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Pharmacology, Biochemistry and Behavior 77 (2004) 183–190
PHARMACOLOGY BIOCHEMISTRY AND BEHAVIOR
www.elsevier.com/locate/pharmbiochembeh

Intrahippocampal administration of the α-keto acids accumulating in maple syrup urine disease provokes learning deficits in rats

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Received 2 April 2003; received in revised form 6 August 2003; accepted 22 October 2003

Abstract

Learning disability is a common feature of patients affected by maple syrup urine disease (MSUD). However, the pathomechanisms underlying learning deficit in this disorder are poorly known. In the present study, we investigated the effect of acute administration of the α-keto acids accumulating in MSUD into the hippocampus on the behavior of rats in the open field and in the inhibitory avoidance tasks. Adult male Wistar rats received intrahippocampal injections of α-ketoisocaproic acid (KIC, 8 μmol), α-ketoisovaleric acid (KIV, 5 μmol), α-keto-β-methylvaleric acid (KMV, 5 μmol), or NaCl (8 μmol) (controls) immediately after or 10 min before training. Testing session was performed 24 h later. Posttraining administration of the keto acids had no effect on learning in the open-field task. In contrast, pretraining administration of KIV and KMV impaired habituation in the open field. Similarly, pretraining administration of KIC, KIV, and KMV affected rat performance in the inhibitory avoidance task, suggesting disruption of acquisition. The results indicate that the α-keto acids accumulating in MSUD induce learning deficits in aversive and nonaversive tasks. We therefore suggest that these findings may be related to the psychomotor delay/mental retardation observed in MSUD, and may indicate the contribution of increased brain concentrations of these organic acids to the pathophysiology of the neurological dysfunction of MSUD patients.

Keywords: Maple syrup urine disease; α-Ketoisocaproic acid; α-Keto-β-methylvaleric acid; α-Ketoisovaleric acid; Rat behavior

1. Introduction

Maple syrup urine disease (MSUD), or branched-chain keto aciduria, is an inborn error of metabolism caused by severe deficiency of the branched-chain L-α-keto acid dehydrogenase complex (BCKAD, E.C. 1.2.4.4) activity (Chuang and Shih, 2001). The inability of this enzyme complex to oxidize α-ketoisocaproic acid (KIC), α-keto-β-methylvaleric acid (KMV), and α-ketoisovaleric acid (KIV) leads to tissue accumulation of these metabolites and their precursor amino acids leucine, isoleucine, and valine, respectively. MSUD consists of heterogeneous clinical and molecular phenotypes. Severity of the disease, ranging from classical to mild variant types, is commonly classified on the basis of indirect parameters, such as onset, leucine tolerance, and/or residual enzyme activity. Based on the mode of clinical presentation and biochemical responses to thiamine administration, MSUD patients can be divided into five clinical and biochemical phenotypes (Chuang and Shih, 2001). Patients with classical MSUD usually present poor feeding, apnea, ketoacidosis, convulsions, coma, and psychomotor delay. This variant is commonly manifested at the neonatal period, whereas presentation of the other forms of the disease usually occurs a few months after birth. The prevalence of the classical variant of MSUD is about 1 in 185,000 worldwide. CNS imaging reveals low density of white matter corresponding to hypomyelination/demyelination and cerebral atrophy. The disease causes a fatal outcome in a considerable number of patients during the first year of life if not diagnosed and treated promptly.

Abbreviations: MSUD, maple syrup urine disease; BCAA, branched chain amino acids; BCKA, branched-chain keto acids; KIC, α-ketoisocaproic acid; KMV, α-keto-β-methylvaleric acid; KIV, α-ketoisovaleric acid; CNS, central nervous system; GABA, gamma-aminobutyric acid.

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0091-3057/$ – see front matter © 2003 Elsevier Inc. All rights reserved.
doi:10.1016/j.pbb.2003.10.013

Those who survive present a variable degree of mental retardation (Chuang and Shih, 2001).

