIST; FISONE, 2007; NISHI; SHUTO, 2017; SCHEGGI *et al.*, 2011; SCHEGGI; RAUGGI; GAMBARANA; *et al.*, 2004). Therefore, the sustained dopaminergic activity during the chronification process may lead to the homeostatic compensatory increase in DAT and P-Thr75-DARPP-32 as a way to decrease such activity. Indeed, their increased expression is expected to increase dopamine reuptake and decrease dopamine signaling, respectively, which may contribute to the hypodopaminergic state characteristic of chronic pain. Importantly, DAT expression

was decreased by SR independent on PGE_2 injections, while it was increased by PGE_2 injections independent on SR. Therefore, during the PGE_2 injections, SR may have maintained dopamine levels low enough to prevent acute hyperalgesia from becoming chronic. Indeed, DAT expression in animals submitted to SR and PGE_2 injections were similar to that of control animals.

Second study: The paradoxical protective effect of long-term SR associated with nociceptive stimulation on the subsequent development of pain.

The nociceptive system shows remarkable plasticity developing central and peripheral sensitization in response to previous activity history and this plasticity has been implicated in the development of chronic pain (FINNERUP; KUNER; JENSEN, 2021; LATREMOLIERE; WOOLF, 2009; PACE *et al.*, 2018). Therefore, in our second study we investigated if a previous history of SR and nociceptive stimulation would impact the subsequent development of pain. Initially, SR and daily PGE_2 injections were combined following the same protocols employed in the first study, then the animals were left to recover baseline nociceptive threshold to be challenged with a new round of PGE_2 injections or subjected to different animals' models of pain.

The previous exposition to 7 days of SR and daily PGE_2 injections did not impact nociceptive response induced by a new round PGE_2 injections, showing no behavioral evidence of nociceptive plasticity (Fig. 5A). However, when SR period was increased to 14 days associated with 7 (Fig. 5B) or 14 (Fig. 5C) days of daily PGE_2 injections, the decrease in nociceptive threshold in response to a new round of PGE_2 injections was suppressed. Importantly, this suppression occurred only when long-term SR (14, but not 7 days) is associated with daily PGE_2 injections (7 or 14 days) and extends to both the acute and chronic hyperalgesic response induced by PGE_2. The resistance to the development of chronic hyperalgesia can be identified after only two injections because when chronic hyperalgesia is developing the nociceptive threshold remains low from the second injection onwards (see results, description of figure 1). However, to demonstrate such resistance, the new round of PGE_2 injections was maintained for 14 days in some groups; the nociceptive threshold decreased only moderately and only after the tenth injection (Fig. 5B). These unexpected results support that animals previously subjected to long-term SR and nociceptive stimulation develop a kind of resistance to the subsequent development of chronic pain. It is improbable that any secondary effect on the animal's ability to

withdraw the paw contribute to this result, because their locomotor activity is preserved (table 1) and they have not undergone any experimental intervention in the last 10 days, during which they were recovering the basal nociceptive threshold. It is noteworthy that this is a different protective effect from the first study. In the former, animals *under* long-term SR are protected from developing chronic pain, in the present, animals *under free sleep condition*, but which have undergone a previous period of SR associated with nociceptive stimulation, are protected from developing chronic pain.

A role for dopamine

Once again, our hypothesis was contradicted and our data point to an additional protective effect of SR on pain development. SR induces a significant disturbance of homeostasis and, therefore, its protective effect should not be seen from a therapeutic perspective, but rather as a tool to understand the neuroadaptations responsible for potential benefits. As discussed above, literature data converge to support that the dopaminergic mesolimbic system undergoes opponent changes in neuroplasticity in the face of SR and pain chronification. Therefore, the key to begin to understand the paradoxical phenomena we are showing may lie in the dopaminergic system. To explore this possibility, we used two pharmacological strategies to increase dopaminergic function during the recovery period, i.e., after the end of SR and the first round of PGE_2 injections and before the beginning of second round of PGE_2 injections. The oral administration of Prolopa® (L-DOPA plus benserazide hydrochloride) or the intra-NAc microinjections of GBR (a dopamine reuptake inhibitor) during 4 days of the recovery period, restored the nociceptive phenotype, normalizing the hyperalgesic response to the new round of PGE_2 injections (Fig. 6A and 6B). Therefore, the pharmacological increase in dopaminergic function, either systemically or in the NAc, prevents the paradoxical protective effect of SR associated with nociceptive stimulation on the subsequent development of pain. A possible explanation is that both dopaminergic interventions may have rescued the mesolimbic system that had been blunted by the opponent plasticity induced by SR and persistent nociception.

The effects of SR on dopaminergic activity are not clear, but data previously published by our lab (SARDI; TOBALDINI; *et al.*, 2018) that finds substantial support in the literature (ALEXANDRE *et al.*, 2017; KRAUSE *et al.*, 2017; SARDI; TOBALDINI; *et al.*, 2018; TOMASI; WANG; VOLKOW, 2016; UGALDE-MUÑIZ *et al.*,

2022; VOLKOW *et al.*, 2012; WIERS *et al.*, 2016) support that the decrease in sleep time decreases dopaminergic D_2 activity. Therefore, we sought to test whether the pharmacological suppression of D_2 activity could somehow replicate the effect of SR on nociceptive responses. Our choice fell on haloperidol, a typical antipsychotic in current clinical use, with antagonistic action at D_2 receptors (KONRADI; HECKERS, 2001; XIBERAS *et al.*, 2001). The oral administration of Haldol® (0.1 or 1mg/kg) was performed daily concomitantly with PGE_2 injections for 14 days and mimic the effect of SR in nociceptive response (Fig. 7). At the lower dose, Haldol® did not significantly affect PGE_2-induced hyperalgesia throughout the induction phase, but prevented its chronification during the maintenance phase, as it happened with SR (Fig. 2C). This finding supports that long-term (14 days) suppression of D_2 activity may protect from developing chronic pain. Although Haldol® has a limited indication for clinical pain control (CENDÁN *et al.*, 2005; DÉCIGA-CAMPOS *et al.*, 2021; LEPPERT *et al.*, 2014; SHAHSAVARI *et al.*, 2021), its apparent analgesic effect at the higher dose (1 mg/kg) should be discarded, because it also decreased locomotion (Tab. 1), which prevents the differentiation between analgesic and motor depressant effects through this test. Since both effects are well documented in literature (KAŹMIERCZAK; NICOLA, 2022; RECH *et al.*, 2022) and go beyond the aim of this study, we will refrain from discussing them. During the challenge phase, animals that have previously received Haldol®, at the higher dose, associated with PGE_2 injections, became resistant to the subsequent acute and chronic hyperalgesic effects of PGE_2, again as it happened with SR (Fig. 5C). This finding supports that the previous suppression of D_2 activity associated with nociceptive stimulation protects from developing subsequent acute and chronic pain. Although its antipsychotic effects are attributed mostly to the antagonism at D_2 receptors, other actions have been attributed to haloperidol (CENDÁN *et al.*, 2005; DÉCIGA-CAMPOS *et al.*, 2020; ENTRENA *et al.*, 2009). Therefore, a question to be addressed in the future is whether the effects induced by Haldol® on nociceptive response are restricted to D_2 blockade.

