PONTIFÍCIA UNIVERSIDADE CATÓLICA DO RIO GRANDE DO SUL
PROGRAMA DE PÓS-GRADUAÇÃO EM BIOLOGIA CELULAR E MOLECULAR

PRISCILLA BATISTA PAIL

COMPARAÇÃO DOS EFEITOS DOS DERIVADOS DA CATINONA, METEDRONA E MEFEDRONA EM CAMUNDONGOS: DETERMINAÇÃO DOS EFEITOS COMPORTAMENTAIS E BIOQUÍMICOS

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Dissertação apresentada como requisito para obtenção do grau de Mestre pelo Programa de Pós-Graduação em Biologia Celular e Molecular da Pontifícia Universidade Católica do Rio Grande do Sul.

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Instituto de Toxicologia e Farmacologia

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2014

À minha família que sempre esteve ao meu lado, me ensinando a retirar o máximo de alegria, aprendizado e felicidade das experiências vividas.

Amo vocês!

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We’re all mad here.

Lewis Carroll.

RESUMO

O presente estudo comparou os efeitos das catinonas sintéticas metiladas, mefedrona e metedrona, sobre diversos parâmetros comportamentais e bioquímicos em camundongos, além de avaliar alguns dos possíveis mecanismos relacionados com os efeitos *in vivo* da metedrona. Além disso, também foram analisados os efeitos da estimulação semelhante a *nightclubs* sobre os efeitos comportamentais das duas substâncias de abuso. Os efeitos das catinonas, mefedrona e metedrona, foram avaliados em diversos paradigmas comportamentais e, ainda, sobre os níveis cerebrais de monoaminas em camundongos, através de HPLC. Considerando a correlação entre *nightclubs* e o consumo de drogas recreacionais, alguns grupos experimentais foram pré-expostos a um ambiente com temperatura elevada, música eletrônica e luzes estroboscópicas, durante sete dias antes da administração das substâncias-teste, a fim de reproduzir o local de consumo das catinonas. Os derivados das catinonas, mefedrona e metedrona, causaram hiperlocomoção, associada com sinais de redução da coordenação motora, durante 30 min após os tratamentos. Além disso, a mefedrona causou efeitos ansiolíticos, enquanto a metedrona induziu comportamento ansiogênico. Ambos os derivados da catinona causaram um aumento da latência à estimulação térmica na placa-quente, acompanhado de redução marcante do tempo de imobilidade no teste de suspensão da cauda. A administração de mefedrona induziu um aumento rápido dos níveis de dopamina e serotonina no *nucleus accumbens* (2 e 3 vezes, respectivamente), com um aumento na dopamina de 1,5 vezes, no córtex frontal. Por outro lado, a metedrona causou uma elevação de duas vezes nos níveis de dopamina no *nucleus accumbens* e no estriado, além de um aumento de 1,5 vezes dos conteúdos de serotonina, no hipocampo e no estriado. A utilização de diferentes ferramentas farmacológicas demonstrou que parte dos efeitos da metedrona está relacionada com a modulação dos sistemas dopaminérgico e serotoninérgico. Finalmente, foi demonstrado que a estimulação ambiental do semelhante a *nightclubs* produziu um aumento dos efeitos analgésicos da mefedrona e da metedrona no teste de placa-quente. A mefedrona e a metedrona induziram uma série de alterações comportamentais, que foram semelhantes ou distintas, dependendo do paradigma experimental avaliado. Os efeitos destas catinonas sintéticas parecem estar diretamente relacionados com a modulação dopaminérgica e serotoninérgica. Curiosamente, o aumento da latência à estimulação térmica às catinonas foi potencializado pela ambientação tipo *nightclub*.

Palavras chaves: catinona; mefedrone; metedrone; designer drug; depressão; anxiedade; música

SUMMARY

We have compared the behaviour and neurochemical effects of synthetic cathinones mephedrone and methedrone in mice, with attempts to evaluate some the mechanisms of action of methedrone, as well as the influence of nightclub-like stimulation on the behaviour. The effects of cathinone derivatives were examined in a series of behavioural tests in mice, and monoamine brain levels were determined by HPLC. Since there is a correlation between club parties and consume of recreational drugs, separated groups were pre-exposed to nightclub-like environment. Cathinone derivatives caused marked hyperlocomotion, allied to motor coordination inability, through 30 min after injection. Moreover, mephedrone caused anxiolytic-like effects, while methedrone induced anxiogenic actions. Both cathinone derivatives increased the latency in the hot-plate test, with a significant reduction of immobility time in tail suspension test. Mephedrone triggered 2- and 3-fold increase of dopamine and serotonin levels, respectively, in the *nucleus accumbens*, with 1.5-fold elevation of dopamine contents in the frontal cortex. Methedrone caused a 2-fold increase of dopamine in the *nucleus accumbens* and striatum, and 1.5-fold increase of serotonin levels in the hippocampus and striatum. Part of methedrone effects appear to be dependent on dopamine and serotonin modulation. Noteworthy, nightclub-like stimulation produced a further increase of latency to thermal stimulation, in both mephedrone and methedrone-treated mice. Mephedrone and methedrone induced a series of distinct behavioural changes likely by modulation of dopamine and serotonin systems. Curiously, increased latency to thermal stimulation elicited by cathinones was intensified by nightclub-like environment.

Keywords: cathinone; mephedrone; methedrone; designer drug; dopamine; serotonin; norepinephrine; depression; anxiety; music

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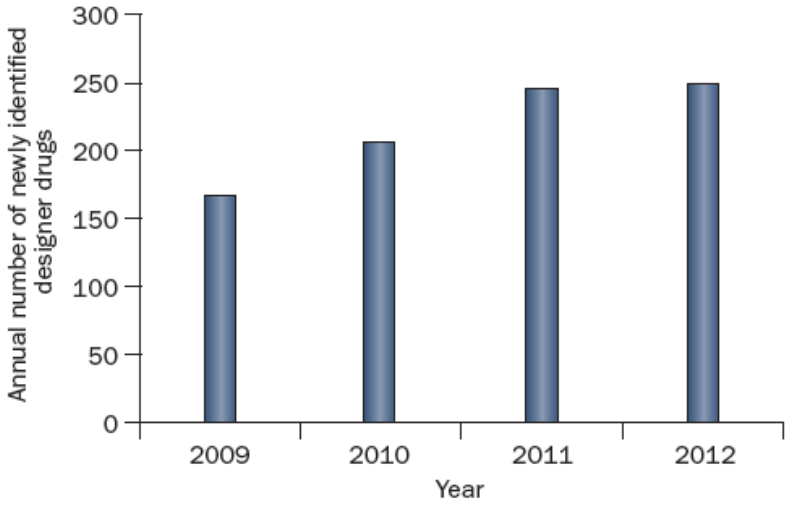
1 CARACTERIZAÇÃO GERAL DO PROBLEMA

Legal highs ou *designer drugs* são substâncias psicoativas (SPA) sintéticas que mimetizam o efeito de SPA usadas desde a década de 1980, como maconha, cocaína, heroína, anfetamina e seus derivados. O número de novas substâncias ofertadas teve aumento significativo a partir 1990. Apenas em 2012, a União Europeia identificou 236 novas SPA sintéticas, um valor alarmante comparado com as 14 substâncias do mesmo gênero identificadas em 2005. Como mostra a Figura 1, esse mercado tende a aumentar, uma vez que sua comercialização é feita com facilidade e com pouca fiscalização (Iversen *et al*., 2014; Luciano e Perazella, 2014). Em outras palavras, é como se para cada substância proibida, novas drogas surgissem com pequenas modificações em suas estruturas químicas, tornando-as "não ilegais".

Figura 1 - Índice do crescimento de novas *designer drugs*



Fonte: Luciano e Perazella (2014).

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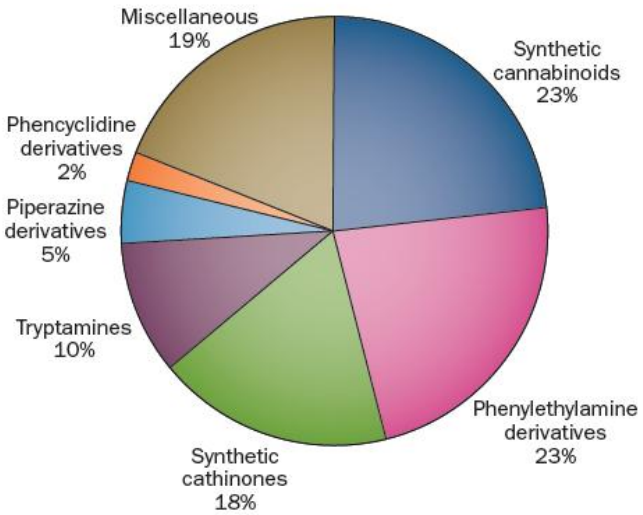
Em uma revisão recente, os autores salientam que dois terços das substâncias notificadas ao *European Monitoring Centre for Drugs and Drug Addiction* (EMCDDA) eram canabinoides sintéticos ou análogos da catinona (Figura 2), o princípio ativo majoritário do arbusto conhecido popularmente como Khat, citando vinte e oito derivados sintéticos desta (Valente *et al*., 2014; Wood e Dargan, 2012).

As folhas do Khat (*Catha edulis*), natural da África e da Península Arábica, são utilizadas por suas propriedades estimulantes na Somália e no Iêmen. No final do século XVIII, o arbusto Khat tornou-se conhecido na Europa, após ser catalogado pelo botânico sueco Peter Forsskal. Em 1887, Fluckiger e Gerock isolaram o alcaloide "katin", e, depois de algumas dificuldades, em 1930, conseguiu-se isolar e purificar o princípio ativo denominado catina, o componente psicoativo. No século XX, após se descobrir que os efeitos da catina eram modestos, iniciaram-se novas investigações e, em 1975, isolou-se a catinona de folhas frescas do Khat. A partir dessa descoberta, surgiram muitos derivados das catinonas para uso recreacional ou terapêutico: a) a metcatinona, descoberta em 1928, antes da própria catinona, começou a ser utilizada como droga de abuso, em 1970; b) a metilona foi patenteada em 1996, como antidepressivo e, em 2004, começou a ser vendida como uma *Legal High* em lojas especializadas e pela internet; c) a fleferona apareceu pela primeira vez em 2009 e seu consumo cresceu significativamente no primeiro semestre de 2010; d) a mefedrona, descoberta em 1929, se tornou comum em 2007 e 2008; e) a metedrona passou a ser relatada em 2009. A Figura 3 representa a estrutura química da catinona; nos pontos destacados, são adicionados os substituintes para formar seus os derivados (James *et al*., 2011; Kelly, 2011; McElrath e O'Neill, 2011).

Figura 2 - Classificação de novas *designer drugs*



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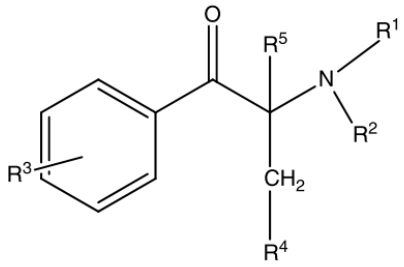
Embora muitas destas substâncias tenham surgido com o intuito de trazer benefícios à população, com o objetivo final de descobrir novos medicamentos, seu uso indevido pode desencadear grave risco à saúde dos usuários. O EMCDDA, por exemplo, objetiva compreender a toxicidade desses agentes para assim recomendar ou não o controle de tais SPA (Wood e Dargan, 2012).

As *legal highs* são um grupo de substâncias sintéticas que produzem quadros clínicos com consequências graves, chegando a convulsões e/ou óbitos. Entretanto, não existem muitos estudos *in vitro* ou *in vivo* acerca do mecanismo de ação destes agentes, uma vez que o número de novas substâncias desenvolvidas e comercializadas aumenta rapidamente, o que faz com que existam muitos efeitos ainda desconhecidos, dificultando o serviço dos profissionais que prestam atendimento aos usuários nas emergências hospitalares ou nos departamentos de informações relacionados com drogas de abuso (Schifano *et al*., 2011).

Figura 3 - Estrutura da catinona



Fonte: Kelly (2011).

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1.1 INFLUÊNCIA DOS AMBIENTES FREQUENTADOS PELOS USUÁRIOS SOBRE OS EFEITOS DAS *LEGAL HIGHS*

Assim como algumas outras drogas recreacionais, as *legal highs* são substâncias utilizadas principalmente em festas. Como citado por Almeida e Silva (2000), os ambientes aos quais os usuários de SPA se expõem é composto por música alta e ininterrupta, iluminação com luzes negras, coloridas, estroboscópicas e laser. O ambiente é propício para se dançar por horas seguidas, contendo centenas de pessoas, com temperatura elevada e, por vezes, pouca ventilação. Essa situação pode resultar em aumento dos efeitos tóxicos e comportamentais provocados pelas SPA.

De acordo com Green e Nutt (2014), essas SPA são consumidas em ambientes específicos; logo, os experimentos que utilizam modelos *in vivo* deveriam mimetizar condições similares. Por exemplo, a temperatura de ratos aumenta após a administração de metilenodioximetanfetamina (MDMA), mas quando os animais são mantidos em uma temperatura ambiente mais elevada (aproximadamente 30 °C), o quadro de hipertermia tende a se agravar. Além disso, em relação ao comportamento, deve-se considerar o impacto da música sobre o cérebro. Estudos mostram que músicas relaxantes podem ser tão efetivas quanto benzodiazepínicos no controle de ansiedade no pré-operatório em humanos (Berbel *et al*., 2007; Nociti, 2010; Sanchez *at al*., 2004). Por outro lado, de acordo com Pimentel e Günther (2009), músicas de *rap* e *heavy metal* podem ser inspiradoras de alterações marcantes dos comportamentos sociais. Por exemplo, determinados estilos musicais associados ao uso indevido do álcool podem acarretar incidentes violentos (Forsyth, 2009). Em um estudo pré-clínico, ratas foram submetidas ao tratamento com anfetaminas e estímulos sonoros. Após sete dias de estímulos, com duração de 90 min cada, os animais foram testados, demonstrando aumento da atividade locomotora, bem como, dos níveis de dopamina (DA), destacando o impacto musical no que diz respeito ao abuso de SPA (Polston *et al*., 2011). Em outra pesquisa realizada em 2011, mais de 700 usuários de SPA relataram preferir o estilo *dance music*, seguido por frequentadores de *goa parties* ou *clubs*. Entretanto, os autores descrevem que o uso de SPA está intimamente relacionado com a alta frequência da vida noturna dos jovens. Curiosamente, os entrevistados que relataram gostar de rock, administram drogas ilícitas, como a cocaína, com menos frequência do que aqueles que apreciam outros estilos de músicas relatados anteriormente. Os autores ainda salientam que os usuários não devem ser colocados apenas em uma categoria, uma vez que frequentam ambientes noturnos variados (Havere *et al*., 2011). No que se refere aos usuários de *legal highs*, a preferência musical corrobora estudos anteriores relacionadas às SPA que estimulam o sistema nervoso central (SNC), sendo a *dance music* o estilo mais ouvido (Winstock *et al*., 2010).

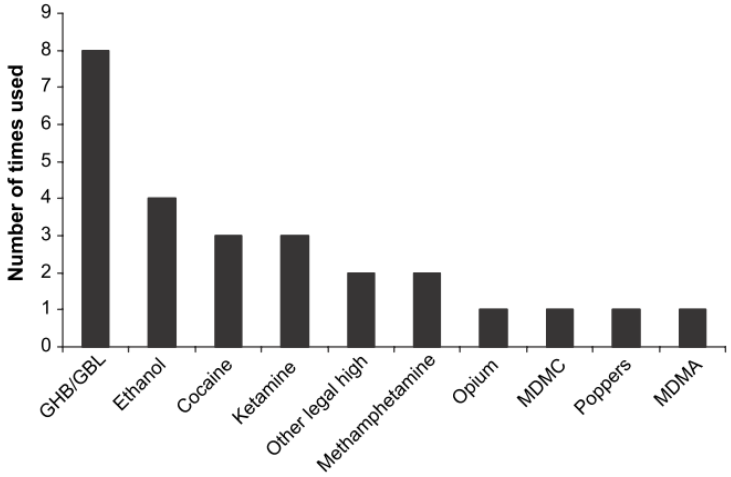
1.2 DERIVADOS DA CATINONA

Dentre as *legal highs*, os derivados da catinona chamam a atenção, em especial a 4-metilmetcatinona (mefedrona). Essa teve sua disseminação em lojas especializadas e pela internet entre 2007 e 2008. Originalmente, sua produção foi iniciada em Israel, sendo proibida em 2008. Após esse evento, a substância alastrou-se para inúmeras partes do mundo (Vardakou *et al*., 2011). Apesar de ter sido proibida no Reino Unido, em 2010, essa droga

continua sendo vendida pela internet e em lojas especializadas, com o apelo de ser um produto para plantas ou como "sais de banho", contendo a seguinte frase nos rótulos: "não recomendado para consumo humano" (McElrath e O'Neill, 2011).

No estudo feito por Vardakou *et al.* (2011), os usuários relataram o uso de outras substâncias associadas com os derivados da catinona, como mostrado na Figura 4. As mais utilizadas foram os depressores, como gama-hidroxibutirato (GHB) e gama-butirolactona (GBL), seguidos pelo etanol, que segundo os entrevistados da pesquisa de McElrath e O'Nell (2011), diminuía as reações adversas causadas pela mefedrona. Shifano *et al*. (2011) citam que na Romênia, onde a via endovenosa é mais popular, é comum a associação com heroína e, que esta associação, foi a causa da primeira morte relacionada à mefedrona nos Estados Unidos. Em relação ao gênero, foi constatado que homens utilizam a substância com maior frequência, em relação ao público feminino (Wood *et al*., 2011). Este dado foi confirmado e complementado por Vardakou *et al*. (2011), estendendo o perfil dos usuários para jovens, entre 12 e 24 anos de idade, de áreas urbanas, principalmente em danceterias.

Figura 4 - Substâncias associadas ao uso de mefedrona



Fonte: Wood *et al*., (2011).

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Em um estudo feito por Carhart-Harris *et al*. (2011), foram coletadas informações com 1506 usuários da mefedrona, a respeito de sua opinião sobre a droga e seu comportamento sob efeito desta. Pânico, ansiedade e palpitação foram reações negativas descritas por 20% dos entrevistados. Ademais, 28% sabiam de algum amigo ou conhecido que havia experimentado uma reação negativa ao uso da mefedrona. Em outra pesquisa,

Wood e Dargan (2012) expõem alguns dos efeitos relatados pelos usuários, incluindo: aumento da temperatura corporal, dor de cabeça, dor no peito, convulsões, sudorese, ansiedade, alucinações, paranoia, bruxismo, náuseas, má circulação nos dedos e dores, além de hemorragias nasais. Lusthof *et al*. (2011) relataram que psicose e agressividade são outros efeitos e, pessoas com problemas psiquiátricos, cardíacos e neurológicos pré-existentes podem ser mais propensas aos efeitos adversos nocivos (Tabela 1). Quando algumas mortes foram associadas a esta droga, chamando a atenção da mídia, o governo do Reino Unido a incluiu na Classe B, a mesma de outras substâncias psicoativas, como as anfetaminas (Carhart-Harris *et al*., 2011).

