to 2 ml with type I ultrapure water and heated at 95 °C for 90 min in a water bath using a glass ball as a condenser. After cooling to room temperature, absorbance was measured in the supernatant at 532 nm [29].

2.5. Protein carbonyl assay ex vivo

Immediately after the behavioral evaluation, the animals were killed by decapitation and had their forebrain removed. Tissues were homogenized in 10 volumes (w/v) of 10 mM Tris–HCl buffer, pH 7.4, using a glass homogenizer and its carbonyl protein content was determined by the method described by Yan et al. [44], adapted for brain tissue, as below. Briefly, homogenates were diluted to 750–800 μg/ml of protein in each sample, and 1 ml of aliquots was mixed with 0.2 ml of 2,4-dinitrophenylhydrazine (DNPH, 10 mM) or 0.2 ml HCl (2 M). After incubation at room temperature for 1 h in a dark ambient, 0.6 ml of denaturing buffer (150 mM sodium phosphate buffer, pH 6.8, containing 3% SDS), 1.8 ml of heptane (99.5%) and 1.8 ml of ethanol (99.8%) were added sequentially, and mixed with vortex agitation for 40 s and centrifuged for 15 min. Next, the protein isolated from the interface was washed two times with 1 ml of ethyl acetate/ethanol 1:1 (v/v) and suspended in 1 ml of denaturing buffer. Each DNPH sample was read at 370 nm in a Hitachi U-2001 spectrophotometer against the corresponding HCl sample (blank), and total carbonylation calculated using a molar extinction coefficient of 22,000 $M^{-1}cm^{-1}$, as described by Levine et al. [22].

2.6. Protein determination

Protein content was measured by the method of Bradford [4] using bovine serum albumin (1 mg/ml) as standard.

2.7. Statistical analysis

Behavioral and biochemical data were analyzed by one-way or two-way analysis of variance (ANOVA), depending on the experimental design. Post hoc analysis was carried out by the Duncan multiple range test, when appropriate. A P value less than 0.05 was considered significant.

3. Results

Fig. 1A shows the effect of increasing doses of succinate (0.7, 1.0 and 1.7 μmol/5 μl icv) on the content of TBARS in forebrain ex vivo. Statistical analysis (one-way ANOVA followed by Duncan multiple range test) revealed that while the intracerebroventricular administration of 0.7 and 1.0 μmol succinate increased TBARS content compared with the control group ($F_{(3,23)} = 29.76$; $P < 0.0001$), 1.7 μmol succinate had no effect on forebrain TBARS content. The effect of increasing doses of succinate (0.7, 1.0 and 1.7 μmol/5 μl icv) on total protein carbonyl content ex vivo is shown in Fig. 1B. Statistical analysis (one-way ANOVA followed by analysis Duncan multiple range test) revealed that while the intracerebroventricular administration of 0.7 and 1.0 μmol succinate increased total protein carbonyl content compared with the control group ($F_{(3,23)} = 3.26$; $P < 0.05$), 1.7 μmol succinate had no effect on forebrain total protein carbonyl content. The effect of succinate on the locomotor behavior of the animals was also investigated. Statistical analysis (one-way ANOVA followed by Duncan multiple range test) revealed that intracerebroventricular administration of 0.7 and 1.0 μmol succinate decreased the number of areas crossed ($F_{(3,23)} = 7.58$; $P < 0.001$), the number of rearing responses ($F_{(3,23)} = 4.62$; $P < 0.01$) and increased time spent in immobility ($F_{(3,23)} = 13.65$; $P < 0.0001$) compared with the control group (Table 1).