The paradoxical protective effect extends to other pain models.

So far, the protective of the association of SR and nociceptive stimulation was demonstrated in PGE_2-induced acute and chronic hyperalgesia. To rule out that this effect is limited to the nociceptive response induced by PGE_2, we sought to test its expression in other animals' models of pain. We started with two classical models:

the carrageenan model of hyperalgesia and the formalin test. Formalin-induced overt nociception was similar in control animals and in those previously submitted to SR associated with PGE_2 injections (Fig. 8A). In contrast, carrageenan-induced hyperalgesia was suppressed in animals previously submitted to SR associated with PGE_2 injections (Fig. 8B). These findings may indicate that the development of hyperalgesia, but not overt nociception is hampered after long-term SR associated with nociceptive stimulation.

Hyperalgesia and overt nociception result from different mechanisms in the periphery, while overt nociception results from the activation of primary afferents; hyperalgesia results from their sensitization, i.e, a decrease in their firing threshold (BASBAUM *et al.*, 2009). Consequently, the resultant nociceptive input to the CNS is qualitatively and quantitatively different. Although the influence of sleep on pain probably lies in its central processing, this processing is affected by peripheral neural activity. Therefore, we sought to use an additional allogenic agent and test the protective effect on the resulting hyperalgesia and nociception. Capsaicin, the prototypical TRPV1 (transient receptor potential vanilloid 1) agonist seemed a reasonable choice because it induces both a short-term overt nociception followed by a mid-term hyperalgesia (FRIAS; MERIGHI, 2016). Capsaicin-induced nociception was similar in control animals and in those previously submitted to SR and PGE_2 injections (Fig. 9A); while capsaicin-induced hyperalgesia was suppressed in these animals (Fig. 9B). These findings provide additional support to the idea that the development of hyperalgesia, but not overt nociception is hampered after long-term SR associated with nociceptive stimulation.

The mechanisms underlying nociceptive sensitization, which result in hyperalgesia and allodynia (defined as a nociceptive response elicited by non-noxious stimuli) are widely variable. While PGE_2- and carrageenan-induced hyperalgesia share much of their mechanistic basis (FERREIRA; LORENZETTI; POOLE, 1993); capsaicin acts through quite different mechanisms (MOTTA; CHICHORRO; RAE, 2009; SCHWARTZ *et al.*, 2008). However, all three recruit inflammatory mechanisms to induce hyperalgesia and we wondered whether previous SR associated with nociceptive stimulation might also hamper the development of a subsequent neuropathic injury-induced sensitization. The infraorbital nerve constriction model (CION) of trigeminal neuropathic pain seemed an excellent choice because it includes the additional feature of inducing

sensitization in a region not yet tested in this study, the orofacial region. Before CION, SR decreased orofacial thermal withdrawal latency (Fig. 10A), showing for the first time that the decrease in sleep time increases thermal sensitivity in the orofacial region. This is in agreement with previous studies showing increased mechanical and thermal orofacial sensitivity in insomnia patients (SMITH *et al.*, 2009) and increased mechanical orofacial sensitivity after experimental sleep deprivation (KAMIYAMA *et al.*, 2019). Animals were left to recover and CION was performed four days later. CION-induced thermal allodynia, demonstrated by the significant decrease in orofacial thermal withdrawal latency in all groups, except in that previously submitted to SR associated with PGE_2 injections (Fig. 10B). This finding demonstrates that previous exposition to SR associated with nociceptive stimulation may protect from the subsequent development of orofacial neuropathic pain symptoms.

The above findings support that nociceptive sensitization induced by three different inflammatory agents and a neuropathic injury is hampered in animals previously submitted to long-term SR associated with nociceptive stimulation. We called this phenomenon “the paradoxical protective effect of long-term SR associated with nociceptive stimulation on the subsequent development of pain”. The mechanisms underlying this effect are unknown, but we have demonstrated that the pharmacological increase in dopaminergic function after the end of SR prevents it, restoring the nociceptive phenotype. Some important features of this phenomenon should be highlighted (1) it is necessary the association of SR with nociceptive stimulation to prevent the subsequent nociceptive sensitization; (2) SR must be long, at least longer than seven days; (3) Finally and perhaps the most important, the disruption of nociceptive processing is selective. Responses to notably nociceptive stimuli (noxious or potentially harmful) are unaffected, overt nociception (induced by formalin or capsaicin) and the escape response to potentially harmful mechanical and thermal stimuli (the escape threshold does not rise above that of saline treated or sham controls even in animals under the protective effect) are preserved. The protective effect is apparently restricted to nociceptive sensitization, no matter if it is inflammatory or neuropathic, mechanical or thermal, in the spinal or trigeminal region. It is as if the CNS no longer responded to the exacerbation of nociceptive activity in the face of peripheral injury.

In summary, this study demonstrated two paradoxical effects of SR on pain processing. Specifically long-term SR (1) prevented the transition from acute to

chronic hyperalgesia and (2) its association with nociceptive stimulation induced resistance to the subsequent hyperalgesic effect of several inflammatory agents and a neuropathic injury. This data must be interpreted with caution, because as hyperalgesia is a key manifestation of clinical pain, they support that, under determined conditions, SR may protect from developing pain. The physical discomfort and homeostatic disturbance induced by a sustained decrease in sleep time rule out any clinical application of these findings. However, this study supports SR as a tool to advance our understanding on the neuroadaptations that emerge from the complex interrelationship between decreased sleep time and pain chronification.