Tabela 1: Efeitos relatados por usuários de mefedrona

Efeitos Desejados	Aumento de energia, da socialização e da libido.	
Efeitos Adversos	Psíquicos	Pânico, psicose, agressividade, ansiedade, alucinações, paranoia, euforia.
	Físicos	Sangramento do nariz, midríase, visão turva, boca ressecada, sede, tensão muscular, aumento da temperatura corpórea, aceleração cardíaca, retração de genitais masculinos, dores de cabeça, dores no peito, convulsões, sudorese, bruxismo, náuseas, má circulação nos dedos, vômito.
	Pós-administração	Fadiga, tontura, depressão.

Fonte: Carhart-Harris *et al*., (2011); Lusthof *et al*., (2011); McElrath e O'Neill (2011); Vardakou *et al*., (2011); Wood e Dargan (2012).

NOTA: Dados baseados em pesquisas com relatos de usuários.

Semelhante às SPA mais conhecidas, como a anfetamina e seus derivados, a mefedrona parece atuar nas mesmas vias de neurotransmissão, estimulando principalmente o sistema límbico, tendo alto poder de permeação pela barreira hematoencefálica (German *et al*., 2014; Simmler *et al*., 2013). Baumann *et al*. (2012) mostraram, em ratos machos da linhagem Sprague-Dawley, que a injeção de 1 mg/kg de mefedrona, através de microdiálise no *nucleus accumbes*, aumenta três vezes os níveis de DA e, até sete vezes os níveis de serotonina (5-HT), consistente com outros dados da literatura que apontam maior ação nos

neurônios serotoninérgicos (Martínez-Clemente *et al*., 2012; Simmler *et al*., 2013). Entretanto, por causa de sua ação marcante sobre o *nucleus accumbens*, é destacado o grande risco dessa substância para dependência (Robinson *et al*., 2012). Esses dados, a respeito da ação nos terminais dopaminérgicos, noradrenérgicos e serotoninérgicos, estão de acordo com relatos em usuários intoxicados, uma vez que apresentaram efeitos simpatomiméticos, como, por exemplo, taquicardia, hipertensão e agitação, com quadro clínico semelhante ao de outras SPA simpatomiméticas, tais como o MDMA e a cocaína (Tabela 1) (Wood *et al*., 2012). Uma pesquisa em camundongos machos demonstrou os efeitos estimulantes relatados pelos usuários, ocorrendo aumento da atividade locomotora (Marusich *et al*., 2012). Essa hiperlocomoção foi observada por outros autores que administraram mefedrona, em ratos Wistar e Sprague-Dawley, com doses variáveis (via subcutânea 1-10mg/kg) (Wright Jr. *et al*., 2012). Em relação à neurotoxicidade, a monoaminooxidase degrada a DA e a 5-HT, gerando o peróxido de hidrogênio, que por sua vez forma o radical hidroxila (OH•). Como a mefedrona aumenta os níveis desses neurotransmissores no meio extracelular (a 5-HT em maior quantidade que a DA), sugere-se que esta SPA teria efeito neurotóxico (Colleen *et al*., 2008; Green *et al*., 2012; Gudelsky *et al*., 1994). Martínez-Clemente *et al*., (2012) demonstram, em ratos, que a mefedrona pode causar, em doses elevadas e a longo prazo, problemas cognitivos e de memória, além de cardiotoxicidade e efeitos alucinógenos (Hadlock *et al*., 2011; Motbey *et al*., 2012). Entretanto, Angoa-Pérez *et al*. (2012) analisaram fêmeas da linhagem C57BL/6, tratadas com doses de 20 e 40 mg/kg, em intervalos de duas horas, mostrando que a mefedrona não causou neurotoxicidade das terminações dopaminérgicas, dois dias após a última administração. Para assegurar que não haveria uma resposta tóxica tardia, sete dias após, foi feita outra análise com uma dose mais elevada, na qual foram observadas hipertermia e hiperatividade, mas não houve ativação de astrócitos e microglia no estriado, indicando ausência de danos às terminações nervosas de DA. Os mesmos autores ainda citam que a mefedrona pode ser mais seletiva que o MDMA, direcionando a neurotoxicidade exclusivamente para 5-HT. De modo geral, pode-se dizer que a mefedrona causa alterações agudas, mas não persistentes das concentrações de DA e 5-HT (Motbey *et al*., 2012). Entretanto, uma vez que há grande associação de SPA pelos usuários junto com a mefedrona, consciente ou não, um estudo realizado em 2013 (Angoa-Pérez *et al*., 2013) demonstrou que a mistura da mefedrona com metanfetamina, anfetamina e MDMA desencadeia danos nos terminais dopaminérgicos mais acentuados do que essas três últimas substâncias isoladas, potencializando sua neurotoxicidade.

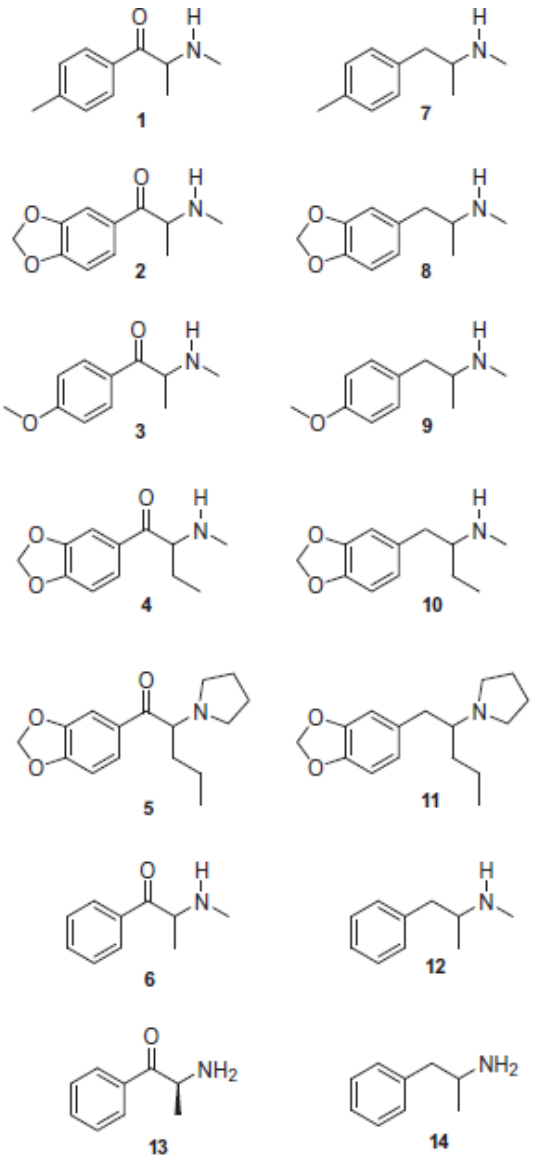
Apesar da catinona e seus derivados serem comparados com outras SPA estimulantes, sua principal relação se dá com as anfetaminas e seus derivados, por serem estruturalmente semelhantes: a metilona é análoga ao MDMA, a metcatinona, à metanfetamina e, a mefedrona, à 4'-metilmetanfetamina (Figura 5). Entretanto, há algumas diferenças nos efeitos fisiológicos entre essas classes de substâncias: Shortall *et al*. (2013) demonstraram, em ratos, que a catinona possui efeitos sobre a termorregulação, diferentes do MDMA. Também demonstraram que, enquanto o MDMA diminui as concentrações de 5-HT em várias regiões do cérebro e reduz os níveis de ácido homovanílico (HVA) no estriado, 2 h após a administração, a catinona aumenta os níveis de HVA e ácido 5-hidroxiindolacético (5-HIAA) no estriado e aumenta a 5-HT no estriado e no hipotálamo. Eles também relataram que a mefedrona elevou os níveis de noradrenalina (NA).

No estudo de Huang *et al*. (2012), os autores enfatizam que nem todas novas *legal highs* derivadas da catinona se tornaram populares e que algumas são mais semelhantes às anfetaminas, bem como, outras podem ter efeitos únicos, salientando que há tanto semelhanças quanto diferenças nos efeitos desencadeados por essas SPA. Por exemplo, a mefedrona que provoca uma despolarização nos neurônios dopaminérgicos, ao contrário da metilenodioxipirovalerona (MDPV) que causa uma hiperpolarização da membrana neuronal (Figura 6) (Cameron *et al*., 2013).

Corroborando os dados acerca das diferenças nas respostas comportamentais e bioquímicas de derivados distintos da catinona, Lópes-Arnau *et al*. (2012) demonstraram em camundongos machos Swiss CD-1, que a butilona e a metilona desencadearam aumento da atividade locomotora maior que a mefedrona, quando administradas na mesma dose (25 mg/kg), sendo que a locomoção de todas foi maior do que a dos animais tratados com MDMA (5 mg/kg). Com relação aos aspectos farmacológicos, foi visto que esses três derivados da catinona, na dose 5 mg/kg, tiveram seu efeito revertido quase que completamente quando pré-tratados com quetanserina e haloperidol, mas apenas a mefedrona teve a atividade locomotora revertida por $_{DL}$-ρ-clorofenilalanina metil ester (ρCPA) e, a butilona por SB-216641, destacando as diferenças entre essas *legal highs.*

Outra *legal high* utilizada, mas pouco estudada, é a metedrona, estruturalmente análoga da mefedrona. Poucas pesquisas foram publicadas a respeito desta; quando se faz uma busca em uma base de dados (PubMed, agosto de 2014) encontra-se dezesseis artigos disponíveis, um número modesto se comparado com as 272 publicações referentes a mefedrona ou, com as 40206 ligadas as anfetaminas.

Figura 5 - Derivados da Catinona e Anfetaminas



Fonte: Gibbons e Zloh (2010).

Nota: Mefedrona**(1**), metilona (**2**), metedrona (**3**), butilona (**4**), metilenodioxipirovalerona (MDPV, **5**), metcatinona (**6**), 4'-metilmetanfetamina (**7**), metilenodioximetanfetamina (MDMA, 'ecstasy', **8**), 4'-metoximetamfetamina (4'-MMA, **9**), metilenedioxietilanfetamina (MDEA, **10**), metilbenzodioxazolilbutamina (MBDB, 'Eden', **11**), metanfetamina ('crystalmeth', **12**), catinona(**13**), anfetamina (**14**).

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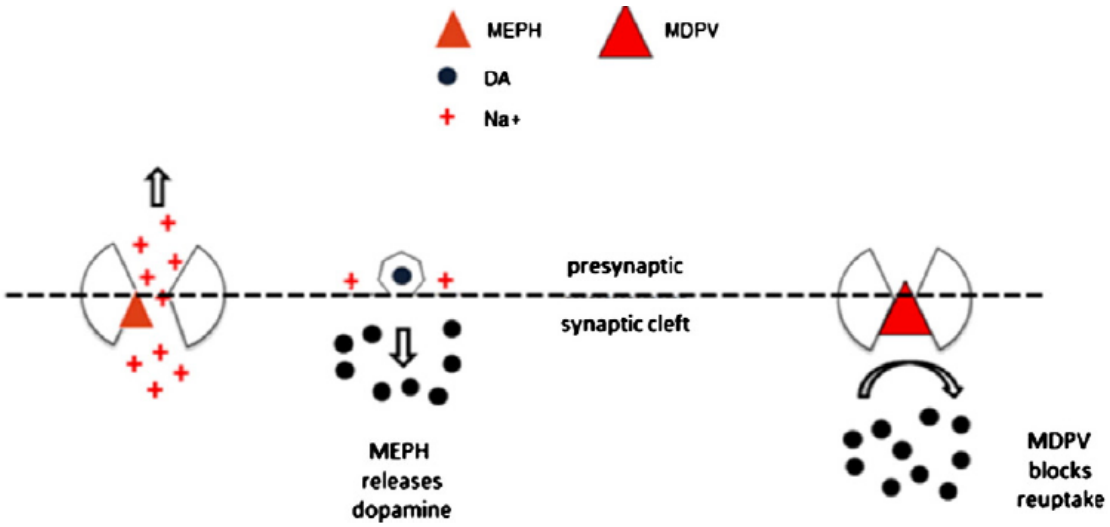
Em 2009, o uso da metedrona (4-metoximetcatinona) começou a ser relatado em hospitais e a mesma a ser apreendida pela polícia (Wikström *et al*., 2010). Essa SPA é um derivado da catinona que está intimamente relacionado com a mefedrona. Seu nome químico corresponde a 1-(4-metoxifenil)-2-(metilamina)-1-propanona, seu peso é de 229,7 g/mol e sua fórmula é $C_{11}H_{15}NO_2$ (Figura 5) (Cayman Chemical, 2012). Um dos poucos estudos

realizados indica que a metedrona causa aumento da circulação sanguínea, salivação e hiperatividade (Marusich *et al*., 2012). Em um estudo de caso, Wikström *et al*., (2010) relataram duas mortes relacionadas à metedrona. No primeiro caso, um homem de 23 anos foi a óbito 16 h após a admissão no hospital; sua temperatura corporal havia atingido 42˚C e houve falência múltipla de órgãos. No exame *posmortem*, foram documentados edema e congestão pulmonar. A amostra sanguínea *antemortem* registrou 13,2 µg/g de metedrona e, na amostra *posmortem*, foram encontrados 8,4 µg/g. No segundo caso, um homem de 19 anos teve convulsões antes de ir a óbito. Nos exames *posmortem*, foram documentados edema e congestão pulmonar; no sangue, foram encontrados 9,6 µg/g de metedrona e, amostras de cabelo revelaram o uso crônico desta substância. Os autores relatam que as doses normalmente encontradas em outros usuários variaram de 0,2 a 4,8 µg/g, de forma semelhante aos dois casos descritos acima, indicando uma janela terapêutica estreita, enfatizando os perigos associados com a metedrona.

Figura 6 - Mecanismo de ação da mefedrona e da MDPV



Fonte: De Felice *et al*., (2013).

NOTA: Mefedrona (MEPH).

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Sabe-se que essa substância induz a liberação moderada de DA e, libera quantidades significativas de NA e 5-HT, tendo um perfil farmacológico semelhante ao MDMA. Com relação à afinidade por receptores, parecer ter preferência pelo receptor α_{1A} (*Ki* > 6) e D_2 (*Ki* > 10), em relação a outros receptores como 5-HT_{1A} e α_{2A} (*Ki* > 20) e, 5-HT_{2A}, 5-HT_{2C}, D_1 (*Ki* > 12) (Simmler *et al*., 2014).

Como relatado na literatura, as *legal highs* estão associadas a quadros clínicos graves. O consumo da mefedrona tem aumentado, bem como, os casos de intoxicações e óbitos devido a sua administração. A metedrona, embora não tão conhecida, tem relatos tanto de sua apreensão, quanto de usuários seriamente intoxicados, salientando seu potencial prejuízo à saúde. Apesar dos estudos disponíveis sobre a catinona e seus derivados, o tratamento para os usuários não está bem estabelecido. Existe semelhança com outras SPA, como as anfetaminas e a cocaína, mas devido às diferenças entre estas substâncias e seus efeitos adversos, se fazem necessárias opções terapêuticas adicionais.

Portanto, com o intuito de fornecer mais conhecimentos à comunidade científica, no que diz respeito às *legal highs*, o presente estudo procurou ampliar o que se sabe sobre os efeitos comportamentais, assim como, os mecanismos bioquímicos e farmacológicos dos derivados da catinona, mefedrona e metedrona. Conhecendo-se melhor os efeitos *in vivo* de tais substâncias, aumentam as possibilidades de melhorar o tratamento para os usuários intoxicados que dão entrada nas emergências hospitalares ou, até mesmo, para os profissionais que buscam orientações em centros de informações toxicológicas.

2 OBJETIVOS

2.1 OBJETIVOS GERAIS

Avaliar e comparar os efeitos comportamentais e bioquímicos dos derivados da catinona, mefedrona e metedrona, após a administração aguda em camundongos, bem como, analisar o impacto do ambiente condicionado, semelhante à *nightclub*, sobre os animais tratados com as catinonas.

2.2 OBJETIVOS ESPECÍFICOS

a) Comparar os efeitos comportamentais desencadeados pela mefedrona e pela metedrona após a administração aguda dessas substâncias;
b) Quantificar os níveis de DA, 5-HT e glutamato após a administração aguda da mefedrona ou metedrona;
c) Identificar alguns mecanismos de ação da metedrona por meio do uso de ferramentas farmacológicas;
d) Comparar os efeitos comportamentais desencadeados pela mefedrona e pela metedrona, na presença e na ausência de estímulos visuais, sonoros e temperatura elevada (ambiente semelhante à *nightclub*).

3 RESULTADOS: **ARTIGO CIENTÍFICO**

Comparative Pharmacological Evaluation of the Cathinone Derivatives, Mephedrone and Methedrone, in Mice: Impacts of Environmental Conditioning

Artigo submetido ao periódico *British Journal of Pharmacology*®

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Comparative Pharmacological Evaluation of the Cathinone Derivatives, Mephedrone and Methedrone, in Mice: Impacts of Environmental Conditioning

Running title: Comparison of Cathinone Derivatives in Mice

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PB Pail, KM Costa and CE Leite performed the research

PB Pail and MM Campos designed the research study

MM Campos contributed essential reagents or tools

PB Pail and MM Campos analysed the data

PB Pail and MM Campos wrote the paper

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Abstract

Background and Purpose: We compared the behavioural and the neurochemical effects of the synthetic cathinones, mephedrone and methedrone, attempting to evaluate some mechanisms of action of methedrone, and the influence of nightclub-like stimulation on behavioural changes elicited by both cathinones in mice.

Experimental Approach: The effects of cathinones were examined in a series of behavioural tests in mice, and monoamine brain levels were determined by HPLC. Since there is a correlation between club parties and the consumption of recreational drugs, separated groups were pre-exposed to a nightclub-like environment.

Key Results: Cathinones caused marked hyperlocomotion, allied to motor coordination inability, until 30 min of evaluation. Mephedrone caused anxiolytic-like effects, while methedrone induced anxiogenic actions. Both of the cathinones increased latency in the hot-plate test, with reduced immobility time in the tail suspension test. Mephedrone triggered a 2- and 3-fold increment of dopamine and serotonin levels, respectively, in the *nucleus accumbens*, with a 1.5-fold elevation of dopamine in the frontal cortex. Methedrone caused a 2-fold increment of dopamine in the *nucleus accumbens* and striatum, and 1.5-fold increment of serotonin levels in the hippocampus and striatum. Part of the methedrone effects were inhibited by dopamine and serotonin modulators. Nightclub-like stimulation produced a further increment of latency to thermal stimulation, in both mephedrone and methedrone-treated mice.

Conclusions and Implications: Mephedrone and methedrone induced distinct behavioural changes most likely caused by the modulation of dopamine and serotonin systems. Curiously, an increased latency to thermal stimulation elicited by the cathinones was intensified by a nightclub-like environment.

Keywords: cathinone; mephedrone; methedrone; dopamine; serotonin; norepinephrine; music

Abbreviations: DA, dopamine; 5-HT, 5-hydroxytryptamine; NE, norepinephrine.

Introduction

Designer drugs are a group of synthetic substances with stimulant, hallucinogenic and/or entactogenic effects, which are classified according to their chemical structure (Luciano and Perazella, 2014). Cathinone (Figure 10A) is the main naturally-occurring psychoactive constituent obtained from the leaves of Khat (*Catha edulis*), which served as the basis for the development of a series of synthetic molecules (Baumann *et al*., 2012). Synthetic cathinones easily permeate the blood-brain barrier, eliciting sympathomimetic and psychostimulant actions. Their effects in humans might include paranoia, hallucinations, euphoria, aggressiveness, psychosis and an increment of libido, in addition to the more serious occurrence of hypertension, hyperthermia, seizures, respiratory distress, with reports of fatal outcomes (Luciano and Perazella, 2014; Valente *et al.*, 2014).

