UNIVERSIDADE FEDERAL DO PARANÁ

GABRIEL CAMARGO DE OLIVEIRA

INFLUÊNCIA DA RESTRIÇÃO DO SONO NO DESENVOLVIMENTO DE RESPOSTAS ASSOCIADAS A MIGRÂNEA EM RATOS MACHOS E FÊMEAS

CURITIBA

2025

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Dissertação apresentada ao curso de Pós-Graduação em Farmacologia, Setor de Ciências Biológicas, Universidade Federal do Paraná, como requisito parcial à obtenção do título de Mestre em Farmacologia

Orientadora: Profa. Dra. Juliana Geremias Chichorro

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TERMO DE APROVAÇÃO

Os membros da Banca Examinadora designada pelo Colegiado do Programa de Pós-Graduação FARMACOLOGIA da Universidade Federal do Paraná foram convocados para realizar a arguição da Dissertação de Mestrado de **GABRIEL CAMARGO DE OLIVEIRA**, intitulada: **INFLUÊNCIA DA RESTRIÇÃO DO SONO NO DESENVOLVIMENTO DE RESPOSTAS ASSOCIADAS A MIGRÂNEA EM RATOS MACHOS E FÊMEAS**, sob orientação da Profa. Dra. JULIANA GEREMIAS CHICHORRO, que após terem inquirido o aluno e realizada a avaliação do trabalho, são de parecer pela sua APROVAÇÃO no rito de defesa.

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CURITIBA, 10 de Dezembro de 2025.

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JULIANA GEREMIAS CHICHORRO
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Dedico este trabalho à minha esposa, por estar ao meu lado em cada etapa desta jornada, ouvindo, incentivando e apoiando com amor e paciência em todos os momentos. Aos meus pais, que me deram as bases, o exemplo e as oportunidades que me permitiram chegar até aqui. A todos que contribuíram de alguma forma para a conclusão do mesmo.

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“Deus não precisa das tuas boas obras, mas o teu próximo precisa.”

— Martinho Lutero, Tratado sobre as Boas Obras (1520)

RESUMO

A migrânea é uma síndrome neurológica incapacitante que afeta aproximadamente 15% da população mundial, com prevalência de duas a três vezes maior em mulheres do que em homens. Dentre os mecanismos subjacentes à migrânea, a liberação de peptídeos vasoativos por neurônios do gânglio do trigêmeo (GT), tais como o peptídeo relacionado ao gene da calcitonina (CGRP) e o polipeptídeo ativador da adenilato ciclase hipofisária (PACAP), desempenham um papel crucial. Esses peptídeos contribuem para a inflamação neurogênica, a sensibilização periférica e central, bem como para a vasodilatação, processos que participam da geração e manutenção da crise migranosa. Vários gatilhos ambientais, incluindo privação de alimentos, estresse, exposição à luz e restrição do sono (RS), têm sido associados ao início das crises de migrânea. No entanto, os mecanismos pelos quais esses fatores modulam a sensibilização nociceptiva permanecem pouco compreendidos. Este estudo investiga a relação entre sono e migrânea, avaliando se a RS pode alterar o limiar mecânico nociceptivo na região periorbital de ratos machos e fêmeas ou atuar como agente sensibilizador do sistema trigeminovascular. Ratos *Wistar* machos e fêmeas foram submetidos à RS por 6 horas diárias durante três dias consecutivos, utilizando o método *Gentle Handling*. Os grupos controle foram mantidos sob as mesmas condições, mas sem restrição do sono. No primeiro experimento, a alodinia mecânica periorbital foi mensurada com filamentos de von Frey antes e após cada dia de RS. Nos experimentos subsequentes, CGRP (38 ng/10 µL), PACAP (0,1 ng/10 µL) ou os veículos correspondentes (10 µL) foram administrados no GT no terceiro dia de RS, seguidos pela avaliação do limiar mecânico periorbital. Vinte e quatro horas após a injeção, os mesmos animais foram expostos à luz intensa (~ 6000 lux) por 1 hora para avaliação de uma possível reativação do limiar mecânico periorbital. Por fim, avaliou-se a influência da cafeína no protocolo descrito acima, mediante administração oral diária dessa substância (50 mg/kg) por três dias consecutivos antes da RS. Os resultados demonstraram que a RS isoladamente não alterou o limiar mecânico periorbital em ratos machos ou fêmeas, mesmo após três dias consecutivos. No entanto, a administração de CGRP ou PACAP em doses baixas, quando combinada à RS, induziu alodinia mecânica significativa em ratas fêmeas na segunda hora após a injeção, mas não em ratos machos. A exposição à luz aversiva reativou a alodinia mecânica periorbital em fêmeas tratadas com CGRP ou PACAP, por uma e duas horas, respectivamente, sem efeito em machos. A cafeína potencializou ainda mais os efeitos sensibilizantes de CGRP e PACAP sobre a alodinia e a fotossensibilidade, gerando respostas nociceptivas em animais de ambos os sexos. Esses achados indicam que a restrição de sono facilita a sensibilização do sistema trigeminovascular, promovendo respostas semelhantes à migrânea de maneira dependente do sexo, e destacam a cafeína como um modulador dessa interação.

Palavras-chave: Cafeína; Restrição de sono; Migrânea; Alodinia; CGRP; PACAP; Fotossensibilidade.

ABSTRACT

Migraine is a debilitating neurological syndrome that affects approximately 15% of the global population, with a prevalence two to three times higher in women than in men. Among the underlying mechanisms of migraine, the release of vasoactive peptides by trigeminal ganglion (TG) neurons—such as calcitonin gene-related peptide (CGRP) and pituitary adenylate cyclase-activating polypeptide (PACAP)—plays a crucial role. These peptides contribute to neurogenic inflammation, peripheral and central sensitization, and vasodilation, all processes involved in the generation and maintenance of migraine attacks. Several environmental triggers, including food deprivation, stress, light exposure, and sleep restriction (SR), have been associated with the onset of migraine crises. However, the mechanisms by which these factors modulate nociceptive sensitization remain poorly understood. This study investigates the relationship between sleep and migraine, assessing whether SR can alter the periorbital mechanical nociceptive threshold in male and female rats or act as a sensitizing agent of the trigeminovascular system. Male and female Wistar rats were subjected to SR for 6 hours per day over three consecutive days using the Gentle Handling method. Control groups were maintained under the same conditions without SR. In the first experiment, periorbital mechanical allodynia was assessed using von Frey filaments before and after each SR session. In subsequent experiments, CGRP (38 ng/10 µL), PACAP (0.1 ng/10 µL), or their respective vehicles (10 µL) were administered into the TG on the third day of SR, followed by evaluation of the periorbital mechanical threshold. Twenty-four hours after injection, the same animals were exposed to bright light (~6000 lux) for one hour to assess possible reactivation of the periorbital mechanical threshold. Finally, the influence of caffeine on this protocol was evaluated through daily oral administration (50 mg/kg) for three consecutive days prior to SR. The results showed that SR alone did not alter the periorbital mechanical threshold in either male or female rats, even after three consecutive days. However, the administration of subthreshold doses of CGRP or PACAP combined with SR induced significant mechanical allodynia in female rats two hours after injection, but not in males. Exposure to aversive light reactivated periorbital allodynia in females treated with CGRP or PACAP for one and two hours, respectively, with no effect in males. Caffeine further enhanced the sensitizing effects of CGRP and PACAP on allodynia and photosensitivity, eliciting nociceptive responses in both sexes. These findings indicate that sleep restriction facilitates sensitization of the trigeminovascular system, promoting migraine-like responses in a sex-dependent manner, and highlight caffeine as a modulator of this interaction.

Keywords: Caffeine; Sleep restriction; Migraine; Allodynia; CGRP; PACAP; Photosensitivity.

APRESENTAÇÃO

Esta dissertação é apresentada em formato alternativo, de acordo com as normas do Programa de Pós-Graduação em Farmacologia da UFPR. Este trabalho está composto por uma introdução e objetivos. Na sequência, é apresentado o artigo científico, resultante deste período de mestrado, organizado conforme as normas da revista pela qual foi submetida e com suas respectivas referências. Há, também, a sessão do material suplementar submetido em conjunto com o artigo. Por fim, são apresentadas as considerações finais do trabalho, seguida pelas referências utilizadas nestas seções fora do artigo científico.

LISTA DE FIGURAS E TABELAS

LISTA DE ABREVIATURAS OU SIGLAS

A1	–Adenosine receptor A1
A2A	–Adenosine receptor A2A
AMPc	–Adenosina monofosfato cíclico
CGRP	–Calcitonin gene-related peptide
CLR	– Calcitonin receptor-like receptor
CSD	– Cortical Spreading Depression
GT	– Gânglio do trigêmeo
PAC1	– Pituitary adenylate cyclase-activating polypeptide receptor type 1
PACAP	– Pituitary adenylate cyclase-activating polypeptide
PDE	– Fosfodiesterase
PKA	– Proteína Quinase A
RAMP1	– Receptor activity-modifying protein 1
RS	– Restrição de sono
STV	– Sistema trigeminovascular
VPAC1	– Vasoactive intestinal peptide/pituitary adenylate cyclase receptor type 1
VPAC2	– Vasoactive intestinal peptide/pituitary adenylate cyclase receptor type 2

SUMÁRIO

1. INTRODUÇÃO

1.1 MIGRÂNEA

A migrânea é um distúrbio neurológico complexo e recorrente caracterizado por episódios de dor de cabeça intensa, geralmente unilateral e pulsátil, frequentemente acompanhada de sintomas como náusea, fotofobia e fonofobia (ICHD-3, 2018). É considerada uma das doenças neurológicas mais incapacitantes do mundo, afetando aproximadamente 15% da população global, sendo de duas a três vezes mais prevalente em mulheres do que em homens (CEN et al., 2021).

A fisiopatologia da migrânea envolve múltiplas fases interligadas. A fase prodrômica, que antecede a dor, é marcada por alterações autonômicas, de humor e apetite, indicando ativação hipotalâmica precoce. Na fase de aura, presente em cerca de 30% dos pacientes, ocorrem sintomas neurológicos transitórios — como distúrbios visuais ou sensoriais — decorrentes da depressão cortical alastrante. A fase de cefaleia é mediada principalmente pela ativação do sistema trigeminovascular (STV), no qual terminações nociceptivas das fibras do nervo trigêmeo que inervam as meninges e vasculatura craniana liberam neuropeptídeos vasoativos, especialmente o peptídeo relacionado ao gene da calcitonina (CGRP) e o polipeptídeo ativador da adenilato ciclase hipofisária (PACAP) (KUBURAS & RUSSO et al., 2023). A fase de pósdromo da migrânea, frequentemente chamada de "ressaca da enxaqueca", ocorre após a dor ter diminuído e pode durar horas ou até dias. Nessa fase, os indivíduos costumam relatar sintomas residuais como fadiga intensa, dificuldade de concentração, lentidão mental, hipersensibilidade sensorial, alterações de humor e mal-estar geral (GOADSBY et al., 2017). Embora a dor de cabeça em si tenha cessado, ainda existem alterações na neurobiologia central — incluindo a persistência da sensibilização neural e problemas na modulação sensorial — que explicam a continuidade do comprometimento funcional (GOADSBY et al., 2017).

O CGRP desempenha papel fundamental na vasodilatação e na sensibilização periférica e central, amplificando a excitabilidade neuronal. O PACAP, anteriormente considerado apenas um mediador secundário, passou a ser reconhecido como um elemento-chave da resposta migranosa, com evidências apontando que suas vias de sinalização diferem daquelas já descritas para o CGRP. Essa diferença pode

explicar a variabilidade na resposta clínica aos anticorpos monoclonais anti-CGRP, que, apesar de amplamente utilizados, apresentam eficácia limitada em parte dos pacientes (KUBURAS et al., 2021). Atualmente, anticorpos direcionados ao PACAP estão em estudos clínicos de fase II (ASHINA et al., 2024), com o objetivo de promover benefício aos pacientes não responsivos às terapias anti-CGRP. Estudos sugerem que, mesmo durante a crise migranosa, os anticorpos têm penetração muito baixa na barreira hematoencefálica (CRESTA et al., 2024), o que indica que o efeito dessas terapias é periférico. Nesse sentido, o TG tem sido considerado um potencial sítio para a ação das terapias anti-CGRP. Estudos mostram que cerca de 50% dos neurônios do gânglio do trigêmeo (GT) expressam CGRP (EFTEKHARI et al., 2011), enquanto o PACAP é expresso apenas em um subconjunto distinto de neurônios trigeminais que não expressam CGRP, correspondendo a aproximadamente 20% (DELLA PIETRA; KUBURAS; RUSSO, 2025). Ambos os neuropeptídeos são liberados no GT após a ativação de aferentes trigeminais e promovem vasodilatação e sensibilização neuronal (DELLA PIETRA; KUBURAS; RUSSO, 2025). O PACAP interage com os receptores PAC1, VPAC1 e VPAC2 nas células musculares lisas vasculares, iniciando assim vias dependentes de AMPc que abrem canais de potássio ativados por cálcio sensíveis a ATP, resultando em uma vasodilatação. Por outro lado, o CGRP causa vasodilatação meníngea via receptores CLR/RAMP1 além de agir também em células gliais satélites e mastócitos, levando à liberação de mediadores pró-inflamatórios que contribuem para a sensibilização periférica, bem como para a inflamação neurogênica local (DELLA PIETRA; KUBURAS; RUSSO, 2025). Nesse sentido, nosso grupo desenvolveu um modelo de migrânea que consiste na administração intraganglionar de CGRP, o qual reproduz em ratos fenótipos robustos de migranea, como alodinia mecânica periorbital e fotossensibilidade, com diferenças sexuais quanto à intensidade da resposta (ARAYA et al., 2020). Além disso, reforça a relevância translacional, pois reproduz a sinalização entre neurônios e células gliais no GT – um dos principais locais de iniciação da sensibilização periférica e central. Além do CGRP, um estudo recente do nosso grupo mostrou que a injeção de PACAP no GT de ratos também é capaz de induzir alodinia periorbital e fotosenssibilidade, porém apenas em fêmeas (DA LUZ et al., 2025). É valido mencionar que esse modelo apresenta validade farmacológico, pois tanto as respostas evocadas por CGRP quanto por PACAP são inibidas por sumatriptano, um fármaco anti-migranoso

seletivo, corroborando observações clínicas (ARAYA et al., 2020; DA LUZ et al., 2025; ASGHAR et al., 2010; WIENHOLTZ et al., 2021).