5 DISCUSSÃO

A hipótese deste trabalho foi pautada na restrição de sono atuando como facilitadora no processo de cronificação da dor. Esta hipótese foi desenvolvida a partir do entendimento sobre a relação entre dor e sono, sendo que a presença de distúrbios de sono parece aumentar a incidência de dor crônica (AFOLALU; RAMLEE; TANG, 2017; AGMON; ARMON, 2014; ANDERSEN *et al.*, 2018; CHOUCHOU *et al.*, 2014; CREMEANS-SMITH *et al.*, 2006; GENERAAL *et al.*, 2017; GUPTA *et al.*, 2007; JONES *et al.*, 2009; LINDELL; GRIMBY-EKMAN, 2022), e, complementarmente, diversos estudos já demonstraram que a baixa qualidade de sono aumenta a percepção dolorosa, principalmente por diminuir o limiar nociceptivo (AFFLECK *et al.*, 1996; HAMBRECHT-WIEDBUSCH *et al.*, 2017; HUANG *et al.*, 2014; JENNUM *et al.*, 2013; LI *et al.*, 2019; MORPHY *et al.*, 2007; O'BRIEN *et al.*, 2011; PAGE; OPP; KOZACHIK, 2014; STONE *et al.*, 1997; SUTTON; OPP, 2014; TANG *et al.*, 2012; TOMIM *et al.*, 2016; VANINI, 2016; XUE *et al.*, 2018).

De fato, foi observado que a restrição de sono prolonga a hiperalgesia induzida por apenas 7 injeções de PGE_2 (Fig. 2A e 3). Contudo, paradoxalmente, um longo período de restrição de sono (14 dias) impediu o desenvolvimento da hiperalgesia crônica induzida por PGE_2 (Fig. 2C), contrariando a hipótese inicial do trabalho. Não menos surpreendente, posterior à manipulação de 14 dias de restrição de sono com 7 dias finais de injeções de PGE_2, os animais se tornaram resistentes à hiperalgesia induzida por novas injeções de PGE_2 (aguda e cronicamente) (Fig. 5B), por carragenina (Fig. 8B), por capsaicina (Fig. 9A e B) e até induzida por constrição do nervo infraorbital (hiperalgesia térmica) (Fig. 10). Estes dados contradizem o atual entendimento sobre a relação entre dor e sono e devem ser interpretados com cautela. Os próximos parágrafos tratarão de discutir possíveis mecanismos que explicariam como as observações comportamentais poderiam emergir da complexa relação entre diminuição do tempo de sono e estimulação nociceptiva. É importante salientar que, apesar dos resultados, nós não acreditamos e não vamos defender nenhum efeito benéfico da restrição de sono sobre a dor crônica. Neste caso, a restrição de sono deve ser analisada como uma ferramenta para ampliar o entendimento sobre o processo de cronificação da dor. Mais estudos precisam ser realizados para ampliar o entendimento sobre como alterações induzidas pela diminuição no tempo de sono interagem com o processo de cronificação da dor.

Primeiro estudo

No primeiro estudo foram realizados diferentes períodos de restrição de sono com o intuito de facilitar a cronificação da dor, a qual foi avaliada comparando o período de manutenção da hiperalgesia com o que ocorre no modelo de hiperalgesia inflamatória crônica induzida por PGE_2 (Fig. 1). Quando a restrição de sono foi associada com injeções de PGE_2 durante 7 dias, metade do período de injeções preconizado no modelo, foi observado um efeito aditivo, o período de manutenção da hiperalgesia se estendeu (Fig. 2A). Contudo, a associação 7 dias de restrição de sono com injeção diária de PGE_2, não mimetizou o período de manutenção característico do modelo. Frente a este resultado, ampliamos período de restrição de sono para 14 dias, associado a 7 dias finais de injeção de PGE_2. Esta associação não induziu um prolongamento evidente do período de manutenção da hiperalgesia, de fato, o período de manutenção da hiperalgesia foi menor quando comparado ao que ocorreu com apenas 7 dias de restrição de sono (Fig. 3). Estes resultados demonstram que, com o aumento do período de restrição de sono, o efeito aditivo entre restrição de sono e estimulação nociceptiva parece perder potência. A partir deste resultado, foi realizado um experimento associando restrição de sono com 14 injeções de PGE_2 (protocolo completo de hiperalgesia inflamatória crônica). O resultado desse experimento foi paradoxal, os animais não demonstraram o período de manutenção da hiperalgesia característico do modelo. O período de manutenção foi evidentemente menor comparado ao grupo que recebeu apenas 14 injeções de PGE_2, contradizendo a hipótese inicial do trabalho. A partir das observações comportamentais do primeiro estudo surgiu a necessidade de buscar mecanismos que poderiam explicar os achados. Em razão da literatura, que será exposta nos próximos parágrafos, buscamos explicações em alterações no sistema dopaminérgico mesolímbico.

O sistema dopaminérgico mesolímbico é um importante núcleo relacionado com motivação e aprendizagem (BABILONI *et al.*, 2021; ELMAN; BORSOOK, 2016; LEKNES; TRACEY, 2008). Possui a função de integrar informações sensoriais e atribuir saliência aos estímulos, com o objetivo de selecionar a resposta comportamental mais apropriada para cada situação (PADGETT *et al.*, 2012; THOMPSON; NEUGEBAUER, 2018). Este sistema é responsável pela motivação para alcançar uma recompensa ou evitar um estimulo doloroso (BABILONI *et al.*,

2021; ELMAN; BORSOOK, 2016; LEKNES; TRACEY, 2008). Ou seja, tanto estímulos aversivos quanto estímulos associados à recompensa liberam dopamina no NAc (ELMAN; BORSOOK, 2016). Assim como as drogas de abuso, a dor aguda ativa a neurotransmissão dopaminérgica (ELMAN; BORSOOK, 2016), sendo esta ativação envolvida na dimensão afetiva da dor (HUNT; MANTYH, 2001), motivação de escape, planejamento para cessar o dano e aprendizagem/reforço negativo relacionado ao dano (APKARIAN *et al.*, 2005; BALIKI *et al.*, 2010). Em resumo, a informação nociceptiva, através de alterações no tônus dopaminérgico, modula o comportamento frente a estímulos nocivos.