There is a current interest in the pharmacological effects of synthetic cathinones, considering their widespread recreational use. Among them, mephedrone (4-methylmethcathinone; Figure 10B) has been the target of extensive research in the last few years, being characterised as a potent synthetic cathinone, which displays amphetamine-like psychostimulant properties (Green *at al*., 2014). *In vitro* and *in vivo* studies have revealed that mephedrone is able to induce a rapid extracellular increment of norepinephrine (NE), dopamine (DA) and serotonin (5-HT) levels, by interfering with the mechanisms of release, uptake, and the transportation of neurotransmitters (Kehr *et al*., 2011; Cameron *et al*., 2013; López-Arnau *et al*., 2012; Simmler *et al*., 2013; Iversen *et al*., 2014). After several cases of intoxication and deaths, mephedrone has been banned in different countries, although it continues to be illicitly commercialised worldwide, especially on the Internet and in smartshops (Green *et al*., 2014; Valente *et al*., 2014).

The less studied cathinone methedrone (4-methoxymethcathinone; Figure 10C) has been demonstrated to evoke hyperlocomotion and stereotyped behaviour in rodents (Marusich *et al*., 2012); additionally, *in vitro* experiments have indicated predominant serotoninergic effects for this drug (Simmler *et al.*, 2014). A case-report study has described two fatal intoxications, following methedrone consumption, which were preceded by hyperthermia, pulmonary oedema and seizures (Wilkström *et al.*, 2010), reinforcing the need for additional studies on the abuse of this drug.

This study assessed the effects of mephedrone and methedrone in mice, aiming to better characterise and understand the mechanisms of action of the less studied substance methedrone. Considering the extensive recreational use of synthetic

cathinones, we have also attempted to evaluate the impacts of environmental nightclub-like stimulation on behavioural effects of mephedrone and methedrone. Our data extends previous studies on the actions of mephedrone, bringing novel evidence on the pharmacological and neurochemical effects of methedrone.

Materials and Methods

Drugs

Mephedrone (2-(methylamino)-1-(4-methylphenyl)-1-propanone hydrochloride) and methedrone (1-(4-methoxyphenyl)-2-(methylamino)-1-propanone hydrochloride) were obtained from the Cayman Chemical Company (Ann Arbor, Michigan, USA) with purity >98%. Haloperidol, naloxone and $_{DL}$-ρ-chlorophenylalanine methyl ester (ρCPA) were obtained from the Sigma Chemical Company (St. Louis, Missouri, USA). These substances were dissolved in saline solution (NaCl 0.9%) for i.p. administration (10 ml kg^{-1}). The doses of the cathinones were selected on the basis of literature data and pilot experiments (Lisek *et al.*, 2012; den Hollander *et al.*, 2013). During the development of this study, the sales of cathinone derivatives were forbidden in Brazil, impeding us to perform complete dose-response studies.

Protocols of Treatment

The behavioural effects of a single administration (i.p.) of methedrone (15 or 30 mg kg^{-1}), were assessed during 60 min, in several experimental paradigms, as will be described in the next sections, and compared with the effects elicited by mephedrone (30 mg kg^{-1}) (Lisek *et al.*, 2012; Marusich *et al.*, 2012). In some experimental sets, the mice were pre-exposed to a nightclub-like stimulation, to mimic the environment of the users of cathinones (see details below). In separate groups, the brain levels of neurotransmitters were quantified 20 min after the single (i.p.) administration of methedrone or mephedrone (both at 30 mg kg^{-1}). To evaluate some of the mechanisms underlying the effects of methedrone, different groups of mice were treated with the non-selective DA receptor antagonist haloperidol (0.1 mg kg^{-1}, i.p.) or with the non-selective opioid receptor antagonist naloxone (1 mg kg^{-1}, i.p.), given 30 min before the injection of methedrone (30 mg kg^{-1}, i.p.). Separately, the animals received the inhibitor of 5-HT synthesis ρCPA (100 mg kg^{-1}, i.p.), administered over a period of 4 days, once a day, with the last administration 18 h before the methedrone injection (30 mg kg^{-1}, i.p.). The control groups received saline solution at the same intervals of time, before the methedrone administration. Parallel control groups received saline plus saline, or the pharmacological modulators plus saline.

Body Temperature Assessment

Rectal temperature was recorded (°C) using a commercially available thermometer (Pro-check®). The body temperature was measured 30 min before the injection of substances, and at 30 min and 60 min after the administration.

Open-Field Test

The animals were evaluated in the open-field test, according to the methodology as described by Holland and Weldon (1968). Immediately after the cathinone administration, the mouse was placed in the centre of an acrylic box (20 x 40 x 30 cm), with the floor divided into 12 squares. The animals were observed at 10, 20, 30, and 60 min, after the administration of the cathinones. The following parameters were registered: (i) the number of squares crossed with their four paws, (ii) the duration of grooming (s), and (iii) the number of rearings. Stereotyped movements, walking-backwards, round-back position, Straub tail, piloerection, and tremors, were also evaluated, and expressed as a positive, when more than 30% of the animals exhibited the behaviour.

Elevated Plus-Maze

Anxiety was measured in the elevated plus-maze, according to the methodology as described before (Karim *et al*., 2012; den Hollander *et al*., 2013). Thirty min after the administration of the cathinones, the mouse was placed on the central platform, facing a closed-arm, and was allowed free exploration of the maze for five min. The apparatus consisted of a central platform (5 × 5 cm), from which two open-arms (5 × 30 cm), and two closed-arms (5 × 30 × 15 cm), extended. The number of entries in the open- and closed-arms, and their head-dipping was registered, and was considered as the exploration index. The time spent in the open- and closed-arms, and centre platform, was recorded as a measure of an anxiety state.

Hot-Plate Test

Latency to thermal stimulation was measured 35 min after the injection of cathinones, following the method as described by Ferguson *et al*. (1969), with minor modifications. The plate temperature was maintained at 50 ± 1 ºC (Ugo Basile 7280 Hot Plate®), and the latency to licking the forepaw (s) was registered. A latency period (cut-off) of 30 s was adopted to prevent any tissue damage.

Tail Suspension Test

To further assess the anti-immobility effects of the cathinones, we employed the tail suspension test, according to the methodology as described by Steru *et al*. (1985). Forty min after the injection of the cathinones, the mouse was suspended 100 cm above the floor, by means of adhesive tape, and placed approximately 1 cm from the tip of the tail. The time during which the mice remained immobile was quantified (s) during a period of 6 min.

Determination of the Levels of Monoamines: Dopamine, Serotonin and Glutamate

This analysis was performed using previously described methods (Hows *et al*., 2004; González *et al*., 2011), with minor modifications. Striatum, hippocampus, frontal cortex, and *nucleus accumbens*, were bilaterally dissected from the brain, after 20 min of the treatment with mephedrone or methedrone (both 30 mg kg^{-1}; i.p.), and were stored at -80°C until analysis. Frozen tissues were homogenised in a 15-fold volume of a solution of formic acid (0.1 M), whereas the *nucleus accumbens* was homogenised in 400 µl of the same solution, and then centrifuged at 18000 x g for 20 min at 4°C. The supernatants were filtered (0.22-µm filter), and were used for chromatographic analysis. A stock solution containing a mixture of DA, 5-HT, and glutamate, was prepared in methanol (1 mg ml^{-1}). The equipment UHPLC 1290/MS 6460 TQQQ-Agilent (all HPLC components with MassHunter software were from Agilent Technologies®) was used. Chromatographic separations were performed using a Zorbax Eclipse Plus C18 2.1 x 50 mm 1.8-µm column. When evaluating the flow rate of methanol (eluent A):formic acid 0.05% with 1 mM of heptafluorobutyric acid (HFBA) (eluent B), the mobile phase was 0.2 m min^{-1}, with a column temperature of 30°C. A gradient was used, starting at 95% of eluent B constant for 0.5 min, and subsequently decreasing to 0% in 3.5 min. Five microliters of the samples were injected into the UHPLC system. The monitored transitions were: DA (154>137 and 154>91), 5-HT (177>160) and glutamate (148>130 and 148>84). The results were expressed in ng g^{-1} tissue for DA and 5-HT, and in µg g^{-1} tissue for glutamate.

Nightclub-Like Stimulation

Since there is a correlation between acoustic and visual events, and the abuse of psychoactive substances, some animals were exposed to nightclub-like stimulation, to mimic the environment of drug users, to better characterise the effects of cathinones. Separated groups of mice were exposed to electronic music (70-85 decibels), flashing lights, and high temperatures (26 ± 2°C), for 1 h, during seven days, before the treatment with cathinones

(Figure S1). On the day of the experiment, the cathinones were administered immediately after stimulus. The parameters of environmental conditioning were chosen on the basis of previous literature data (Green and Nutt, 2014; Polston *et al*., 2011; Sanchez *et al*., 2004).

Animals

All animal care and experimental procedures were in accordance with the Guidelines for the Use and Care of Laboratorial Animals, as stated by the National Institutes of Health and their Ethical Guidelines for the investigation of experimental pain in conscious animals. All procedures were approved by the Local Animal Ethics Committee (CEUA protocol 13/00336), and the study was accomplished according to ARRIVE recommendations.

Female C57BL-6 mice (total=254 animals) were used, as they are known to be very sensitive to neuronal damage by amphetamines (Angoa-Pérez *et al*., 2012). The animals were obtained from the Central Animal House of the Universidade Federal de Pelotas (UFPEL, Brazil). The mice (17-23 g; 9-11 weeks-old) were housed in groups of four per cage, and were maintained in controlled temperature (22 ± 1 °C) and humidity (60-70%), under a 12 h light/dark cycle, with food and water *ad libitum*. The animals were acclimatised to the laboratory for at least 1 h before testing and all of the tests were performed between 8:00 AM and 3:00 PM. For the behavioural tests, the animals were visually and acoustically isolated during the experimental sessions. An observer, blind to the treatments, analysed all the experiments.

Statistical Analysis

Results are presented as mean±standard error mean (SEM). The statistical comparison of the data was performed by unpaired Student's *t*-test or by one-way analysis of variance (ANOVA), followed by Bonferroni´s post-hoc test. *P* values of less than 0.05 ($P < 0.05$) were considered to be an indicative of significance (GraphPad Prism 5.0, La Jolla, CA, USA).

Results

Acute Behavioural Effects Elicited by Mephedrone and Methedrone

On the basis of literature data, we initially assessed the effects of mephedrone (20 and 30 mg kg^{-1}) in a series of *in vivo* experimental models, from 20 to 360 min after injection of this cathinone (Lisek *et* al., 2012; López-Arnau *et al*., 2012). Surprisingly, there was no significant alteration of locomotor parameters, latency to heat stimulus, or anxiety indexes, according to the evaluation from 60 to 90 min after the mephedrone administration (Figure S2 A-C; E-G). Also, no significant change of rectal temperature was detected following the mephedrone treatment, from 20 to 360 min (Figure S2 H). Nevertheless, it was possible to observe a significant reduction of grooming duration in the mephedrone (30 mg kg^{-1})-treated mice, when analysed from 0 to 20 min after treatment ($P<0.01$; Figure S2 D). This prompted us to carry out an additional evaluation of mephedrone effects at earlier time-points. Notably, the administration of mephedrone (30 mg kg^{-1}) resulted in a marked and time-related increase of the crossing number, which peaked at 10 min, remained significant until 30 min, and returned to control levels at 60 min (at 10 and 20 min, $P<0.01$; at 30 min, $P<0.05$; Figure 1A). This correlated well to the significant reduction of grooming time (at 10 min, $P<0.01$; at 30 min, $P<0.05$; Figure 1C) and a great inhibition of rearing numbers (at 60 min, $P<0.01$; Figure 1B). In the open-field, some of the animals treated with mephedrone (30 mg kg^{-1}) displayed peculiar behaviour, such as walking-backwards and Straub tail (Table 1). In the elevated plus-maze, the mephedrone-treated animals (at 30 min) displayed an increased time that was spent in the open-arms ($P<0.05$; Figure 1D), and a significant increase of head-dipping behaviour ($P<0.05$; Figure 1E). The administration of mephedrone also resulted in a significant increment of latency in licking forepaws in the hot-plate test ($P<0.05$; Figure 1F), and a marked reduction of immobility time in the tail suspension test ($P<0.05$; Figure 1G), as evaluated at 35 and 40 min, respectively. However, the rectal temperature was not affected by the mephedrone administration (30 mg kg^{-1}), at either 30 or 60 min (Figure 1H).

For the purposes of comparison, the behavioural effects of methedrone (15 and 30 mg kg^{-1}) were also evaluated from 10 to 60 min after injection. The injection of methedrone (30 mg kg^{-1}) caused a time-dependent increase of the crossing number, peaking at 10 min, and remaining significant until 30 min (from 10 to 30 min, $P<0.01$; Figure 2A). This behavioural change was accompanied by a marked reduction of rearing numbers (at 20 min, $P<0.01$; at 30 min, $P<0.05$; Figure 2B) and of grooming duration (at 10, 20 and 60 min, $P<0.01$; at 30 min, $P<0.05$; Figure 2C). The dosage of 15 mg kg^{-1} of methedrone displayed similar effects on the locomotor parameters in the open-field test, although the increment of crossing numbers was

only significant at 10 min (P<0.05), the rearing numbers were significant at 20 min (P<0.01), and the grooming time at 10 min (P<0.01) and 30 min (P<0.05) (Figure 2A-C). When observed in the open-field, methedrone (15 and 30 mg kg^{-1})-treated mice presented a series of behavioural features, including piloerection, tremors, round-back position and Straub tail (Table 1).

The administration of methedrone (30 mg kg^{-1}) caused a significant decrement of time spent in the open-arms (P < 0.05) and on the centre platform (P<0.01), that was associated to increased time spent in the closed-arms of the elevated plus-maze (at 30 min, P<0.01; Figure 2D). Likewise, this dose of methedrone elicited a marked reduction of head-dipping behaviour (P < 0.05; Figure 2E). In this experimental paradigm, a dosage of 15 mg kg^{-1} of methedrone was able to induce a significant increment of head-dipping (P<0.05), without altering any additional parameters (Figure 2D-E). Conversely, the mice treated with either doses of methedrone (15 and 30 mg kg^{-1}), showed a significant increment of latencies to heat stimulation in the hot-plate experiment (at 35 min, P<0.01; Figure 2F); either dose was also associated with a marked reduction of immobility time in the tail suspension test (at 35 min, P<0.01; Figure 2G). There was no significant change of rectal temperature after the methedrone administration (15 and 30 mg kg^{-1}), according to the evaluation at 30 or 60 min (Figure 2H).

Assessment of Mephedrone and Methedrone Effects on Brain Neurotransmitters

Next, we performed an evaluation of neurotransmitter levels throughout different brain regions, after the administration of methedrone or mephedrone (both 30 mg kg^{-1}), at 20 min after treatment. Mephedrone elicited a 3-fold increase of DA levels (P<0.05; Figure 3A), and 2-fold elevation of 5-HT (P<0.01; Figure 3B) in the *nucleus accumbens*, although glutamate contents remained unaltered in this region (Figure 3C). The administration of mephedrone also induced a 1.5-fold increase of DA levels in the frontal cortex (P<0.05; Figure 3D), whereas the 5-HT and glutamate contents remained unaffected in this region (Figure 3E and F). There was no significant change of DA, 5-HT, or glutamate levels, in the striatum or hippocampus, after the mephedrone treatment (Figure 3G-I; J-L).

Methedrone displayed a 2-fold increase in DA levels in the *nucleus accumbens* (P<0.05; Figure 4A), accompanied by a slight, but not significant increment of 5-HT levels in this structure (P=0.1414; Figure 4B); whereas glutamate levels were not significantly altered when compared to control (Figure 4C). Neurotransmitter levels were not significantly changed in the frontal cortex, when in relation to control (Figure 4D-F). In the hippocampus,

methedrone caused a 1.5-fold increase of 5-HT levels (P<0.01; Figure 4H), but this particular cathinone did not alter DA or glutamate contents (Figure 4G and I). In the striatum, the methedrone treatment elicited a significant 1.5-fold elevation of DA (P<0.05; Figure 4J) and 5-HT levels (P<0.01; Figure 4K), whilst the glutamate contents were not significantly affected (Figure 4L).

Pharmacological Characterization of Mechanisms Underlying Methedrone Behavioural Effects

In view of the few literature studies regarding methedrone effects, we decided to investigate some of the possible mechanisms of this specific cathinone. We tested the effects of a low dose of the non-selective dopamine receptor antagonist haloperidol (0.1 mg kg^{-1}), given 30 min before the methedrone injection (30 mg kg^{-1}). Haloperidol was able to significantly revert the increased locomotor activity induced by methedrone in mice, according to the evaluation of the crossing numbers (from 10 to 30 min, P<0.01; at 60 min, P<0.05; Figure 5A). However, this dose of haloperidol was also associated with significant alterations of locomotor parameters in the saline-treated mice (Figure 5A-C). The pre-treatment with haloperidol was able to revert the anxiogenic-like effects induced by methedrone in the elevated plus-maze, according to the assessment of time spent in the open- (P<0.05) and closed-arms (P<0.01), whereas haloperidol did not interfere with these parameters in the saline-treated control groups (Figure 5D). The exploration indexes, the latency to heat stimulation, the immobility time, or the rectal temperature, in the methedrone-treated mice, were not significantly altered by the haloperidol dosage (Figure 5E-H).

Next, we assessed the effects of repeated administration of the 5-HT synthesis inhibitor ρCPA (100 mg kg^{-1}, for 4 days) on the methedrone (30 mg kg^{-1})-induced behavioural effects in mice. This pharmacological tool significantly prevented the increment of crossing activity allied to the methedrone administration (at 10 and 20 min, P<0.05; Figure 6A), and inhibited the increment of latency to heat stimulation caused by methedrone (P<0.05; Figure 6F). The other behavioural changes induced by the methedrone dosage were not significantly modified by the ρCPA treatment (Figure 6B-E; G and H).

Considering the observation of the methedrone-induced Straub tail, we also decided to test the effects of pre-treatment with the non-selective opioid antagonist naloxone (1 mg kg^{-1}), dosed 30 min before the methedrone (30 mg kg^{-1}) administration. Naloxone was unable to significantly change any behavioural alteration elicited by the methedrone dosage (Figure 7).

Neither of the tested pharmacological tools prevented the occurrence of piloerection, tremors, round-back position or Straub tail, in the methedrone-treated mice (Table 1).

Impacts of Nightclub-Like Stimulation on Cathinone-Induced Behavioural Changes

The behavioural effects of nightclub drugs, such as cathinones, are commonly associated with the environments in which these substances are used. To experimentally evaluate this hypothesis, we tested the effects of a conditioned environment, by exposing the mice to electronic music (70-85 decibels), flashing lights, and high temperatures (26 ± 2°C), for 1 h, over seven days (Figure S1 A-D), and monitored the cathinone effects. In the saline-treated control animals, the nightclub-like stimulation did not cause any significant alteration of locomotor activity, anxiety-like behaviour, immobility time in the tail suspension test, or in rectal temperature (Figure S3 A-E; G and H), whereas it significantly increased the latency to thermal stimulation in the hot-plate test ($P < 0.01$; at 35 min; Figure S3 F), and the frequency of walking-backwards behaviour (Table 1). The nightclub stimulation did not modify the behavioural changes elicited by mephedrone (Figure 8A-E; G and H) or methedrone (Figure 9A-E; G and H), both dosed at 30 mg kg^{-1}, except for a significant increment of crossing activity in the methedrone-treated mice (but only at 60 min, $P < 0.05$; Figure 9A). However, the environmental conditioning significantly increased the latency to heat stimulation in both the mephedrone and methedrone-treated mice (at 35 min, $P < 0.05$; Figure 8F and 9F, respectively).

