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Holeboard Maze-Learning Deficits and Brain Monoaminergic Neurotransmitter Concentrations in Rats After Intracerebroventricular Injection of 3-Bromopyruvate

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FROELICH, L., A. DING AND S. HOYER. *Holeboard maze-learning deficits and brain monoaminergic neurotransmitter concentrations in rats after intracerebroventricular injection of 3-bromopyruvate.* PHARMACOL BIOCHEM BEHAV **51**(4) 917–922, 1995.—3-Bromopyruvate is a suicide inhibitor of pyruvate dehydrogenase complex in brain homogenates, and after intracerebral injection reduces acetylcholine tissue content and muscarinic cholinergic receptors in brain cortex and hippocampus for extended periods of time. A stereotaxic injection of 0.2 μmol 3-bromopyruvate was given twice into the cerebral ventricles of male Wistar rats. Ten weeks later, the animals were tested for learning deficits in a food-motivated complex holeboard test. 3-Bromopyruvate-treated rats showed an increased number of visits to nonfood-baited holes over a 5-day testing period (four trials per day) compared to sham-operated control rats, an increased number of visits to food-baited holes over the first 2 days of the testing period and an increased time for completing the task. There were no changes in brain monoaminergic neurotransmitter concentrations compared to controls. The results indicate that long-term learning deficits in a spatial discrimination paradigm are caused by 3-bromopyruvate, which might be related to a cholinergic deficit induced by a primary inhibition of brain glucose metabolism at the step of pyruvate dehydrogenase complex. This animal model may be useful for behavioral studies in relation to neurodegenerative diseases like dementia of Alzheimer type.

3-Bromopyruvate Pyruvate dehydrogenase complex Rats Holeboard maze Learning deficits
Noradrenalin Serotonin Dopamine Neurotransmitters Cholinergic deficit Dementia of Alzheimer type

IN DEMENTIA of the Alzheimer type (DAT), abnormalities in several neurotransmitter systems have been reported (11). Among the neurotransmitter deficits, the partial cholinergic deafferentation of the cortical mantle is the most pronounced, and the cholinergic deficit may be linked most directly to the deterioration of cognitive function typical for dementia (3,4). Such changes have been artificially induced in rodents and are used as animal models of DAT for pathogenetic and pharmacological studies (12,22).

The synthesis of acetylcholine depends not only on the availability of choline as one precursor, but, to the same extent, on the activity of the pyruvate dehydrogenase complex (PDHc), which is one of the regulatory enzymes of the oxidative glucose metabolism and provides acetyl-CoA for the mito-

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chondrial tricarboxylic acid cycle to gain ATP and acetylcholine (20,23,26). This enzyme is of particular interest in DAT, because its activity is reduced in postmortem brain tissue (20,21) and it is relatively enriched in the cholinergic cells of the nucleus basalis of Meynert complex that are characteristically affected in the disease (17). PDHc may be inhibited by 3-bromopyruvate in vitro (1) and in vivo in the rat brain (2,7). The resulting decrease in pyruvate oxidation has been reported to cause a proportional decrease in the synthesis of acetylcholine in vitro (10) and in vivo in the rat brain (2). Thus, the in vivo application of 3-bromopyruvate in rats might induce a functional cholinergic deficit by a primary inhibition of brain glucose metabolism at the step of pyruvate dehydrogenase complex. This model appears to some extent comparable to metabolic and behavioral changes induced by the cholinergic neurotoxin AF64A (8).

In the present experiments we have investigated which deficits in learning and memory are induced by the intracerebroventricular injection of 3-bromopyruvate in rats, as measured by a complex holeboard task 10 weeks after intracerebroventricular injection. To investigate the specificity of the biochemical alterations after 3-bromopyruvate, brain monoaminergic neurotransmitters and metabolites were determined in several brain areas and compared to controls.

METHOD

3-Bromopyruvate was prepared from 3-bromopyruvic acid (Sigma Chemicals, Germany), which was brought to pH 6.8 by 1 M NaOH and lyophilized immediately afterwards. For intracerebroventricular injection, freshly dissolved lyophilisate in artificial CSF (14) was used in a concentration of 40 mM 3-bromopyruvate.