Recentemente muitos estudos focaram na estimulação nociceptiva induzindo alterações plásticas no sistema mesolímbico (CHANG *et al.*, 2014; CHEN *et al.*, 2022; DIAS *et al.*, 2015; LI *et al.*, 2020; MANSOUR *et al.*, 2014; TAYLOR *et al.*, 2016; VERGARA *et al.*, 2020). Diversos estudos demonstraram que o aumento da sinalização dopaminérgica mesolímbica atenua a resposta nociceptiva (ALTIER; STEWART, 1998; DIAS *et al.*, 2015; GEAR; ALEY; LEVINE, 1999; TAYLOR; JOSHI; UPPAL, 2003; WANG *et al.*, 2021; WATANABE *et al.*, 2018; WOOD, 2006). Entretanto, de forma contrastante, o sistema dopaminérgico mesolímbico é recrutado durante o processo de cronificação da dor e necessário para induzir um estado de dor crônica (APKARIAN, 2008; CHANG *et al.*, 2014; DIAS *et al.*, 2015; LI *et al.*, 2020; MANSOUR *et al.*, 2014; VERGARA *et al.*, 2020). Mais especificamente, estudos do nosso laboratório já demonstraram que, no modelo de hiperalgesia crônica induzida por PGE_2, a transição da dor aguda para dor crônica depende da integridade do sistema dopaminérgico mesolímbico (VERGARA *et al.*, 2020).

O sistema dopaminérgico mesolímbico é formado a partir de neurônios dopaminérgicos da VTA que projetam para o NAc, e, um dano aos neurônios dopaminérgicos da VTA (VERGARA *et al.*, 2020) ou o bloqueio farmacológico dos receptores dopaminérgicos no NAc (DIAS *et al.*, 2015), previnem a transição da hiperalgesia aguda para crônica. Contudo, quando a hiperalgesia crônica já está estabelecida, a integridade da função dopaminérgica mesolímbica não afeta a manutenção da hiperalgesia (VERGARA *et al.*, 2020). Em outras palavras, estes dados suportam a ideia de que a função dopaminérgica mesolímbica é essencial para a transição da dor aguda para crônica, mas não é necessária para a manutenção do estado de dor crônica. Em contraste, o estado de dor crônica é caracterizado pela diminuição da função dopaminérgica (CHANG *et al.*, 2014; REN

et al., 2015; SCHWARTZ *et al.*, 2014; TAYLOR *et al.*, 2016; VERGARA *et al.*, 2020; WOOD *et al.*, 2007). O sistema dopaminérgico passa a apresentar menor ligação aos receptores do tipo D_2 de dopamina (TAYLOR *et al.*, 2016; WOOD *et al.*, 2007), menor expressão de receptores de dopamina dos tipos D_1 e D_2 (CHANG *et al.*, 2014), redução de dopamina (CHANG *et al.*, 2014; VERGARA *et al.*, 2020) e regulação tônica de dopamina deficiente (WOOD et al., 2007). Mais especificamente, dados do nosso laboratório já demonstraram que os níveis de dopamina no NAc diminuem progressivamente ao longo do processo de cronificação da dor, se mantendo abaixo do normal ao longo da fase de manutenção da hiperalgesia crônica (VERGARA *et al.*, 2020). O que se relaciona com a diminuição da motivação observada nos pacientes com dor crônica (DENK; MCMAHON; TRACEY, 2014; MARGOLIS; KARKHANIS, 2019; SEIXAS; PALACE; TRACEY, 2016; SERAFINI; PRYCE; ZACHARIOU, 2020).

Como discutido, é possível que ocorra uma mudança no tônus dopaminérgico à medida que a dor se torna crônica, resultado da neuroplasticidade do sistema mesolímbico (CHANG *et al.*, 2014; CHEN *et al.*, 2022; DIAS *et al.*, 2015; KUNER; KUNER, 2021; RECKZIEGEL *et al.*, 2019; SERAFINI; PRYCE; ZACHARIOU, 2020; VERGARA *et al.*, 2020). Isto ocorre provavelmente em decorrência do estado de constante estimulação nociceptiva e há muitas semelhanças com o processo de adição (CHEN *et al.*, 2022; KUNER; KUNER, 2021; SERAFINI; PRYCE; ZACHARIOU, 2020). Uma possível explicação recai às alterações na sinalização molecular do NAc, e, contribuindo para esta ideia, neste estudo foi observado um aumento na expressão de DAT (transportador de dopamina, do inglês, “dopamine transporter”) e na fosforilação de DARPP-32 (fosfoproteína regulada por dopamina e AMP-c com peso molecular de 32KDa, do inglês, “dopamine and cyclic-AMP-regulated phosphoprotein of molecular weight 32KDa”) no resíduo Thr-75 no NAc ao final da fase de indução da hiperalgesia crônica induzida por 14 injeções de PGE_2.

O funcionamento de DAT é modulado a depender da demanda fisiológica que, por sua vez, influencia na quantidade de dopamina disponível na sinapse (VAUGHAN; FOSTER, 2013). A ativação do receptor D_2 de dopamina no NAc, necessária para a cronificação da dor (DIAS *et al.*, 2015), pode estar relacionada com o aumento da expressão de DAT encontrada neste trabalho, isto porque a ativação de D_2 sinaliza para o aumento da atividade e expressão de DAT (RAMAMOORTHY; SHIPPENBERG; JAYANTHI, 2011), possivelmente como um

mecanismo compensatório. Complementarmente, a ativação do receptor KOR no NAc também parece ser necessária para a cronificação da dor (CAHILL *et al.*, 2014, 2022; LIU *et al.*, 2019; MARTINA *et al.*, 2019; MASSALY; MORÓN; AL-HASANI, 2016; VERGARA *et al.*, 2020), e, também sinaliza para o aumento da recaptação de dopamina (CHEN *et al.*, 2022; MARGOLIS; KARKHANIS, 2019; RAMAMOORTHY; SHIPPENBERG; JAYANTHI, 2011). Como consequência, aumento da expressão de DAT tem relação direta com a redução da sinalização dopaminérgica, isto porque à medida que a recaptação de dopamina aumenta, menos dopamina fica disponível na sinapse.