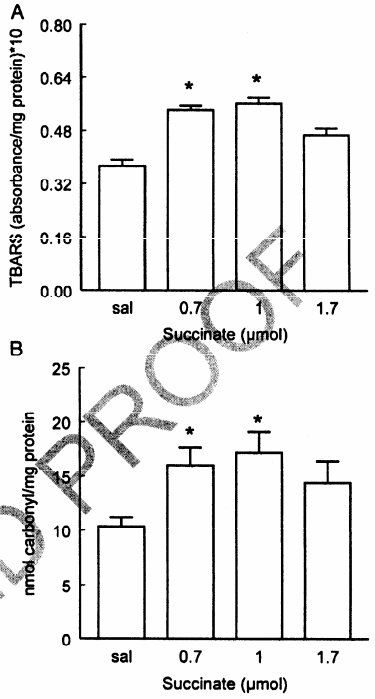

Fig. 1. (A) Effect of succinate administration (icv) on TBARS content ex vivo. *$P < 0.0001$ compared with control by Duncan multiple range test. Data are the means T SEM; $n = 6–7$ animals in each group. (B) Effect of succinate administration (icv) on total protein carbonylation content ex vivo. *$P < 0.05$ compared with control by Duncan multiple range test. Data are the means T SEM; $n = 6–7$ animals in each group.

The involvement of NMDA receptors in the increase of total protein carbonyl content induced by succinate was assessed by co-administrating MK-801 (7 nmol/2.5 μl icv), a noncompetitive NMDA receptor antagonist, with succinate (1.0 μmol/2.5 μl icv). Statistical analysis (two-way ANOVA) of total protein carbonyl content data revealed a

Table 1
Effect of succinate injection (0.7, 1.0 and 1.7 μmol/5 μl icv) on exploratory activity of mice

Treatment	Rearing responses	Number of crossing	Immobility time
0.9% NaCl	39.42 T 12.00	188.42 T 20.47	124.50 T 19.86
succinate (μmol)			
0.7	4.71 T 4.06*	31.57 T 22.99*	516.14 T 1.02#
1.0	7.50 T 2.76*	81.33 T 29.51*	386.66 T 52.90#
1.7	23.00 T 6.44	121.57 T 25.61	171.17 T 77.60

Data are the means T SEM for $n = 6-7$ in each group.
* $P < 0.01$.
# $P < 0.0001$ compared with 0.9% NaCl group (Duncan multiple range test).

significant pretreatment (0.9% NaCl or 1.0 μmol succinate) by treatment (0.9% NaCl or 7 nmol MK-801) interaction ($F_{(3,36)} = 9.545$; $P < 0.01$, Fig. 2), suggesting the involvement of NMDA receptors in the protein carbonylation induced by succinate.

Statistical analysis (two-way ANOVA) of the number of crossing responses and time spent in immobility in the open field revealed a significant pretreatment (0.9% NaCl or 1.0 μmol succinate) by treatment (0.9% NaCl or 7 nmol MK-801) interaction: ($F_{(1,38)} = 4.61$; $P < 0.05$ and $F_{(1,38)} = 4.99$; $P < 0.05$), respectively, since MK-801 prevented the inhibitory effect of succinate on locomotor activity (Table 2).

4. Discussion

In the present study, we showed, for the first time, that the intracerebroventricular administration of succinate decreases exploratory activity and increases forebrain thiobarbituric acid reactive substances (TBARS) and protein carbonyl content biphasically. Interestingly, succinate doses (0.7 and 1.0 μmol), which decreased locomotor activity, also increased the biochemical markers of oxidative damage.

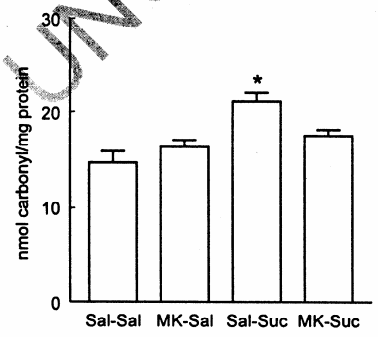

Fig. 2. Effect of MK-801 administration (7 nmol/2.5 μl icv) on succinate-induced (1.0 μmol/2.5 μl) total protein carbonylation content ex vivo. *$P <$ 0.01 compared with 0.9% NaCl plus 0.9% NaCl by Duncan multiple range test. Data are the means T SEM; $n = 10-11$ animals in each group.