At the schedules of administration tested in the present study, mephedrone or methedrone did not elicit any significant change of total, or differential, blood cell counts (Figure S4 A and B). The acute administration of both cathinones did not induce liver oxidative stress effects, as indicated by the assessment of catalase activity and lipid peroxidation (Figure S4 C-J).

Discussion

The prevalence of cathinone consumption among young people has surpassed the use of methamphetamine and heroin in the USA (Dybdal-Hargreaves *et al*., 2013; Luciano and Perazella, 2014). Mephedrone and methedrone display similar chemical structures, representing synthetic methylated cathinones. However, methedrone presents a methoxy radical in position 4 of the aromatic ring (Figure 10C), that might imply distinct pharmacological actions in relation to mephedrone (Valente *et al.*, 2014). This study compared the behavioural and neurochemical effects of mephedrone and methedrone in mice, attempting to evaluate some of the mechanisms of action of methedrone, and the influence of environmental nightclub-like stimulation on the behavioural alterations elicited by cathinones.

The acute administration of mephedrone and methedrone (both at 30 mg kg^{-1}), to female C57BL/6 mice, elicited a marked and rapid increase of locomotion, associated with some grade of motor coordination inability. These results are quite consistent with the reports of users, describing increased alertness, and euphoria, after the recreational consumption of cathinones (Winstock *et al.*, 2011). The acute treatment with mephedrone induced hyperlocomotion in mice, or rats, when administered at similar doses (Lisek *et al.*, 2012; López-Arnau *et al*., 2012). Another study carried out with mephedrone and methedrone also demonstrated a rapid increase of general locomotion in mice, until 90 min of evaluation (Marusich *et al*., 2012). Herein, we also observed that both of the tested cathinones induced other behavioural alterations in the open-field arena, such as walking-backwards, piloerection, tremors, round-back position, and Straub tail. Marusich *et al*. (2012) demonstrated the acute effects of methedrone and mephedrone in a functional observational battery, indicating the occurrence of stereotyped head weaving, and head circling, for both cathinones, contributing to better understanding of the behavioural effects elicited by these illicit substances.

In the elevated plus-maze, mephedrone provoked anxiolytic-like effects, as indicated by a significant increment in the time spent in the open-arms, and in head-dipping numbers. This was similar to that seen in the mice treated with the cathinone-related antidepressant drug bupropion (Biala and Kruk, 2009). In contrast, methedrone caused anxiogenic-like actions, with a marked increase in the time spent in the closed-arms, and with a reduction of head-dipping counts. A similar anxiogenic effect was noted in the mice pre-treated with cocaine, or the dopamine reuptake inhibitor JHW007 (Velázquez-Sánchez *et al*., 2010). This is the first pre-clinical report, showing the distinct behavioural profiles, for mephedrone and methedrone in the elevated plus-maze.

It was demonstrated before, that mice pre-treated with a high dose of Khat extract (1,800 mg kg^{-1}), displayed a significant increase in the latency to heat stimulus in hot-plate and tail-flick tests (Connor *et al*., 2000). Our results confirm and extend this data, showing that mephedrone or methedrone caused antinociceptive effects in the hot-plate test. Furthermore, both synthetic cathinones produced a significant reduction of immobility time in the tail suspension test, displaying an antidepressant-like profile. Accordingly, the immobility time was also reduced following a treatment with *d*-amphetamine in mice (Cryan *et al*., 2005). Of note, some synthetic cathinones have been initially developed as possible therapeutic options for treating depression (Valente *et al.*, 2014). Also, it has been demonstrated that antidepressant drugs, such as bupropion, display analgesic effects in rats (Naderi *et al*., 2014). The reduction of immobility time was more pronounced in the methedrone-treated mice, when in relation to the animals that received mephedrone, further confirming the different profiles of the tested cathinones. In fact, the methedrone-treated mice displayed struggling behaviour during the test sessions (results not shown). A similar effect has been demonstrated before in mice pre-treated with desipramine, but not fluoxetine, suggesting that struggling is a noradrenergic-mediated alteration (Lockridge *et al*. 2013).

Previous literature data has revealed that the administration of low doses of mephedrone, in a binge-like schedule of treatment, was related to hyperthermia (4 injections of 20 mg kg^{-1}, at 2-h intervals), whilst higher doses (4 injections of 40 mg kg^{-1}, at 2-h intervals), induced alternate periods of hyperthermia and hypothermia in female C57BL-6 mice (Angoa-Perez *et al.*, 2012). The acute administration of mephedrone caused distinct effects on body temperature, depending on the tested rat strain (Wright *et al.*, 2012). However, we did not detect any significant alteration of rectal temperature, in mice acutely treated with either mephedrone or methedrone. The discrepancy between our data and the literature results can be explained by the differences in the protocols of administration and the rodent species. We also tested low doses of mephedrone (20 mg kg^{-1}) and methedrone (15 mg kg^{-1}), but the effects were generally modest when compared to the dosage of 30 mg kg^{-1} of both substances, justifying the use of this latter dose in additional experiments.

Previous *in vivo* studies have shown variable effects for mephedrone on rodent brain neurotransmitter levels (Motbey *et al.*, 2012; Shortall *et al.*, 2013a; 2013b), although the actions of methedrone were assessed only in HEK 293 cultured cells (Simmler *et al.*, 2014). Herein, mephedrone and methedrone induced a significant increase in DA and 5-HT contents in the mouse *nucleus accumbens*. This data is similar to that reported by Wright *et al*. (2012), which showed an increment of neurotransmitter levels in this region, after an acute

mephedrone administration in rats. The elevation of DA and 5-HT levels in the *nucleus accumbens* might support the stimulant effects of both mephedrone and methedrone, according to the evaluation of locomotor parameters. Prior literature evidence has suggested a positive relationship between the levels of DA and 5-HT in the *nucleus accumbens*, and the locomotor activity indexes in mephedrone-treated rats (Kehr *et al*., 2011; Baumann *et al*., 2012). Furthermore, these results can be correlated to the analgesic-like actions of mephedrone and methedrone in the hot-plate test, when considering the effects of neurotransmitters in descending inhibitory pathways of pain (Bannister *et al.*, 2009), although this hypothesis remains to be further investigated. The measurement of brain neurotransmitters also demonstrated that mephedrone induced an increment of DA levels in the frontal cortex, whereas methedrone caused an elevation of 5-HT levels in the hippocampus, and increased DA and 5-HT levels in the striatum. These differences emphasise the distinct effects of mephedrone and methedrone in the elevated plus-maze and the tail suspension test.

In comparison to mephedrone, only a few literature studies evaluated the effects of methedrone, with 272 versus 16 publications, respectively (PubMed, August 2014). To gain further insights into the mechanisms of action of methedrone, we tested the effects of some pharmacological tools, on the behavioural changes elicited by this specific cathinone. A pre-treatment with haloperidol was able to prevent both hyperlocomotion and anxiogenic-like effects induced by a methedrone dosage in mice. According to the literature, a pre-treatment with haloperidol, with the same range of doses used by us, also prevented hyperlocomotion caused by mephedrone in mice (López-Arnau *et al*., 2012). Additionally, it has been demonstrated that mephedrone-induced increase in locomotor activity was inhibited by the selective D_1 receptor antagonist SCH 23390, whereas the selective D_2 receptor blocker sulpiride, failed to alter this parameter in rats (Lisek *et al.*, 2012). Our results clearly suggest the relevance of DA system for the increased locomotor activity induced by an acute administration of methedrone. Regarding the implication of DA in methedrone anxiogenic effects, our data is supported by a previous publication, showing that a pre-treatment with haloperidol was able to reverse the anxiogenic effects elicited by the repeated administration of amphetamine in rats (Cancela *et al.*, 2001).

A pre-treatment with ρCPA reduced methedrone-induced hyperlocomotion, and prevented the increased latency to thermal stimulation in the hot-plate test. These results extend and amplify the literature evidence showing the involvement of 5-HT in mephedrone-induced hyperlocomotion in mice (López-Arnau *et al*. 2012). Moreover, the involvement of 5-

HT in mephedrone-elicited increase in the latency to thermal stimulation in mice, is consistent with previous studies showing analgesic effects, in mice, for the selective 5-HT_{1A} agonist 8-OH-DPAT in the hot-plate test (Galeotti *et al*., 1997). Our pharmacological and neurochemical results support the relevance of DA and 5-HT in hyperlocomotion, induced by both mephedrone and methedrone, with distinct roles for these neurotransmitters, in the anxiogenic and analgesic effects of methedrone.

When considering the occurrence of Straub tail in methedrone-treated mice, we have also assessed the effects of a pre-treatment with naloxone, on the behavioural changes elicited by this substance. Surprisingly, a naloxone administration was not able to significantly affect any of the evaluated behavioural parameters in our study. This contrasts somewhat with the clinical use of naloxone in patients intoxicated with synthetic cathinones (Woo and Hanley, 2013). Further investigations with more selective opioid antagonists might be useful.

Recreational drugs are commonly consumed at club parties, in environments with high temperatures, with electronic music, and with stroboscopic lights. These conditions might potentiate the effects of designer drugs, in addition to increasing the number of violent events, and alcoholic consumption (Forsyth, 2009; Green and Nutt, 2014; Klein *et* al., 2009; Van Havere *et al*., 2011). To explore this notion, we performed separate experiments, in which the animals were submitted to a nightclub-like environment, for 1 h, over seven days. Unexpectedly, this stimulation did not modify any behavioural alteration, elicited by the tested cathinones, except by a slight increase in the latency to thermal stimulation in the methedrone and mephedrone-treated mice.

The main results of the present study are summarised in Figure 10. Our data has revealed similarities, and differences, between mephedrone and methedrone, in both behavioural and biochemical assays. The nightclub-like environment only modified the latency to painful stimulation. Additional studies are, therefore, still necessary to better characterise the pre-clinical effects of cathinone derivatives, and to understand the impacts of environmental conditioning on the effects of synthetic cathinones.

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Conflicts of interest statement

The authors declare that there are no conflicts of interest.

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Table 1. General effects of cathinone derivates in open-field test

Treatment	Walking-backward	Piloerection	Tremor	Round-back	Straub tail
Saline	-	-	-	-	-
Saline stimulation[a]	+	-	-	-	-
Mephedrone (30 mg kg^{-1})	+	-	-	-	+
Mephedrone (30 mg kg^{-1}) stimulation	+	-	-	-	+
Methedrone (15 mg kg^{-1})	-	+	+	+	+
Methedrone (30 mg kg^{-1})	-	+	+	+	+
Methedrone (30 mg kg^{-1}) stimulation	+	+	+	+	+
Haloperidol (0.1 mg kg^{-1}) plus methedrone (30 mg kg^{-1})	-	+	+	+	+
ρCPA (100 mg kg^{-1}) plus methedrone (30 mg kg^{-1})	-	+	+	+	+
Naloxone (1 mg kg^{-1}) plus methedrone (30 mg kg^{-1})	-	+	+	+	+

Considered (+) when more than 30% of the animals exhibited the behavior. [a]Stimulation refers to nightclub-like exposition.

Figure legends

Fig. 1 The behavioural effects of a single administration of mephedrone (30 mg kg^{-1}; i.p.) into female C57/BL6 mice. (A) The number of lines crossed, (B) rearing numbers, and (C) grooming (s) in the open-field test; (D) the time spent (s) in the open-arms, closed-arms, and the centre platform, of the elevated plus-maze; (E) the exploration index: the number of entries into the open- and closed-arms, and the head-dipping numbers in the elevated plus-maze; (F) a latency to licking the forepaws in the hot-plate test; (G) the immobility time (s) in the tail suspension test; (H) rectal temperature is expressed in °C. Data is expressed as the mean ± SEM of 6-8 mice per group. **P*<0.05; ***P*<0.01, was significantly different from the control group (unpaired Student's *t*-test).

Fig. 2 The behavioural effects of a single administration of methedrone (15 or 30 mg kg^{-1}; i.p.) into female C57/BL6 mice. (A) The number of lines crossed, (B) rearing numbers, and (C) grooming (s) in the open-field test; (D) the time spent (s) in the open-arms, closed-arms, and the centre platform, of the elevated plus-maze; (E) the exploration index: the number of entries into the open- and closed-arms, and the head-dipping numbers in the elevated plus-maze; (F) a latency to licking the forepaws in the hot-plate test; (G) the immobility time (s) in the tail suspension test; (H) rectal temperature is expressed in °C. Data is expressed as the mean ± SEM from the two separate experiments. Saline *n*=12, methedrone 15 and 30 mg kg^{-1} *n*=5-6. **P*<0.05; ***P*<0.01, was significantly different from the control group (one-way ANOVA followed by Bonferroni's *post-hoc* Test).

Fig. 3 The effect of a single administration of mephedrone (30 mg kg^{-1}, i.p.), 20 min after injection, on the brain levels of the neurotransmitters. (A, B and C) the levels of dopamine, serotonin, and glutamate, in the *nucleus accumbens*, respectively; (D, E and F) the levels of

dopamine, serotonin, and glutamate, in the frontal cortex, respectively; (G, H and I) the levels of dopamine, serotonin, and glutamate, in the hippocampus, respectively; (J, K and L) the levels of dopamine, serotonin, and glutamate, in the striatum, respectively. Data is expressed as the mean ± SEM of 4 mice per group; for the *nucleus accumbens*, a "pool sample" of two animals was used, n=2. *P<0.05; **P<0.01, was significantly different from the control group (unpaired Student's t-test).

Fig. 4 The effect of a single administration of methedrone (30 mg kg^{-1}, i.p.), 20 min after injection, on the brain levels of the neurotransmitters. (A, B and C) the levels of dopamine, serotonin, and glutamate, in the *nucleus accumbens*, respectively; (D, E and F) the levels of dopamine, serotonin, and glutamate, in the frontal cortex, respectively; (G, H and I) the levels of dopamine, serotonin, and glutamate, in the hippocampus, respectively; (J, K and L) the levels of dopamine, serotonin, and glutamate, in the striatum, respectively. Data is expressed as the mean ± SEM of 4 mice per group; for the *nucleus accumbens*, a "pool sample" of two animals was used, n=2. *P<0.05; **P<0.01, was significantly different from the control group (unpaired Student's t-test).

Fig. 5 The effects of a single administration of haloperidol (0.1 mg kg^{-1}; i.p.), 30 min before, on the behavioural changes caused by methedrone (30 mg kg^{-1}; i.p.), in female C57/BL6 mice. (A) The number of lines crossed, (B) rearing numbers, and (C) grooming (s) in the open-field test; (D) the time spent (s) in the open-arms, closed-arms, and the centre platform, of the elevated plus-maze; (E) the exploration index: the number of entries into the open- and closed-arms, and the head-dipping numbers in the elevated plus-maze; (F) a latency to licking the forepaws in the hot-plate test; (G) the immobility time (s) in the tail suspension test; (H) rectal temperature is expressed in °C. Data is expressed as the mean ± SEM of 5-6 mice per

group. *P<0.05; **P<0.01, was significantly different from the control group (saline plus saline). §P< 0.05; §§P<0.01, was significantly different from the haloperidol plus saline group. †P<0.05; ††P<0.01, was significantly different from the saline plus methedrone group (one-way ANOVA followed by Bonferroni's post-hoc Test).

Fig. 6 The effects of a repeated administration of ρCPA (100 mg kg^{-1}; i.p.), one injection a day, over 4 days, on the behavioural effects caused by methedrone (30 mg kg^{-1}; i.p.). Eighteen h after the last administration of ρCPA, the female C57/BL6 mice received methedrone (30 mg kg^{-1}; i.p.). (A) The number of lines crossed, (B) rearing numbers, and (C) grooming (s) in the open-field test; (D) the time spent (s) in the open-arms, closed-arms, and the centre platform, of the elevated plus-maze; (E) the exploration index: the number of entries into the open- and closed-arms, and the head-dipping numbers in the elevated plus-maze; (F) a latency to licking the forepaws in the hot-plate test; (G) the immobility time (s) in the tail suspension test; (H) rectal temperature is expressed in °C. Data is expressed as the mean ± SEM of 5-6 mice per group. *P<0.05; **P<0.01, was significantly different from the control group (saline plus saline). §P<0.05; §§P<0.01, was significantly different from the ρCPA plus saline group. †P<0.05; ††P<0.01, was significantly different from the saline plus methedrone group (one-way ANOVA followed by Bonferroni's post-hoc Test).

Fig. 7 The effects of a single administration of naloxone (1 mg kg^{-1}; i.p.), 30 min before, on the behavioural changes caused by methedrone (30 mg kg^{-1}; i.p.) in female C57/BL6 mice. (A) The number of lines crossed, (B) rearing numbers, and (C) grooming (s) in the open-field test; (D) the time spent (s) in the open-arms, closed-arms, and the centre platform, of the elevated plus-maze; (E) the exploration index: the number of entries into the open- and closed-arms, and the head-dipping numbers in the elevated plus-maze; (F) a latency to licking

the forepaws in the hot-plate test; (G) the immobility time (s) in the tail suspension test; (H) rectal temperature is expressed in °C. Data is expressed as the mean ± SEM of 5-6 mice per group. *P<0.05; **P<0.01, was significantly different from the control group (saline plus saline). §P<0.05; §§P<0.01, was significantly different from the naloxone plus saline group (one-way ANOVA followed by Bonferroni's post-hoc Test).

Fig. 8 The behavioural effects of a nightclub-like stimulation for seven days, plus a mephedrone administration (30 mg kg^{-1}; i.p.). (A) The number of lines crossed, (B) rearing numbers, and (C) grooming (s) in the open-field test; (D) the time spent (s) in the open-arms, closed-arms, and the centre platform, of the elevated plus-maze; (E) the exploration index: the number of entries into the open- and closed-arms, and the head-dipping numbers in the elevated plus-maze; (F) a latency to licking the forepaws in the hot-plate test; (G) the immobility time (s) in the tail suspension test; (H) rectal temperature is expressed in °C. Data is expressed as the mean ± SEM of 6-8 mice per group. *P<0.05; **P<0.01, was significantly different from the control group (saline non-stimulated). §§P<0.01, was significantly different from the saline stimulated-group. †P<0.05, was significantly different from the mephedrone group (one-way ANOVA followed by Bonferroni's post-hoc Test).

Fig. 9 The behavioural effects of a nightclub-like stimulation for seven days, plus a methedrone administration (30 mg kg^{-1}; i.p.). (A) The number of lines crossed, (B) rearing numbers, and (C) grooming (s) in the open-field test; (D) the time spent (s) in the open-arms, closed-arms, and the centre platform, of the elevated plus-maze; (E) the exploration index: the number of entries into the open- and closed-arms, and the head-dipping numbers in the elevated plus-maze; (F) a latency to licking the forepaws in the hot-plate test; (G) the immobility time (s) in the tail suspension test; (H) rectal temperature is expressed in °C. Data

is expressed as the mean ± SEM of 6-8 mice per group. *P<0.05; **P<0.01, was significantly different from the control group (saline non-stimulated). §§P<0.01, was significantly different from the saline stimulated-group. †P<0.05, was significantly different from the mephedrone group (one-way ANOVA followed by Bonferroni's post-hoc Test).