The reduction of plasma branched-chain amino acids (BCAA) concentrations to normal levels by special dietary products has been considered the main target to treat MSUD patients (Danner and Elsas, 1989), whenever the disease is not responsive to thiamine (Chuang and Shih, 2001). Although this approach has contributed decisively to the survival of the affected individuals, a considerable number of the "well-treated" patients present a variable degree of developmental delay/mental retardation associated to chronic brain structural changes. This may occur because the pathophysiology of the neurological dysfunction of MSUD is still poorly known. However, there is a large body of evidence associating defective leucine metabolism and the neurological symptoms of the affected patients. In this context, leucine and/or its keto acid, KIC, are considered the main neurotoxic metabolites in MSUD (Snyderman et al., 1964; Efron, 1965; Chuang and Shih, 2001). Accordingly, we have previously shown that chronic subcutaneous administration of high doses of leucine to young rats induces learning/memory deficits in the open field and in the shuttle avoidance tasks during adult age (Mello et al., 1999), indicating that high leucine levels during brain development may significantly contribute to the learning and memory deficits. On the other hand, convulsive properties have been demonstrated for KIV, suggesting that this organic acid is probably involved in the pathophysiology of the convulsions, characteristic of MSUD patients (Coitinho et al., 2001). In addition, it has been postulated that brain energy deficit provoked by KIV and KIC (Howell and Lee, 1963; Land et al., 1976; Danner and Elsas, 1989), competition of KIV, KIC, and their hydroxyderivatives with L-glutamate for decarboxylation with the consequent reduction of γ-aminobutyric acid (GABA) brain concentration (Tashian, 1961), impairment of myelin development (Appel, 1966; Taketomi et al., 1983; Tribble and Shapira, 1983; Treacy et al., 1992), and low plasma and brain levels of essential amino acids (Wajner and Vargas, 1999; Wajner et al., 2000) may contribute to brain injury. A recent study observed that the α-branched-chain keto acids (BCKA) that accumulate in MSUD trigger apoptosis in glial and neuronal cells, making these compounds more toxic than the corresponding BCAA (Jouvet et al., 2000). However, these investigators used much higher concentrations of the BCKA than those found in MSUD.

The effect of the accumulating organic acids in MSUD on learning and memory processes has not been so far investigated. Therefore, in order to determine the specific participation of the BCKA on the learning deficit characteristic of this disorder, the objective of the present study was to study the effects of acute intrahippocampal administration of the α-keto acids KIC, KIV, and KMV on adult male rat cognitive behavior in nonaversive (open field) and aversive (step-down inhibitory avoidance) learning tasks.

We used the hippocampus as the target tissue for drug administration because this cerebral structure is essential for learning and memory formation, is sensitive to various neurotoxins eliciting metabolic inhibition and free radicals, and, because of these properties, the hippocampus has been used to detect behavioral and biochemical alterations attributed to various substances in the rat (Izquierdo and Medina, 1997; Saransaari and Oja, 2001). Furthermore, lesions of the hippocampus lead to impairment in novel exploration (Harley and Martin, 1999; Moses et al., 2002) and step-down avoidance task which are dependent on the integrity of the hippocampus (Antoniadis and McDonald, 2000; Fanselow, 2000).

2. Material and methods

2.1. Subjects and reagents

A total of 170 sixty-day-old male Wistar rats (180–230 g) from our own breeding stock were used. The animals were housed five per cage with food and water freely available under a 12-h light/12-h darkness cycle (lights on at 7:00 a.m.) at a constant temperature of 22 ± 1 °C. The experimental protocol was approved by the Ethics Committee for animal research of the Federal University of Rio Grande do Sul, Porto Alegre, Brazil, and followed the *Principles of Laboratory Animal Care* (NIH publication no. 85-23, revised 1985). All reagents were purchased from Sigma (St. Louis, MO, USA), unless otherwise stated.

2.2. Surgery

To deliver the compounds to be tested, rats were bilaterally implanted under deep anaesthesia (a mixture of ketamine and xilazine; 75 and 10 mg/kg ip, respectively) with 27-gauge stainless cannulae (0.6 mm O.D.) with an inner needle guide (diameter, 0.3 mm) aimed 1.0 mm above the pyramidal cell layer of the CA1 region of the dorsal hippocampus (A: −4.2, L: ±3.0, V: +1.3 mm, according to Paxinos and Watson, 1986) (Fig. 1). The cannulae were fixed to the skull with dental cement (Izquierdo and Medina, 1997). All procedures were performed under aseptic conditions. The animals were allowed to recover unrestrained for 3 days after surgery until the day of the experiment.