Por fim, diversos gatilhos ambientais e comportamentais são reconhecidos por precipitar crises migranosas, embora sua relação causal nem sempre seja direta. Entre os fatores mais frequentemente relatados estão alterações no sono, estresse emocional, exposição à luz intensa, flutuações hormonais, mudanças climáticas, consumo de álcool, jejum prolongado e certos alimentos, especialmente aqueles ricos em tiramina, cafeína, nitratos ou aspartame (KARSAN & GOADSBY, 2021). Entretanto, estudos recentes têm questionado o papel causal dos chamados “gatilhos alimentares”. Evidências indicam que o desejo súbito por alimentos específicos, como chocolate, café ou queijos, pode representar manifestações precoces da fase pródromica da migrânea, resultantes de alterações hipotalâmicas que modulam o apetite, o humor e a homeostase energética antes do início da dor, mas não como um gatilho causal (KARSAN et al., 2021; CHARLES et al., 2018). De forma geral, a suscetibilidade individual a gatilhos parece refletir uma interação complexa entre predisposição genética, estado hormonal e modulação homeostática do sono e da dor, reforçando a ideia de que a migrânea é um distúrbio de processamento sensorial mais do que uma simples resposta a estímulos externos (KARSAN & GOADSBY, 2021). Dentre os gatilhos mais frequentemente reportados pelos pacientes está a privação do sono, que atua não apenas como fator precipitante isolado, mas como um potente modulador do limiar de ativação das vias trigeminovasculares e da excitabilidade cortical. Estudos em modelos humanos e animais mostram que a falta de sono reduz a inibição intracortical, aumenta a atividade glutamatérgica e favorece a suscetibilidade a *Cortical Spreading Depression* (CSD), fenômeno intimamente ligado à fisiopatologia da enxaqueca (NEGRO et al., 2020). Além disso, a privação de sono está relacionada ao aumento da adenosina extracelular e à disfunção nos mecanismos de modulação do sono e da dor, o que pode reduzir o limiar para o desencadeamento de crises migranosas (TORRENTE et al., 2024)

1.2 RELAÇÃO ENTRE MIGRÂNEA E DISTÚRBIOS DO SONO

O sono desempenha um papel vital na manutenção da homeostase neural e na modulação da dor. Alterações em sua quantidade ou qualidade têm sido

associadas ao aumento da sensibilidade à dor e ao surgimento de condições de dor crônica (RUNGE et al., 2024). Estima-se que de 30% a 50% da população mundial sofra de privação ou distúrbio do sono de qualquer natureza, seja por causas clínicas, como insônia e apneia obstrutiva do sono, ou por fatores sociais, como trabalho em turnos e uso excessivo de dispositivos eletrônicos (CANEVER et al., 2024; MORIN et al., 2015). A alta prevalência desse problema torna ainda mais relevante a investigação dos mecanismos pelos quais a restrição do sono (RS) influencia a nocicepção e contribui para o desenvolvimento de condições dolorosas, incluindo a migrânea.

Estudos epidemiológicos indicam que o sono é tanto causa quanto efeito da migrânea, ou seja, pacientes que sofrem de migrânea apresentam maior prevalência de distúrbios do sono, enquanto a redução da duração do sono é um gatilho para as crises, bem como para o aumento da frequência das crises e do risco de cronificação (TISEO et al., 2020). Pacientes com migrânea apresentam maior prevalência de distúrbios como insônia, síndrome das pernas inquietas, apneia obstrutiva e sonolência diurna excessiva, em comparação com a população geral (STANYER et al., 2021; KIM et al., 2018).

A ativação hipotalâmica durante a fase prodrômica da migrânea é um dos achados mais consistentes em estudos de neuroimagem funcional, evidenciando o papel dessa estrutura como elo central entre o metabolismo, o sono e a dor (CHARLES et al., 2018; SCHULTE & ASHINA, 2020). Alterações nos níveis de adenosina — um neuromodulador crucial na regulação do sono e da dor — foram descritas em pacientes migranosos, sugerindo que a privação de sono altera o limiar de excitabilidade neuronal em núcleos trigeminais e talâmicos (BROWN et al., 2012; FRIED et al., 2017). Essa disfunção neuroquímica contribui para o estado de hipersensibilidade característico da migrânea, tornando o sistema trigeminovascular mais responsivo a estímulos internos e externos.

Em modelos experimentais, a restrição de sono tem se mostrado um potente fator de sensibilização da via trigeminovascular. Protocolos de restrição de sono por seis horas diárias durante três dias consecutivos, utilizando o método de *gentle handling*, foram capazes de reduzir progressivamente o limiar nociceptivo mecânico em roedores e induzir alodinia significativa (ALEXANDRE et al., 2017). Esses achados corroboram a hipótese de que a falta de sono atua como um agente facilitador da sensibilização periférica e central, preparando o sistema nociceptivo

para responder de forma exacerbada a mediadores inflamatórios ou vasodilatadores endógenos. De fato, foi recentemente demonstrado que a restrição de sono intensifica a sensibilização dos circuitos de dor a estímulos subliminares. No estudo de Lillo Vizin et al. (2024), a privação aguda de sono em camundongos fêmeas resultou em respostas nociceptivas intensificadas a baixas doses de CGRP ou nitroglicerina, revelando uma relação direta entre sono insuficiente e maior suscetibilidade à dor migranosa.

Além de potencializar a resposta aos mediadores da migrânea, a restrição de sono promove desequilíbrios neuroquímicos que afetam a modulação descendente da dor. O acúmulo de adenosina extracelular em regiões corticais e hipotalâmicas durante a privação prolongada de sono exerce efeito bifásico sobre a excitabilidade neuronal, podendo, em concentrações elevadas, ativar receptores de adenosina A_{2A} e aumentar a liberação de CGRP (BROWN et al., 2012).

Por fim, a relação entre sono e migrânea ultrapassa a dimensão puramente fisiológica, alcançando implicações clínicas e terapêuticas. O manejo comportamental do sono — incluindo higiene do sono, regularidade nos horários de repouso e tratamento de distúrbios como insônia ou apneia — tem se mostrado eficaz na redução da frequência e intensidade das crises migranosas (SULLIVAN et al., 2019; CALHOUN et al., 2007). A melhora da qualidade do sono parece restaurar parcialmente os mecanismos endógenos de analgesia e reduzir a hiperexcitabilidade cortical, funcionando como um modulador preventivo natural. Dessa forma, compreender os mecanismos que conectam o sono à migrânea, tanto em humanos quanto em modelos experimentais, é fundamental para o desenvolvimento de estratégias terapêuticas mais eficazes, capazes de atuar não apenas sobre os sintomas, mas sobre os fatores de vulnerabilidade subjacentes à doença.

1.3 INFLUÊNCIA DA CAFEÍNA NA MODULAÇÃO DO SONO E DA DOR

A cafeína é a substância psicoativa mais consumida no mundo, presente em bebidas, alimentos e produtos farmacêuticos, sendo ingerida diariamente por cerca de 80% da população (HECKMAN et al., 2010). Seu principal mecanismo de ação consiste no antagonismo competitivo dos receptores de adenosina, especialmente os subtipos A_1 e A_{2A} o que resulta em aumento da excitabilidade neuronal e modulação de diversos sistemas de neurotransmissão, incluindo dopaminérgico e

serotoninérgico (FREDHOLM et al., 1999). Em condições fisiológicas, a adenosina se acumula durante a vigília, promovendo o sono e atuando como modulador inibitório da atividade neural (BROWN et al., 2012). Ao bloquear esses receptores, a cafeína impede a ação sedativa da adenosina, prolongando o estado de alerta e interferindo na regulação da homeostase do sono e da dor.

No contexto da nocicepção, a cafeína apresenta um perfil bifásico. Em doses moderadas e uso agudo, atua como adjuvante analgésico, potencializando o efeito de fármacos como paracetamol e ácido acetilsalicílico (DIENER et al., 2020). Essa potencialização ocorre por mecanismos farmacodinâmicos e farmacocinéticos: o bloqueio dos receptores A_{2A} reduz a vasodilatação mediada por adenosina nos vasos cranianos, atenuando o componente vascular da cefaleia; além disso, a cafeína promove maior biodisponibilidade dos analgésicos e acelera sua absorção gastrintestinal, contribuindo para um início de ação mais rápido (DIENER et al., 2020). Por outro lado, o consumo crônico elevado, bem como a interrupção abrupta do uso, está associado ao desencadeamento de cefaleia ou de crises migranosas, evidenciando uma relação bidirecional entre cafeína e dor (ZDUŃSKA et al., 2023; ALSTADHAUG et al., 2020, NOWACZEWSKA et al., 2020). Esse efeito é atribuído à supersensibilidade adenosinérgica compensatória que ocorre após a retirada da cafeína, levando a vasodilatação excessiva e aumento da excitabilidade neuronal (KIM et al., 2021; SAWYNOK, 2011).

A interferência da cafeína na arquitetura do sono é bem documentada. O bloqueio dos receptores de adenosina reduz a pressão homeostática do sono e aumenta a latência para adormecer, reduz o tempo total e a eficiência do sono e suprime a atividade de ondas lentas, essenciais para o sono restaurador (BROWN et al., 2012). Estudos populacionais indicam que o consumo de cafeína em horários próximos ao período de repouso está associado à menor qualidade de sono e aumento da sonolência diurna (WATSON et al., 2015). Essa alteração é relevante no contexto da dor, pois o sono profundo participa da regulação dos sistemas endógenos de analgesia, e sua fragmentação reduz a capacidade do organismo de modular estímulos nociceptivos (SMITH et al., 2020). Assim, a perturbação crônica do sono induzida pela cafeína pode contribuir para um estado de hipersensibilidade neural e, consequentemente, para maior suscetibilidade a crises migranosas. Esses efeitos podem estar relacionados à interação entre o sistema adenosinérgico e a liberação de neuropeptídeos vasoativos, uma vez que a ativação prolongada de

receptores A_{2A} parece aumentar a liberação de CGRP em fibras trigeminais (FRIED et al., 2017). Além disso, é importante ressaltar sua ação potencial na inibição não seletiva das fosfodiesterases (PDEs), enzimas responsáveis pela degradação de nucleotídeos cíclicos, como o monofosfato de adenosina cíclico (AMPc). A inibição das PDEs pela cafeína leva a um aumento do AMPc intracelular, ativando assim a proteína quinase A (PKA) e modulando vias de sinalização envolvidas na excitabilidade neuronal e no controle do tônus vascular (DALY, 1987; FREDHOLM et al., 1999). Nos tecidos vasculares, o aumento do AMPc está classicamente associado ao relaxamento da musculatura lisa e à vasodilatação, um efeito que pode se tornar relevante em condições de exposição a doses elevadas ou repetidas de cafeína (FREDHOLM et al., 1999). Embora em doses usuais o principal efeito da cafeína seja mediado pelo bloqueio dos receptores de adenosina A_1 e A_2A, a contribuição da inibição da PDE e o consequente aumento do cAMP podem atingir significado fisiopatológico em contextos específicos, como a modulação da reatividade vascular e da sinalização nociceptiva associada à enxaqueca (NEHLIG, 2010).

Embora o uso de cafeína como adjuvante analgésico seja comprovadamente eficaz em cefaleias episódicas, a sua administração crônica ou em condições de privação de sono requer cautela. Evidências sugerem que o uso contínuo pode alterar a resposta a mediadores endógenos e comprometer os mecanismos de analgesia descendente (FRIED et al., 2017). Além disso, a cafeína influencia a secreção de dopamina e serotonina em regiões corticais e talâmicas, neurotransmissores diretamente envolvidos na modulação da dor e na fisiopatologia da migrânea (NEHLIG et al., 1992). A dopamina, em especial, tem sido relacionada à fase pródrômica da migrânea, e a interferência da cafeína em seus níveis sinápticos pode alterar a transição entre os estágios da crise (CHARLES et al., 2018).

Portanto, a cafeína exerce efeitos multifacetados sobre o sono e a dor. Seu antagonismo adenosinérgico explica tanto sua ação estimulante e analgésica quanto seus efeitos adversos sobre o ciclo sono-vigília e a sensibilização trigeminovascular. No contexto da migrânea, pode atuar como fator modulador de risco ou como coadjuvante terapêutico, dependendo da dose, do tempo de uso e do estado fisiológico do indivíduo. Assim, compreender a interação entre cafeína e migrânea é essencial para delinear estratégias terapêuticas mais seguras e eficazes,

particularmente em condições nas quais o sono e a dor estão intrinsecamente relacionados.

2. OBJETIVO

2.1 OBJETIVO GERAL

Avaliar os efeitos da restrição de sono, isoladamente ou em associação a intervenções farmacológicas, sobre respostas nociceptivas relacionadas à migrânea em ratos machos e fêmeas.