DARPP-32 é uma fosfoproteína abundante nos neurônios de projeção do estriado e possui papel chave na integração dos efeitos de diversos neurotransmissores sobre os neurônios de projeção do estriado (GIRAULT, 2012; GREENGARD *et al.*, 1998; SVENNINGSSON *et al.*, 2004). A fosforilação do resíduo Thr-34 é mediada por PKA (proteína quinase A, do inglês, “protein kinase A”), uma proteína quinase dependente de cAMP (adenosina 3,5-monofosfato cíclico, do inglês, “Cyclic adenosine monophosphate”) (GREENGARD *et al.*, 1998). Como resultado, mecanismos de sinalização intracelular que aumentem cAMP, como ativação de receptores excitatórios D_1 de dopamina e A_{2A} de adenosina, promovem um aumento da fosforilação de DARPP-32 no resíduo Thr-34 (GREENGARD *et al.*, 1998; GREENGARD; ALLEN; NAIRN, 1999; NEVE; SEAMANS; TRANTHAM-DAVIDSON, 2004). Quando fosforilada no resíduo Thr-34, DARPP-32 se liga à proteína fosfatase 1 (PP-1, do inglês, “protein phosphatase 1”) e a inativa (GREENGARD *et al.*, 1998; SCHEGGI; DE-MONTIS; GAMBARANA, 2018). Considerando a hipótese acima mencionada de que a estimulação nociceptiva crônica aumentaria o tônus dopaminérgico a nossa hipótese era de que o protocolo de 14 dias de injeções de PGE_2 induziria um aumento da fosforilação de DARPP-32 no resíduo thr 34. Contudo, não foram observadas alterações em nenhum dos diferentes tratamentos (Fig. 4B). Uma possível explicação para isso decorre da interrelação entre a fosforilação de DARPP-32 nos diferentes resíduos, possivelmente parte de um complexo mecanismo de *feedback* negativo (resumido na figura B). Há um fator de transcrição, CREB (proteína de ligação ao elemento de resposta ao cAMP, do inglês, “cAMP response element-binding protein”), que se torna mais ativo quando há um aumento de DARPP-32 Thr-34 (SVENNINGSSON *et al.*, 2004). Em consequência, CREB aumenta a transcrição de Fos B (FIENBERG;

GREENGARD, 2000; IMBE; KIMURA, 2015), o que, por meio do aumento da transcrição de CDK5 (proteína quinase dependente de ciclína, do inglês, “cyclin dependent kinase”) (SCHEGGI; RAUGGI; NANNI; *et al.*, 2004; SVENNINGSSON *et al.*, 2004), aumenta a fosforilação de DARPP-32 no resíduo Thr-75 (SCHEGGI; RAUGGI; NANNI; *et al.*, 2004; SVENNINGSSON *et al.*, 2004). Em resumo, se ocorrer um aumento da ativação de receptores excitatórios por período prolongado ocorrerá um aumento de DARPP-32 Thr-75, mesmo que por vias de aumento de cAMP e não de diminuição do mesmo. Isso torna passível de se esperar que manipulações crônicas que aumentem a ativação neuronal induzam alterações apenas na fosforilação do resíduo Thr-75, o que de fato ocorre com o uso crônico de drogas de abuso (BIBB *et al.*, 2001; BORGKVIST, 2007; SCHEGGI *et al.*, 2004) e, como observado pela primeira vez neste estudo, após a estimulação nociceptiva crônica (Fig. 4C).

Por fim, o aumento na expressão de DARPP-32 Thr-75 observado ao final do período de indução da hiperalgesia crônica, juntamente com o aumento da expressão de DAT, estão de acordo com a hipótese de que a dor crônica resulta em um estado de hipofunção dopaminérgica possivelmente em decorrência de um estado inicial de aumento da sinalização dopaminérgica. Isto porque a fosforilação de DARPP-32 no resíduo Thr-75 resulta em um inibidor de PKA (GREENGARD *et al.*, 1998; SVENNINGSSON *et al.*, 2004), e acaba por reduzir a sinalização dopaminérgica (NISHI *et al.*, 2000; WALAAS *et al.*, 2011). A redução da sinalização dopaminérgica resulta em alterações comportamentais como depressão, ansiedade e redução da motivação (BENAVIDES; BIBB, 2004; SCHEGGI *et al.*, 2011, 2015), comumente observada em pacientes com dor crônica (DENK; MCMAHON; TRACEY, 2014; SEIXAS; PALACE; TRACEY, 2016; SERAFINI; PRYCE; ZACHARIOU, 2020).

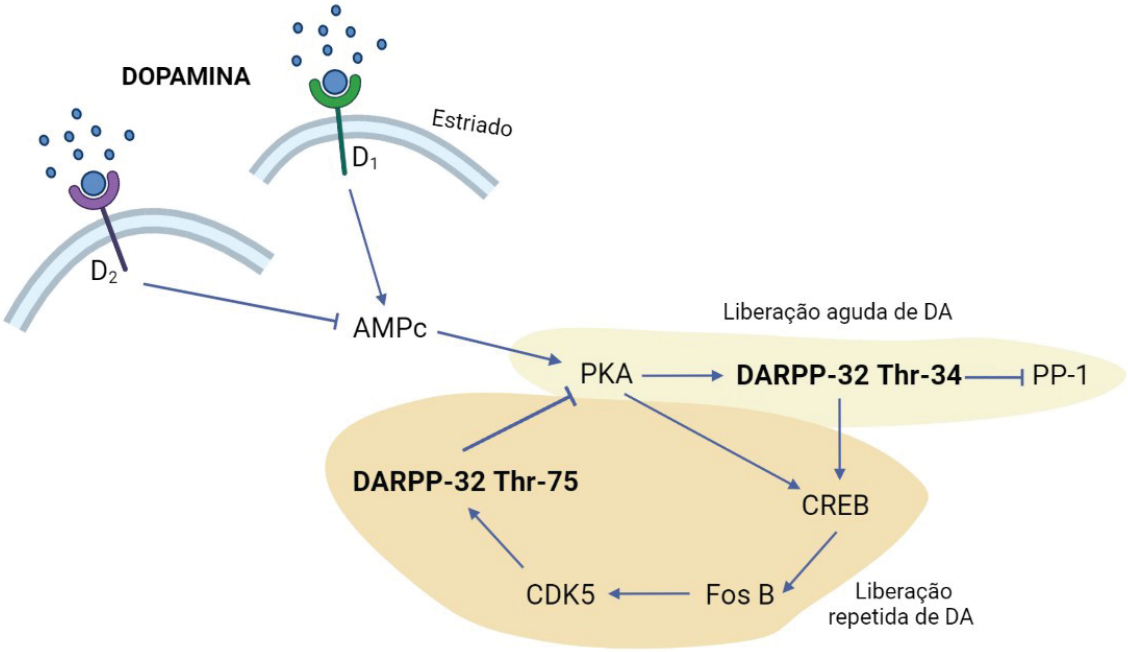

Figura B: Esquema representativo da orquestra de sinalização intracelular que envolve a fosforilação de DARPP em diferentes situações de liberação de dopamina.