Table 2
Effect of MK-801 (7 nmol/2.5 μl icv) on succinate-induced (1 μmol/2.5 μl icv) exploratory behavior in mice

Treatment	Rearing responses	Number of crossing	Immobility time
0.9% NaCl + 0.9% NaCl	27.27 T 4.80	90.45 T 12.80	164.36 T 32.53
0.9% NaCl + succinate	17.30 T 5.38	61.40 T 14.74	249.40 T 40.85
MK-801 + 0.9% NaCl	14.72 T 3.21	71.63 T 14.58	267.45 T 37.89
MK-801 + succinate	20.90 T 8.91	129.40 T 33.59	188.60 T 35.01

Data are the means T SEM for $n = 10-11$ in each group.

MK-801 prevented the increase of total protein carbonylation and the decrease of locomotor activity induced by 1.0 μmol succinate.

The currently reported succinate-induced increase in TBARS production ex vivo suggests that this organic acid causes oxidative damage, because TBA adducts are mainly formed from the reaction of lipid-derived MDA with TBA [29]. Accordingly, succinate increased total protein carbonylation, a relatively specific marker of oxidative damage. These findings strongly suggest that 0.7 and 1.0 μmol succinate induce oxidative damage. However, since we did not test doses of succinate lower than 0.7 μmol, we cannot rule out that even lower amounts of succinate do not cause oxidative damage. Conversely, a high dose of succinate (1.7 μmol) returned TBARS and total protein carbonyl content to control levels, characterizing a biphasic effect of succinate on the studied oxidation markers. The molecular mechanisms underlying the currently reported antioxidant effect of 1.7 μmol succinate (see Figs. 1A and B) are not established, but it is interesting that a similar biphasic effect of succinate on fEPSPs has been reported. While low concentrations (0.3–1.0 mM) of succinate increase neuronal fEPSPs in hippocampal slices by NMDA-mediated mechanisms, high concentrations of succinate (3–10 mM) decrease fEPSPs and reverse the increase of fEPSPs induced by low concentrations of succinate [33]. In that study, the biphasic effect on fEPSPs was attributed to a putative biphasic effect of succinate on the NMDA receptor. Given the marked similar pattern of variation of the currently reported dose–effect curve for succinate and that reported by Roehrs et al. [33], one might propose that they might occur by similar mechanisms, i.e., NMDA receptors. Therefore, we decided to investigate whether NMDA receptors were involved in the succinate-induced oxidative damage. The co-administration of MK-801, an noncompetitive NMDA receptor antagonist, protected against succinate-induced protein carbonylation increase, suggesting the involvement of this subtype of glutamate receptors in the oxidative damage induced by succinate.

One remarkable finding in this study is that succinate-induced oxidative damage was accompanied by significant behavioral alterations, whose pattern of variation along succinate doses coincided with the markers of oxidative damage. More specifically, succinate caused forebrain oxidative damage markers increase, which coincided with

a decrease in locomotor activity. Accordingly, increasing succinate doses returned locomotor activity scores and oxidative damage markers to control levels. These interesting results reveal a parallelism between these events and tempt us to propose that some relationship may exist between them. Additional evidences that oxidative damage increase and locomotor activity decrease are somehow related come from the experiment that showed that MK-801 prevents succinate effects on both a biochemical and a behavioral parameter (Fig. 2). Since pharmacological treatment affected both behavioral and biochemical parameters in the same direction, to a similar extent, and did not dissociate them, it is rather possible that the currently reported biochemical and behavioral effects of succinate are related. However, if there is a cause–effect relationship between them cannot be established at the moment, and a more detailed analysis of the variation of cerebral oxidative stress markers levels and convulsive activity along time has to be performed, in order to determine whether a cause–effect relationship between these events does exist. In addition, we did not assess if succinate increases oxidative damage markers other than TBARS and protein carbonyl. Therefore, it remains to be determined if other important cellular targets for oxidative damage, such as DNA, are affected by succinate, and in what extent our results comparatively apply to the human condition, because species-specific susceptibility is possible and succinate concentrations in the white matter are not known.