2.2 OBJETIVOS ESPECÍFICOS

- Avaliar a influência da restrição de sono *per se* no limiar mecânico periorbital de ratos machos e fêmeas.
- Investigar um possível sinergismo entre restrição de sono e injeção intraganglionar de CGRP no desenvolvimento de respostas nociceptivas associadas à migrânea em ratos machos e fêmeas.
- Investigar um possível sinergismo entre restrição de sono e injeção intraganglionar de PACAP no desenvolvimento de respostas nociceptivas associadas à migrânea em ratos machos e fêmeas.
- Avaliar a influência da cafeína no contexto da restrição de sono em ratos machos e fêmeas submetidos à injeção intraganglionar de CGRP ou PACAP.

ARTIGO CIENTÍFICO

Submetido

Lifestyle triggers of migraine: sleep restriction and caffeine lower the threshold for migraine-like responses in rats in a sex-specific manner

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All authors declare no conflicts of interest related to this work.

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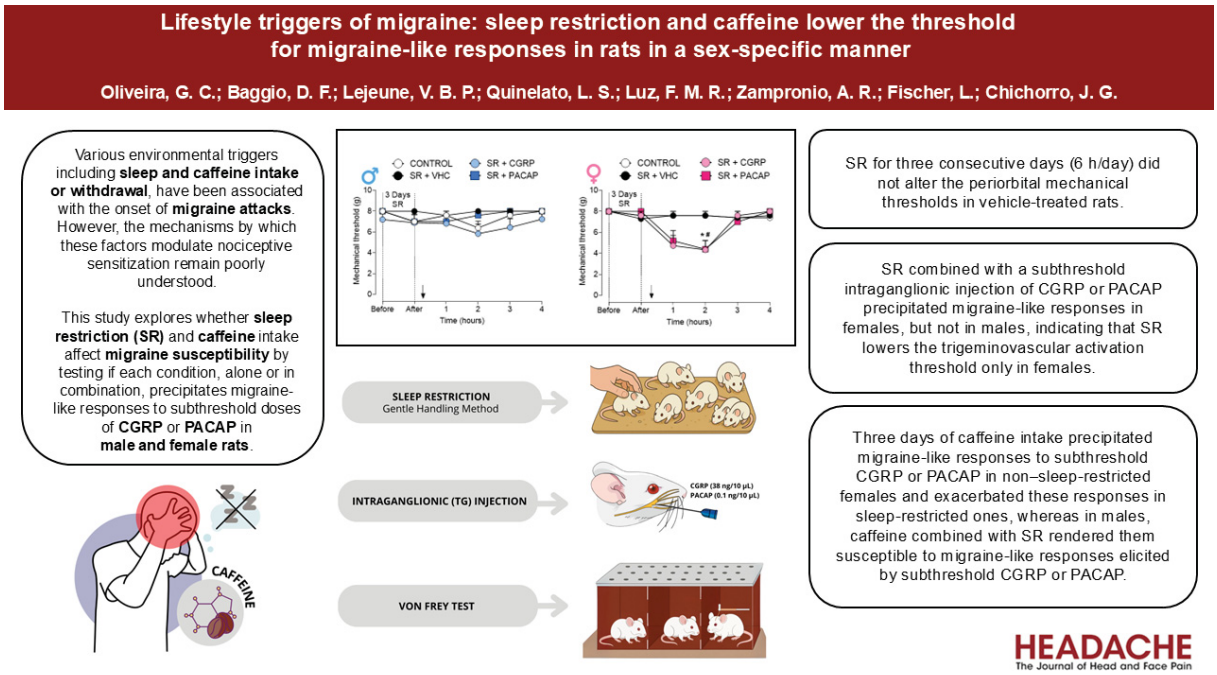
Ethics approval:

All procedures were approved by the Ethics Committee on Animal Use of the Federal University of Paraná (CEUA-BIO-UFPR, protocol #1507) and followed national and international guidelines for animal research.

GRAPHICAL ABSTRACT



Abbreviations:

CGRP - Calcitonin gene-related peptide

PACAP - Pituitary adenylate cyclase-activating polypeptide TG

SR - Sleep restriction

CEUA-BIO-UFPR - Comissão de Ética no Uso de Animais do Setor de Ciências Biológicas da Universidade Federal do Paraná

α-CGRP - Alpha isoform of calcitonin gene-related peptide

PACAP-38 - 38-amino acid isoform of pituitary adenylate cyclase-activating polypeptide

CAPES - Coordination for the Improvement of Higher Education Personnel (Brazil) CNPq - National Council for Scientific and Technological Development (Brazil)

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ABSTRACT

Objective. This study explores whether sleep restriction (SR) and caffeine intake affect migraine susceptibility by testing if each condition, alone or in combination, precipitates migraine-like responses to subthreshold doses of CGRP or PACAP in male and female rats. **Background.** Migraine is a debilitating neurological syndrome that affects approximately 15% of the global population, with a threefold higher prevalence in women compared to men. Among the peripheral mechanisms underlying migraine, the release of vasoactive peptides by trigeminal ganglion (TG) neurons, such as calcitonin gene-related peptide (CGRP) and pituitary adenylate cyclase-activating polypeptide (PACAP), plays a crucial role. Various environmental triggers - including sleep or food deprivation, caffeine intake or withdrawal, stress and light exposure - have been associated with the onset of migraine attacks. However, the mechanisms by which these factors modulate nociceptive sensitization remain poorly understood. **Methods.** Male and female Wistar rats were subjected to SR for 6 hours daily over three consecutive days using the gentle handling method and the periorbital mechanical allodynia was assessed using von Frey filaments before and after each day of SR. Next, a subliminal dose of CGRP (38 ng/ 10 µL) or PACAP (0.1 ng/ 10 µL) was administered into the TG on the third day of SR to evaluate whether sleep loss enhances the susceptibility to migraine-like responses. Finally, two additional experiments were conducted to investigate the influence of caffeine (50 mg/kg) exposure in combination of SR in CGRP and PACAP effects. In all experiments, on day 4 (i.e. 24 h after the last SR), the animals were exposed for 1 hour to an aversive light for verification of latent sensitization. **Results.** The results demonstrated that SR alone did not alter the periorbital mechanical threshold in either male or female rats. However, when SR was combined with the administration of CGRP or PACAP at subliminal doses, a significant periorbital mechanical allodynia developed in female, but not in male rats. The exposure to light in the subsequent day caused a transitory reactivation of

mechanical allodynia only in females. In well-rested animals, a three-day caffeine regimen enabled behaviorally subthreshold doses of CGRP or PACAP to elicit migraine-like responses in females, but not in males. In sleep-restricted animals, combining caffeine with subthreshold doses of CGRP or PACAP rendered males susceptible to migraine-like responses and markedly exacerbated these responses in females, including one day later, after light exposure. **Conclusion.** These findings suggest that SR facilitates trigeminovascular sensitization, promoting migraine-like responses in a sex-specific manner and highlighting caffeine as an enhancer of this interaction. Beyond reinforcing the association between poor sleep and migraine, the data offer new insights into the involvement of the purinergic system and sex differences in migraine pathophysiology.

Plain Language Summary: Sleep restriction increases the sensitivity to migraine triggers. In this study, rats exposed to sleep restriction and caffeine showed more intense and longer-lasting pain-related responses after the administration of migraine-induced peptides. These findings suggest that poor sleep quality and caffeine, together, may increase the severity of migraine attacks, especially in women.

Keywords: Caffeine; CGRP; Mechanical Allodynia; PACAP; Photosensitivity; Sleep Restriction

3. INTRODUCTION

Migraine is a neurovascular disorder that affects approximately 15% of the global population, making it one of the most prevalent neurological conditions worldwide, being 2–3 times more prevalent in women [1]. Central to its pathophysiology is the trigeminovascular system, where activation of meningeal trigeminal nociceptors triggers the release of vasoactive neuropeptides, most notably calcitonin gene-related peptide (CGRP), which in turn promotes vasodilation, peripheral and central sensitization [2]. More recently, pituitary adenylate cyclase-activating polypeptide (PACAP) has emerged as a parallel effector, inducing mast cell degranulation, and nociceptor sensitization. These pathways converge in a feed-forward loop of neurogenic inflammation [2, 3]. Building on these mechanistic insights, recent advances in migraine therapy have centered on monoclonal antibodies targeting CGRP—now in clinical use—and PACAP, which is currently undergoing phase II trials [3].

Consistent with this pathophysiological framework, rodent models employing neuropeptide-based paradigms have demonstrated strong translational relevance. Recognition that monoclonal antibodies targeting CGRP exhibit minimal penetration of the blood–brain barrier [2, 4] has shifted focus toward peripheral migraine models, with direct injection of these neuropeptides into the TG emerging as particularly advantageous [5, 6]. This approach avoids systemic cardiovascular artifacts that can confound behavioral readouts [7] and enhances the TG's neuron–glia crosstalk, driving ganglion-originated peripheral and central sensitization [5, 6].

Poor sleep, whether from clinical sleep disorders or social demands, has become increasingly common worldwide, with estimated rates ranging from 20% to 70% of the general population [8, 9]. Epidemiological studies reveal a bidirectional link between migraine and sleep: migraineurs are more prone to sleep restriction (SR), while insufficient sleep increases the risk of migraine onset, chronification, and attack frequency [10]. Despite this

strong association, the underlying mechanisms remain largely unexplored, underscoring the need for targeted animal models. One study showed that 6 h of acute SR sensitizes female mice to subthreshold dural CGRP [11], but it remains unknown whether prolonged SR alters susceptibility to migraine-like responses induced by CGRP or PACAP injected into the TG. Caffeine is one of the most widely consumed psychoactive substances worldwide, with approximately 80% of the global population ingesting at least one caffeinated beverage daily [12]. Clinical data reveal a complex, bidirectional role of caffeine in migraine: while withdrawal can trigger migraine attacks [13], its combination with analgesics significantly enhanced treatment efficacy [14]. However, although caffeine consumption is elevated among individuals with poor sleep, its effects on migraine under sleep-deprived conditions remain unexplored.

Therefore, in this study, we investigated whether SR and caffeine intake interact to influence migraine-like pain. First, we tested whether three days of SR (6 h per day) precipitate migraine-like responses in male and female rats receiving subthreshold doses of CGRP or PACAP into the TG. Then, we examined the impact of three days of caffeine intake on the development of migraine-like responses in both sleep-restricted and well-rested animals.

4. MATERIALS AND METHODS

4.1 ANIMALS

A total of 320 male and female adult Wistar rats, weighing 220-300g, were used in this study. The animals were kept in plastic cages (4 per cage) containing wood shavings, in an enriched environment with controlled light (12-hour light/dark cycles) and with food and water ad libitum. All experimental protocols were performed in accordance with ARRIVE guidelines and were previously approved by the UFPR Ethics Committee (CEUA-BIO-UFPR # 1507). The animals were assigned to the different experimental groups by sortition, and the sample size was determined based on the G*Power 3.1 software, defining a large standardized effect of F= 0.5; power of 0.85 and α=0.05, and estimated 10 rats per group.

4.2 Drugs

Rat calcitonin gene-related peptide (α-CGRP) and pituitary adenylate cyclase-activating polypeptide-38 (PACAP-38) were obtained from Sigma-Aldrich (St. Louis, MO, USA). Both were dissolved in 0.9% sodium chloride at concentrations of 38 ng/10 µL and 0.1 ng/10 µL, respectively, immediately before the intraganglionic injection. Both neuropeptides were administered at subthreshold doses, i.e., doses that do not induce behavioral responses, corresponding to one-tenth of the doses previously reported to induce periorbital mechanical allodynia, as established in prior studies [5,6].

Caffeine (Vetec, SP, Brazil) was diluted in 0.9% sodium chloride to a concentration of 25 mg/mL immediately before the administration at a dose of 50 mg/kg [14, 15, 16]. The oral bioavailability of caffeine in rats is around 90% and there is no difference in the pharmacokinetics and behavioral effects compared to the intraperitoneal route. These observations justify the administration by gavage, which is less invasive than the intraperitoneal route for repeated treatments. Halothane (Tanohalo, Cristalia, Brazil) was used for inhalation anesthesia.

4.3 INTRAGANGLIONIC INJECTION

The injection was performed according to previous studies [5, 6]. After brief inhalation anesthesia with halothane (approximately 3 minutes), the animal's head was restrained, and a long sterile 27-G needle connected to a 0.5 mL Hamilton syringe was inserted and positioned at a 10º angle to the midline in the zygomatic process. The needle was inserted through the infraorbital canal until its tip passed the foramen rotundum and reached the TG. Only the right TG was injected with CGRP, PACAP or vehicle and the injection volume was 10 μL. After the injection (performed over approximately 2 minutes), animals were monitored until complete recovery from anesthesia (around 5 min).

4.4 SLEEP RESTRICTION (SR)

The rats were subjected to SR for a period of 6 hours (from 7:00 AM until 1:00 PM) daily for 3 days using the enriched gentle handling method [17, 18]. Briefly, the method consists of using gentle manipulation to prevent the animal from settling down to sleep. To keep the animals awake, they were gently touched with a soft-bristled brush, the experimenter introduced new small objects into the environment and, if necessary, gently moved the home cage in a seesaw motion. The control groups were kept in the same conditions but were allowed to sleep freely.