2.3. Infusion procedure and control of cannula placement

Immediately after or 10 min before training, a 30-gauge needle was inserted into the guide cannula (Izquierdo et al., 1999), protruding 1 mm from the tip of the cannula in order to administer a bilateral infusion of 2 μl of KIC (8 μmol), KIV (5 μmol), KMV (5 μmol), or NaCl (5–8 μmol) into the dorsal hippocampus (CA1 area). The pH of each solution was previously adjusted to 7.4 with 0.1N NaOH or 0.1N HCl. Infusions were performed at a rate of 1 μl/min. After the infusions were completed, the cannula was left in place for an additional 30 s. Thus, the entire bilateral infusion procedure

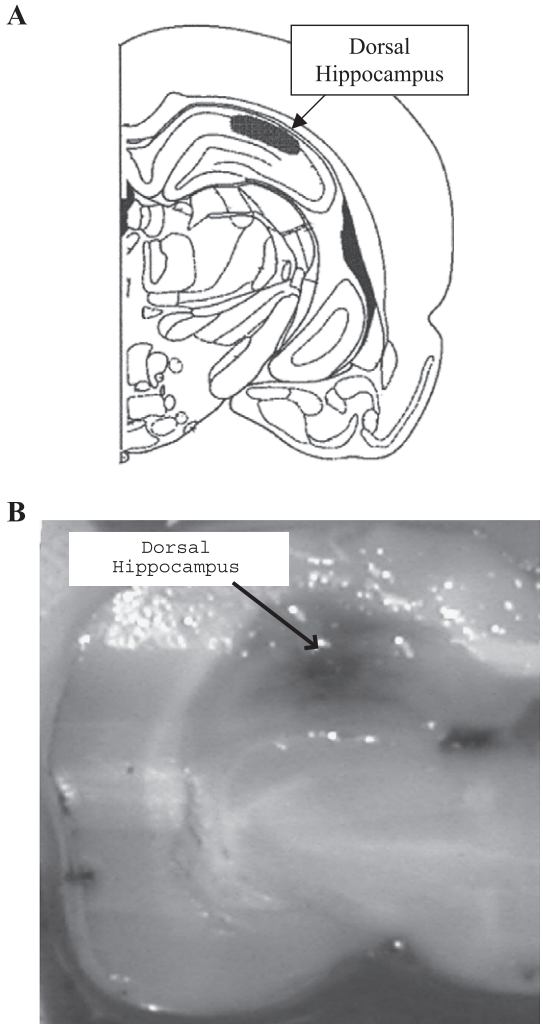

Fig. 1. Schematic drawing (A) and actual picture after methylene blue infusion (B) of a rat brain section at plane −4.20 mm (Paxinos and Watson, 1986) showing the extension of the dorsal hippocampus reached by drug infusion which appears as a filled area. It can be seen in the figure (B) that diffusion of the stain is restricted to the hippocampus.

took 150 s. At the end of behavioral evaluation, the animals were killed by decapitation and infused with 2 μl of 4% methylene blue through the cannula. The brains were removed and stored in 3.7% formalin for histological evaluation of cannula placement (Izquierdo et al., 2000). Fig. 1B, which is an actual picture after methylene blue infusion, shows that diffusion was restricted to the hippocampus. In case the cannula was not in the right place or when the infusion surpassed the limits of the hippocampus, the rats were discarded.

2.4. Behavioral procedures

2.4.1. Open-field habituation

The rats' ability to habituate to a new environment was assessed by subjecting the animals to two consecutive 3-min sessions (training and test) spaced 24 h apart in an open field. The rats were injected with 2 μl of KIC, KIV, KMV, or NaCl into the hippocampus immediately after (59 animals), or 10 min before (57 animals) the training session, as described above. The apparatus consisted of a wooden box measuring 60 × 40 × 50 cm with a frontal glass wall. The floor was divided into 12 equal squares by black lines. The animals were placed gently on the left rear quadrant of the open field, and the number of squares crossed with the four paws, number of rearing responses and number of *fecal boli* recorded by an observer who was not aware of the subject condition (Walsh and Cummins, 1976). The number of *fecal boli* (defecation) at the training session was considered as a measure of emotionality (Archer, 1973; Elias et al., 1975). The number of squares crossed at training session was indicative of motor activity and the reduction in the number of rearing responses along the sessions was considered as a measure of memory retention (habituation) (Walden, 1968; Netto et al., 1986).