Regardless if the currently reported behavioral and neurochemical effects of succinate are causally related to each other or not, this study shows, for the first time, that succinate causes significant oxidative damage and behavioral effects at subconvulsant doses by NMDA receptor-mediated mechanisms. This is an important finding for different areas, particularly those concerned with the study of the succinate-accumulating conditions, such as exposure to exogenous [1,7,17,19,32,40] and endogenous [10,9,12–14,18,24,34,38] SDH inhibitor toxins and inherited deficiencies of SDH [5,6,14,30,31], because it shows that at least some of the deleterious effects of these conditions may arise from secondary succinate accumulation, a possibility that has been overlooked in the literature.

Acknowledgments

Research supported by CNPq (grant: 500120/2003-0 and 500096/2003-1) and CAPES. Carlos Fernando de Mello and Maribel Antonello Rubin are the recipients of CNPq fellowships (grant: 500120/2003-0 and 500096/2003-1). Valéria Dornelles Gindri Sinhorin and Juliana Saibt Martins Pasin are the recipients of CAPES fellowships. We thank Florindo Gonçalves Duarte and Dinah Barbara Pathek for capable technical assistance.

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4 - DISCUSSÃO

4. DISCUSSÃO

A ação do L-glutamato, um aminoácido de cadeia acíclica e estrutura flexível, nos vários subtipos de receptores ionotrópicos e metabotrópicos tem um importante papel tanto na comunicação neuronal como em doenças que afetam o SNC (Cotman et al., 1995). Em função disso, muitos pesquisadores tem investido na síntese de novos análogos do L-glutamato, tanto agonistas quanto antagonistas seletivos daqueles receptores. Estes novos agonistas são valiosos para caracterizar a farmacologia dos receptores (Willis et al., 1997).

No primeiro artigo, foi demonstrado por meio de experimentos com glutamato rádio-marcado, que o D,L-*cis*-2,3-PDC, um análogo do L-glutamato de cadeia cíclica e estrutura rígida, inibiu a ligação do [^{3}H]-L-glutamato Na^+-independente em preparações de membranas plasmáticas de córtex de ratos, sugerindo uma interação com receptores glutamatérgicos. Da mesma forma, o seu diasteroisômero, o L-*trans*-2,3-PDC também inibe a ligação do [^{3}H]-L-glutamato, preferentemente em receptores NMDA (Willis et al., 1996). Por outro lado, o D,L-*cis*-2,3-PDC não interagiu com sítios de captação de L-glutamato, pois ele não inibiu a ligação do [^{3}H]-L-glutamato Na^+-dependente em preparações de membranas plasmáticas de córtex de ratos. Estes dados contrastam com a capacidade do seu diasteroisômero inibir, embora fracamente, a captação de [^{3}H]-D-aspartato em sinaptossomas de cérebro de ratos (Ki= 33μM) (Willis et al., 1996). Já o derivado pirrolidínico L-*trans*-2,4-PDC, cujos grupos carboxilas distam um do outro três átomos carbonos, é um potente inibidor seletivo do transportador de glutamato Na^+-dependente (Ki= 1μM) (Bridges et al., 1991). Embora, o D,L-*cis*-2,3-PDC não tenha apresentado efeito sobre a ligação do [^{3}H]-L-glutamato Na^+-dependente, isso não quer dizer que tal composto não altere a captação ou liberação de L-glutamato, já que os estudos de ligação não são diretamente usados para avaliar a atividade funcional e sim, identificar sítios específicos de ação. O fato de que o derivado *cis*-dicarboxilato de pirrolidina não ter alterado a ligação do [^{3}H]-L-glutamato Na^+-dependente enquanto o isômero *trans* altera, sugere que a configuração *cis* nos grupamentos carboxilas do anel pirrolidínico lhe atribui alguma seletividade de ligação em sítios para o L-glutamato não-transportáveis. Por isso, foi investigado se o D,L-*cis*-2,3-PDC causava alguma alteração comportamental em camundongos, e qual o possível mecanismo de ação envolvido nessas alterações.