4.5 ASSESSMENT OF PERIORBITAL MECHANICAL ALLODYNIA

The animals were placed individually in 30 cm^3 acrylic observation boxes for at least 1 hour for habituation. The mechanical threshold of the periorbital region was assessed with von Frey filaments (Semmes-Weinstein monofilaments, Stoelting, Wood Dale, IL, USA), applied to the midline of the forehead. Eight filaments, from 0.04 to 8.0 g were applied in

ascending order, 3 times each, respecting an interval of 30 seconds between each application. The filament that evoked two positive behaviors such as attack, escape, and/or rapid withdrawal of the head was considered the periorbital mechanical threshold [5, 6]. Only rats showing baseline responses equal to 8 g were included in the experiments. During this test, the experimenters were blinded to the animal's condition.

4.6 PHOTOSENSITIVITY ASSESSMENT

The photosensitivity protocol was performed based on previously published studies [5, 6], with animals kept in their home cage and exposed to 5,000 lux of illumination for a period of 1 hour, using a 180-watt lamp, with the room lighting during the experiment being 0 to 1 lux. Periorbital cutaneous allodynia was assessed every hour for up to 4 hours following light exposure and, when indicated by the persistence of the behavioral response, additionally at 24 and 48 hours.

4.7 EXPERIMENTAL DESIGN

The experimental procedures are represented in Figure 1.

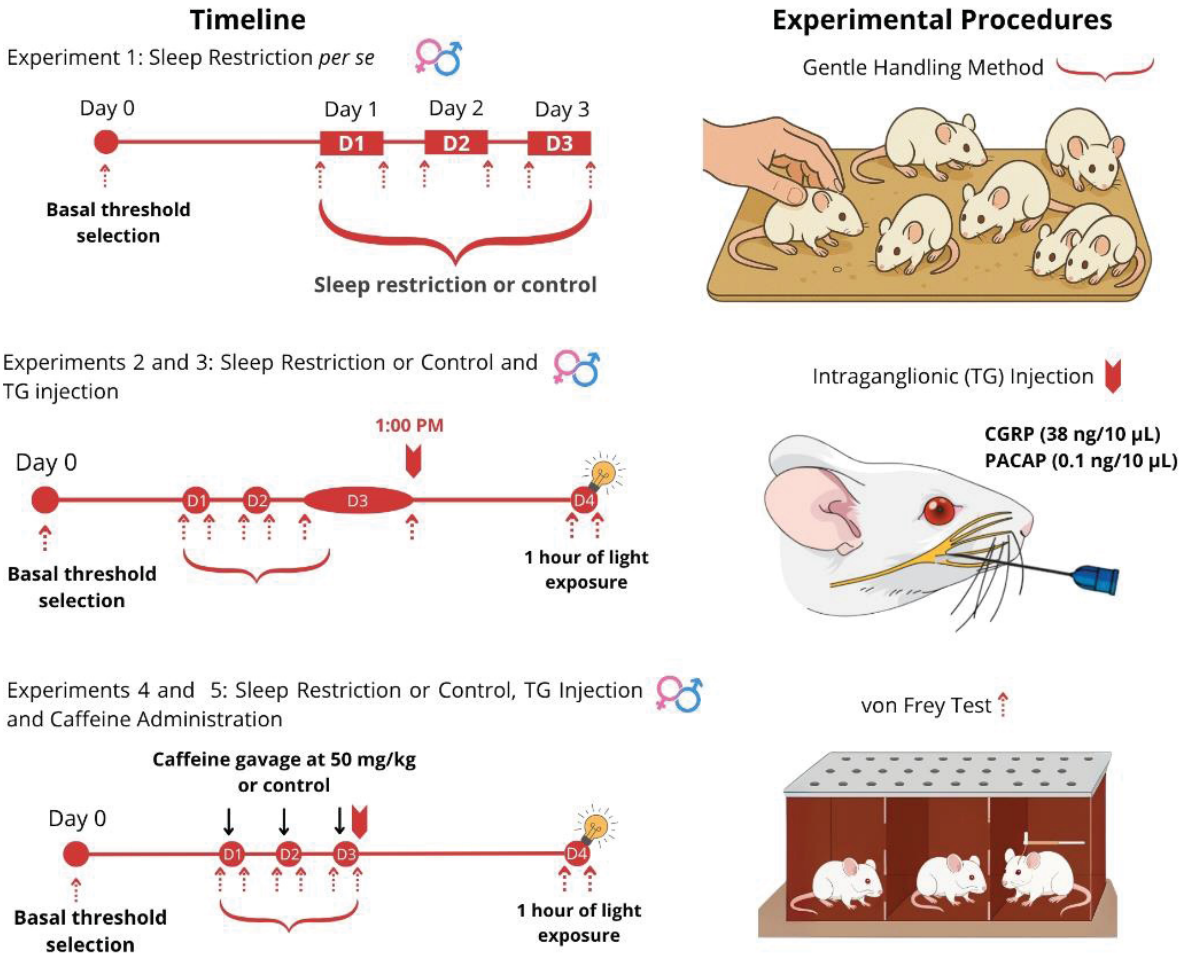

Figure 1. The timeline of experiments 1-5 is depicted on the left column and the experimental procedures are illustrated on the right column. CGRP = calcitonin gene-related peptide; PACAP = pituitary adenylate cyclase-activating polypeptide. Created with Biorender.

Experiment 1 was carried out to determine whether SR per se would influence the periorbital mechanical threshold of male and female rats. The baseline periorbital mechanical threshold of animals was assessed followed by the SR protocol, i.e. 6 hours of SR. At the end of the SR, the von Frey test was repeated each day.

Experiments 2 and 3: Male and female rats were subject to the same procedures described in experiment 1. However, on day 3, independent groups of rats received an

intraganglionic injection of CGRP or vehicle (experiment 2) or PACAP or vehicle (experiment 3) at 1 PM. At the end of the SR period on day 3, the von Frey test was performed at 1-hour intervals up to 4 hours after the injection. On Day 4, the rats were exposed to the aversive light for 1 hour, and the von Frey test was performed before exposure to light, and at 1-hour intervals up to 4 hours.

Experiments 4 and 5 were carried out to test the hypothesis that caffeine would counteract the effect of SR. The control group received caffeine orally for 3 days, and on day 3 at 1 PM, received an intraganglionic injection of CGRP, PACAP or the vehicle, but was not subjected to SR. All additional experimental groups were subjected to SR and received the following treatments: vehicle or caffeine, orally for 3 days, before the SR period; CGRP, PACAP or vehicle, intraganglionic, on day 3 at 1 PM, immediately after the last SR period. The von Frey test was performed on days 1, 2 and 3, before and after the SR period, and on day 3 also at 1-hour intervals up to 4 hours after the intraganglionic treatments. On day 4, the animals were exposed to the aversive light for 1 hour, and the von Frey test was performed before exposure to light, at 1-hour intervals up to 4 hours and again at 24 and 48 h after light exposure.

4.8 STATISTICAL ANALYSIS

Data were expressed as mean ± standard error of the mean (SEM). Two-way repeated measures analysis of variance (ANOVA), with treatment and time as independent factors, followed by Bonferroni's post hoc test, was used to analyze the time course of periorbital mechanical allodynia and photosensitivity. Results were considered statistically significant when $P < 0.05$. All statistical analyses were performed using GraphPad Prism version 9 (GraphPad Software, San Diego, CA, USA) for Windows.

5. RESULTS

5.1. SLEEP RESTRICTION PER SE DOES NOT CHANGE THE PERIORBITAL MECHANICAL THRESHOLD OF MALE AND FEMALE RATS

Six hours a day of SR for 3 consecutive days did not change the periorbital mechanical threshold in male (Figure 2A, $F(6, 108) = 1.913$, $P = 0.085$) and female (Figure 2B, $F(6, 108) = 1.048$, $P = 0.398$) rats.

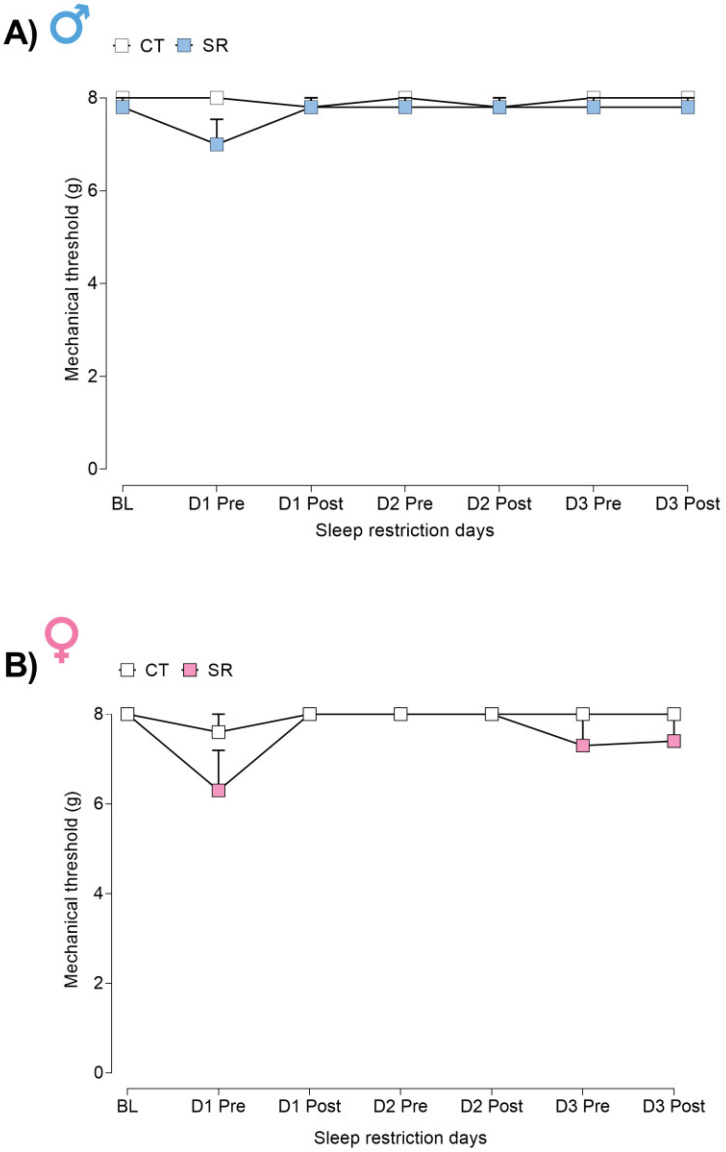

Figure 2. Influence of SR on the periorbital mechanical threshold of male and female rats. The baseline periorbital mechanical threshold was assessed (BL) followed by 3 consecutive days (D1- D3) of sleep restriction. The periorbital mechanical threshold of male (A) and female (B) rats was assessed every day before (Pre) and

after (Post) the SR protocol. Data are expressed as mean ± SEM (n=10/group). Two-way ANOVA with repeated measures followed by Bonferroni post hoc test. CT = control; SR = Sleep Restriction.

5.2. SLEEP RESTRICTION SENSITIZES THE TRIGEMINAL SYSTEM TO CGRP INJECTED INTO THE TG OF FEMALE BUT NOT MALE RATS

Six hours a day of SR for 3 consecutive days did not change the periorbital mechanical threshold in male rats that received a behaviorally ineffective low dose of CGRP into the TG (Figure 3A, $F(10, 135) = 0.895$, $P = 0.539$). Similarly, exposure to an intense, aversive light stimulus, 24 h after the injection, failed to alter periorbital mechanical threshold in males (Figure 3B, $F(8, 108) = 1.609$, $P = 0.130$).

In contrast, the same SR protocol rendered female rats sensitive to that otherwise behaviorally ineffective low dose of CGRP administered into the TG. Periorbital mechanical threshold was significantly decreased two hours after injection in sleep deprived females (Figure 3C, $F(10, 135) = 6.819$, $P < 0.001$). Twenty-four hours after CGRP injection, exposure to an aversive light stimulus reinstated periorbital mechanical allodynia in female rats (Figure 3D, $F(8, 108) = 2.771$, $P = 0.008$), with significant effects at 1 h and 2 h post-exposure.

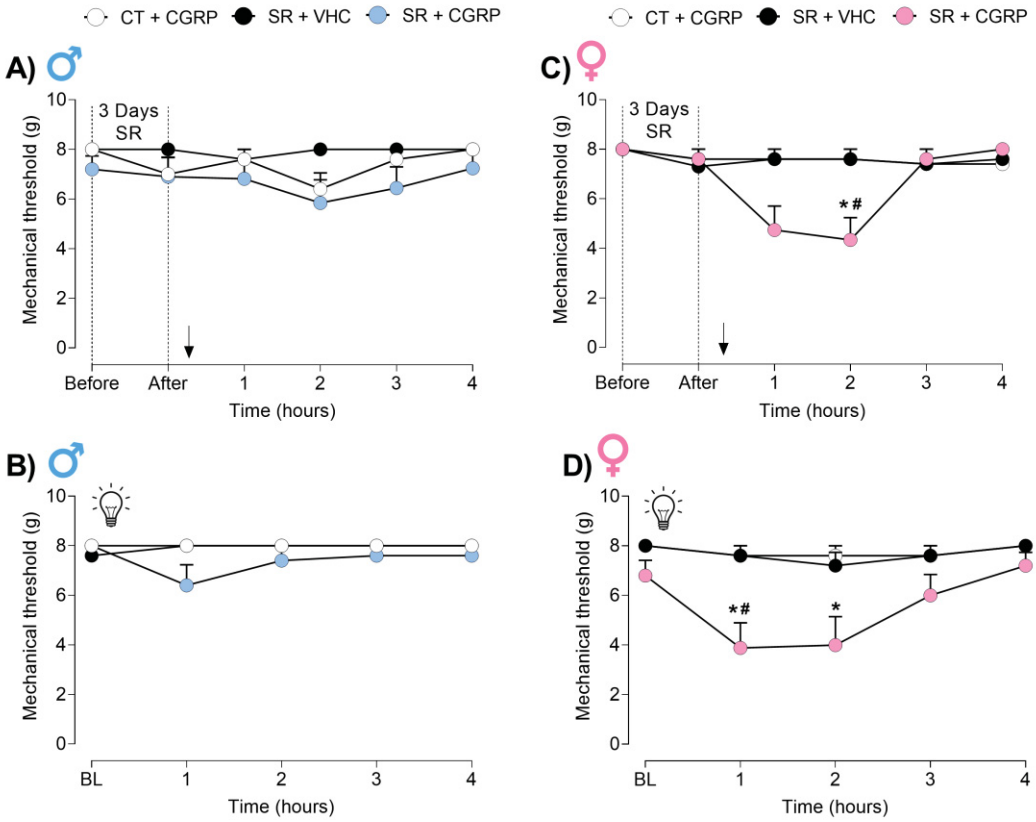

Figure 3. Influence of SR on the effects of CGRP in male and female rats. The baseline periorbital mechanical threshold was assessed (BL), followed by 3 consecutive days of SR. CGRP was injected into the TG at the end of the restriction period (indicated by the arrow). The periorbital mechanical threshold of male (A) and female (C) rats was assessed before (Before) and after (After) the SR, and at 1, 2, 3, and 4 hours following CGRP injection. Twenty-four hours after injection, males (B) and females (D) were exposed to light, and the mechanical threshold was reassessed up to 4 hours. Data are expressed as mean ± SEM (n = 10/group). *P < 0.05 compared to the CT + CGRP group and $^{\#}$P < 0.05 compared to the SR + VEH group. Two-way ANOVA with repeated measures followed by Bonferroni post hoc test. VEH = vehicle; CT = control; SR = Sleep Restriction.