2.4.2. Step-down inhibitory avoidance

The task was carried out using a 45 × 30 × 30 cm metal box with a frontal glass wall. The right side of the floor consisted of a grid (43 × 25 cm) of 0.2 cm diameter bronze bars spaced 1 cm apart. The rats (54 animals) were injected with the BCKA or NaCl in the same way as previously described (Section 2.3). Ten minutes after injection, the animals were gently placed on a 25 × 12 × 4-cm wooden platform facing the rear left corner of the box (Barros et al., 2000). At the very moment the animal descended from the platform and put its four paws on the grid it received a 0.4-mA, 2-s scrambled footshock. Latency to step down onto the grid with all four paws was measured and compared to that obtained in a test session performed 24 h later. In the test session, the procedure was identical to that used during training, except that the footshock was omitted (Izquierdo and Medina, 1997; Izquierdo et al., 2000). The difference between training and testing step-down latencies was taken as a measure of retention. Comparisons between groups in step-down latency at the testing session were also evaluated.

2.5. Statistical analysis

The number of rearing responses in the open field and inhibitory avoidance step-down latencies were analyzed by two-way analysis of variance (ANOVA) 2 (NaCl or α-keto acid treatment) × 2 (training and testing sessions) with the sessions variable treated as a within-subject factor. Results are shown as mean ± standard error of the mean (S.E.M.). In order to determine which of the groups varied the scores along the sessions, post hoc analysis was carried out by the *F* test for simple effects (Bruning and Kintz, 1968). Number of crossing responses and *fecal boli* at training were expressed by mean ± S.E.M. and analyzed by the Student's *t* test for nonpaired samples.

The statistical analyses were performed using the *SPSS* (Statistical Package for the Social Sciences) and Microsoft

Excel softwares in a PC-compatible computer. $P<.05$ was considered significant.

3. Results

3.1. Open field

Fig. 2 shows the effect of the α-keto acids KIC, KIV, and KMV on the number of rearing responses in the open-field task. Drug administration immediately after training caused no effect on the number of rearings in the testing session, i.e., all groups of animals significantly reduced the number of rearing scores along the sessions [significant main effect of sessions for KIC: $F(1,17)=14.11$, $P<.025$; KIV: $F(1,17)=23.23$, $P<.001$; and KMV: $F(1,23)=13.76$, $P<.005$], indicating habituation to the task.

However, when drugs were administered before training, statistical analysis revealed a significant treatment (NaCl or α-keto acid) by session interaction for KIV-treated animals [$F(1,21)=9.15$, $P<.01$] and KMV-treated animals [$F(1,17)=18.63$, $P<.001$]. Post hoc analysis (F test for simple effect) revealed that, while saline-injected animals decreased [$F(1,21)=9.41$, $P<.01$], KIV-injected animals did not vary rearing responses scores along the sessions [$F(1,21)=0.99$, $P>.1$].