The study was carried out in 20 1-year-old male Wistar rats (Breeder: Zentralinstitut für Versuchstierzucht, Hannover, Germany). All animals had free access to food and water and were housed individually in Macrolon-type III cages in a temperature-controlled room (21 ± 1°C) under a reversed light: dark cycle (lights off at 0800 h, lights on at 2000 h), and had free access to food and water with the exception of the period of holeboard testing, when they were maintained on a restricted feeding schedule (5 g standard rat chow/day). All behavioral testing was carried out in a separate testing room between 0900 and 1700 h. Animals were allotted randomly to groups of bromopyruvate-treated and sham-operated control animals before behavioral testing. For substance injection, animals were anesthetized with chloral hydrate (8 ml/kg body weight of a 4% solution IP) and positioned in a Kopf® small animal stereotaxic frame. A burr hole was drilled in the skull and a 30 gauge injection needle was positioned according to a stereotaxic atlas for rats (19) (stereotaxic coordinates: 1.9 mm lateral, 3.0 mm posterior from bregma, 3.0 mm ventral from dura). Differences in the size of rat were compensated according to Whishaw et al. (25). The accuracy of the injection to the cerebral ventricles was confirmed with blue dye in several animals not included in the investigation. Bromopyruvate (0.2 μmol), in 10 μl artificial cerebrospinal fluid (CSF) (14), was injected into the left lateral cerebral ventricle over 10 min; in sham-operated controls, equal amounts of artificial CSF were injected into the same ventricle. A second intracerebroventricular injection of bromopyruvate or artificial CSF, respectively, was given after 6 weeks. The infusion needle was left in place for 2 min after the end of injections. Until behavioral training, animals were handled daily for 10 min. Ten weeks after the first injection, behavioral training in the holeboard procedure was begun after a neurological examination according to Tupper and Wallace (24).

The holeboard was adapted with minor modifications from Oades and Isaacson (18). An open field (70 × 70 cm) surrounded by Plexiglas walls (40 cm high) contains 16 holes in a 4 × 4 array. On one side of the walls, an attached starting box is separated from the testing area by a guillotine door, which can be operated from a distance. Each of the holes contains a metal cup (2 cm deep), which has a perforated bottom, under which 20 food pellets of the type used for reinforcement (Altromin®) are placed to minimize odoric cues. After each trial, visible traces of urine and feces are removed with a dry cloth and the maze is sponged clean once every day after the last trial.

Over 5 days, the animals were reduced to 85% of their previous body weight and during this period were allowed to explore the holeboard with all 16 holes baited with food. The animals were placed into the starting box, the guillotine door was opened, and a trial started after closing the guillotine door, when the animal had entered the testing area. The trial ended after 10 min or after all food pellets had been collected. The number of hole visits was recorded by a microcomputer, which identified nose pokes by light beam crossings. From day 6 through 10, a fixed order of four cups were baited with one food pellet. For every session (four trials of 10 min per day with an intertrial interval of 1 min), the latency to enter the open field (time from opening to closing the guillotine door), the number of visits to food-baited holes (hits), the number of visits to unbaited holes (errors), and the time to complete the trial were recorded. All animals completed the task (collection of four food pellets) before 10 min.

After completion of behavioral testing (12 weeks after the first injection of 3-bromopyruvate), the animals were sacrificed by freezing their brains in situ with liquid nitrogen under steady-state conditions (anesthesia with artificial ventilation containing 0.5% halothane, nitrous oxide/oxygen 70:30) in arterial normotension, normocapnia, normoxemia, normoglycemia, and normothermia and then stored at −80°C until preparation of brain areas. Samples from the frontal cortex, the temporal cortex, the parietal cortex, the dorsolateral striatum, and the anterior part of the hippocampus were dissected from horizontal brain slices (1.5 mm thick) in a glove box maintained at −15°C. After weighing, the frozen brain samples were homogenized with a potter and sonicated for 1 min in 0.7 ml of an ice-cold HPLC buffer, as described below. After centrifugation at 50,000 × *g* for 20 min and extraction with a twofold volume of *n*-heptane (Merck) to remove cell fragments, denaturated proteins, and lipids, the aequous phase was filtered through a 0.45 μm Millipore membrane filter, after with 10–30 μl of the filtrate was injected directly into the chromatographic system (except for the striatum samples, which were diluted 10-fold).