5.3. SLEEP RESTRICTION SENSITIZES THE TRIGEMINAL SYSTEM TO PACAP INJECTED INTO THE TG OF MALE AND FEMALE RATS

Three days after SR, intraganglionic injection of PACAP at a low dose did not change the periorbital mechanical threshold in male rats (Figure 4A, F (10, 135) = 1.061, P = 0.397).

Similarly, after light exposure, male rats showed no change in their periorbital mechanical threshold (Figure 4B, $F (8, 108) = 6.000$, $P < 0.001$).

In females subjected to SR for 3 days, intraganglionic injection of PACAP caused a significant reduction of the periorbital mechanical threshold 2 hours after the injection (Figure 4C, $F (10, 135) = 5.846$, $P < 0.001$). One day after PACAP injection, the exposure of female rats to an aversive light caused a reactivation of periorbital mechanical allodynia, which was significant at 2 hours (Figure 4D, $F (8, 108)$ 4.500, $P < 0.001$).

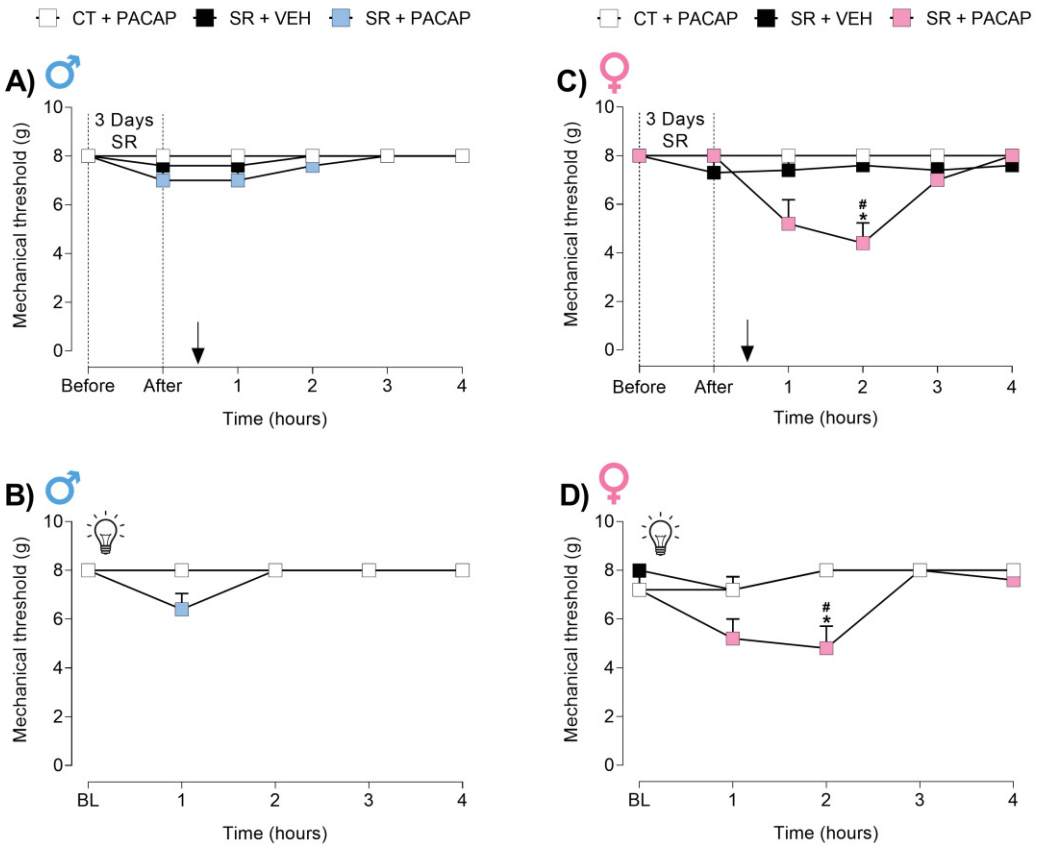

Figure 4. Influence of SR on the effects of PACAP in male and female rats. The baseline periorbital mechanical threshold was assessed (BL), followed by 3 consecutive days of SR. PACAP was injected into the TG at the end of the restriction period (indicated by the arrow). The periorbital mechanical threshold of male (A) and female (C) rats was assessed before and after the SR, and at 1, 2, 3, and 4 hours following PACAP injection. Twenty-four hours after injection, males (B) and females (D) were exposed to light, and the mechanical threshold was reassessed up to 4 hours. Data are expressed as mean ± SEM (n = 10/group). *P < 0.05 compared to the CT + PACAP group and #P < 0.05 compared to the SR + VEH group. Two-way ANOVA with repeated measures followed by Bonferroni post hoc test. VEH = vehicle; CT = control; SR = Sleep Restriction.

5.4 CAFFEINE AMPLIFIES SEX-DEPENDENT SUSCEPTIBILITY TO MIGRAINE-LIKE RESPONSES

When 3 consecutive days of caffeine intake was combined with SR, male rats developed robust periorbital allodynia compared to the non-SR group at 1 h and 2 hours after the subthreshold dose of intraganglionic CGRP (Figure 5A, $F(15, 180) = 11.91$, $P < 0.001$).

Notably, only this triple combination reduced the mechanical periorbital threshold in males, whereas SR alone, SR with subthreshold CGRP, or SR with caffeine (without CGRP) all failed to produce any change. However, even this combination failed to reinstate periorbital allodynia, as males showed no significant change in mechanical threshold after light exposure 24 hours later (Figure 5B, $F(12, 144) = 4.102$, $P < 0.001$).

In female rats given a subthreshold CGRP dose, three consecutive days of caffeine intake triggered migraine-like symptoms in well-rested control animals compared to the animals that were subjected to SR and received caffeine, but not CGRP, at 1- and 2-hours' time points (Figure 5C, $F(15, 180) = 6.047$, $P < 0.001$). Moreover, in those subjected to SR, caffeine plus CGRP combination evoked a robust and long-lasting mechanical allodynia, which was still significant 4 h after injection. At this point, this group differed statistically from the SR groups that receive either CGRP or caffeine. By contrast, caffeine combined with SR had no effect on the periorbital mechanical thresholds in the absence of CGRP at any point evaluated.

One day after CGRP injection, exposure of female rats to the aversive light reinstated periorbital allodynia in a treatment-dependent manner. The triple combination of caffeine, SR, and subthreshold CGRP produced a robust decrease in the periorbital mechanical threshold that remained significant 48 hours after the light exposure (Figure 5D, $F(18, 216) = 2.170$, $P = 0.005$). At the 24- and 48- hours' time points, this group differed statistically from the non-restricted group treated with caffeine and CGRP, as well as from the SR groups that received either CGRP or caffeine.

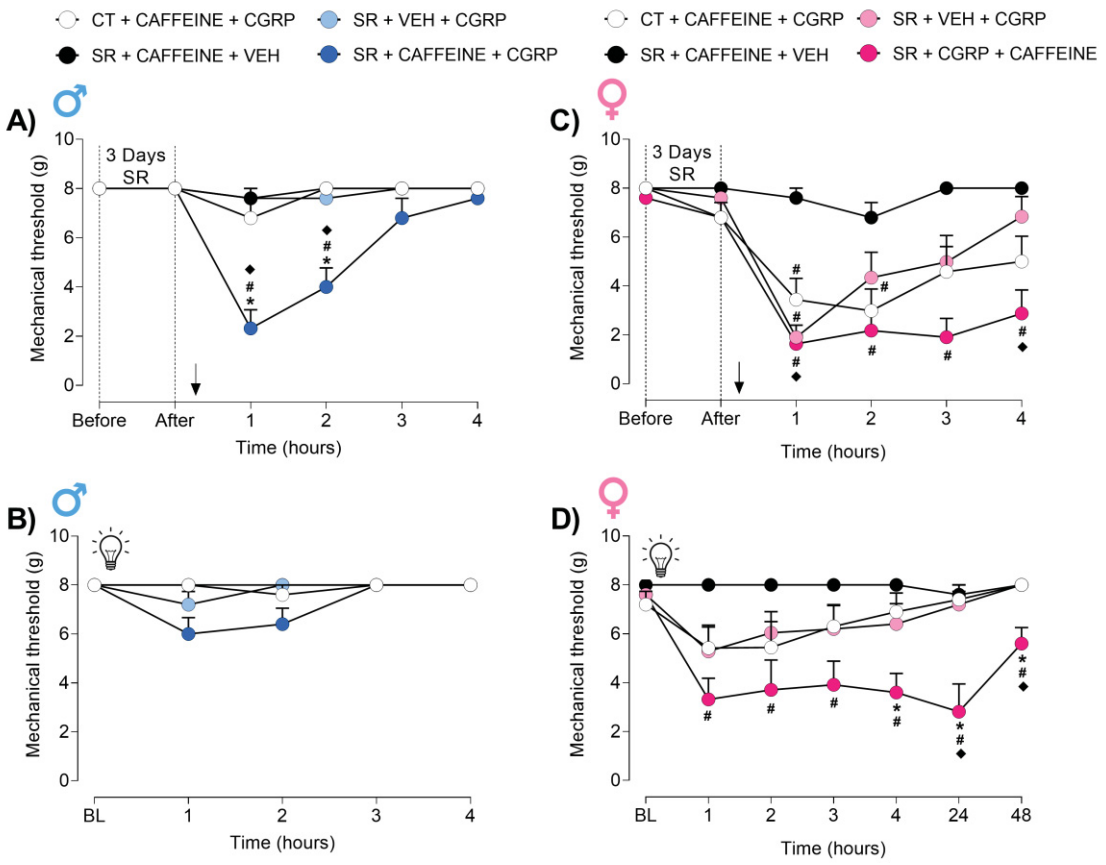

Figure 5. Influence of caffeine on the effects of CGRP in male and female rats subjected to SR. The baseline periorbital mechanical threshold was assessed before 3 consecutive days of caffeine (50 mg/kg) or vehicle (1 mL/kg) oral administration and SR. CGRP was injected into the TG on day 3 (indicated by the arrow). The periorbital mechanical threshold of male (A) and female (C) rats was assessed after the SR, and at 1, 2, 3, and 4 hours following CGRP injection. Twenty-four hours after injection, males (B) and females (D) were exposed to light, and the mechanical threshold was reassessed up to 48 hours. Data are expressed as mean ± SEM (n = 10/group). *P < 0.05 compared to CT + CAFFEINE + CGRP group, $^{\#}$P < 0.05 compared to SR + CAFFEINE + VEH group and $^{\blacklozenge}$P < 0.05 compared to SR + VEH + CGRP group. Two-way ANOVA with repeated measures followed by Bonferroni post hoc test. VEH = vehicle; CT = control; SR= Sleep Restriction.

5.5 CAFFEINE ENHANCES PACAP-INDUCED PERIORBITAL ALLODYNIA FOLLOWING SLEEP RESTRICTION

In male rats the combination of SR plus caffeine or SR plus PACAP did not change the periorbital mechanical threshold. However, the combination of SR plus caffeine and PACAP, induced periorbital mechanical allodynia which was significant from 1 up to 3 hours after PACAP injection into the TG (Figure 6A, $F(15, 180) = 7.344$, $P < 0.001$). The exposure of male rats to light in the following day had no significant effect on the periorbital mechanical threshold (Figure 6B, $F(12, 144) = 1.262$, $P = 0.247$).

In female rats that were subject to SR and received caffeine for 3 consecutive days, the periorbital mechanical threshold was not changed. The combination of SR plus PACAP transitorily decreased the periorbital mechanical threshold compared to the SR group that received caffeine at 1 hour and 2 hours' time points (Figure 6C, $F(15, 180) = 10.59$, $P < 0.001$). Moreover, the combination of caffeine and a subthreshold dose of PACAP triggered migraine-like symptoms in well-rested control animals, as well in the animals subjected to the SR protocol. The responses persisted for at least 4 hours for both groups.

One day later, after exposure of female rats to the light, only the SR group that received PACAP showed a reduction in the periorbital mechanical threshold, which was significantly different from the SR group that received only caffeine at the 2-hours' time point (Figure 6D, $F(12, 144) = 1.262$, $P = 0.247$).