Fig. 10 The chemical structures of Cathinone (A), Mephedrone (B) and Methedrone (C). A schematic view of the acute effects of mephedrone and methedrone (30 mg kg¹). (D) The behavioural and biochemical effects, following the acute treatment with synthetic cathinones (orange box), plus a nightclub-like stimulus (blue box), and the brain levels of neurotransmitters (purple box). *Nucleus accumbens*, NA; frontal cortex, FC; hippocampus, HIP. (E) The possible mechanisms of action of methedrone, which seems to be dependent on the activation of the dopamine and serotonin receptors.

Fig. 1

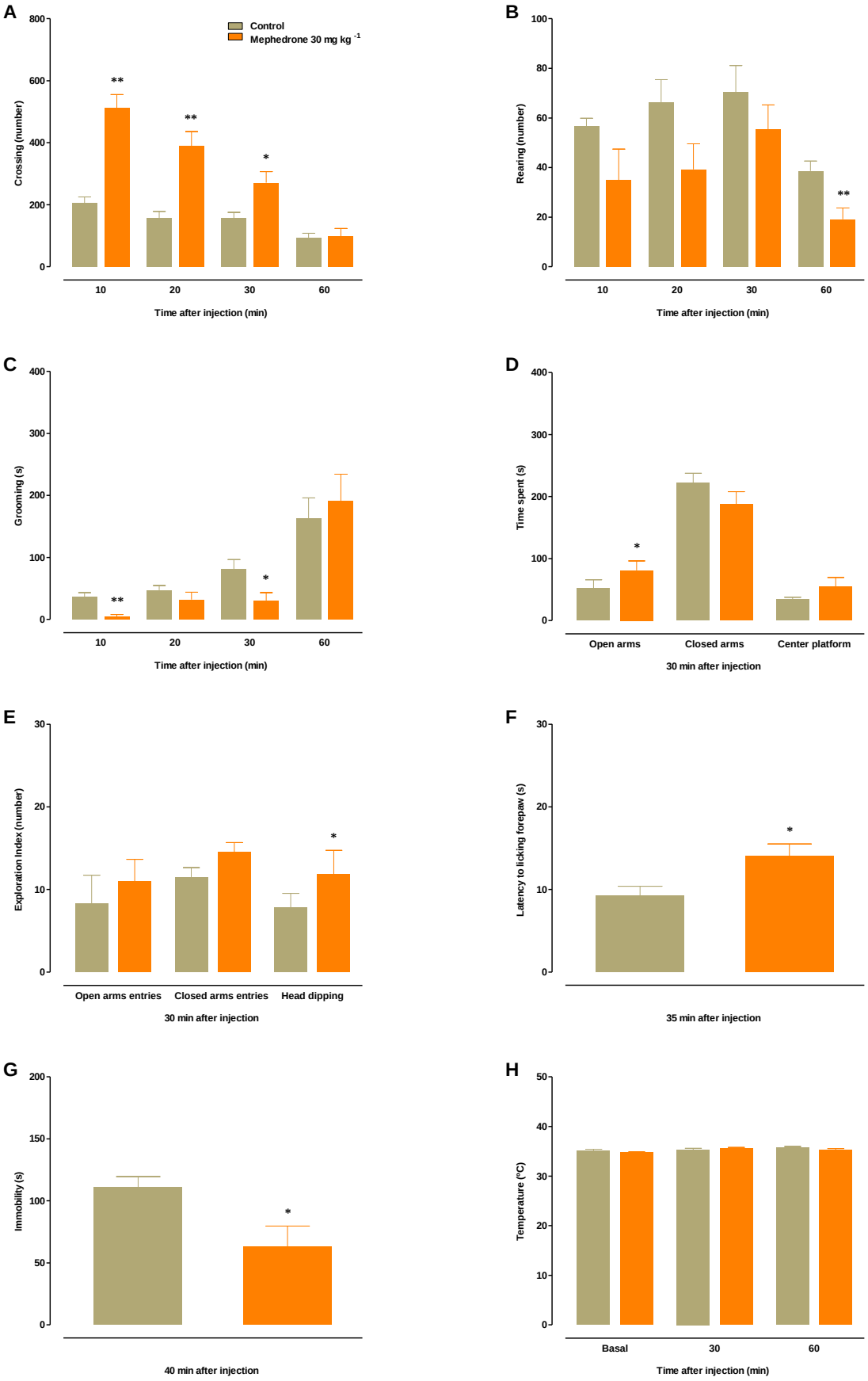

A
Control
Mephedrone 30 mg kg -1
Crossing (number)
Time after injection (min)
B
Rearing (number)
Time after injection (min)
C
Grooming (s)
Time after injection (min)
D
Time spent (s)
Open arms
Closed arms
Center platform
30 min after injection
E
Exploration index (number)
Open arms entries
Closed arms entries
Head dipping
30 min after injection
F
Latency to licking forepaw (s)
35 min after injection
G
Immobility (s)
40 min after injection
H
Temperature (°C)
Basal
30
60
Time after injection (min)

Fig. 2

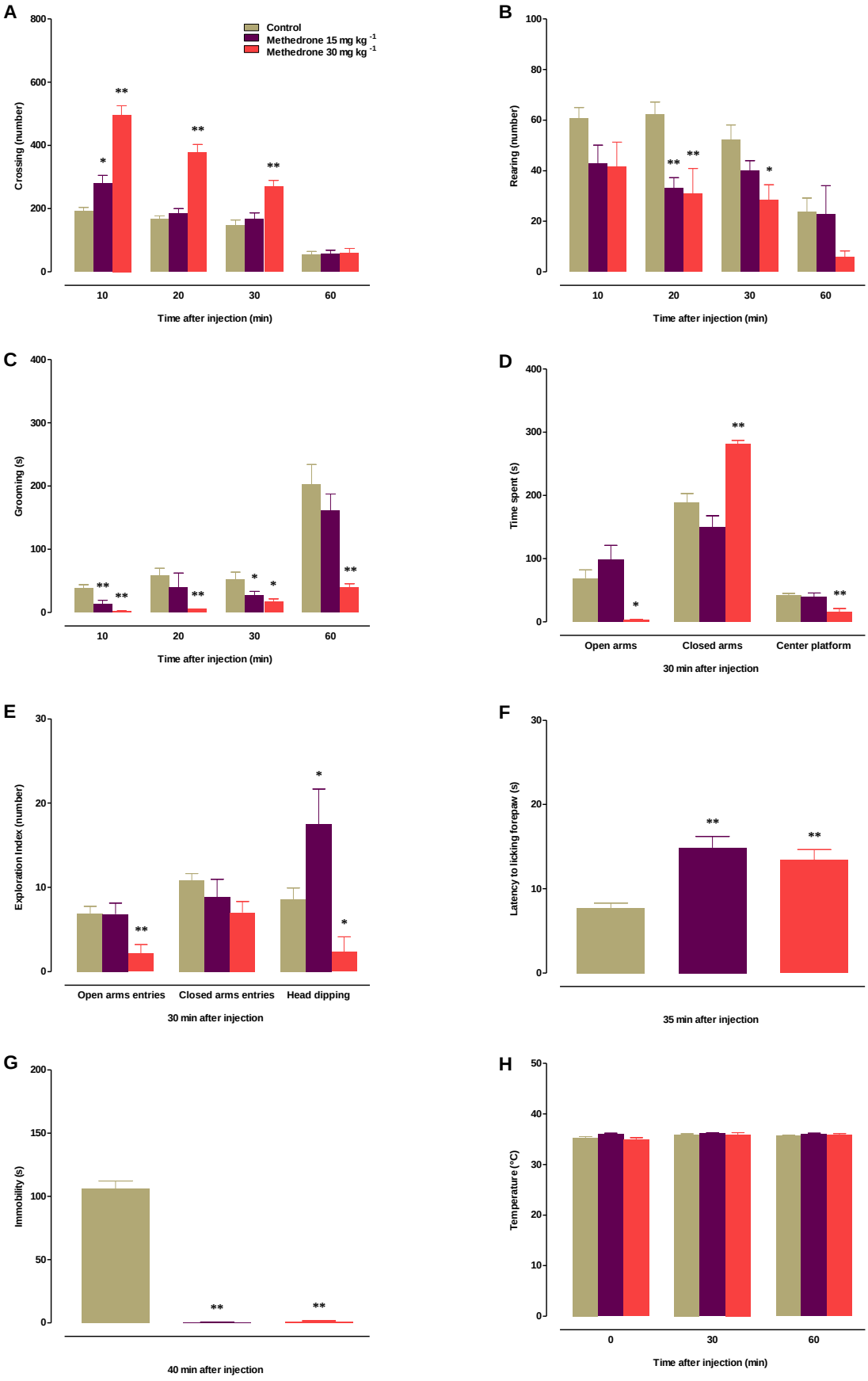

A
Control
Methedrone 15 mg kg -1
Methedrone 30 mg kg -1
Crossing (number)
Time after injection (min)
B
Rearing (number)
Time after injection (min)
C
Grooming (s)
Time after injection (min)
D
Time spent (s)
Open arms
Closed arms
Center platform
30 min after injection
E
Exploration index (number)
Open arms entries
Closed arms entries
Head dipping
30 min after injection
F
Latency to licking forepaw (s)
35 min after injection
G
Immobility (s)
40 min after injection
H
Temperature (°C)
Time after injection (min)

Fig. 3

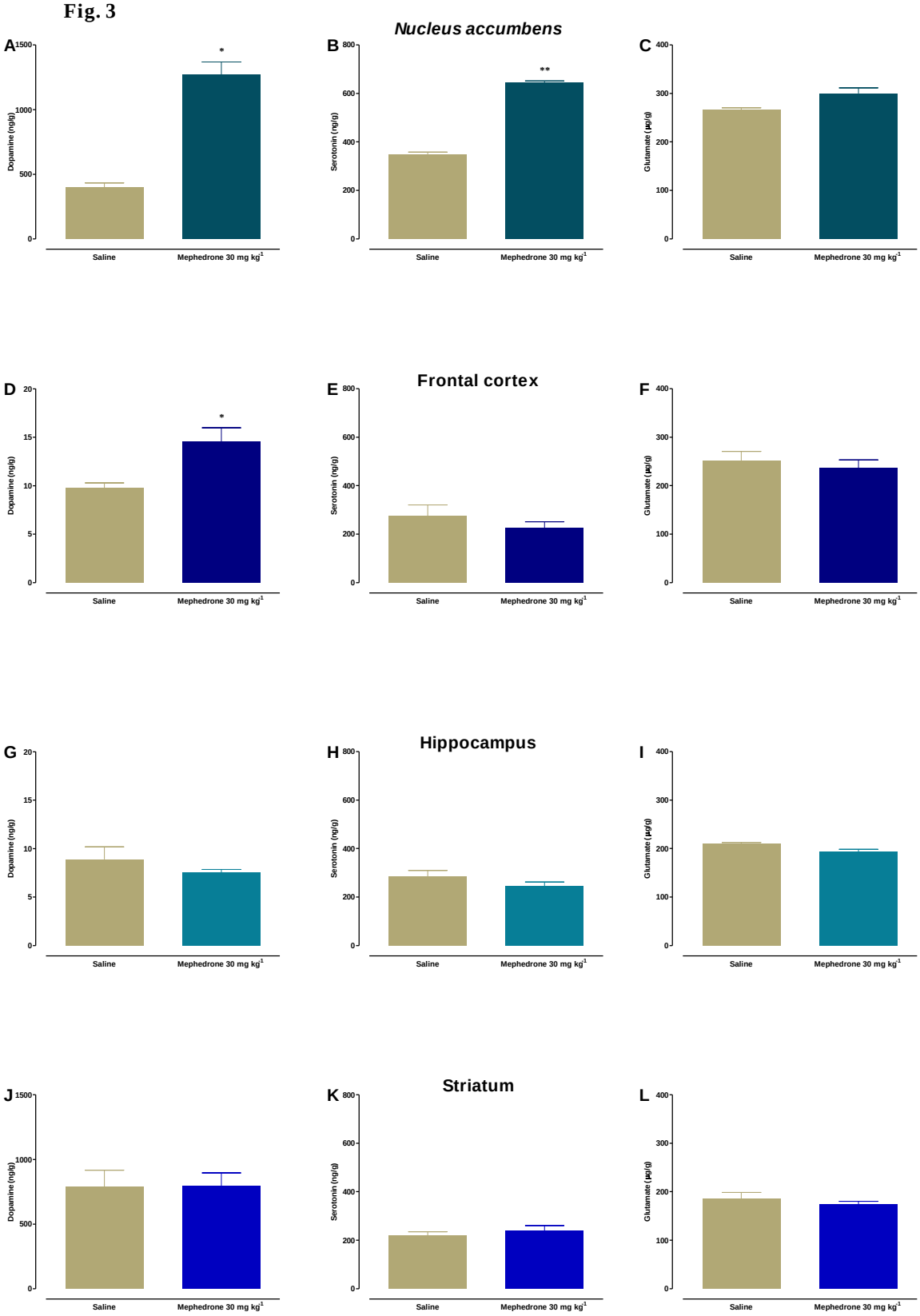

Nucleus accumbens
Frontal cortex
Hippocampus
Striatum
A
B
C
D
E
F
G
H
I
J
K
L
Dopamine (ng/g)
Serotonin (ng/g)
Glutamate (µg/g)
Saline
Mephedrone 30 mg kg-1
*
**

Fig. 4

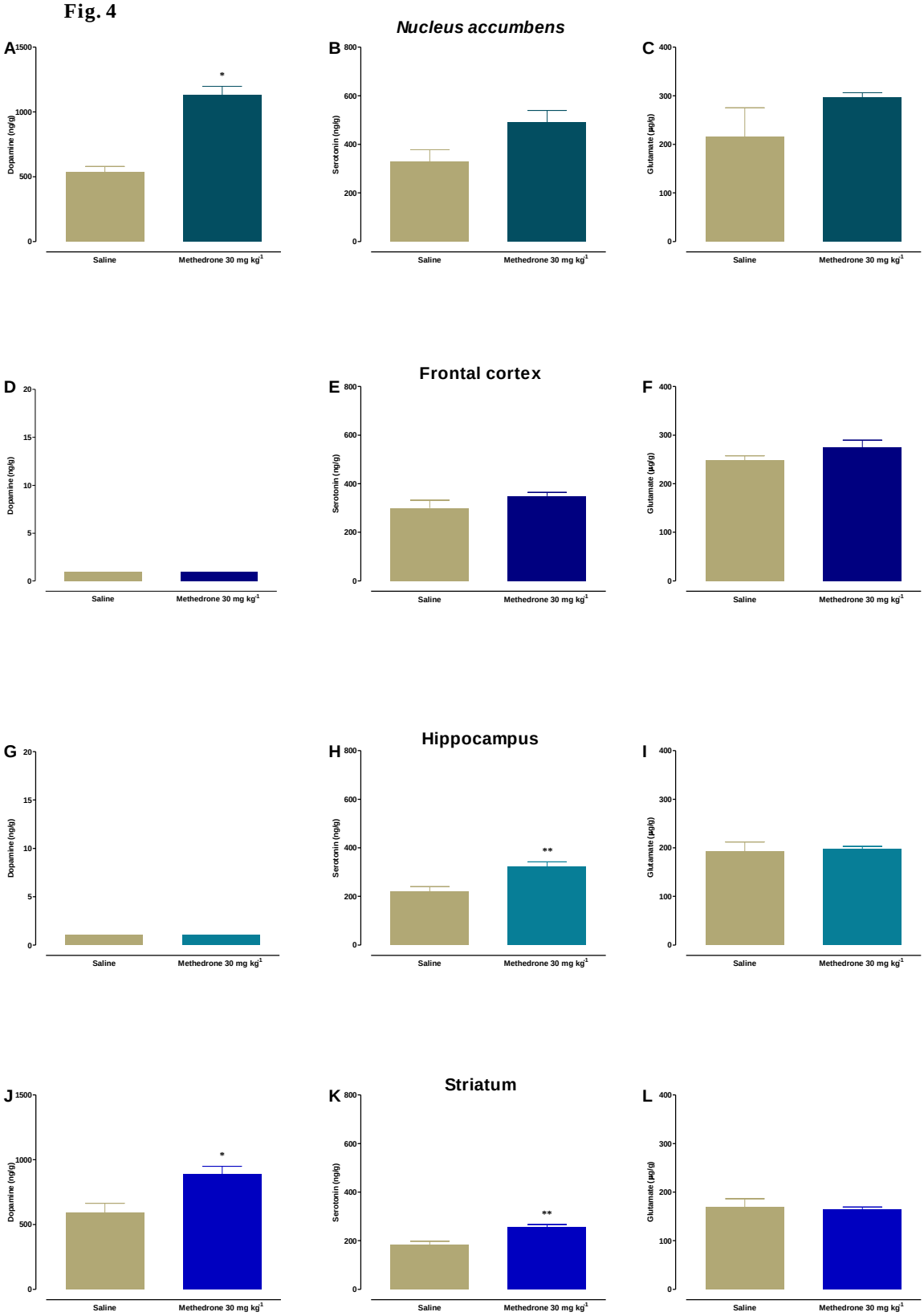

Nucleus accumbens
Frontal cortex
Hippocampus
Striatum
A
B
C
D
E
F
G
H
I
J
K
L
Dopamine (ng/g)
Serotonin (ng/g)
Glutamate (µg/g)
Saline
Methedrone 30 mg kg-1
*
**