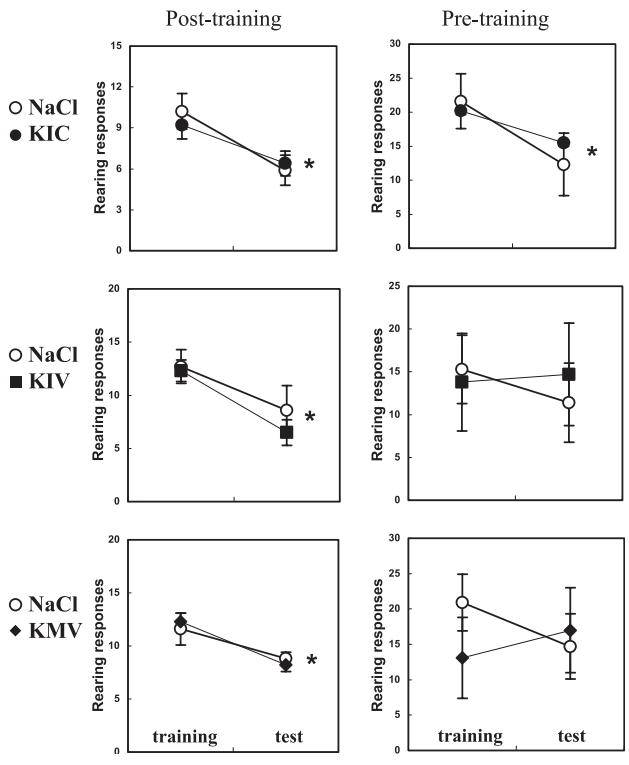

Fig. 2. Effect of intrahippocampal administration of the α-keto acids accumulating in MSUD on the number of rearing responses in the open-field performance of adult rats on two consecutive days. Rats were injected immediately after training or 10 min before training. ○ NaCl 8 μmol; ● KIC 8 μmol; ■ KIV 5 μmol; ◆ KMV 5 μmol. Data are mean ± S.E.M. for $n=7–14$ rats per group. F values are given in the text.

Table 1
Effect of intrahippocampal administration of the α-keto acids accumulating in MSUD on the number of crossing responses in the open-field performance of adult rats at the training session

Treatment	Injection time	
	0 min after training	10 min before training
Control	53.8 ± 4.0	93.3 ± 5.31***
KIC	57.8 ± 4.8	104.0 ± 6.9***
Control	68.2 ± 8.7	68.1 ± 6.1
KIV	59.7 ± 3.2	76.6 ± 6.4*
Control	73.7 ± 4.0	101.7 ± 8.0**
KMV	85.1 ± 9.7	119.0 ± 8.7*

Data are mean ± S.E.M. for $n=7–14$ rats per group.
There were no significant differences between NaCl-treated (control) and α-keto acid-treated groups 10 min before or immediately after the training session (Student's t test for paired samples).
* Significant differences from posttraining at $P<.05$ as calculated by Student's t test for nonpaired samples.
** Significant differences from posttraining at $P<.01$ as calculated by Student's t test for nonpaired samples.
*** Significant differences from posttraining at $P<.001$ as calculated by Student's t test for nonpaired samples.

Post hoc analysis also revealed that, while saline-injected animals decreased the number of rearing responses along the sessions [$F(1,17)=11.27$, $P<.005$], KMV-injected animals increased [$F(1,17)=7.45$, $P<.025$] the number of rearing responses along the sessions. These results indicate that rats receiving KIV or KMV did not habituate to the novel environment. In contrast, statistical analysis of data from the experiments with KIC-treated rats showed only a significant effect of sessions [$F(1,16)=30.3$, $P<.001$]. These results indicate that both groups (control and KIC-injected) similarly altered the number of rearing responses along the sessions. In other words, KIC-treated animals had a normal habituation in the open-field task.

The effect of pretraining intrahippocampal injection of KIC, KIV, or KMV on the number of crossing responses in the open-field task at training session (motor activity) is depicted in Table 1. Statistical analysis showed a significant

Table 2
Effect of intrahippocampal administration of the α-keto acids accumulating in MSUD on the number of fecal boli in the open-field performance of adult rats at the training session

Treatment	Injection time	
	0 min after training	10 min before training
Control	5.9 ± 0.6	4.4 ± 0.9
KIC	5.0 ± 1.3	3.3 ± 0.7
Control	3.6 ± 0.8	2.8 ± 0.9
KIV	2.2 ± 0.8	2.1 ± 0.7
Control	3.4 ± 0.8	3.2 ± 0.9
KMV	2.6 ± 0.8	2.1 ± 0.6

Data are mean ± S.E.M. for $n=7–14$ rats per group. No significant differences between NaCl- and α-keto acids-treated groups were detected at the training session and between pre- and posttraining injected groups (Student's t test for nonpaired samples).

increase of the number of crossing responses in pretraining injected rats compared with rats injected immediately after training [KIC-vehicle: $t(14) = 6.540$, $P < .001$; KIC-treated: $t(17) = 5.134$, $P < .001$; KIV-treated: $t(22) = 2.097$, $P < .05$; KMV-vehicle: $t(18) = 3.292$, $P < .01$; KMV-treated: $t(16) = 2.532$, $P < .05$]. However, no significant differences between NaCl-treated rats and the animals which received the keto acids were observed at training session when drugs were administered immediately after or 10 min before training. These results indicate that the administered drugs did not alter motor activity of the animals.

The effect of the metabolites on the number of *fecal boli* is displayed in Table 2. Defecation of rats submitted to the open field was similar in all groups at training session, since no significant treatment (NaCl, KIC, KIV, or KMV) effect was observed on the number of *fecal boli* when drugs were administered immediately after or 10 min before training. These data suggest a normal emotionality for the animals in the open-field task.