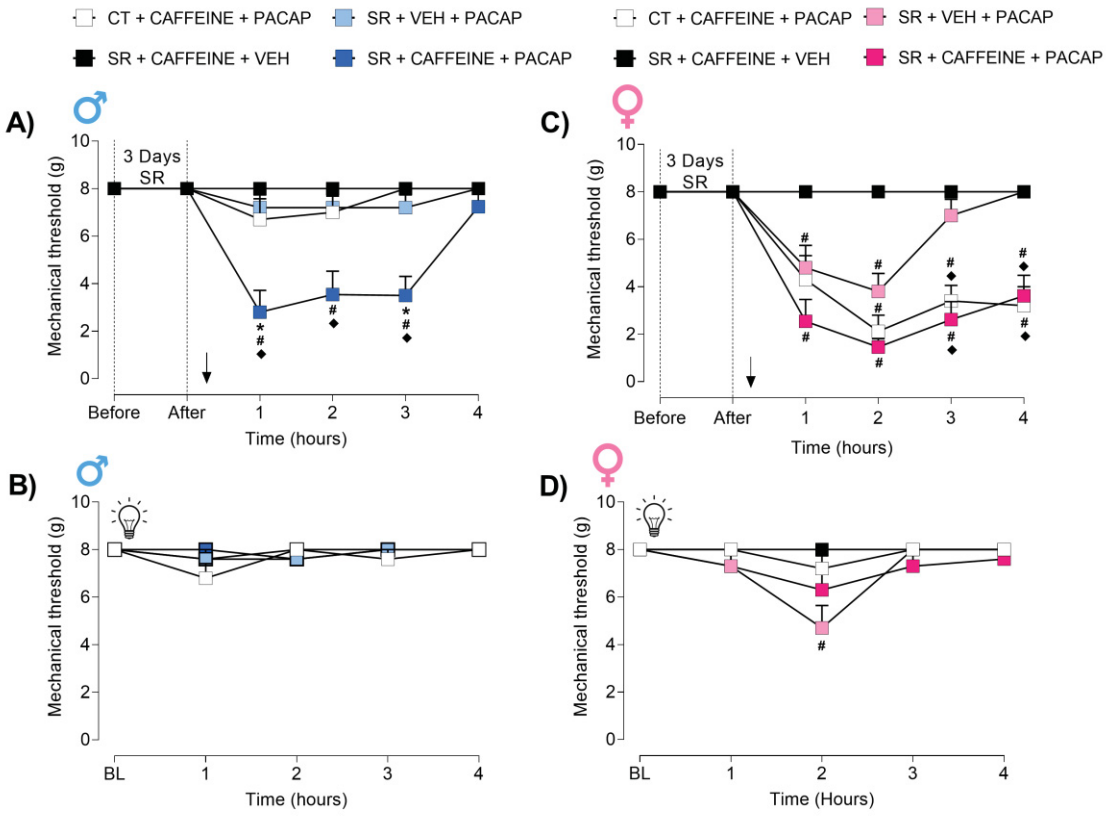

Figure 6. Influence of caffeine on the effects of PACAP in male and female rats subjected to SR. The periorbital mechanical threshold was assessed before 3 consecutive days of caffeine (50 mg/kg) or vehicle (1 mL/kg) oral administration and SR. PACAP was injected into the TG on day 3 (indicated by the arrow). The periorbital mechanical threshold of male (A) and female (C) rats was assessed after the SR, and at 1, 2, 3, and 4 hours following PACAP injection. Twenty-four hours after injection, males (B) and females (D) were exposed to light, and the mechanical threshold was reassessed up to 4 hours. Data are expressed as mean ± SEM (n = 10/group). *P < 0.05 compared to CT + CAFFEINE + PACAP group, $^{\#}$P < 0.05 compared to SR + CAFFEINE + VEH group and $^{\blacklozenge}$P < 0.05 compared to SR + VEH + PACAP group. Two-way ANOVA with repeated measures followed by Bonferroni post hoc test. VEH = vehicle; CT = control; SR = Sleep Restriction.

6. DISCUSSION

There is a well-documented bidirectional relationship between migraine and poor sleep, but its underlying mechanisms remain elusive. In previous studies, we modeled migraine in rats by injecting CGRP or PACAP into the TG. In these models, intraganglionic administration produces sumatriptan-sensitive periorbital allodynia that is subsequently accompanied by light-evoked photosensitivity, which are more pronounced and longer-lasting in females [5, 6]. In the present study we administered behaviorally ineffective (i.e., subthreshold) doses of CGRP or PACAP into the TG to assess whether SR, caffeine intake, or their combination increase susceptibility to migraine-like responses in both sexes. The main findings indicate that (i) SR for three consecutive days (6 h/day) did not alter the periorbital mechanical thresholds in vehicle-treated rats; (ii) SR combined with a subthreshold intraganglionic injection of CGRP or PACAP precipitated migraine-like responses in females, but not in males, indicating that SR lowers the trigeminovascular activation threshold in females; and (iii) three days of caffeine intake precipitated migraine-like responses to subthreshold CGRP or PACAP in non–sleep-restricted females and exacerbated these responses in sleep-restricted ones, whereas in males, caffeine combined with SR rendered them susceptible to migraine-like responses elicited by subthreshold CGRP or PACAP.

Sleep deficit is known to enhance pain sensitivity, though the underlying mechanisms remain poorly understood [17, 19]. In the protocol of SR used herein the animals were prevented from sleeping during the first six hours of the light phase, when sleep pressure is highest in rodents [17]. This procedure has been shown to reduce approximately 98% of slow-wave sleep and abolishes REM sleep [17], but the consequences in the induction of nociceptive responses are variable. Previous studies showed that 6 hours a day of total SR over three days progressively lowered the mechanical nociceptive threshold in the paw of rats, with no sex differences observed [19]. Likewise, acute (9–12 h) or repeated (6 h/day for five days) SR

reduced both thermal and mechanical paw thresholds in mice [17]. These observations led us to first investigate whether SR would also decrease the periorbital mechanical threshold. According to our results, 6 hours of SR for 3 consecutive days did not change the periorbital mechanical threshold in male and female rats. This finding corroborates previous demonstrations that acute SR for 6 hours did not change the hind paw or periorbital mechanical thresholds in male and female mice [10, 17]. One possible reason for this difference is the type of mechanical stimulus applied. According to Alexandre and collaborators, after SR, only sensitivity to high-intensity mechanical stimulation increases, whereas responses to non-noxious mechanical stimuli, such as gentle brush or low intensity von Frey filaments, remain unchanged [17].

Previous studies of our group have demonstrated that the injection of CGRP into the TG cause periorbital mechanical allodynia and photosensitivity in male and female rats, but the responses are more pronounced and longer lasting in females [5, 20]. In the present study, a subthreshold dose of CGRP was injected into the TG at the end of the 3-day SR protocol. The combination of SR and subthreshold CGRP precipitated migraine-like responses only in females, corroborating previous reports of higher sensitivity of females to CGRP and of female-selective mechanisms promoting migraine [20, 21, 22, 23]. These findings indicate that SR can act as a priming mechanism to induce a state of vulnerability to the development of migraine-like responses. When the same animals were exposed to an aversive light for 1 hour, one day after CGRP injection, the mechanical allodynia was reactivated, indicating long-lasting trigeminal sensitization. Exposure to aversive light has been widely used to reveal latent sensitization in migraine models [5, 6, 20, 24, 25]. It has been shown that the exposure of sensitized animals to the bright light has been shown to cause c-Fos activation in neurons of the trigeminal nucleus caudalis, an indicative of central sensitization [25]. In addition, it caused peripheral changes in the trigeminal system including enhanced expression of CGRP and

nitric oxide synthase, as well as glial cell in the TG that contribute to enhanced neuron excitability [26]. Photophobia (i.e., light-aversion) is a commonly reported symptom in migraineurs [27], and different protocols are used in pre-clinical settings to evaluate photosensitivity in animals subjected to stimuli that induce migraine-like responses [22]. We have previously demonstrated that CGRP injection can induced photosensitivity in male and female rats [5], but in the present study the combination of SR plus a subthreshold dose of CGRP caused photosensitivity only in females, suggesting sex differences in the sensitizing effect of SR.

Like CGRP, PACAP is a vasodilatory peptide that can cause migraine-like attacks when infused into patients and migraine-like responses when injected into rodents, but mechanistic studies suggest that these peptides largely act independently from one another [6, 28, 29]. We have also previously shown that PACAP injection into the TG causes mechanical allodynia and photosensitivity, assessed 24 hours after injection, in female, but not in male rats [6]. When injected systemically, PACAP induced light aversion in male and female mice, through CGRP-independent mechanisms [28]. In the present study, a subthreshold dose of PACAP was administered into the TG at the end of the 3-day SR protocol, leading to an outcome comparable to that of the CGRP injection. Only female rats developed significant transitory periorbital mechanical allodynia, which was reactivated 24 hours later by aversive light exposure. To our knowledge, this is the first study to evaluate how SR interacts with PACAP to induce migraine-like symptoms, showing that SR sensitizes the trigeminovascular system to subthreshold PACAP and selectively elicits migraine-like responses in females.

Few preclinical and clinical studies have explored how sleep loss contributes to migraine development in susceptible individuals. Although these studies relied on short (6–12 h) SR protocols, their findings are largely consistent with our results. Acute SR followed by subthreshold dose of either systemic NTG or supradural CGRP induced periorbital and hind

paw mechanical allodynia in female mice, which was observed at the end of the 6-h SR period and became more pronounced 24 h later [10]. Interestingly, when the migraine provoking stimulus was administered before SR, sleep parameters remained unchanged, indicating that migraine-like pain does not significantly disrupt sleep [10]. Additionally, acute SR (6 h or 12 h) significantly increased the frequency of cortical spreading depression induced by application of KCl to the pial surface [30]. Human transcranial magnetic stimulation studies indicate that insufficient sleep perturbs cortical inhibitory/excitatory balance in migraine patients [31, 32], consistent with a state of heightened cortical responsivity that could facilitate cortical spreading depression (and thus aura) and promote attack initiation in sleep-deprived individuals. Taken together these results provide convergent evidence that SR, whether acute (6–12 h) or prolonged (3 days), markedly increases susceptibility to migraine. However, our study is the only to directly contrast male and female responses under the same SR protocol, and it found that SR-induced susceptibility occurred exclusively in females. It therefore remains unresolved whether the absence of effect in males reflects true resistance or merely a higher sensitivity threshold. Addressing this question will require systematic dose–response experiments that escalate CGRP and PACAP doses. It is important to mention that differential sensitivity of males and females to CGRP have been extensively reported in the literature, and more recently sex differences have also been pointed out for PACAP effects [22, 23, 33]. Selective female and male mechanisms related to migraine have been described [23, 34], but have not been explored in the context of SR. Therefore, it is quite likely that biological differences between the sexes contribute to the outcome caused by SR in males and females, but this issue still needs to be investigated.

Caffeine is one of the most widely consumed psychoactive substances worldwide [35] and the consumption tends to be higher among individuals with poor sleep [36]. Habitual or high-dose intake has been associated with increased headache frequency and severity [12, 37].

Despite these interconnections, no studies to date have examined the combined effects of caffeine intake, poor sleep, and migraine-related outcomes across human or animal subjects. Our data indicate that caffeine lowers the trigeminovascular activation threshold for migraine-like symptoms. The dose of caffeine used herein has been shown to produce stable plasmatic levels (25 μg/ml) up to 4 hours [15]. As the behavioral responses were evaluated at least 6 hours after caffeine administration or in the absence of the treatment (day 4), it is unlikely that caffeine causes a direct effect, but it somehow modulates the sensitivity of the trigeminal neurons favoring a pronociceptive state. Females were markedly more susceptible: three days of caffeine intake precipitated migraine-like responses in non–sleep-restricted female rats receiving subthreshold doses of CGRP or PACAP into the TG. These findings suggest that, in susceptible females, caffeine consumption alone can act as a risk factor for developing migraine. When caffeine intake is combined with SR, their effects are synergic, further increasing susceptibility in females and leading to significantly exacerbated migraine-like responses. Notably, the combination of SR and caffeine intake uniquely renders male rats susceptible to migraine-like responses triggered by subthreshold CGRP or PACAP, but this effect is transitory, since it was not reactivated by aversive light exposure. These findings underscore the role of caffeine as a modulator of trigeminovascular sensitivity, highlighting a pronounced sex-dependent effect.

Caffeine exerts an ambivalent role in migraine: it can enhance acute analgesic responses yet act as a trigger or withdrawal precipitant in susceptible individuals [12, 13]. Mechanistically, caffeine is a potent antagonist of adenosine receptors with roughly equally high affinity for both A_1 and A_{2A} receptors, which are widely expressed in the trigeminovascular system [38]. Several studies have reported antinociceptive effects of adenosine that are likely to be mediated by A_1 receptors [39]. In migraine models, activation of A_1 receptors can inhibit neurogenic vasodilation and CGRP release, while A_2 receptors

activation results in dural vasodilation, but does not modulate trigeminal CGRP release [16, 40, 41]. Thus, it is possible that caffeine-induced blockade of A_1 receptors within the trigeminovascular system impairs the inhibitory influence driven by A_1 receptor activation, thereby potentializing the pronociceptive effects of CGRP.