Fig. 5

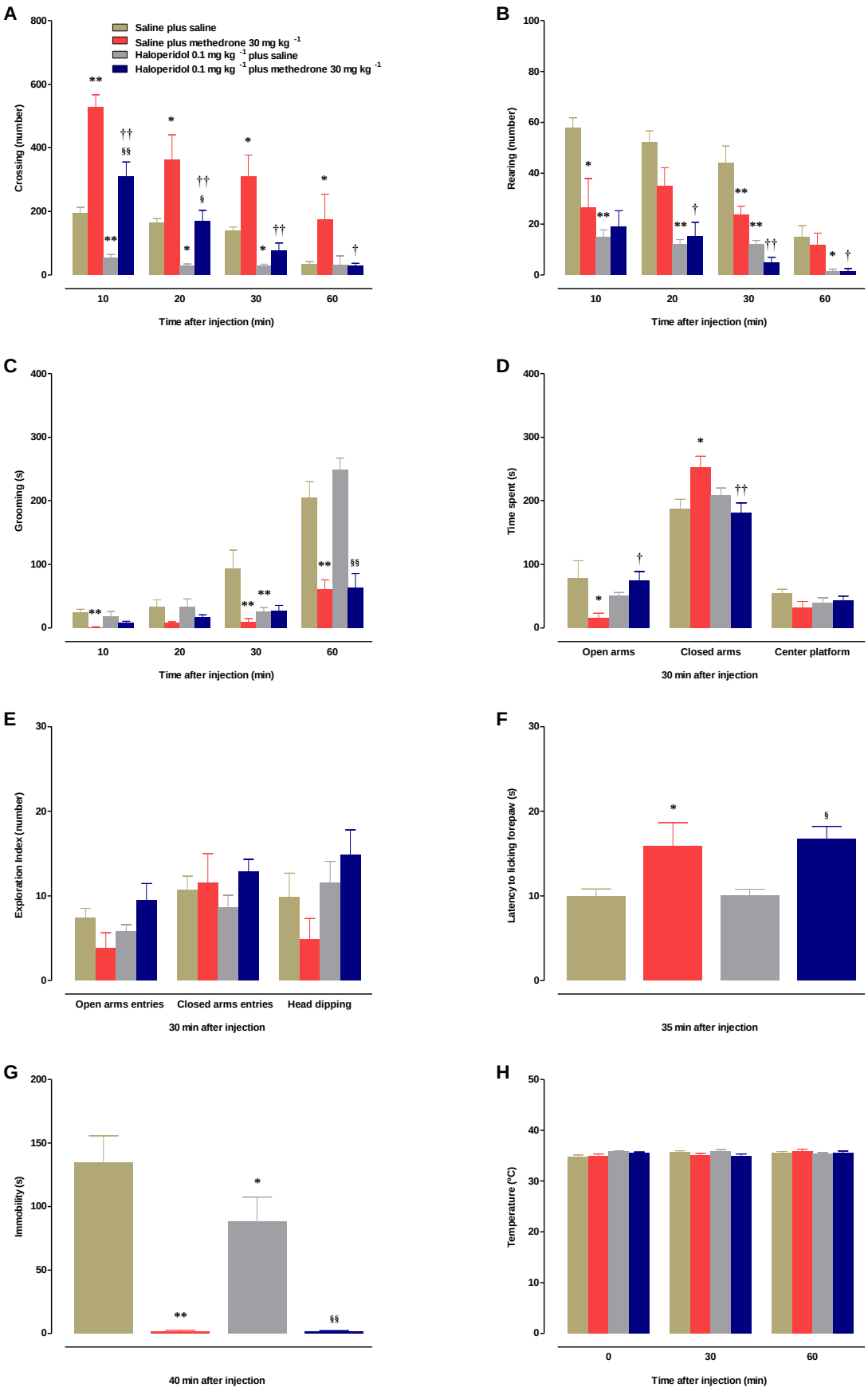

A
Saline plus saline
Saline plus methedrone 30 mg kg -1
Haloperidol 0.1 mg kg -1 plus saline
Haloperidol 0.1 mg kg -1 plus methedrone 30 mg kg -1
Crossing (number)
Time after injection (min)
B
Rearing (number)
Time after injection (min)
C
Grooming (s)
Time after injection (min)
D
Time spent (s)
Open arms
Closed arms
Center platform
30 min after injection
E
Exploration Index (number)
Open arms entries
Closed arms entries
Head dipping
30 min after injection
F
Latency to licking forepaw (s)
35 min after injection
G
Immobility (s)
40 min after injection
H
Temperature (°C)
Time after injection (min)

Fig. 6

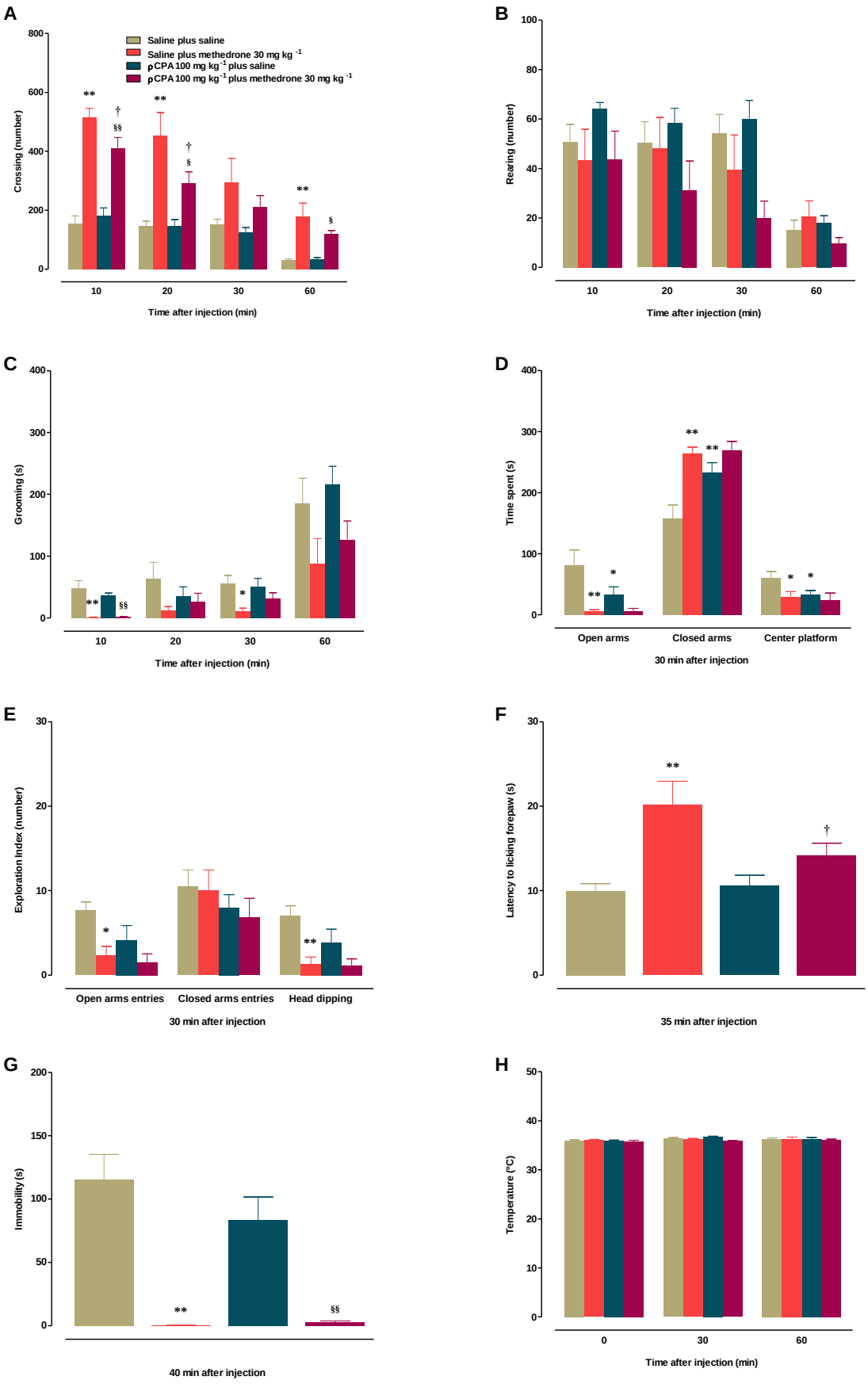

A
Saline plus saline
Saline plus methedrone 30 mg kg -1
ρCPA 100 mg kg -1 plus saline
ρCPA 100 mg kg -1 plus methedrone 30 mg kg -1
Crossing (number)
Time after injection (min)
B
Rearing (number)
Time after injection (min)
C
Grooming (s)
Time after injection (min)
D
Time spent (s)
Open arms
Closed arms
Center platform
30 min after injection
E
Exploration Index (number)
Open arms entries
Closed arms entries
Head dipping
30 min after injection
F
Latency to licking forepaw (s)
35 min after injection
G
Immobility (s)
40 min after injection
H
Temperature (°C)
Time after injection (min)

Fig. 7

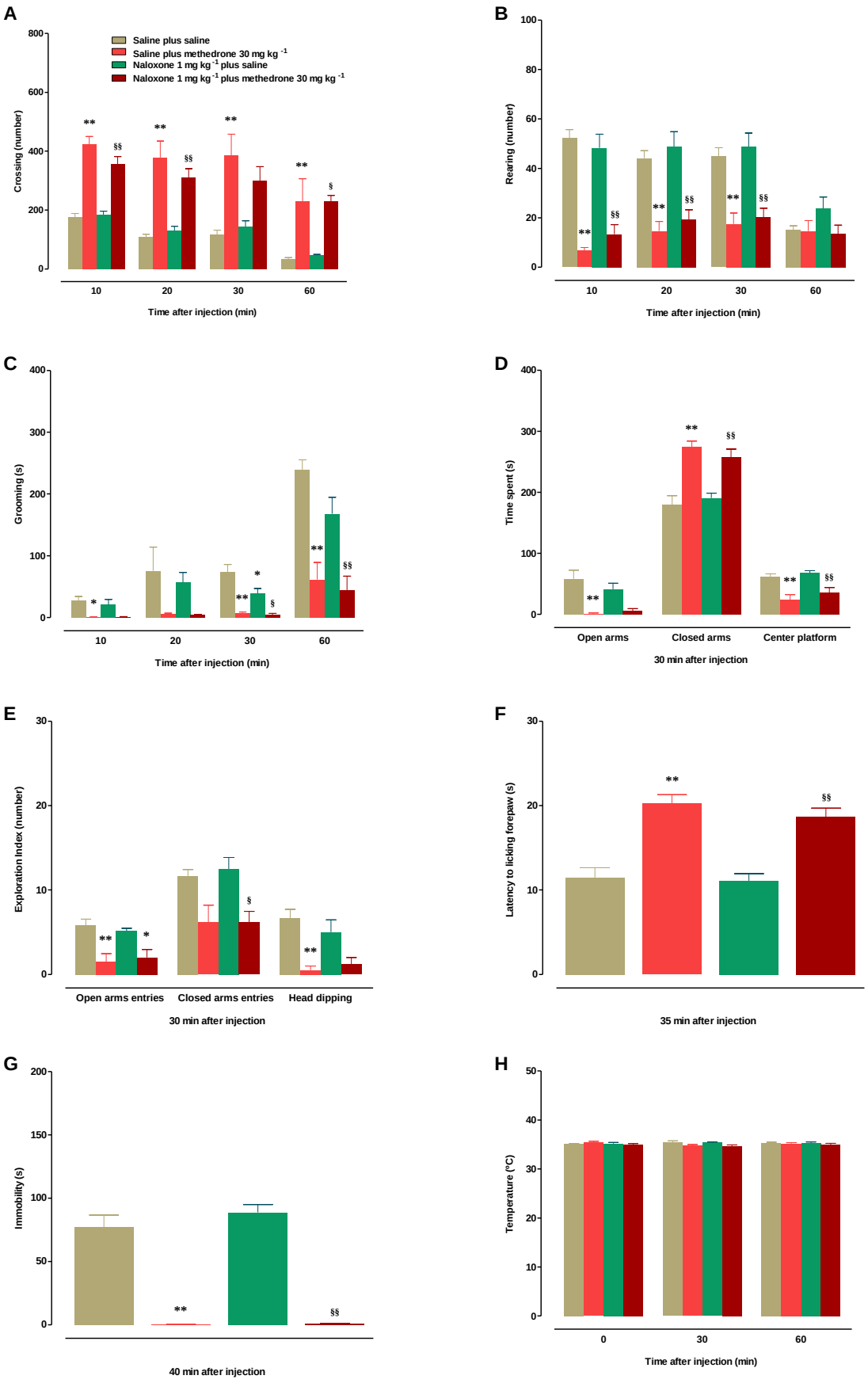

A
Saline plus saline
Saline plus methedrone 30 mg kg -1
Naloxone 1 mg kg -1 plus saline
Naloxone 1 mg kg -1 plus methedrone 30 mg kg -1
Crossing (number)
Time after injection (min)
B
Rearing (number)
Time after injection (min)
C
Grooming (s)
Time after injection (min)
D
Time spent (s)
Open arms
Closed arms
Center platform
30 min after injection
E
Exploration Index (number)
Open arms entries
Closed arms entries
Head dipping
30 min after injection
F
Latency to licking forepaw (s)
35 min after injection
G
Immobility (s)
40 min after injection
H
Temperature (°C)
Time after injection (min)

Fig. 8

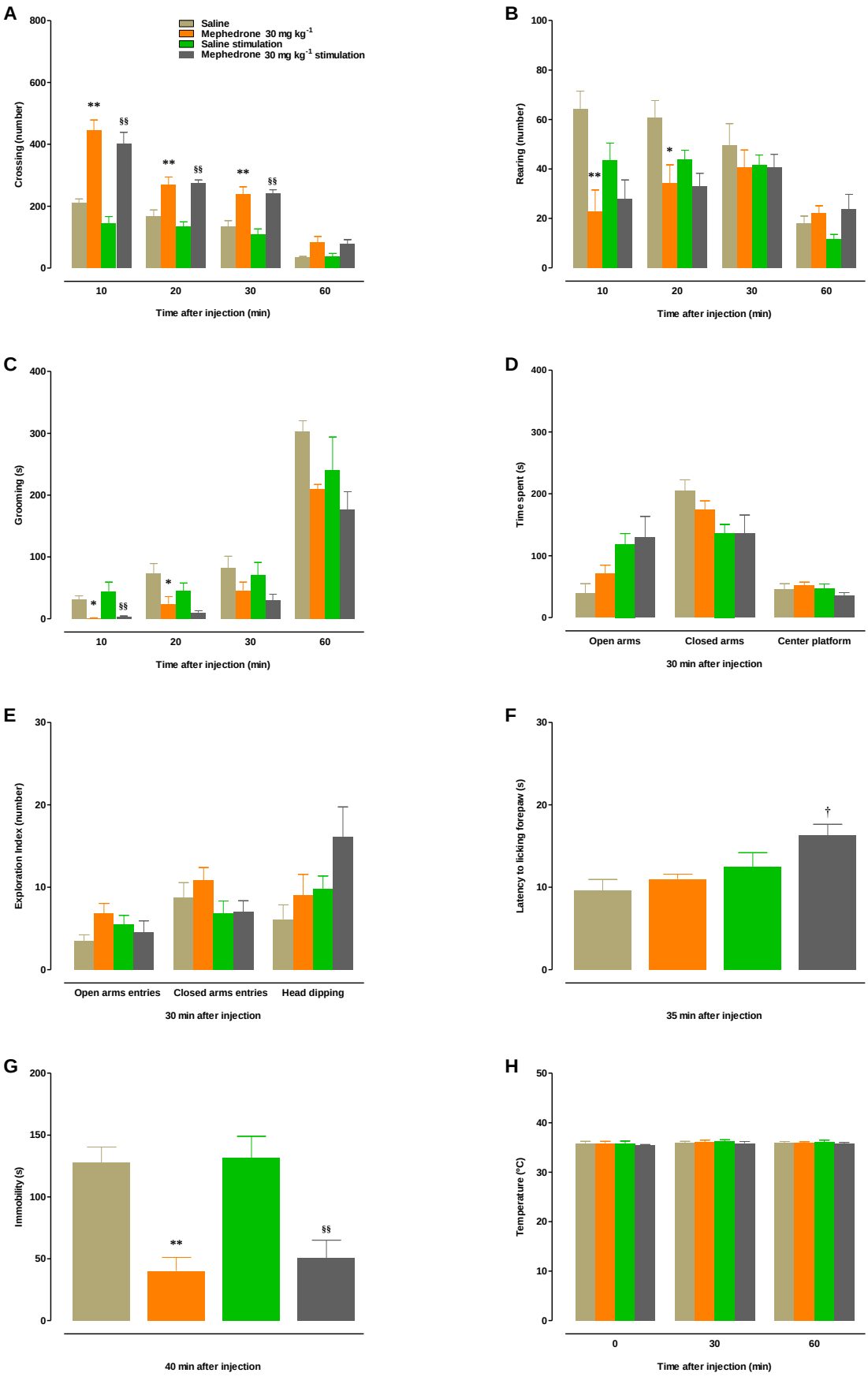

A
Saline
Mephedrone 30 mg kg-1
Saline stimulation
Mephedrone 30 mg kg-1 stimulation
Crossing (number)
Time after injection (min)
B
Rearing (number)
Time after injection (min)
C
Grooming (s)
Time after injection (min)
D
Time spent (s)
Open arms
Closed arms
Center platform
30 min after injection
E
Exploration Index (number)
Open arms entries
Closed arms entries
Head dipping
30 min after injection
F
Latency to licking forepaw (s)
35 min after injection
G
Immobility (s)
40 min after injection
H
Temperature (°C)
Time after injection (min)