A injeção intracerebroventricular de D,L-*cis*-2,3-PDC causou convulsões tônico-clônicas generalizadas nos camundongos de uma maneira dose-dependente. Entretanto, estas convulsões foram prevenidas pela co-administração de MK-801, um antagonista não-competitivo dos receptores NMDA. Por outro lado, a co-administração de DNQX, um antagonista dos receptores AMPA e KA, causou apenas uma ligeira proteção das convulsões induzidas pelo D,L-*cis*-2,3-PDC, sugerindo que o D,L-*cis*-2,3-PDC causa convulsões por ativar os receptores NMDA, possivelmente por se ligar no mesmo sítio que o L-glutamato, e que o envolvimento dos receptores não-NMDA é de menor relevância para o seu efeito convulsivante. Da mesma forma, o diasteroisômero L-*trans*-2,3-PDC causa neurotoxicidade quando injetado no hipocampo de ratos *in vivo* e também em culturas corticais de cérebro de ratos, os quais são revertidos pela co-administração de MK-801 (Willis et al., 1996). Um fato interessante é que o L-*trans*-2,3-PDC também apresenta alguma reatividade com os receptores AMPA e KA (Willis et al., 1996) *in vitro*. Esses resultados sugerem que embora haja uma restrição conformacional imposta pelo anel pirrolidínico este pode sofrer uma interconversão do tipo envelope-envelope o que permite a esses compostos assumir conformações nos três tipos de receptores ionotrópicos (Chamberlin & Bridges, 1993). Da mesma forma, uma relação similar entre estrutura/atividade foi observada em outros análogos do L-glutamato com conformação restrita, tal como carboxiciclopropil-glicinas (Ishida et al., 1991; Kawai et al., 1992).

O sucinato é um metabólito endógeno do ciclo de Krebs e também pode ser produzido por meio de uma rota alternativa a partir do GABA (Sanders et al., 1969). Este composto está largamente distribuído no SNC, como na matéria branca e córtex cerebrais e no cerebelo (Petroff et al., 1988).

No segundo artigo, foi demostrado que a administração ICV de sucinato diminuiu a atividade exploratória, aumentou as Substâncias que reagem ao Ácido Tiobarbitúrico (TBARS) e carbonilação protéica bifasicamente. O sucinato nas doses de 0,7 μmol e 1,0 μmol diminuiu a atividade locomotora e aumentou os marcadores bioquímicos do dano oxidativo. MK-801 preveniu o aumento da carbonilação proteica total e a diminuição da atividade locomotora induzida por 1,0 μmol de sucinato.

O aumento na produção de TBARS induzido por sucinato sugere que este ácido orgânico causa dano oxidativo já que a substância formada durante a