Collectively, our data demonstrate that poor sleep and caffeine intake, individually and particularly in combination, are key determinants of migraine susceptibility, with females being markedly more sensitive. The findings highlight the importance of considering lifestyle factors in migraine risk assessment and prevention and provide a translational framework for understanding how environmental and behavioral triggers interact with trigeminovascular pathways. Future studies should investigate the underlying mechanisms of these sex-specific responses and evaluate whether interventions targeting sleep quality and caffeine consumption can mitigate migraine risk in susceptible populations.

While our findings provide valuable insights into the interaction between SR, caffeine intake, and migraine susceptibility, some limitations should be noted. First, as with all animal studies, species-specific differences in physiology and hormonal regulation may limit the direct translation of these results to humans. Second, migraine-like responses were inferred from specific behavioral endpoints, which may not fully capture the complete spectrum of migraine phenotypes, highlighting an inherent limitation of preclinical models. Finally, although clear sex-specific differences in susceptibility were observed, the precise hormonal, neuronal, and molecular mechanisms underlying these effects remain to be elucidated, warranting further mechanistic investigation.

7. FUNDING

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8. FIGURES

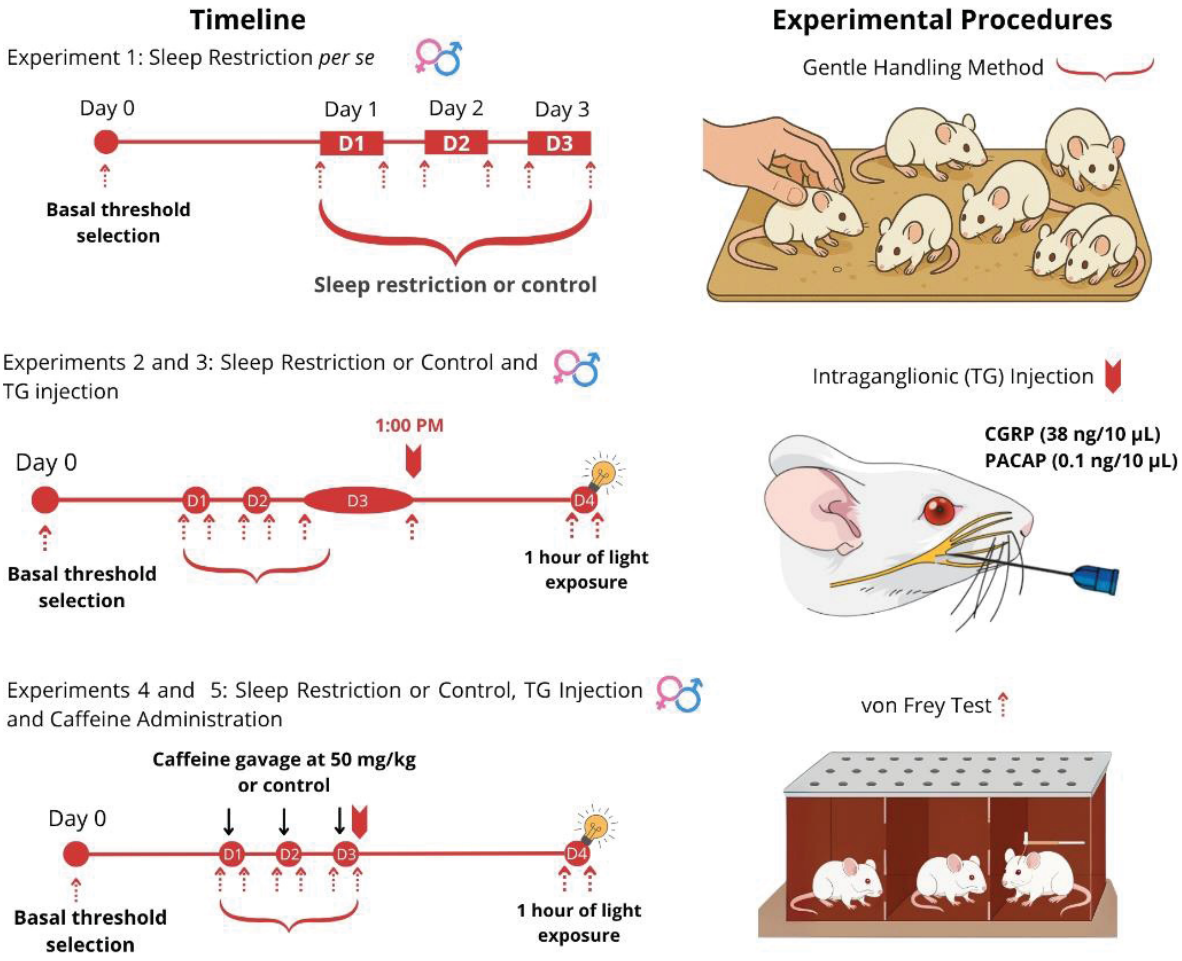

Figure 1. The timeline of experiments 1-5 is depicted on the left column and the experimental procedures are illustrated on the right column. CGRP = calcitonin gene-related peptide; PACAP = pituitary adenylate cyclase-activating polypeptide. Created with Biorender.

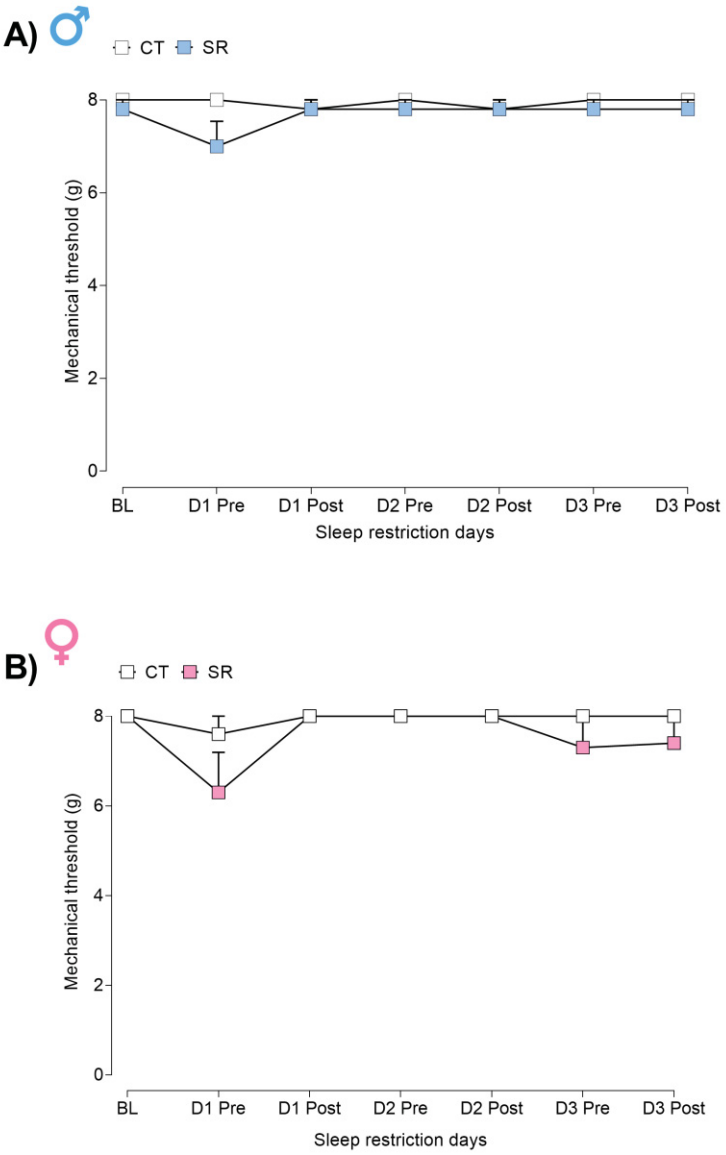

Figure 2. Influence of SR on the periorbital mechanical threshold of male and female rats. The baseline periorbital mechanical threshold was assessed (BL) followed by 3 consecutive days (D1- D3) of sleep restriction. The periorbital mechanical threshold of male (A) and female (B) rats was assessed every day before (Pre) and after (Post) the SR protocol. Data are expressed as mean ± SEM (n=10/group). Two-way ANOVA with repeated measures followed by Bonferroni post hoc test. CT = control; SR = Sleep Restriction.

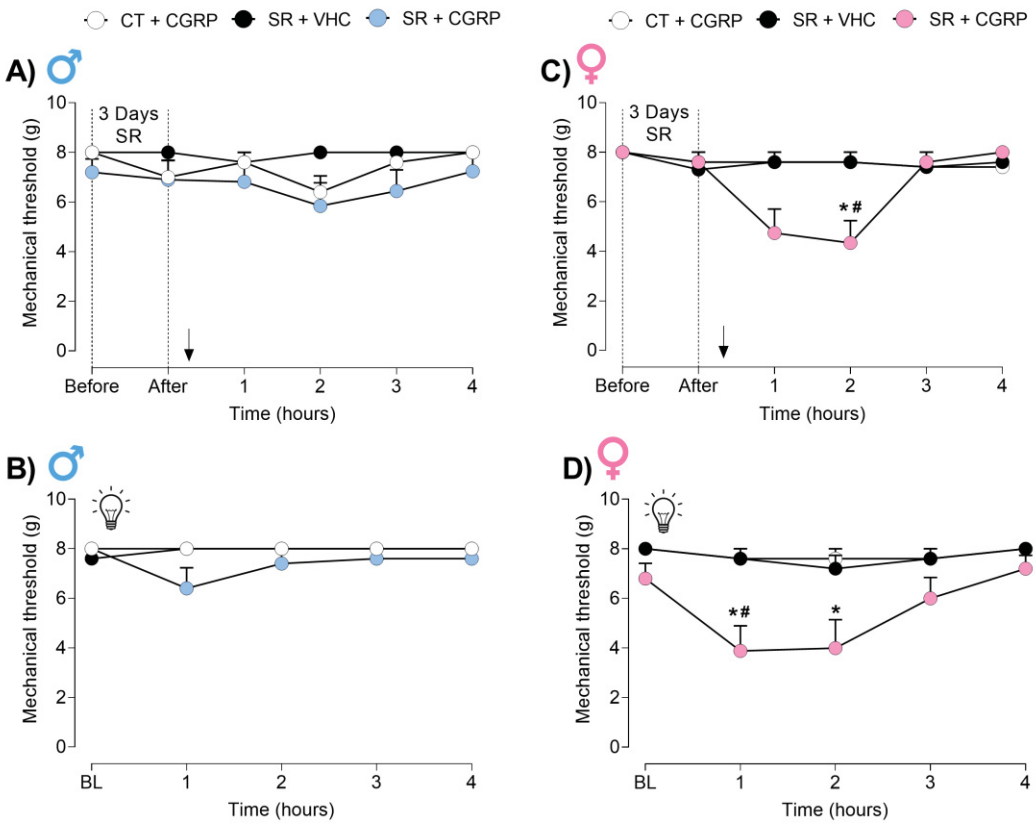

Figure 3. Influence of SR on the effects of CGRP in male and female rats. The baseline periorbital mechanical threshold was assessed (BL), followed by 3 consecutive days of SR. CGRP was injected into the TG at the end of the restriction period (indicated by the arrow). The periorbital mechanical threshold of male (A) and female (C) rats was assessed before (Before) and after (After) the SR, and at 1, 2, 3, and 4 hours following CGRP injection. Twenty-four hours after injection, males (B) and females (D) were exposed to light, and the mechanical threshold was reassessed up to 4 hours. Data are expressed as mean ± SEM (n = 10/group). *P < 0.05 compared to the CT + CGRP group and $^{\#}$P < 0.05 compared to the SR + VEH group. Two-way ANOVA with repeated measures followed by Bonferroni post hoc test. VEH = vehicle; CT = control; SR = Sleep Restriction.

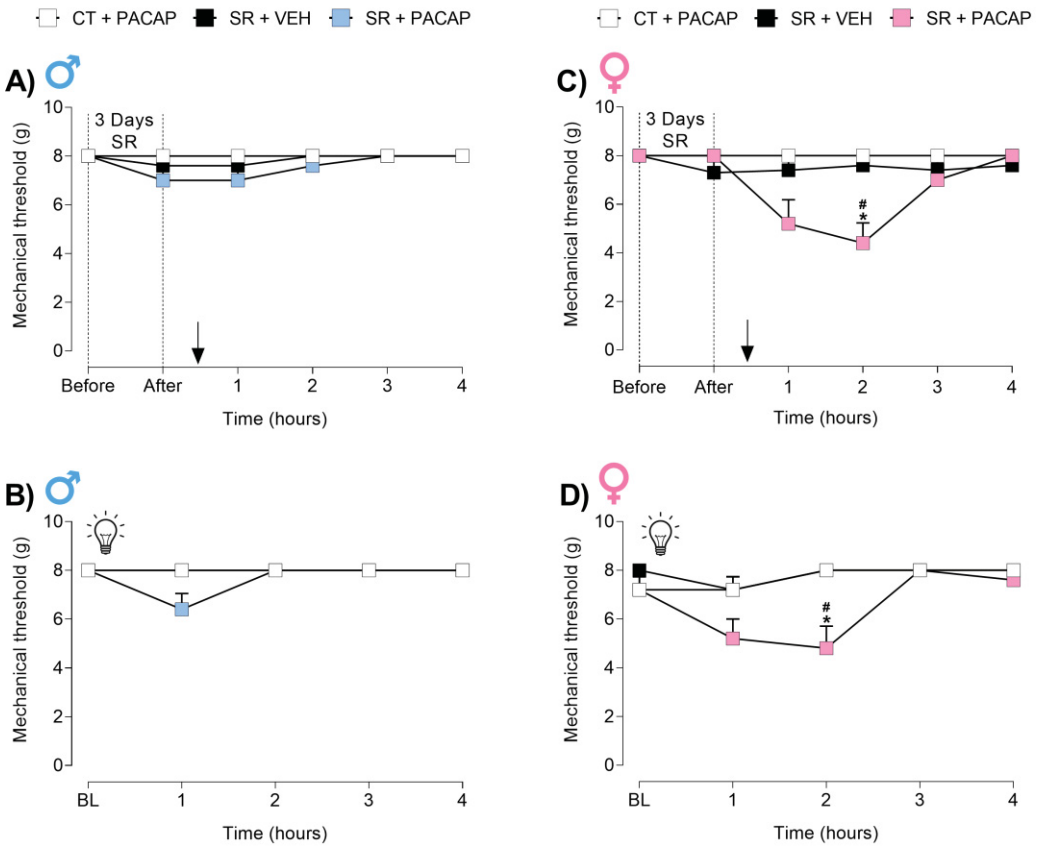

Figure 4. Influence of SR on the effects of PACAP in male and female rats. The baseline periorbital mechanical threshold was assessed (BL), followed by 3 consecutive days of SR. PACAP was injected into the TG at the end of the restriction period (indicated by the arrow). The periorbital mechanical threshold of male (A) and female (C) rats was assessed before and after the SR, and at 1, 2, 3, and 4 hours following PACAP injection. Twenty-four hours after injection, males (B) and females (D) were exposed to light, and the mechanical threshold was reassessed up to 4 hours. Data are expressed as mean ± SEM (n = 10/group). *P < 0.05 compared to the CT + PACAP group and $^{\#}$P < 0.05 compared to the SR + VEH group. Two-way ANOVA with repeated measures followed by Bonferroni post hoc test. VEH = vehicle; CT = control; SR = Sleep Restriction.