The HPLC system contained a precolumn, a Nucleosil 100-$5C_{18}$ analytical column, and a Coulochem ESA detector for electrochemical detection. Separation of the biogenic amines, noradrenaline (NA), dopamine (DA), 3,4-dihydroxyphenylacetic acid (DOPAC), homovanillic acid (HVA), serotonin (5-HT), 5-hydroxyindoleacetic acid (5-HIAA), was obtained with a 0.1 M phosphate buffer ($NaH_2PO_4 \cdot H_2O$; Merck), pH 3.1, containing 3 mM 1-octane sulfonic acid Na salt (Janssen, Jeel, Belgium), 0.5 mM disodium ethylendiaminetetraacetate (Merck), 1.5 mM NaN_3 (Ferak, Berlin, Germany), and 24 vol% methanol (Merck) within an 18-min run time. All chemi-

cals were of analytical grade; standards were obtained from Serva (Heidelberg, Germany). [For a detailed description of the chromatographic setup, see (4).]

Data are displayed as mean values ± SEM. A two-factorial analysis of variance (ANOVA) with repeated measures (factor I: bromopyruvate vs. control; factor II with repeated measures: learning session 1 through 5) was calculated for each of the dependent variables. Although it cannot be assumed that the data follow a normal distribution, ANOVA had to be chosen, because no other statistical test allows an adequate assessment of this experimental design. Furthermore, ANOVA is robust against a violation of the requirement for a parametric distribution of the data. Post hoc comparisons and statistical calculations regarding the neurochemical variables were made with Mann-Whitney *U*-tests. Differences were considered significant at $p < 0.05$. Calculations were performed with the SPSS-PC package.

RESULTS

All animals showed a normal neurological examination after injection, and all animals had learned to eat all 16 pellets from the food cups during the exploration period. The latency to enter the field decreased continously and significantly over the 5 days of testing, $F(4) = 8.22$, $p < 0.0001$. There were no significant group differences between 3-bromopyruvate-lesioned and control animals, $F(1) = 0.08$, $p > 0.1$ (Fig. 1). The visits to food-baited holes (hits) also decreased over the 5 days of testing, $F(4) = 10.05$, $p < 0.0001$, and there were significant differences between the experimental groups on first two days of testing, $F(1) = 40.58$, $p < 0.0001$, post hoc Mann-Whitney *U*-test $p < 0.01$ (Fig. 2). The major finding was a significant difference in the number of visits to unbaited-holes (errors) both, between the experimental groups over all days of testing, i.e., significantly more errors in the 3-bromopyruvate-lesioned group, $F(1) = 53.40$, $p < 0.0001$, post hoc Mann-Whitney *U*-test $p < 0.05$, and decreasing errors over the learning period, $F(4) = 24.04$, $p < 0.0001$ (Fig. 3). The time until completion of the task also was significantly

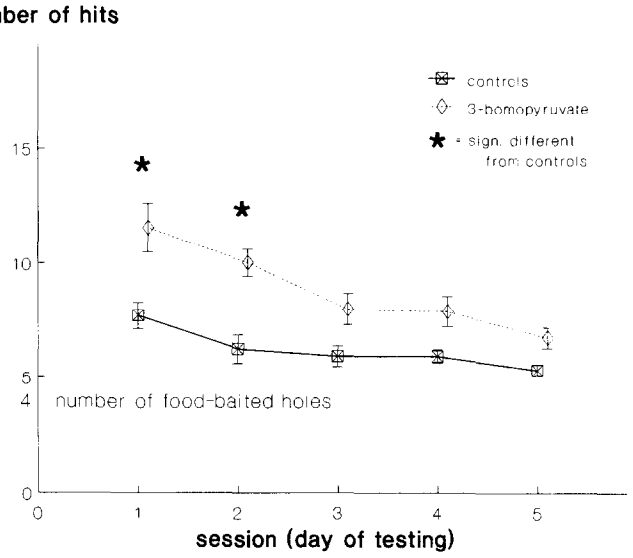

FIG. 2. Number of visits to food-baited holes (hits) in a holeboard spatial learning test for adult male Wistar rats. 4 out of 16 holes were baited with food. Data are displayed as means ± SEM for a 3-bromopyruvate-lesioned group ($n = 10$) and a sham-operated control group ($n = 10$). Hits were recorded over five sessions (four trials per session) on 5 consecutive days. Significant differences between the experimental groups are marked by asterisk.