A atividade dopaminérgica mesolímbica também participa da modulação do ciclo sono-vigília (OISHI *et al.*, 2017; QIU *et al.*, 2010; SCAMMELL; ARRIGONI; LIPTON, 2017; TAYLOR *et al.*, 2016) e, o NAc, cada vez mais, parece ser uma importante região para a transição entre o sono e a vigília (OISHI *et al.*, 2017; QIU *et al.*, 2012; QIU *et al.*, 2010). A dopamina é um conhecido neurotransmissor promotor da vigília e sua sinalização diminui com o aumento da pressão de sono (MENON *et al.*, 2019; OISHI *et al.*, 2017; SCAMMELL; ARRIGONI; LIPTON, 2017). Contudo, o impacto da diminuição no tempo de sono na função dopaminérgica se encontra sob debate no meio científico. Apesar de dados contraditórios já terem sido publicados (ANDERSEN *et al.*, 2005; BERRO *et al.*, 2014; ZANT *et al.*, 2011), a maioria dos recentes trabalhos concordam que a diminuição do tempo de sono está associada à diminuição da função do sistema dopaminérgico mesolímbico (ALEXANDRE *et al.*, 2017; EACRET *et al.*, 2022; FIFEL; MEIJER; DEBOER, 2018; HAACK *et al.*, 2020; HANLON *et al.*, 2010; KRAUSE *et al.*, 2017, 2019; ROODSARI *et al.*, 2022; SARDI; TOBALDINI; *et al.*, 2018; SKINNER *et al.*, 2011; TOMASI; WANG; VOLKOW, 2016; UGALDE-MUÑIZ *et al.*, 2022; VOLKOW *et al.*, 2012; WIERS *et al.*, 2016; ZANT *et al.*, 2011). Por exemplo, a privação de sono diminui a atividade neuronal na VTA

(FIFEL; MEIJER; DEBOER, 2018), reduz a atividade dos receptores D_2 de dopamina no estriado (KRAUSE *et al.*, 2017; VOLKOW *et al.*, 2012; WIERS *et al.*, 2016) e induz alterações comportamentais semelhantes ao que ocorre em um estado de hipofunção dopaminérgica, como a redução da motivação e alterações cognitivas (EACRET *et al.*, 2022; HANLON *et al.*, 2010; KRAUSE *et al.*, 2017; ROODSARI *et al.*, 2022; TOMASI; WANG; VOLKOW, 2016; UGALDE-MUÑIZ *et al.*, 2022). Ainda, diversos estudos já demonstraram que o aumento da sinalização dopaminérgica reverte os efeitos da diminuição do tempo de sono (ALEXANDRE *et al.*, 2017; HANLON *et al.*, 2010; SARDI; TOBALDINI; *et al.*, 2018; SKINNER *et al.*, 2011; UGALDE-MUÑIZ *et al.*, 2022).

De acordo com a hipótese de que a diminuição no tempo de sono leva à hipofunção dopaminérgica, neste trabalho foi observado uma redução da expressão de DAT ao final de 14 dias de restrição de sono (Fig. 4A). A explicação mais simplista para este achado seria de que uma redução na sinalização dopaminérgica, induzida por 14 dias de restrição de sono, resultaria na redução do transporte de dopamina, possivelmente para manter o nível de dopamina necessário para o estado de vigília forçada. De fato este mecanismo compensatório é observados em estados de hipofunção dopaminérgica, como na doença de Parkinson (ALONSO *et al.*, 2021; PALERMO *et al.*, 2020) e após lesão nos terminais dopaminérgicos do estriado (AFONSO-ORAMAS *et al.*, 2010). De maneira complementar, alguns estudos já demonstraram uma redução da atividade de DAT em casos de distúrbios de sono (HUANG, Z. *et al.*, 2020; MIYAMOTO *et al.*, 2020) e que o estado de vigília está relacionado à menor taxa de recaptação de dopamina (ALONSO *et al.*, 2021; FERRIS *et al.*, 2014).

Tomados em conjunto, os achados expostos nos parágrafos anteriores fornecem uma possível base neural em comum entre os efeitos da diminuição no tempo de sono e o desenvolvimento da dor crônica, ambos resultam na neuroplasticidade do sistema dopaminérgico mesolímbico. Para além disso, aparentemente ocorrem alterações neuroplásticas opostas em resposta à diminuição no tempo de sono e durante a cronificação da dor. Esta ideia é suportada por evidencias, acima mencionadas, de que a diminuição no tempo de sono reduz a função dopaminérgica mesolímbica (ALEXANDRE *et al.*, 2017; EACRET *et al.*, 2022; HAACK *et al.*, 2020; HANLON *et al.*, 2010; KRAUSE *et al.*, 2017, 2019; ROODSARI *et al.*, 2022; SARDI; TOBALDINI; *et al.*, 2018; SKINNER *et al.*, 2011; TOMASI;