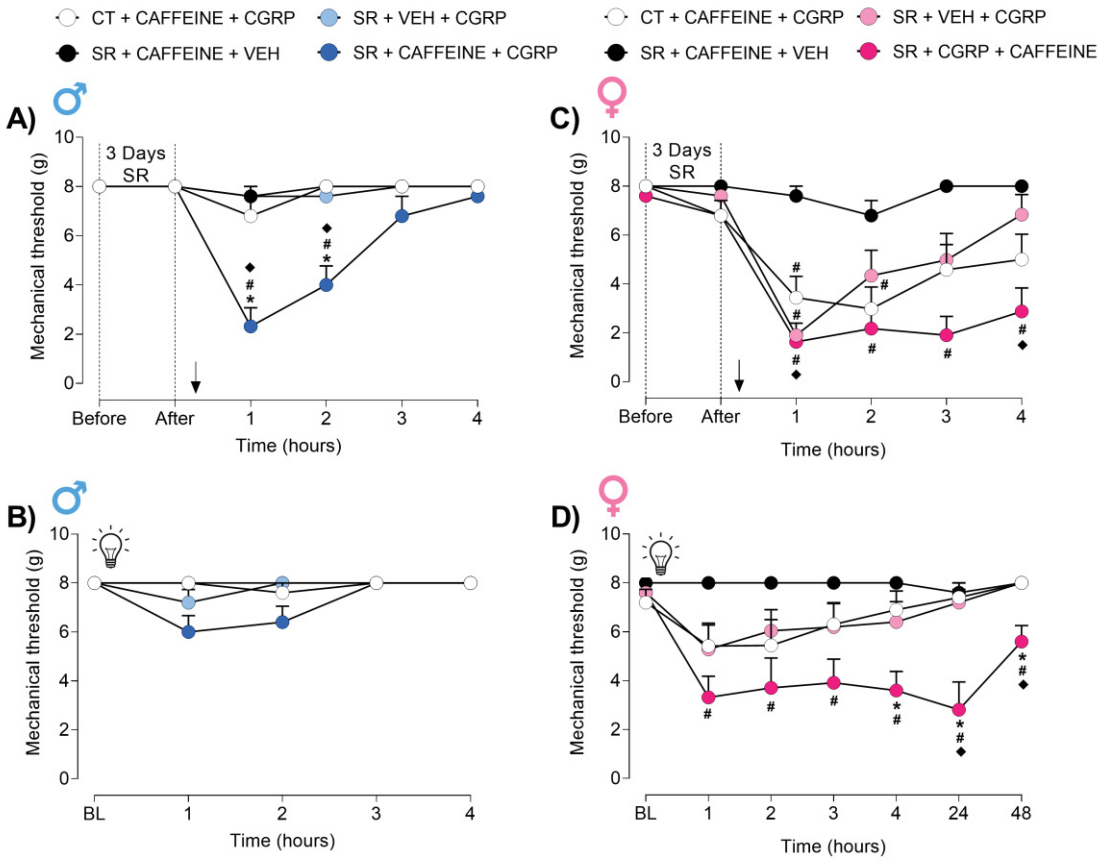

Figure 5. Influence of caffeine on the effects of CGRP in male and female rats subjected to SR. The baseline periorbital mechanical threshold was assessed before 3 consecutive days of caffeine (50 mg/kg) or vehicle (1 mL/kg) oral administration and SR. CGRP was injected into the TG on day 3 (indicated by the arrow). The periorbital mechanical threshold of male (A) and female (C) rats was assessed after the SR, and at 1, 2, 3, and 4 hours following CGRP injection. Twenty-four hours after injection, males (B) and females (D) were exposed to light, and the mechanical threshold was reassessed up to 48 hours. Data are expressed as mean ± SEM (n = 10/group). *P < 0.05 compared to CT + CAFFEINE + CGRP group, $^{\#}$P < 0.05 compared to SR + CAFFEINE + VEH group and $^{\blacklozenge}$P < 0.05 compared to SR + VEH + CGRP group. Two-way ANOVA with repeated measures followed by Bonferroni post hoc test. VEH = vehicle; CT = control; SR= Sleep Restriction.

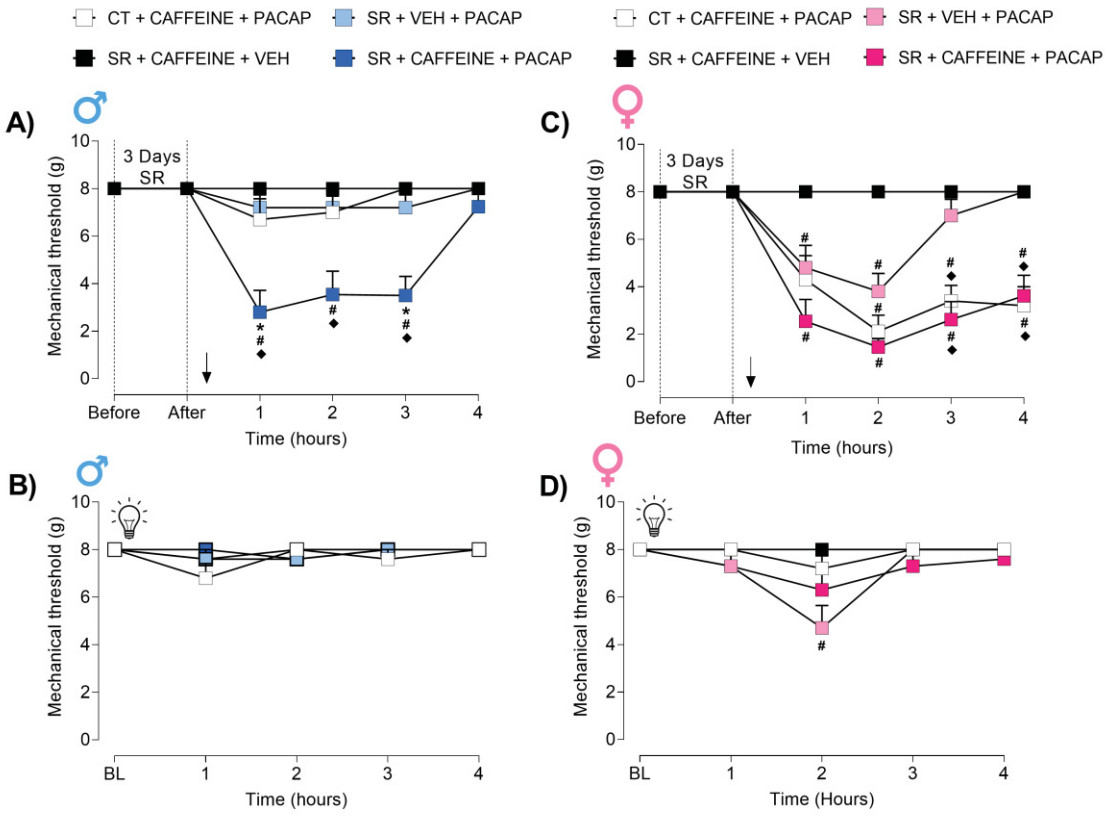

Figure 6. Influence of caffeine on the effects of PACAP in male and female rats subjected to SR. The periorbital mechanical threshold was assessed before 3 consecutive days of caffeine (50 mg/kg) or vehicle (1 mL/kg) oral administration and SR. PACAP was injected into the TG on day 3 (indicated by the arrow). The periorbital mechanical threshold of male (A) and female (C) rats was assessed after the SR, and at 1, 2, 3, and 4 hours following PACAP injection. Twenty-four hours after injection, males (B) and females (D) were exposed to light, and the mechanical threshold was reassessed up to 4 hours. Data are expressed as mean ± SEM (n = 10/group). *P < 0.05 compared to CT + CAFFEINE + PACAP group, $^{\#}$P < 0.05 compared to SR + CAFFEINE + VEH group and $^{\blacklozenge}$ P < 0.05 compared to SR + VEH + PACAP group. Two-way ANOVA with repeated measures followed by Bonferroni post hoc test. VEH = vehicle; CT = control; SR = Sleep Restriction.

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10. CONSIDERAÇÕES FINAIS

O presente estudo investigou a relação entre a restrição de sono e a sensibilização trigeminovascular, avaliando o impacto de diferentes moduladores neuroquímicos — CGRP, PACAP e cafeína — na gênese de respostas semelhantes à migrânea em ratos. A partir de um protocolo controlado de restrição de sono utilizando o método *gentle handling*, foi possível caracterizar efeitos comportamentais e temporais da restrição de sono sobre o limiar mecânico periorbital e a fotossensibilidade. Os resultados demonstraram que, isoladamente, a restrição de sono não foi suficiente para induzir alodinia ou fotossensibilidade, mas quando associada à administração de doses subliminares de CGRP ou PACAP, promoveu uma acentuada redução do limiar nociceptivo em ratas fêmeas, mas não em machos. Esse achado reforça o papel do sono como modulador crítico da excitabilidade trigeminal e aponta para uma diferença sexual na vulnerabilidade à uma crise de migrânea.

A associação entre restrição de sono e administração de cafeína revelou ainda um efeito sinérgico na alodinia e fotossensibilidade induzidas pelos peptídeos, sugerindo que a cafeína pode amplificar a excitabilidade neuronal em um contexto de restrição de sono. Esses resultados apontam para um papel duplo da cafeína: enquanto seu consumo agudo pode ter efeito antinociceptivo em alguns contextos, a exposição contínua, associada a distúrbios do sono, pode contribuir para a facilitação da dor. Essa dualidade reforça a importância de compreender os mecanismos adenosinérgicos na modulação da nocicepção e na fisiopatologia da migrânea.

Este trabalho contribui de maneira significativa para a compreensão dos mecanismos periféricos da sensibilização trigeminovascular, demonstrando que o gânglio trigeminal é um sítio de integração sensorial capaz de responder a múltiplos estímulos moduladores. Algumas limitações devem, contudo, ser reconhecidas. O modelo de restrição de sono empregado representa uma condição de restrição moderada e de curta duração, o que pode não abranger as alterações neuroendócrinas observadas em condições crônicas. Ademais, a avaliação concentrou-se em respostas comportamentais, sem mensurações moleculares complementares capazes de confirmar alterações em marcadores neuronais, gliais ou vasculares. Com os dados obtidos, ensaios futuros poderão buscar com maior

segurança respostas para o impacto da restrição de sono prolongada, bem como realizar análises proteômicas e imunohistoquímicas no gânglio do trigêmeo e no tronco encefálico para identificar as vias celulares envolvidas no processo de sensibilização.

Outro ponto relevante refere-se à possibilidade de avaliar a interação entre o sexo e o ciclo hormonal, buscando trazer respostas para o comportamento diferenciado observado entre machos e fêmeas, o qual sugere influência de hormônios sexuais nessa interação. Estudos futuros que integrem o monitoramento do ciclo estral e intervenções hormonais em modelos de restrição de sono crônica poderão esclarecer de forma mais precisa a contribuição dessas variações para a susceptibilidade às crises de migrânea.

De forma geral, os resultados obtidos indicam que a restrição de sono atua como um fator de sensibilização do sistema trigeminovascular, capaz de reduzir o limiar para o desencadeamento de crises de migrânea por mediadores vasoativos. A cafeína, por sua vez, mostrou-se um elemento modulador dessa interação, exercendo efeito potencializador sobre as respostas nociceptivas. Assim, o presente estudo reforça a importância de considerar distúrbios de sono e os hábitos de consumo de cafeína na compreensão e manejo clínico da migrânea.

Em conjunto, esses achados oferecem subsídios importantes para futuras investigações sobre a interface entre sono, dor e neuropeptídeos vasoativos, ampliando o entendimento sobre os mecanismos que tornam o sistema trigeminal vulnerável a gatilhos ambientais e farmacológicos. O delineamento empregado, aliado à reprodutibilidade do modelo, contribui para o desenvolvimento de estratégias terapêuticas mais direcionadas e para a consolidação de abordagens experimentais que integrem aspectos fisiológicos, sexuais e ambientais na gênese da migrânea.

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