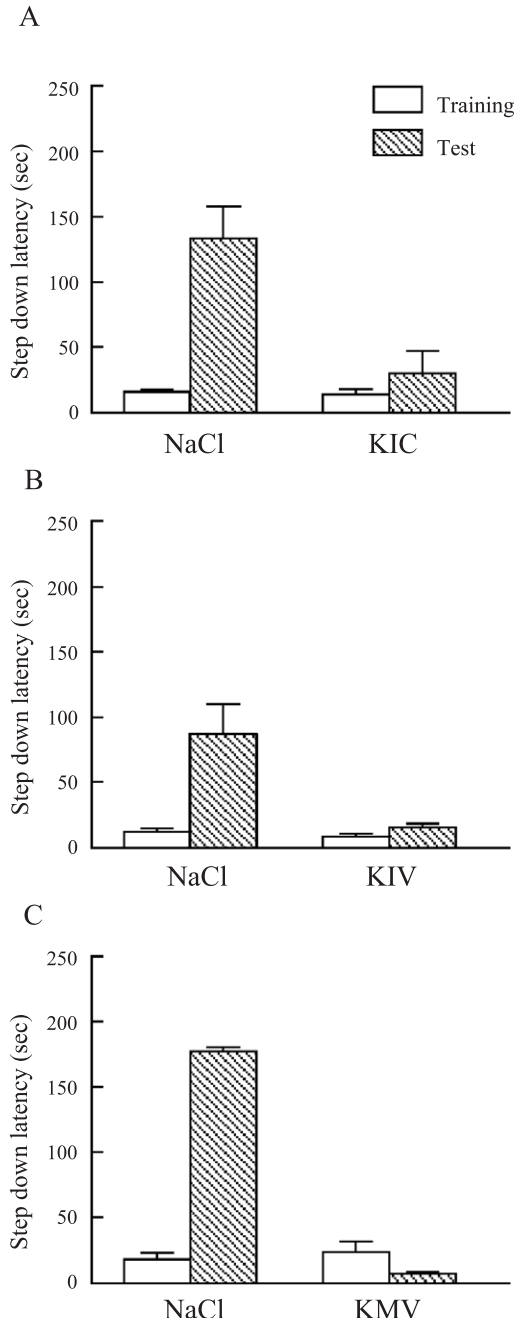

Fig. 3. Effect of intrahippocampal administration of the α-keto acids accumulating in MSUD on step-down latency in the inhibitory avoidance task of adult rats. Ordinates represent mean latency, in seconds (± S.E.M.). $N = 8–11$ per group. F values are given in the text. The treatments were given by bilateral 2-μl infusions into the dorsal CA1 region of the hippocampus 10 min prior to training session. The treatments were NaCl 8 μmol; KIC 8 μmol (A); KIV 5 μmol (B); and KMV 5 μmol (C).

3.2. Inhibitory avoidance

Considering that posttraining injection of the α-keto acids did not elicit any alteration in the performance of animals in the open-field task, in the inhibitory avoidance sessions rats were injected with each of the metabolites 10 min before the training session. The effect of intrahippocampal injection of the α-keto acids accumulating in MSUD on the step-down latency in the inhibitory avoidance task is displayed in Fig. 3. Statistical analysis of step-down latencies revealed a significant Treatment (NaCl or KIC) × Sessions (training and test) interaction [$F(1,16) = 11.6$, $P < .05$], indicating that pretraining KIC administration altered the variation of step-down scores along the sessions, i.e., that KIC-injected animals did not increase step-down latencies along the sessions as NaCl-injected animals did (Fig. 3A). Statistical analysis also revealed that KIV- and KMV-injected animals did not increase step-down latencies along the sessions as their respective controls [significant Treatment × Sessions interaction for KIV: $F(1,17) = 8.45$, $P < .05$, Fig. 3B; and for KMV: $F(1,16) = 320.6$, $P < .001$, Fig. 3C]. These results reveal an impairment of retrieval (memory retention or acquisition) in this task. It can also be seen in the figure that there were no differences in the latency to leave the platform between groups infused with the α-keto acids or infused with NaCl at the training session, suggesting a similar motor activity between all groups.