WANG; VOLKOW, 2016; UGALDE-MUÑIZ *et al.*, 2022; VOLKOW *et al.*, 2012; WIERS *et al.*, 2016; ZANT *et al.*, 2011), enquanto a cronificação depende da função dopaminérgica (APKARIAN, 2008; CHANG *et al.*, 2014; DIAS *et al.*, 2015; LI *et al.*, 2020; MANSOUR *et al.*, 2014; VERGARA *et al.*, 2020). De fato, neste trabalho observamos alterações antagônicas na expressão de DAT compatíveis com esta hipótese (Fig. 4A). A direção oposta em que ocorrem as alterações no sistema mesolímbico induzidas por restrição de sono e estimulação nociceptiva persistente poderia explicar por que a restrição de sono não apenas não facilita o desenvolvimento de dor crônica (Fig. 2A e 2B), mas surpreendentemente a previne (Fig. 2C). De fato, a associação entre restrição de sono crônica (14 dias) e estimulação nociceptiva persistente equilibrou as alterações antagônicas de DAT, observadas quando restrição de sono ou estimulação nociceptiva foram realizadas isoladamente (Fig. 4A). Resultados semelhantes foram observados em um estudo em que a fragmentação do sono por um período crônico foi associada com administração de cocaína (ROODSARI *et al.*, 2022). Esta associação atenuou os efeitos psicoativos induzidos pela cocaína em camundongos e normalizou o aumento da expressão de DAT induzida pela cocaína (ROODSARI et al., 2022). Há diversas evidências que concordam que a restrição de sono e a estimulação nociceptiva crônica induzem alterações antagônicas no sistema dopaminérgico mesolímbico, contudo, quando ambas as intervenções estão presentes, ainda há muitas dúvidas sobre qual o resultado dessas alterações.

Segundo estudo

O sistema nociceptivo possui grande capacidade de remodelamento, podendo resultar na sensibilização periférica e central em resposta ao histórico prévio de ativação nociceptiva, a qual tem sido associada ao desenvolvimento da dor crônica (FINNERUP; KUNER; JENSEN, 2021; LATREMOLIERE; WOOLF, 2009; PACE *et al.*, 2018). Com o intuito de avaliar alterações persistentes no sistema nociceptivo, na segunda parte deste estudo, investigamos se o histórico prévio de restrição de sono e estimulação nociceptiva impactaria na resposta nociceptiva subsequente, mesmo já resolvida a hiperalgesia inicialmente induzida por restrição de sono e PGE_2. A associação entre 7 dias de restrição de sono e injeção diária de PGE_2 não afetou a resposta nociceptiva frente a novas injeções de PGE_2, não demonstrando

evidência de plasticidade (Fig. 5A). Entretanto, quando o período de restrição de sono foi ampliado para 14 dias e associado a 7 ou 14 dias de injeção diária de PGE_2, a resposta nociceptiva frente a novas injeções de PGE_2 foi abolida (Fig. 5B e 5C). Poderia a oposição das alterações neuroplásticas no sistema dopaminérgico, induzidas por restrição de sono crônica e estimulação nociceptiva persistente, explicar essa supressão? Em parte, acreditamos que sim, isto porque enquanto a restrição de sono crônica parece resultar na hipofunção dopaminérgica, a estimulação nociceptiva persistente, em um primeiro momento, parece estar envolvida no aumento da função dopaminérgica. Contudo, qual seria o efeito resultante das alterações neuroplásticas quando restrição de sono e estimulação nociceptiva ocorrem simultaneamente? Esta é uma questão que não temos dados suficientes para concluir uma explicação plausível. Todavia, realizamos um experimento para avaliar a habilidade da dopamina em restaurar a resposta nociceptiva padrão que ocorre ao intervir com PGE_2.

O resultado que obtivemos foi que a administração oral de Prolopa® ou micro injeções intra-NAc de GBR por 4 dias durante o período de recuperação do limiar nociceptivo normaliza a resposta frente a novas injeções de PGE_2 (Fig. 6A e 6B). Assim, se a função dopaminérgica for aumentada, tanto sistemicamente quanto local, no NAc, a resposta nociceptiva de animais previamente submetidos à restrição de sono associada com injeções de PGE_2 se desenvolve similar aos controles. Uma possível explicação para a habilidade da dopamina em reestabelecer a resposta nociceptiva é que uma intervenção que aumente a neurotransmissão dopaminérgica seja capaz de contrapor as alterações plásticas antagônicas induzidas por restrição de sono e estimulação nociceptiva persistente.

Como já discutido nos parágrafos anteriores, o efeito da restrição de sono na atividade dopaminérgica não está claro, contudo, dados já publicados do laboratório (SARDI; TOBALDINI; *et al.*, 2018) e que encontram suporte na literatura (ALEXANDRE *et al.*, 2017; KRAUSE *et al.*, 2017; TOMASI; WANG; VOLKOW, 2016; UGALDE-MUÑIZ *et al.*, 2022; VOLKOW *et al.*, 2012; WIERS *et al.*, 2016) apoiam que a diminuição no tempo de sono reduz a atividade do receptor D_2 de dopamina. Para tanto, foi realizado um experimento com o intuito de avaliar se a supressão farmacológica da atividade de D_2 poderia replicar o efeito da restrição de sono frente as respostas nociceptivas. O experimento foi conduzido administrando Haldol® concomitante com injeção diária de PGE_2 durante 14 dias. O haloperidol é um

antipsicótico típico em uso clínico, e este exerce seu efeito antagonizando receptores D_2 de dopamina (KONRADI; HECKERS, 2001; XIBERAS *et al.*, 2001). Além disso, é utilizado, com restrições, como antiemético pós-operatório e há alguns estudos indicando um efeito analgésico, principalmente como adjuvante, em associação com opioides (CENDÁN *et al.*, 2005; DÉCIGA-CAMPOS *et al.*, 2020; ENTRENA *et al.*, 2009; LEPPERT *et al.*, 2014; MENA-VALDÉS *et al.*, 2021; SALPETER *et al.*, 2015; SHAHSAVARI *et al.*, 2021). Concordando com a literatura de que a diminuição no tempo de sono reduz a atividade de D_2, foi observado que o Haldol® mimetiza o efeito da restrição de sono em dois momentos do experimento. (1) Foi observado que a menor dose de Haldol® (0.1 mg / kg) não afeta a hiperalgesia induzida por PGE_2 ao longo do período de indução, porém previne a cronificação, assim como o que ocorre com a restrição de sono (Fig. 2C). Este resultado vai ao encontro com a literatura, em que já foi demonstrado que a administração de antagonista D_2 (raclopride) intra-NAc prejudica o desenvolvimento da hiperalgesia crônica induzida por PGE_2 (DIAS *et al.*, 2015). O possível efeito anti-nociceptivo no período de indução observado com a maior dose de Haldol® (1 mg / kg) deve ser interpretado com cautela, visto que foi observado uma redução evidente na locomoção. (2) Foi observado que a maior dose de Haldol® (1 mg / kg) quando associada a injeções de PGE_2 resulta na resistência à hiperalgesia induzida por novas injeções de PGE_2 (aguda e cronicamente), assim como o efeito observado quando a restrição de sono foi associada a injeções de PGE_2 (Fig. 5B e 5C). É relevante discutir se este efeito depende das propriedades de redução da atividade de D_2 ou se outros mecanismos estão envolvidos. Isto porque o haloperidol tem efeitos em outros receptores e há estudos indicando que o efeito antinociceptivo depende de sua ação como antagonista de receptor sigma-1 (CENDÁN *et al.*, 2005; DÉCIGA-CAMPOS *et al.*, 2020; ENTRENA *et al.*, 2009).