Fig. 9

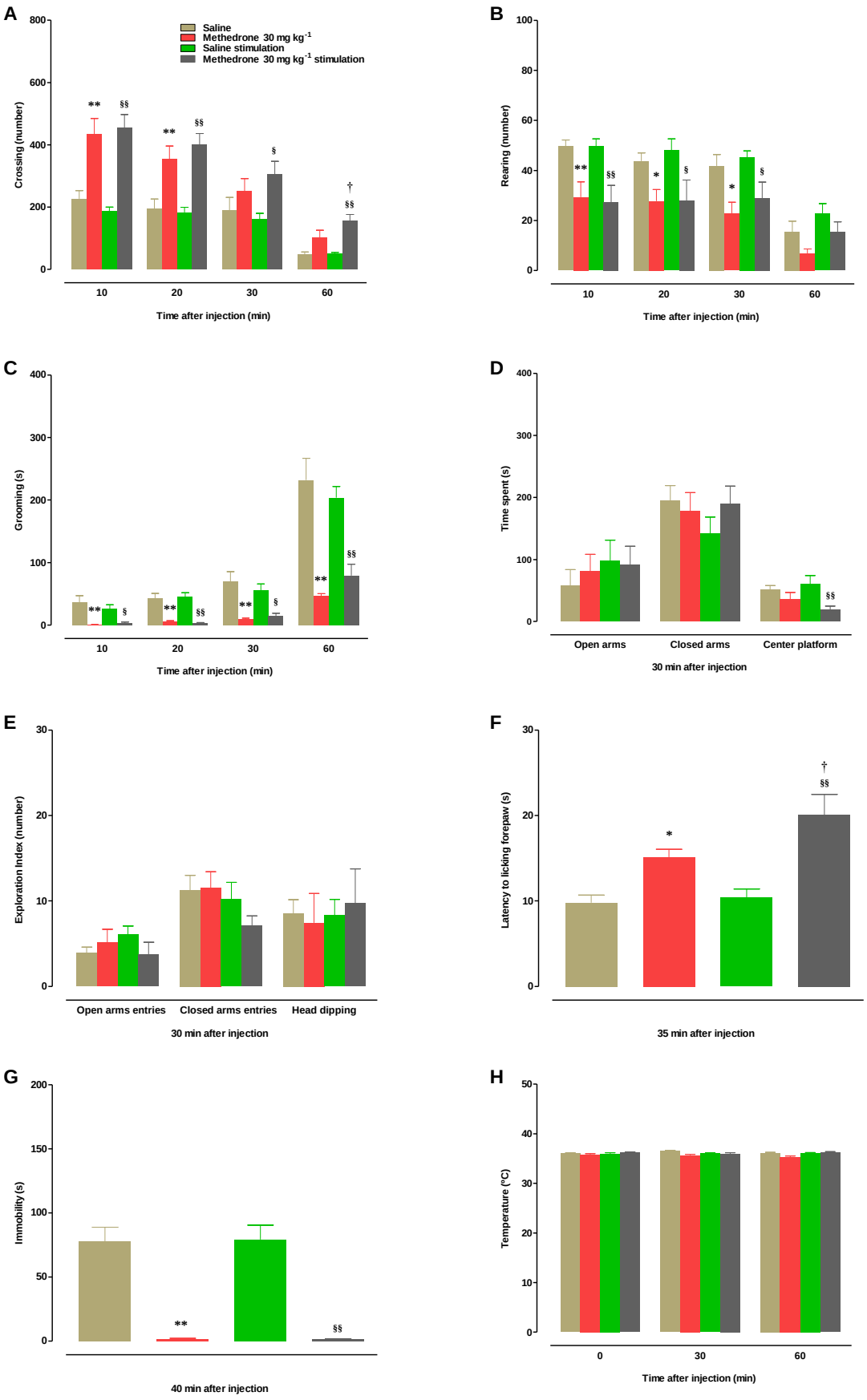
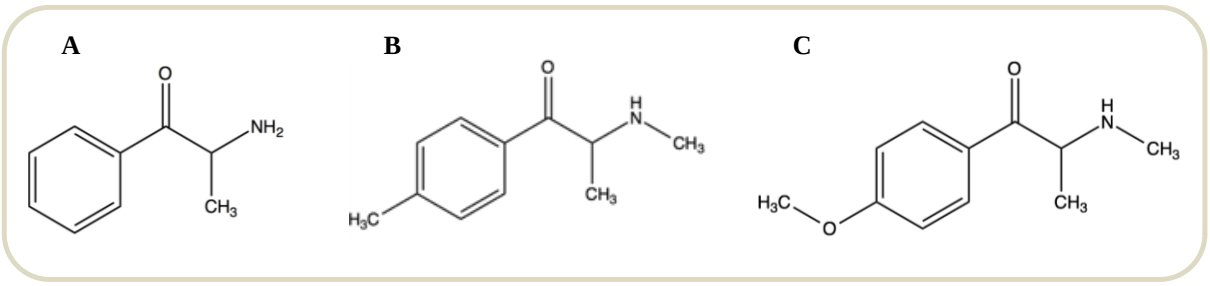
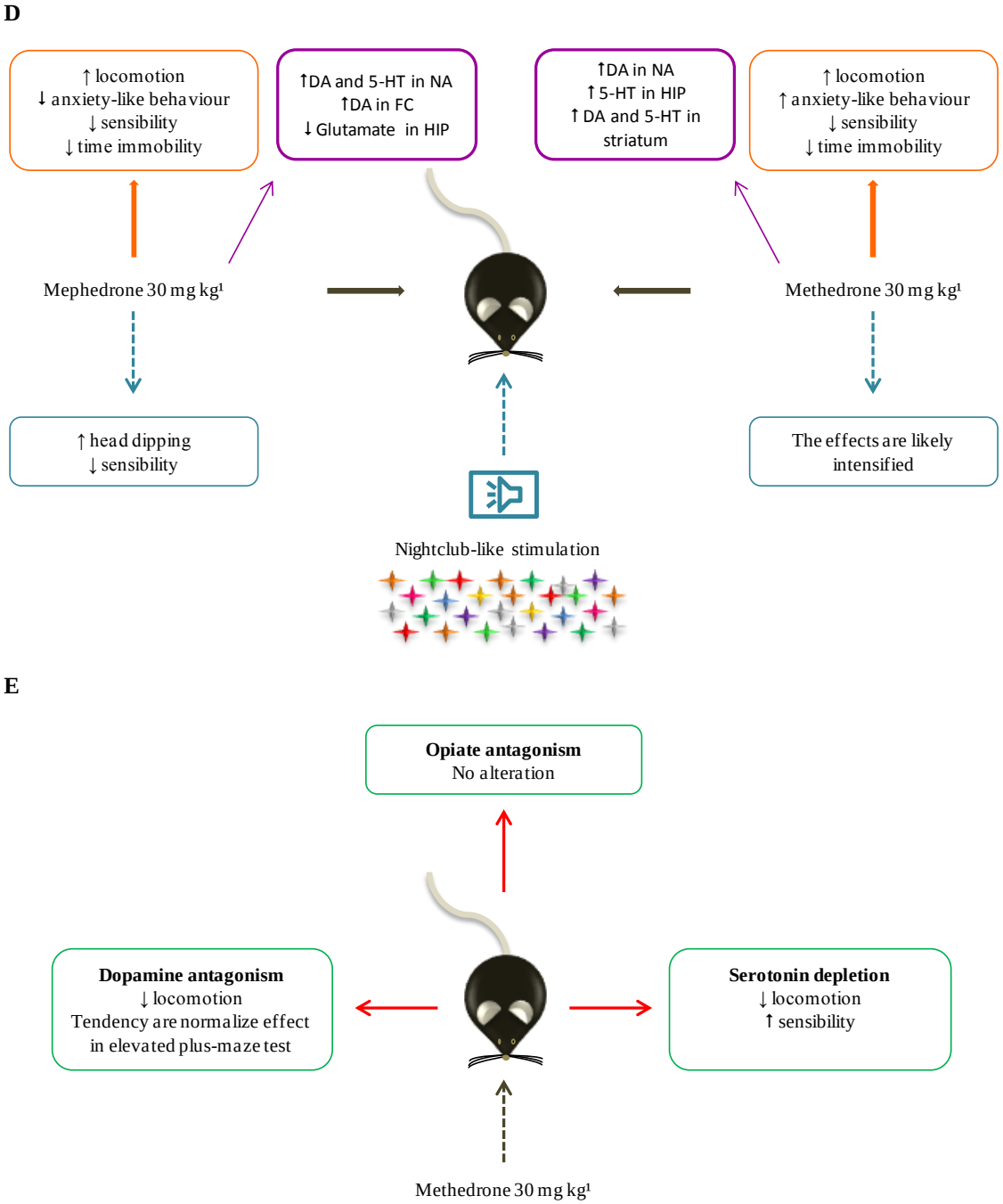

A
Saline
Methedrone 30 mg kg-1
Saline stimulation
Methedrone 30 mg kg-1 stimulation
Crossing (number)
Time after injection (min)
B
Rearing (number)
Time after injection (min)
C
Grooming (s)
Time after injection (min)
D
Time spent (s)
Open arms
Closed arms
Center platform
30 min after injection
E
Exploration Index (number)
Open arms entries
Closed arms entries
Head dipping
30 min after injection
F
Latency to licking forepaw (s)
35 min after injection
G
Immobility (s)
40 min after injection
H
Temperature (°C)
Time after injection (min)

Fig. 10


A
B
C


D
↑ locomotion
↓ anxiety-like behaviour
↓ sensibility
↓ time immobility
↑DA and 5-HT in NA
↑DA in FC
↓ Glutamate in HIP
↑DA in NA
↑ 5-HT in HIP
↑ DA and 5-HT in striatum
↑ locomotion
↑ anxiety-like behaviour
↓ sensibility
↓ time immobility
Mephedrone 30 mg kg¹
Methedrone 30 mg kg¹
↑ head dipping
↓ sensibility
The effects are likely intensified
Nightclub-like stimulation
E
Opiate antagonism
No alteration
Dopamine antagonism
↓ locomotion
Tendency are normalize effect in elevated plus-maze test
Serotonin depletion
↓ locomotion
↑ sensibility
Methedrone 30 mg kg¹

Supporting Information
Comparative Pharmacological Evaluation of the Cathinone Derivatives, Mephedrone and Methedrone, in Mice: Impacts of Environmental Conditioning

P B Pail[1], K M Costa[2], C E Leite[3] and M M Campos[2,3,4]

[1]Postgraduate Program in Cellular and Molecular Biology; [2]Postgraduate Program in Medicine and Health Sciences; [3]Institute of Toxicology and Pharmacology; [4]Faculty of Dentistry, Pontifical Catholic University of Rio Grande do Sul, Porto Alegre/RS, Brazil

INDEX

Figures

Figure S1 Apparatus for nightclub-like stimulation. **(A-B)** The apparatus consisted in an adapted animal cage covered by a coloured-shiny paper, with a pair of sound boxes and a thermometer. **(C-D)** The lights for stimulation were adapted in another cage covered by a series of multi-coloured light bulbs.

Figure S2 Behavioural effects of a single administration of mephedrone in additional time-points. **(A)** Number of lines crossed, **(B)** rearing number, and **(C)** grooming in the open-field test; **(D)** grooming in the glass cylinders; **(E)** latency to licking forepaw in the hot-plate test; **(F)** time spent (s) in the open arms, closed arms and centre platform of elevated plus-maze; **(G)** exploration index: number of entries in the open and closed arms, and head-dipping number in the elevated plus-maze; **(H)** rectal temperature expressed in °C.

Figure S3 Time-related behavioural effects of nightclub-like stimulation. **(A)** Number of lines crossed, **(B)** rearing number, and **(C)** grooming in the open-field test; **(D)** time spent (s) in the open arms, closed arms and centre platform of elevated plus-maze; **(E)** exploration index: number of entries in the open and closed arms, and head-dipping number in the elevated plus-maze; **(F)** latency to licking forepaw in the hot-plate test; **(G)** immobility time (s) in the tail suspension test; **(H)** rectal temperature expressed in °C.

Figure S4 Haematological analysis, and oxidative stress in liver and heart. Effects of mephedrone **(A)** and methedrone **(B)** on the total blood cell counts. Catalase activity in heart **(C-D)** and liver **(E-F)**; the MDA levels in heart **(G-H)** and liver **(I-J).**

A. Liver and heart oxidative stress

1. Preparation of homogenates

The tissue homogenate (10% w/v) was prepared in saline. The sample was centrifuged and, the supernatant was used for the analysis.

2. MDA levels

MDA levels in liver and heart were measured as described by (Boeira *et al*., 2011). Alkaline hydrolysis of protein-bound malondialdehyde (MDA) was achieved by incubating this mixture in a 60°C water bath for 30 min, followed by thiobarbituric acid (39.9 mM, 250 µl) and phosphoric acid (440 mM, 750 µl) addition and incubation in a 95°C water bath for 60 min. The samples were injected into a high-performance liquid chromatograph equipped with ultraviolet detector (Agilent Technologies® Inc., USA). The protein content in the supernatant was determined with a commercial kit (Labtest®). Lipid peroxidation was calculated from the standard curve using the 1,1,3,3- tetraethoxy propane (97%) and expressed as nmol mg protein^{-1}.

3. Catalase activity

Catalase activity in liver and heart was measured as described by (Leite *et al*., 2010). The decomposition of H_2O_2 can be followed directly by the decrease in absorbance at 240 nm (e240 = 0.0394 ± 0.0002 L per mmol L)1 per cm)1). One catalase unit is defined as the enzyme concentration required for the decomposition of 1 lmol of H2O2 per min at 25 C. All assay solutions were prepared at room temperature, as described by Aebi (1984). The complete reaction system for catalase consisted of 0.1 mmol L)1 phosphate buffer, pH 7.4, and 10 mmol L)1 H2O2. The reaction was initiated by the addition of 10 mmol L)1 H2O2, and absorbance was monitored for 2 min at 240 nm.

B. Hematologic parameters

After the end of the experiments, the animals were euthanized and the blood was collected. Immediately after, a small drop of blood was taken for smear evaluation (Pilny, 2008), using May-Grunewald-Giemsa staining. Differential counts (neutrophils, eosinophils, basophils, lymphocytes, monocytes, and immature cells) were estimated under a ×40 objective (Olympus® CH30 model), by counting 100 cells.

Figure S1 The nightclub-like apparatus used for animal stimulation during 1 h, once a day, for 7 days. **(A-B)** The apparatus consisted in an adapted animal cage (20 x 40 x 30 cm) covered by a coloured-shiny paper, with a pair of sound boxes and a thermometer. **(C-D)** The lights for stimulation were adapted in another cage (12 x 27 X 16 cm) covered by a series of multi-coloured light bulbs.

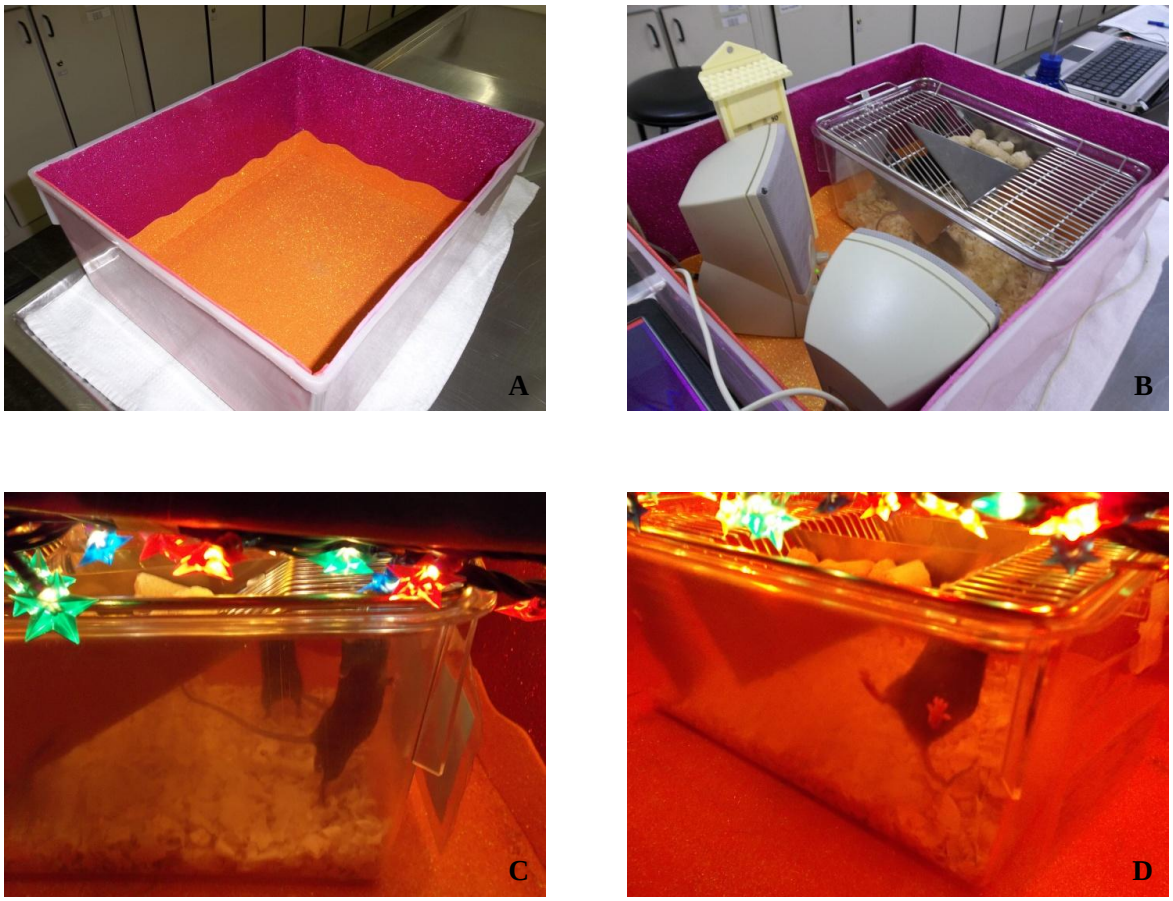


Figure S2 The behavioural effects of a single administration of mephedrone in additional time-points. **(A)** The number of lines crossed, **(B)** rearing numbers, and **(C)** grooming in the open-field test; **(D)** the grooming in the glass cylinders; **(E)** a latency to licking the forepaws in the hot-plate test; **(F)** the time spent (s) in the open-arms, the closed-arms, and the centre platform, of the elevated plus-maze; **(G)** the exploration index: the number of entries into the open-arms, the closed-arms, and the head-dipping numbers in the elevated plus-maze; **(H)** rectal temperature expressed in °C. Data is expressed as the mean ± SEM of 5-17 mice per group, from two separate experiments. **P < 0.01, was significantly different from the control group (unpaired Student's *t*-test).

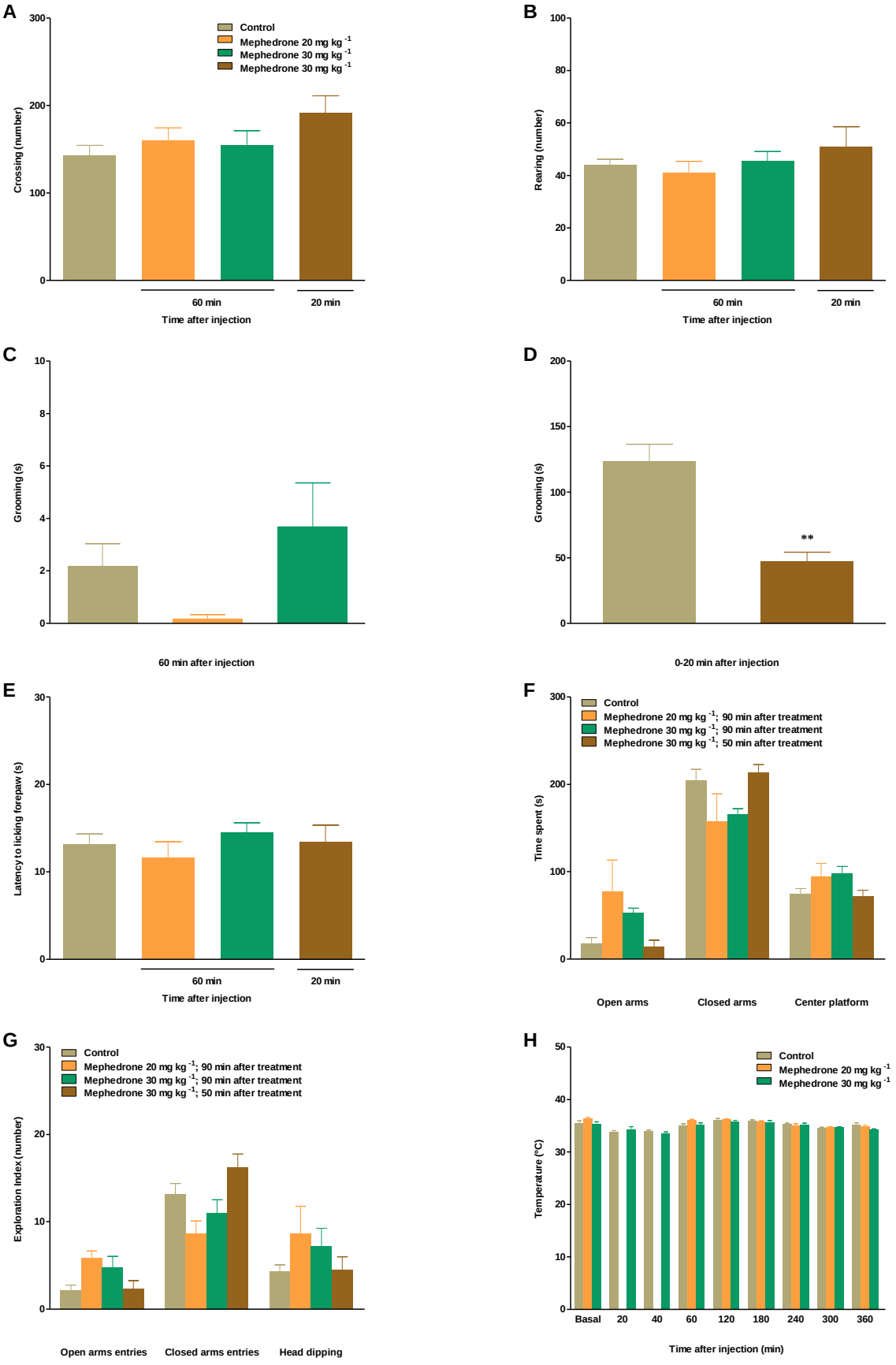

A
Control
Mephedrone 20 mg kg -1
Mephedrone 30 mg kg -1
Mephedrone 30 mg kg -1
Crossing (number)
300
200
100
0
60 min
20 min
Time after injection
B
Rearing (number)
100
80
60
40
20
0
60 min
20 min
Time after injection
C
Grooming (s)
10
8
6
4
2
0
60 min after injection
D
Grooming (s)
200
150
100
50
0
**
0-20 min after injection
E
Latency to licking forepaw (s)
30
20
10
0
60 min
20 min
Time after injection
F
Control
Mephedrone 20 mg kg -1; 90 min after treatment
Mephedrone 30 mg kg -1; 90 min after treatment
Mephedrone 30 mg kg -1; 50 min after treatment
Time spent (s)
300
200
100
0
Open arms
Closed arms
Center platform
G
Control
Mephedrone 20 mg kg -1; 90 min after treatment
Mephedrone 30 mg kg -1; 90 min after treatment
Mephedrone 30 mg kg -1; 50 min after treatment
Exploration Index (number)
30
20
10
0
Open arms entries
Closed arms entries
Head dipping
H
Control
Mephedrone 20 mg kg -1
Mephedrone 30 mg kg -1
Temperature (°C)
50
40
30
20
10
0
Basal
20
40
60
120
180
240
300
360
Time after injection (min)

Figure S3 Time-related behavioural effects of a nightclub-like stimulation. (A) The number of lines crossed, (B) rearing numbers, and (C) grooming (s) in the open-field test; (D) the time spent (s) in the open-arms, closed-arms, and the centre platform, of the elevated plus-maze; (E) the exploration index: the number of entries into the open and closed-arms, and the head-dipping numbers in the elevated plus-maze; (F) a latency to licking the forepaws in the hot-plate test; (G) the immobility time (s) in the tail suspension test; (H) rectal temperature is expressed in °C. Data is expressed as the mean ± SEM of 8 mice per group. $^{**}P < 0.01$, was significantly different from the control group (unpaired Student's *t*-test).