4. Discussion

In the present study, we showed that pretraining intrahippocampal injection of KMV and KIV decreases the number of rearing responses in the open-field testing session. It is well known that rats exhibit a typical exploratory behavior (consisting of increased locomotor activity and rearing responses) when first exposed to an open field. This exploratory activity decreases over time and is thought to be due to the formation of an integrated memory of the context (Harley and Martin, 1999), in which the hippocampus plays an essential role (O'Keefe et al., 1979; Save et al., 1992). Therefore, it has been considered that memory retention or habituation to a novel environment can be determined by a

decrease in the number of rearing responses that the animals exhibit along the training and testing sessions (Netto et al., 1986; Izquierdo, 1994; Rodrigues et al., 1996) in an open field. Therefore, the presently reported lack of habituation of rats, which were injected with KIV or KMV into the hippocampus, indicates that these keto acids disrupt learning or the early consolidation of this task. A major concern in studies investigating the effect of drugs on the acquisition of a given task is to verify whether pharmacological treatment affects motivational aspects of learning, such as emotionality. Here we present evidence that alterations of motor activity or emotionality probably did not contribute to the deficit of habituation detected for KIV and KMV, since all groups of animals behaved similarly as regards to the number of crossing responses and *fecal boli* (Tables 1 and 2) at the training session (Denenberg, 1969). However, pretraining injections increased the number of crossing responses in all (saline- and BCKA-treated) animals at training, as compared to posttraining injections (Table 1), which may be due to the anxiety itself induced by the injections. Considering that this effect occurred in all animal groups, it probably did not contribute to the observed differences in habituation. More importantly, the increase of crossing responses at training was not accompanied by an alteration in the number of rearing responses.

On the other hand, rats administered immediately after training with NaCl and with the α-keto acids behaved similarly and habituated. These negative data are important as far as they indicate that the intrahippocampal injection of 2 μl of the metabolites did not cause long-lasting brain lesions per se, capable of producing behavioral alterations at the testing session. If that was the case, animals injected immediately posttraining with the keto acids should also have their performance affected at the testing session. Furthermore, since only the pretraining injections led to behavioral impairment, it is conceivable that the metabolites had no effect on posttraining memory consolidation. Therefore, one might suggest that KIV and KMV probably disrupt the acquisition of this task.

In the step-down inhibitory avoidance task, animals injected into the hippocampus with each of the α-keto acids before training presented a deficit in the retention of the task, as revealed by the lack of increase in the step-down latencies along sessions, in contrast with the control (saline-treated) animals, which significantly increased the time to step down from the platform at the testing session. Motor activity of all groups of animals, as measured by the period of time they remained in the platform at the training session, was not different. These data suggest that KIC, KIV, and KMV provoke learning deficits in the inhibitory avoidance task and are in agreement with data obtained using the open-field task. Again, alterations in motor activity could not explain these findings.

The step-down avoidance learning task, which may be considered as a variant of contextual fear conditioning, has been demonstrated to be dependent on the hippocampus (Antoniadis and McDonald, 2000; Fanselow, 2000). Therefore, considering that this cerebral structure is also crucial for spatial memory and is most likely involved in the response to spatial novelty (Moses et al., 2002), it is not surprising that intrahippocampal injection of the main metabolites accumulating in MSUD caused memory disruption in the animals.

Taken together, the results of the open-field and step-down inhibitory avoidance tasks demonstrate that administration of the principal metabolites accumulating in MSUD into the hippocampus induces disruption of acquisition. However, the significance of these findings, and their possible association with the human condition, is far from clear. Although the concentrations of the α-keto acids achieved in our model are unknown, we presume that the concentrations achieved by these compounds in the hippocampus are probably sufficient to cause metabolic alterations in pathways involved in learning/memory and in other neurological functions. It is also interesting to note that the infused α-keto acids can easily cross the blood–brain barrier (Conn et al., 1983) and achieve high concentrations inside the brain. Therefore, it is possible that the neurobehavioral alterations in MSUD children, such as psychomotor delay/mental retardation, may result from an increase in KIV, KMV, and KIC concentrations in the brain above a certain threshold that may affect essential pathways necessary for learning/memory.