O último bloco de experimentos tratou de avaliar se o efeito protetor tardio da associação entre restrição de sono e estimulação nociceptiva se estende a outros modelos de nocicepção. Para tanto, realizamos o desafio com três agentes nociceptivos diferentes, formalina, carragenina e capsaicina. No modelo de dor inflamatória induzida por formalina o comportamento nociceptivo foi semelhante ao controle, não havendo efeito protetor da associação prévia entre restrição de sono e injeções de PGE_2 (Fig. 8A). Já no modelo de hiperalgesia inflamatória induzida por carragenina foi observado o efeito protetor semelhante ao que ocorreu com a PGE_2.

Como as agentes pró-nociceptivos PGE_2 e carragenina compartilham parte dos mecanismos de indução de hiperalgesia (FERREIRA; LORENZETTI; POOLE, 1993; VILLARREAL *et al.*, 2013), é possível que a semelhança seja resultado de uma mesma base mecanista. Para descartar esta possibilidade, foi utilizado um agonista de receptor TRPV1 (receptor de potencial transitório vanilóide tipo 1, do inglês, "transient receptor potential vanilloid 1") como desafio, a capsaicina, um agente que induz dor aguda seguida de hiperalgesia mediante mecanismo diferente do compartilhado pela carragenina e pela PGE_2 (MOTTA; CHICHORRO; RAE, 2009; SCHWARTZ *et al.*, 2008). Novamente foi observado efeito protetor contra a hiperalgesia (Fig. 9B), mas não contra a dor aguda (Fig. 9A). Estes resultados indicam que a hiperalgesia, mas não a dor aguda, é sensível ao efeito protetor estudado.

Os modelos acima testados foram avaliados em uma mesma região, face dorsal da pata traseira, e os modelos que demonstraram efeito protetor foram avaliados por um mesmo teste, o limiar mecânico de retirada da pata. Frente a isso, foi realizado um último experimento para avaliar se o efeito protetor da prévia associação entre restrição de sono e estimulação nociceptiva também seria observado em um modelo de neuropatia na região orofacial. Com este propósito foi utilizado o modelo de neuropatia trigeminal induzida por constrição do nervo infraorbital (CION) e foi avaliado o limiar de sensibilidade térmica na região orofacial. Anterior ao CION, realizamos uma avaliação do limiar nociceptivo térmico na região da face no dia 1 de experimento e após os 14 dias de restrição de sono com 7 dias finais de PGE_2. A partir destas avaliações observamos que a restrição de sono reduziu o limiar nociceptivo térmico da região orofacial independente das injeções de PGE_2 (Fig. 10A), demonstrando pela primeira vez, pelo nosso conhecimento da literatura, que a restrição de sono aumenta a sensibilidade térmica na região orofacial. Este resultado está de acordo com estudos prévios que demonstraram um aumento da sensibilidade mecânica e térmica na região orofacial de pacientes com insônia (SMITH *et al.*, 2009) e um aumento da sensibilidade mecânica na região orofacial após experimento com privação de sono (KAMIYAMA *et al.*, 2019). Após os 14 dias de restrição de sono com 7 dias finais de injeção diária de PGE_2, os animais entraram no processo de recuperação, sendo que o CION foi realizado 4 dias após o final da restrição de sono. A constrição do nervo infraorbital induziu significativa

redução do limiar nociceptivo térmico, exceto no grupo previamente submetido à associação entre restrição de sono e estimulação nociceptiva (Fig. 10B).

Estes achados suportam, utilizando cinco modelos diferentes de agentes nociceptivos, que o efeito protetor não é expresso quando os neurônios nociceptivos são ativados, mas apenas quando estão sensibilizados, demonstrado pela redução do limiar nociceptivo. Limiar nociceptivo é o conceito do grau mínimo de estímulo a ser percebido como dor, este é altamente dinâmico e altera a depender da organização dos circuitos relacionados com a percepção dolorosa (APKARIAN; BALIKI; GEHA, 2009; BALIKI; APKARIAN, 2015). O limiar nociceptivo parece ser definido a partir de conexões entre áreas límbicas e corticais, onde as entradas sensoriais são moduladas (APKARIAN, 2008; BALIKI; APKARIAN, 2015; KUNER; KUNER, 2021). Neste ponto o NAc desempenha importante função, estando potencialmente envolvido na redução do limiar nociceptivo observado na dor crônica (APKARIAN; BALIKI; GEHA, 2009; BALIKI; APKARIAN, 2015). Como observado após a manipulação com GBR intra-NAc, é possível que o efeito protetor observado apenas no limiar nociceptivo seja resultado de uma neuroplasticidade no NAc.

Em resumo, este estudo demonstrou dois efeitos paradoxais da restrição de sono no processamento doloroso. Especificamente, (1) um longo período de restrição de sono preveniu a transição da hiperalgesia aguda para crônica e (2) a prévia associação entre restrição de sono e estimulação nociceptiva tornou os animais resistentes a subsequentes estímulos pró-nociceptivos. É importante salientar que estes dados devem ser interpretados com cautela, o desconforto físico e os distúrbios causados pela diminuição no tempo de sono descartam qualquer aplicação clínica destes achados. No entanto, este estudo apoia o uso da restrição de sono como uma ferramenta para avançar nossa compreensão sobre as neuro adaptações que emergem da complexa inter-relação entre diminuição do tempo de sono e cronificação da dor.

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