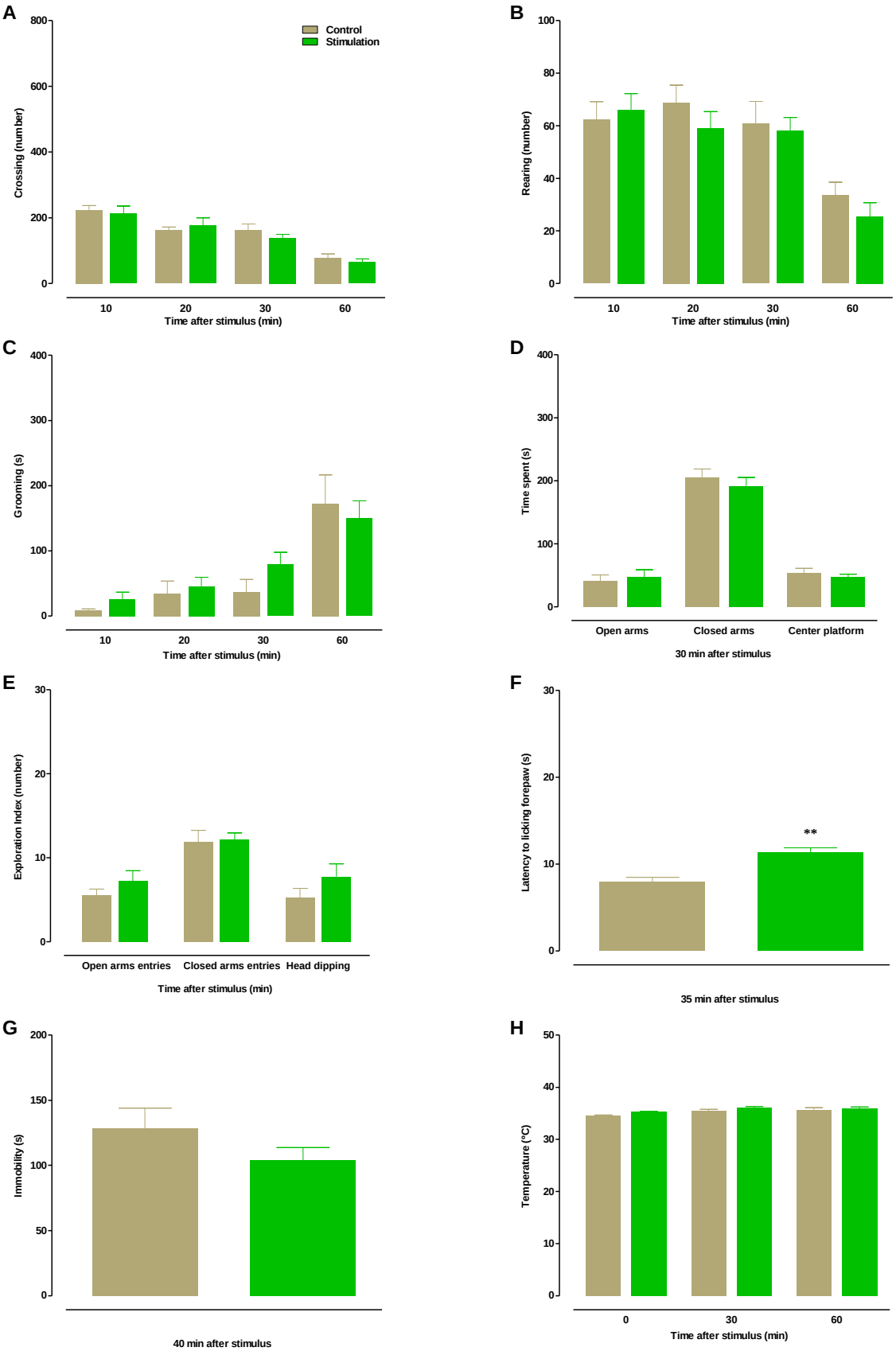
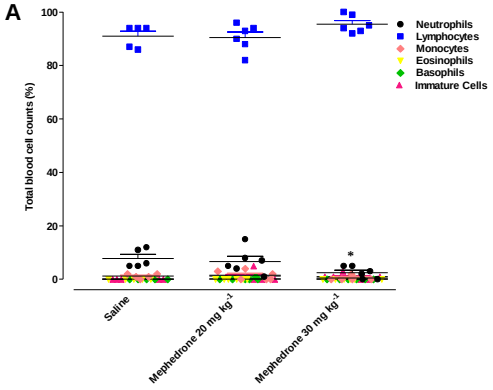
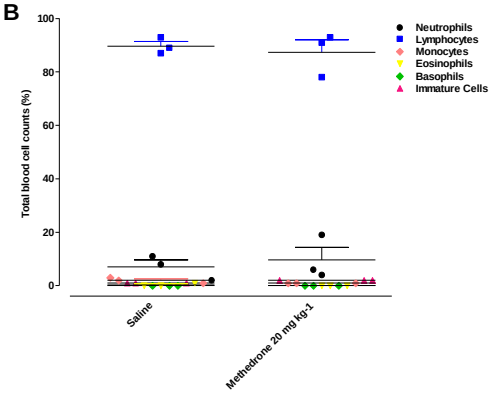
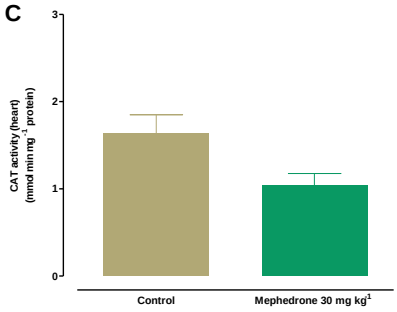
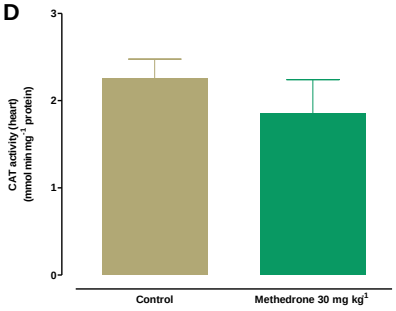

A
Control
Stimulation
Crossing (number)
800
600
400
200
0
10
20
30
60
Time after stimulus (min)
B
Rearing (number)
100
80
60
40
20
0
10
20
30
60
Time after stimulus (min)
C
Grooming (s)
400
300
200
100
0
10
20
30
60
Time after stimulus (min)
D
Time spent (s)
400
300
200
100
0
Open arms
Closed arms
Center platform
30 min after stimulus
E
Exploration index (number)
30
20
10
0
Open arms entries
Closed arms entries
Head dipping
Time after stimulus (min)
F
Latency to licking forepaw (s)
30
20
10
0
**
35 min after stimulus
G
Immobility (s)
200
150
100
50
0
40 min after stimulus
H
Temperature (°C)
50
40
30
20
10
0
0
30
60
Time after stimulus (min)

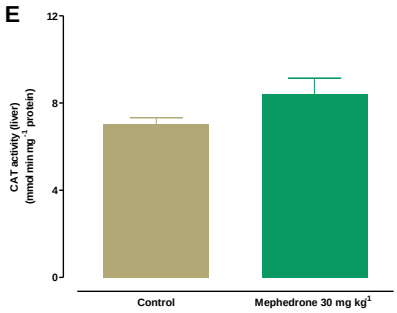
Figure S4 The haematological analysis, and oxidative stress in liver and heart. Effects of mephedrone **(A)** and methedrone **(B)** on the total blood cell counts; the samples were collected 6 h after treatment with cathinones, *n*=3-5 per group. Catalase activity in heart **(C-D)** and liver **(E-F)**; MDA levels in heart **(G-H)** and liver **(I-J);** all the samples were collected 20 min after treatment with cathinones. Data is expressed as the mean ± SEM of 3-5 mice per group (one-way ANOVA followed by Bonferroni's post-hoc Test).

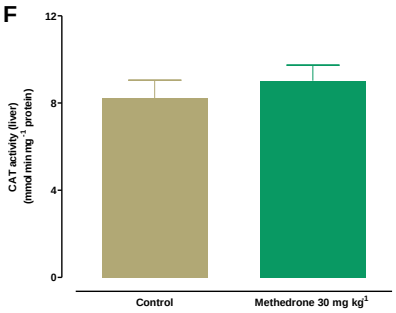

A
Total blood cell counts (%)
Neutrophils
Lymphocytes
Monocytes
Eosinophils
Basophils
Immature Cells
Saline
Mephedrone 20 mg kg-1
Mephedrone 30 mg kg-1

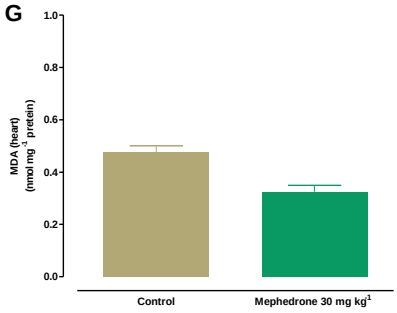

B
Total blood cell counts (%)
Neutrophils
Lymphocytes
Monocytes
Eosinophils
Basophils
Immature Cells
Saline
Methedrone 20 mg kg-1

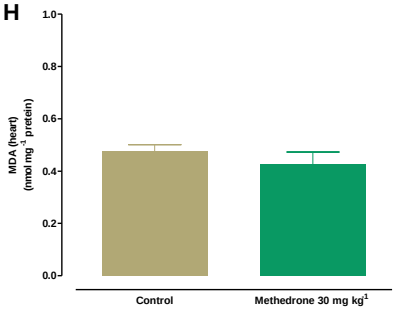

C
CAT activity (heart)
(mmol min mg-1 protein)
Control
Mephedrone 30 mg kg-1

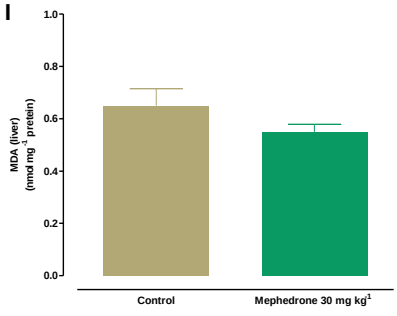

D
CAT activity (heart)
(mmol min mg-1 protein)
Control
Methedrone 30 mg kg-1

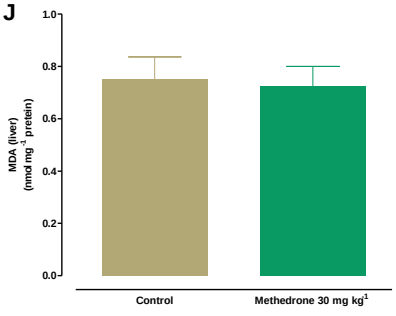

E
CAT activity (liver)
(mmol min mg-1 protein)
Control
Mephedrone 30 mg kg-1


F
CAT activity (liver)
(mmol min mg-1 protein)
Control
Methedrone 30 mg kg-1


G
MDA (heart)
(nmol mg-1 pretein)
Control
Mephedrone 30 mg kg-1


H
MDA (heart)
(nmol mg-1 pretein)
Control
Methedrone 30 mg kg-1


I
MDA (liver)
(nmol mg-1 pretein)
Control
Mephedrone 30 mg kg-1


J
MDA (liver)
(nmol mg-1 pretein)
Control
Methedrone 30 mg kg-1

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4 CONCLUSÕES

O desenvolvimento de novos derivados sintéticos da catinona está ligado a um mercado ilícito em ascensão, representando o terceiro maior grupo dentre as *designer drugs*. Entretanto, embora derivem do mesmo princípio ativo, tenham estruturas químicas similares e sejam substâncias estimulantes do SNC, seus efeitos podem ser distintos. Conforme demonstram os resultados dessa pesquisa, a mefedrona e a metedrona alteram de forma marcante e similar o comportamento de camundongos fêmeos, aumentando o número de quadrantes cruzados no teste de campo aberto e a latência para responder a estímulo térmico. Em relação à ansiedade, estas substâncias apresentaram reações opostas no teste do labirinto em cruz elevado, a mefedrona foi capaz de desencadear comportamento do tipo ansiolítico e, a metedrona, comportamento ansiogênico; outras alterações foram observadas exclusivamente nos animais tratados com metedrona, como a piloereção e tremores, salientando as semelhanças e as diferenças entre esses derivados da catinona.

Corroborando os dados comportamentais, suas ações no cérebro ocorrem em regiões relacionadas ao comportamento, desencadeando alterações nos níveis de 5-HT e DA no *nucleus accumbens*, com ambos os derivados. A mefedrona, na dose de 30 mg/kg, desencadeou aumento da atividade dopaminérgica no córtex frontal e diminuição glutamatérgica no hipocampo. A metedrona, na mesma dose, por outro lado, provocou aumento dos níveis de 5-HT no hipocampo, e DA e 5-HT no estriado, 20 min após a administração, demonstrando rápida ação para ambas as substâncias. As regiões ativadas por estas substâncias estão relacionadas com o comportamento e com o sistema de recompensa, desencadeando alterações marcantes, que levam os usuários a atos que podem prejudicar a si mesmos e a outras pessoas, como a dependência, agressividade, paranoia, ansiedade, entre outros comportamentos pouco esclarecidos, tais como os efeitos em longo prazo.

Após a realização de experimentos adicionais para melhor caracterizar os mecanismos de ação da metedrona, conclui-se que esse derivado aumenta a locomoção devido à atividade dopaminérgica e serotoninérgica. O antagonista não seletivo para os receptores D_1 e D_2, haloperidol, reverteu o efeito ansiogênico, ao passo que o inibidor da síntese de 5-HT, ρCPA, normalizou a latência de resposta ao estímulo térmico. Outros comportamentos apresentados pelos animais tratados com metedrona, como o número de *rearings* e o tempo de *grooming*, não foram alterados por nenhuma das ferramentas farmacológicas avaliadas. O tempo de imobilidade dos camundongos no teste de suspensão da cauda também não foi revertido. Entretanto, com base na literatura (Lockridge *et al*., 2013), acredita-se que possa ter

um possível envolvimento noradrenérgico, devido ao comportamento de “luta” do animal, salientando a importância de mais estudos para melhor explicar seu mecanismo de ação. Em consequência da proibição da comercialização dos derivados da catinona durante a condução deste projeto, não foi possível a realização de experimentos que visassem o bloqueio da transmissão dopaminérgica e serotoninérgica com o a mefedrona, ou noradrenérgica com a metedrona.

Como mencionado anteriormente, as *designer drugs* são utilizadas indevidamente com fins recreacionais em ambientes que podem alterar o comportamento dos usuários, bem como, podem potencializar os efeitos de determinadas SPA. Nas condições utilizadas para indução de estímulos semelhantes à *nightclub*, observaram-se algumas alterações, em especial, a potencialização dos efeitos da mefedrona e metedrona. Estes poderiam ser diferentes se as condições fossem outras, como, por exemplo, com estímulos mais intensos do que os adotados no protocolo desta pesquisa. Salienta-se que o ambiente frequentado pelos usuários consiste em uma série de estímulos estressantes, tais como a alta temperatura do local, às vezes com pouca ventilação, luzes estroboscópicas, consumo indevido de álcool, grande quantidade de pessoas em conjunto com música alta, com aumento de movimentos (aumentando ainda mais sua temperatura), podendo acentuar os efeitos danosos das substâncias consumidas. Logo, devido ao aumento do uso de substâncias psicoativas sintéticas, seu risco à saúde pública e ao número de novas substâncias desenvolvidas e comercializadas, tornam-se necessárias mais pesquisas acerca de seus mecanismos de ações e efeitos desencadeados, em nível fisiológico, bioquímico, comportamental e psíquico.

Com base nos resultados deste trabalho, destacam-se as particularidades entre os derivados sintéticos da catinona e seu risco à saúde. Ambos os derivados alteraram de forma marcante o comportamento de camundongos nas condições testadas, da mesma forma que alteraram significativamente os níveis de neurotransmissores em determinadas regiões do cérebro 20 min após a aplicação. O local frequentado pelos usuários pode ser um fator adicional para o maior risco dessas substâncias, devendo ser considerado em pesquisas que busquem melhor caracterização dos efeitos de SPA utilizadas de modo recreacional. Por fim, este trabalho fornece à comunidade científica mais conhecimento concernente às alterações comportamentais, bioquímicas e fisiológicas desencadeadas pelos derivados sintéticos da catinona, mefedrona e metedrona.

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ANEXO – CARTA DE APROVAÇÃO DO CEUA

Pontifícia Universidade Católica do Rio Grande do Sul
PRÓ-REITORIA DE PESQUISA, INOVAÇÃO E DESENVOLVIMENTO
COMISSÃO DE ÉTICA NO USO DE ANIMAIS

Ofício 43/13 - CEUA

Porto Alegre, 21 de junho de 2013.

Senhora Pesquisadora,

A Comissão de Ética no Uso de Animais da PUCRS apreciou e aprovou seu Protocolo de Pesquisa, registro CEUA 13/00336, **"Comparação dos efeitos dos derivados das catinonas, metedrona e mefedrona, em camundongos: determinação dos efeitos comportamentais e bioquímicos"**.

Sua investigação está autorizada a partir da presente data.

Lembramos que é necessário o encaminhamento de relatório final quando finalizar esta investigação.

Atenciosamente,

Prof. Dr. João Batista Blessmann Weber
Coordenador da CEUA/PUCRS

Ilma. Sra.
Profª. Maria Martha Campos
FABIO
Nesta Universidade