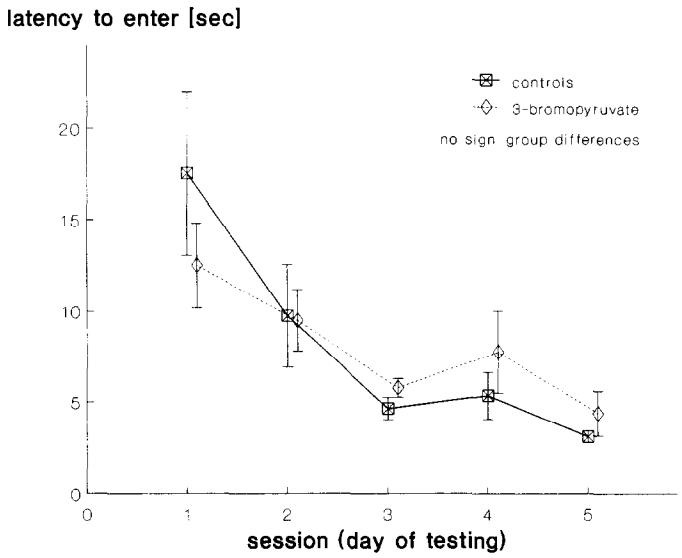

FIG. 1. Latency to enter the open field in a holeboard spatial learning test for adult male Wistar rats. Data are displayed as means ± SEM for a 3-bromopyruvate-lesioned group ($n = 10$) and a sham-operated control group ($n = 10$). Latency was measured in seconds over five sessions (four trials per session) on 5 consecutive days. There were no significant differences between the experimental groups.

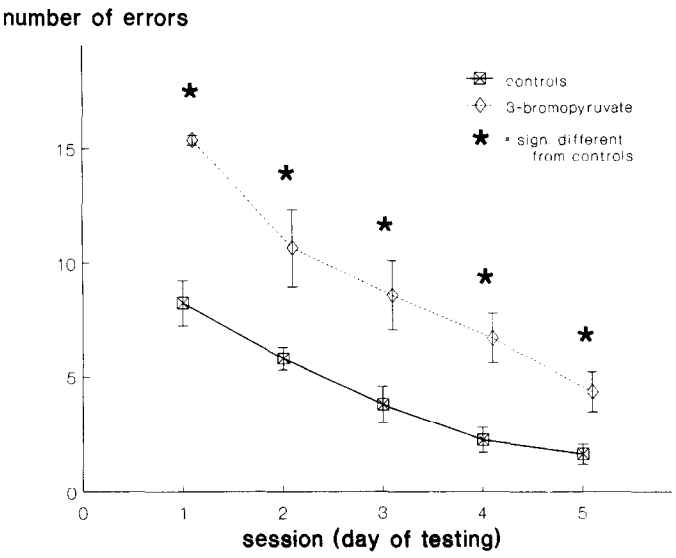

FIG. 3. Number of visits to nonbaited holes (errors) in a holeboard spatial learning test for adult male Wistar rats. Data are displayed as means ± SEM for a 3-bromopyruvate-lesioned group ($n = 10$) and a sham-operated control group ($n = 10$). Errors were recorded over five sessions (four trials per session) on 5 consecutive days. Significant differences between the experimental groups are marked by asterisk.