degradação de lipídios por EAO, o MDA, reage com TBA formando um complexo MDA-TBA de coloração rósea (Kohn & Liversedge, 1944; Patton & Kurtz, 1951; Dahle et al., 1962; Ohkawa et al., 1979). Da mesma forma, sucinato aumentou a carbonilação protéica, um marcador específico do dano oxidativo. Estes resultados fortemente sugerem que as doses de 0,7 μmol e 1,0 μmol induzem dano oxidativo. Por outro lado, uma alta dose de sucinato (1,7 μmol) retornou TBARS e carbonilação protéica aos valores do controle, caracterizando um efeito bifásico do sucinato sobre os marcadores do dano oxidativo estudados. O mecanismo molecular pelo qual a dose de 1,7 μmol apresenta efeito antioxidante não está estabelecido, mas é interessante que um efeito bifásico similar de sucinato sobre os potenciais pós-sinápticos excitatórios de campo (PEPSs) tem sido relatado. Enquanto baixas concentrações (0,3-1,0 mM) de sucinato aumenta os PEPSs em fatias de hipocampo de ratos através da ativação dos receptores NMDA, altas concentrações de sucinato (3-10 mM) diminuem os PEPSs e revertem o aumento destes causados por baixas concentrações de sucinato (Roehrs et al., 2004). Naquele estudo, o efeito bifásico sobre os PEPSs foi atribuído a um efeito bifásico do sucinato sobre o receptor NMDA. Dado o marcado padrão similar de variação da curva dose-efeito para o sucinato o qual foi relatado por Roehrs et al. (2004), pode–se propor que eles podem ocorrer por mecanismos similares, isto é, receptores NMDA. Chen et al. (2003) demonstraram que o sucinato exibe uma curva de dose-resposta na forma de U invertido, atuando como ansiolítico no teste de plus maze, corroborando com os efeitos bifásicos do sucinato já descritos anteriormente.

Portanto, decidiu-se investigar se os receptores NMDA estavam envolvidos no dano oxidativo e, não no dano lipídico, induzido por sucinato utilizando a técnica de carbonilação protéica. Embora a medida de malondialdeído (MDA) tenha sido usada extensivamente nas últimas quatro décadas como um marcador da LPO (Janero, 1990) este método possui algumas peculiaridades e limitações que podem levar a uma interpretação errônea dos resultados. Conseqüentemente, foi avaliado o envolvimento dos receptores NMDA somente sobre a carbonilação protéica induzida por sucinato.

A co-administração de MK-801 protegeu contra o aumento da carbonilação protéica induzida por sucinato, sugerindo o envolvimento deste subtipo de receptor glutamatérgico no dano oxidativo induzido por este ácido dicarboxílico.

Um importante resultado deste estudo é que o dano oxidativo foi acompanhado por significantes alterações comportamentais, cujo padrão de variação ao longo das doses de sucinato coincidiu com os marcadores do dano oxidativo. Mais especificamente, sucinato causou um aumento dos marcadores do dano oxidativo no cérebro, o qual coincidiu com a diminuição na atividade locomotora. Além disso, o aumento nas doses de sucinato retornou os escores da atividade locomotora e marcadores bioquímicos ao nível do controle. Esses resultados revelam um paralelismo entre esses eventos, sendo tentador propor que alguma relação pode existir entre eles. Evidência adicional de que o aumento no dano oxidativo e a diminuição na atividade locomotora estão, de alguma forma, relacionados, é proveniente dos experimentos que mostraram que o MK-801 previne os efeitos do sucinato sobre ambos os parâmetros bioquímicos e comportamental. Uma vez que o tratamento farmacológico afetou ambos os parâmetros (bioquímico e comportamental) na mesma direção, de forma similar, não dissociável, é possível que esses efeitos estejam relacionados. De qualquer maneira, se existe uma relação causa-efeito entre eles ainda não pode ser estabelecido e, uma análise mais detalhada da variação dos níveis de marcadores do estresse oxidativo cerebral e atividade convulsiva ao longo do tempo é necessária para determinar se existe uma relação causa-efeito entre esses efeitos. Além disso, não foi investigado se o sucinato altera outros importantes marcadores do dano oxidativo, tais como o DNA, e em que extensão nossos resultados se comparam às condições humanas.