We cannot establish, at present, the exact underlying mechanisms responsible for the learning/memory deficits induced by the intrahippocampal administration of the BCKA. A recent report demonstrated that all three keto acids accumulating in MSUD cause apoptosis of glial and neuronal cells in the hippocampus in vitro and in vivo and in a dose- and time-dependent manner (Jouvet et al., 2000). The induction of apoptotic cell death was verified by classical morphological criteria, in situ end labeling of apoptotic nuclei, evidence of caspase activation and nucleosome (DNA) laddering. These investigators also found that apoptosis was associated with a significant reduction in cell respiration but without impairment of the respiratory chain function. These interesting observations indicate a link between previous findings of BCKA-impaired energy metabolism (Dreyfus and Prensky, 1967; Land et al., 1976; Walajtys-Rode and Williamson, 1980; Jackson and Singer, 1983; Zielke et al., 1997) and apoptosis.

In our model, we cannot associate apoptosis or other form of cellular death after BCKA administration with the behavioral effects observed. It should not matter if the injection is 10 min before or after the training, given the fact that BCKA-induced cell death impaired memory after 24 h. In either case, the cells exposed to BCKA would be nonfunctioning 24 h later. However, reduced mitochondrial function after acute administration of BCKA into the hippocampus in vivo should be considered as a possible mechanism accounting for the presently reported impaired performance of rats in aversive and nonaversive tasks. In

this context, it has been recently shown that fear conditioning and spatial learning are disrupted in porin-deficient mice (Weeber et al., 2002), further supporting a role for the mitochondrial permeability transition (MPT) pore found in all eukaryotic cells (Levy et al., 2003). In this context, the MTP has been demonstrated to play a role in learning and synaptic plasticity in rodents, as well as in other physiological cellular functions (Pfeiffer et al., 2001). Furthermore, considering that mitochondrial disorders in humans are characterized by various cognitive deficits (Kartsounis et al., 1992), experimental mouse models of mitochondrial disorders have been developed and showed to present learning and memory deficits when analyzed by distinct behavioral tasks including the open field (Graham et al., 2002). All this corroborate with the hypothesis that the BCKA may induce disturbed rat behavior via inhibition of mitochondrial function. However, further experiments are necessary to clarify this point.

It is worth pointing out that we cannot rule out that BCKA products, such as their respective amino acids (given that BCKA transaminases are well expressed in the brain) (Hutson et al., 1998), are involved in the presently reported effects. This hypothesis is currently under investigation in our laboratory.

On the other hand, a major concern in shock-motivated learning tasks, particularly in those that investigate the effect of drugs on the acquisition of a given task, is whether pharmacological treatment affects motivational aspects of learning, such as shock sensitivity and anxiety. Such a concern is particularly relevant in studies involving shock-motivated tasks, since the fear response to acute stressful situations is generally accompanied by anxiety, sympathetic activation, and release of endogenous antinociceptive substances (Jensen and Smith, 1982; Snow et al., 1985; Fanselow, 1985; Lester and Fanselow, 1985; Netto et al., 1987). In the present study, we demonstrate that the keto acids disrupted learning in the acquisition phase when motivational differences may account for different latency scores at testing. Therefore, drug-induced motivational differences cannot be ruled out as possible causes of the presently reported inhibitory avoidance and open-field deficits.

In conclusion, the present study demonstrated significant behavioral alterations of animals after injection of α-keto acids into the hippocampus, which may be of value to elucidate some aspects of the neurological dysfunction occurring in MSUD. Treatment of MSUD patients has been directed to reduce plasma BCAA levels, but despite dietary amino acid and protein restriction, which results in normal plasma BCAA concentrations, ''well-treated'' MSUD patients have a variable degree of psychomotor delay. Thus, we propose that the concentrations of the α-keto acids in plasma, urine, and CSF, which primarily accumulate in this disorder, should also be measured and correlated with the clinical symptoms and brain abnormalities of these patients in order to verify which metabolites (BCAA or BCKA) give the best correlation, and should be measured at regular intervals for the follow up of the affected patients.

Acknowledgements

This work was supported by Conselho Nacional de Desenvolvimento Científico e Tecnológico (CNPq), Coordenação de Aperfeiçoamento de Pessoal de Ensino Superior (CAPES), Financiadora de Estudos e Projetos (FINEP), and Pró-Reitoria de Pesquisa e Pós-Graduação da Universidade Federal do Rio Grande do Sul (PROPESq-UFRGS).

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