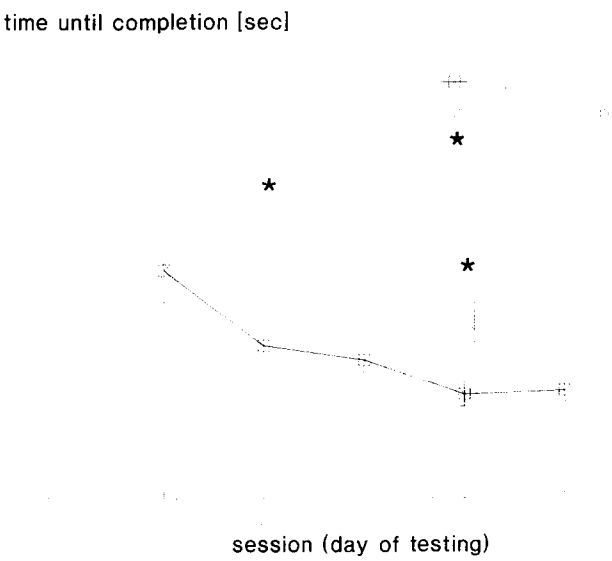

FIG. 4. Time until completion of the task in a holeboard spatial learning test for adult male Wistar rats. Data are displayed as means ± SEM for a 3-bromopyruvate-lesioned group (n = 10) and a sham-operated control group (n = 10). Time in seconds was recorded over five sessions (four trials per session) on 5 consecutive days. Significant differences between the experimental groups are marked by asterisk.

different between the experimental groups on days 2 and 4, $F(1) = 19.67$, $p < 0.0001$, post hoc Mann-Whitney U-test $p < 0.05$, and decreased significantly over the learning period, $F(4) = 15.84$, $p < 0.0001$ (Fig. 4).

NA concentrations showed a regional variation with highest mean values (0.46 ng/mg wet weight) in the anterior hippocampus, and lowest mean values (0.12 ng/mg wet weight) in the striatum. 5-HT mean values varied between 0.60 ng/mg wet weight in the striatum and 0.32/0.31 ng/mg wet weight in the frontal cortex/anterior hippocampus. DA mean values were highest in the striatum (10.6 ng/mg wet weight), and below the detection limit of the chromatographic system in all other regions except the frontal cortex. The metabolites 5-HIAA, DOPAC, and HVA showed a similar regional variation to the respective neurotransmitters. Neither of the monoaminergic neurotransmitters and their metabolites in any of the brain regions investigated here showed a significant change from the control group, while control levels were comparable to earlier studies (4) (Table 1).

DISCUSSION

The intracerebroventricular injection of 3-bromopyruvate in rats impaired learning performance in a complex spatial discrimination paradigm 10 weeks after injection. Because the latency to enter the holeboard field was not significantly altered in 3-bromopyruvate-lesioned rats, and because they did not spend fewer time in the center of the open field (location of the holes), as estimated from a comparable number of total hole visits, any major contribution of motivational factors, such as anxiety, may be excluded. Furthermore, all animals had habituated to the testing apparatus and had eaten all food pellets during the 5-day exploration period. A major contribution of a motor component may also be excluded, because the neurological examination was normal in all animals and because there were no differences in the time to complete the task in the 3-bromopyruvate lesioned rats at day 5 of the testing period. The best discriminating parameter of the holeboard procedure was the number of errors, i.e., visits to unbaited holes, which requires the animals to suppress an unrewarded response.

An impaired passive avoidance response was observed 1 week after an injection of 3-bromopyruvate into the area of the basal forebrain of rats, but not 18 days after injection (2). In the present experiments, the behavioral deficit could be demonstrated 10 weeks after injection. This might be either due to a more widespread damage caused by an intracerebroventricular injection as compared to an injection into the area of the ascending cholinergic neurones, due to the higher amount of 3-bromopyruvate injected in the present experiments [0.2 μmol as compared to 0.02 nmol in (2)], or due to the more complex and more demanding learning task in the present experiments. The holeboard procedure has been shown to be sensitive to detect behavioral consequences of hippocampal destruction (18). Qualitatively similar deficits may be found after lesions of the septo-hippocampal system