Independente se os efeitos neuroquímicos e neurocomportamentais do sucinato aqui descritos estão casualmente relacionados, este estudo mostra que o sucinato causa dano oxidativo e efeitos comportamentais em doses subconvulsivantes, por mecanismos mediados por receptores NMDA. Estes resultados são particularmente importantes para o estudo de condições nas quais acumulam sucinato, tais como a exposição a toxinas exógenas inibidoras da SDH (Riepe et al., 1992; Hassel & Sonnewald, 1995; Greene & Greenamyre, 1996; Urbanska et al., 1998; Alexi et al., 1998; Calabresi et al., 2001) e endógenas (Greene et al., 1993; Davolio & Greenamyre, 1995; Toyoshima et al., 1995; De Mello et al., 1996; Fighera et al., 1999, 2003; Marisco et al., 2003; Royes et al., 2003; Fleck et al., 2004) bem como deficiências da SDH herdadas por erros inatos do metabolismo (Brismar & Ozand, 1994; Ozand et al., 1994; Brockman et al., 2002; Okun et al., 2002; Fleck et al., 2004), pois revela que pelo menos alguns dos efeitos

deletérios dessas condições podem ser decorrentes do acúmulo secundário de sucinato, uma possibilidade que tem sido negligenciada.

Nesse contexto, pode-se concluir do presente estudo que o sucinato, um ácido dicarboxílico de cadeia acíclica e estrutura flexível, provavelmente exerça seus efeitos neurotóxicos por ativar os receptores NMDA e que a formação das EAO podem estar envolvidas nos episódios convulsivos causados por este ácido. Além disso, não se pode descartar que o acúmulo de sucinato seja de grande importância nas acidemias orgânicas nas quais acumulam malonato e metilmalonato, inibidores reversíveis da SDH. E, que o D,L-*cis*-2,3-PDC, um análogo do L-glutamato de cadeia cíclica e estrutura rígida, diasteroisômero do L-*trans*-2,3-PDC, interage com sítios de ligação para o L-glutamato não-transportáveis e causa convulsões em camundongos via ativação dos receptores NMDA. Outros estudos são ainda necessários para determinar se esta nova neurotoxina afeta outras funções do L-glutamato, bem como sua utilidade como uma ferramenta farmacológica.

Esses resultados demonstram que mesmo havendo mudança na cadeia ou estrutura química, os compostos dicarboxílicos parecem modular funcionalmente os receptores glutamatérgicos.

5 - CONCLUSÃO

a) O D,L-*cis*-2,3-PDC inibi a ligação do [^{3}H]-L-glutamato Na^+-independente em receptores de membranas plasmáticas de córtex de ratos e não apresenta efeito sobre a ligação de [^{3}H]-L-glutamato Na^+-dependente.

b) A administração intracerebroventricular de D,L-*cis*-2,3-PDC em camundongos induz convulsões tônico-clônicas generalizadas de uma maneira dose-dependente.

c) A co-administração de MK-801, um bloqueador do canal NMDA, com D,L-*cis*-2,3-PDC totalmente protege os animais das convulsões induzidas por D,L-*cis*-2,3-PDC. A co-administração de DNQX, um antagonista dos receptores AMPA e KA, aumenta a latência para as convulsões induzidas por D,L-*cis*-2,3-PDC, mas não altera a percentagem de animais que apresentam convulsões. Estes resultados sugerem que o D,L-*cis*-2,3-PDC induz seus efeitos preferencialmente por meio da ativação dos receptores NMDA.

d) A administração intracerebroventricular de sucinato diminui bifasicamente a atividade locomotora dos camundongos.

e) A administração intracerebroventricular de sucinato aumenta bifasicamente a produção de Substâncias que reagem ao Ácido Tiobarbitúrico (TBARS) em cérebros de camundongos *ex vivo.*

f) A administração intracerebroventricular de sucinato aumenta bifasicamente a carbonilação de proteínas em cérebros de camundongos *ex vivo*.

g) A co-administração de MK-801 previne a diminuição da atividade locomotora *in viv*o e o aumento da carbonilação de proteínas em cérebros de camundongos *ex vivo.* Estes resultados sugerem o envolvimento dos receptores NMDA no dano oxidativo induzido por sucinato.

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