TABLE 1

TISSUE CONCENTRATIONS OF MONOAMINERGIC NEUROTRANSMITTERS AND THEIR METABOLITES IN SEVERAL RAT BRAIN AREAS

Area	Group	Sample Size	NA	5-HT	5-HIAA	DA	DOPAC	HVA
f.c.	Control	10	0.40 ± 0.07	0.58 ± 0.10	0.51 ± 0.13	0.09 ± 0.07	0.07 ± 0.02	n.d.
	3-BP	10	0.44 ± 0.07	0.56 ± 0.07	0.51 ± 0.12	0.07 ± 0.01	0.08 ± 0.03	n.d.
t.c.	Control	10	0.38 ± 0.06	0.35 ± 0.07	0.40 ± 0.07	n.d.	0.04 ± 0.02	n.d.
	3-BP	10	0.40 ± 0.07	0.36 ± 0.07	0.38 ± 0.04	n.d.	0.05 ± 0.02	n.d.
p.c.	Control	10	0.39 ± 0.06	0.31 ± 0.03	0.29 ± 0.05	n.d.	n.d.	n.d.
	3-BP	10	0.41 ± 0.09	0.30 ± 0.06	0.29 ± 0.04	n.d.	n.d.	n.d.
st.	Control	10	0.12 ± 0.05	0.60 ± 0.17	0.87 ± 0.25	10.6 ± 2.10	2.46 ± 0.48	1.15 ± 0.41
	3-BP	10	0.11 ± 0.03	0.63 ± 0.18	0.88 ± 0.19	11.1 ± 2.10	2.55 ± 0.72	1.23 ± 0.47
hic.	Control	10	0.46 ± 0.09	0.32 ± 0.13	0.45 ± 0.13	n.d.	n.d.	n.d.
	3-BP	10	0.46 ± 0.11	0.31 ± 0.12	0.46 ± 0.11	n.d.	n.d.	n.d.

Data are presented as means ± standard deviation (SD); all values in ng/mg wet weight. f.c. = frontal cortex, t.c. = temporal cortex, p.c. = parietal cortex, st. = striatum, hic. = anterior hippocampus. n.d. = not detected, values below the detection limit of the chromatographic system. There are no statistically significant changes from the control group by Mann-Whitney U-test.

(27), but also after lesioning the ascending cholinergic projection to the cortex (13) or after experimental cholinergic denervation in general (23).

3-Bromopyruvate is known to cause a secondary cholinergic deficit, presumably due to a reduced availability of acetyl-CoA, which is a direct precursor of acetylcholine (2,7). Earlier studies from this laboratory have indicated only α-ketoglutarate in the hippocampus to be significantly elevated 12 weeks after 3-brompyruvate ICV injection, while no significant change in the concentrations of glucose, pyruvate, lactate, and other compounds of the tricarboylic acid cycle were found in brain cortex or hippocampus after the same experimental conditions as used in this study (8), despite short-term reductions of acetylcholine synthesis and PDHc activity (2,7). Neither were there changes in the overall concentrations of the energy-rich phosphates creatine phosphate, ATP, ADP, and AMP in the brain cortex (7). However, a long-term decrease in the number of muscarinic cholinergic receptors has been observed, indicative of a persistent cholinergic deficit (7). In another rat model of an inhibition of brain glucose metabolism induced by intracerebroventricular injection of streptozotocin, deficits in passive avoidance learning (15) and monoaminergic neurotransmitter concentrations (4) have been observed. Conversely, a memory improving action of glucose, presumably due to a facilitation of hippocampal acetylcholine synthesis, has been demonstrated in mice (16). As an indication of some specificity of the biochemical effects of the 3-bromopyruvate injection, the concentrations of the monoaminergic neurotransmitters NA, DA, 5-HT, and their metabolites HVA, DOPAC, and 5-HIAA were found to be unchanged in several brain areas.

Thus, a close interrelationship may exist between brain glucose metabolism and learning and memory functions in such a way that a disruption of glucose-dependent neurotransmitter metabolism precedes energy failure (9) with acetylcholine being predominately affected (6). These experimental findings in rats support the notion that the abnormalities of brain glucose metabolism and cholinergic neurotransmission, which have been observed in human dementing disorders, e.g., sporadic DAT, might indicate a pathophysiologically relevant primary deficit of the carbohydrate metabolic pathway.

In summary, the present experiments demonstrated deficits in spatial learning in rats caused by 3-bromopyruvate without changes in brain monoaminergic neurotransmitters, which may be related to a functional cholinergic deficit induced by a primary inhibition of brain glucose metabolism at the step of pyruvate dehydrogenase complex. This animal model may be a useful tool for behavioral studies in relation to neurodegenerative diseases, like sporadic dementia of Alzheimer type.

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