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Microperfusion of 3-MPA into the brain augments GABA

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Abstract

In vivo effects of microperfusion of a GABA synthesis inhibitor (3-MPA) into the striatum and hippocampus on amino acid concentrations and electrical neuronal activity were investigated.

Paradoxical elevations in GABA in the striatum (5-fold in anesthetized and 50-fold in awake rats) and hippocampus (2-fold in anesthetized and 15-fold in awake rats) were documented under steady-state concentrations of 3-MPA along with expected increases in glutamate (a 15-fold increase and a 250-fold increase in the striatum of anesthetized and awake rats, respectively; a 7-fold increase and a 25-fold increase in the hippocampus of anesthetized and awake rats, respectively). There was no clear epileptiform or seizure activity.

Explanations for the paradoxical increase in GABA are offered, and emphasis is placed on the dependency of disinhibition on the model in which its effects are studied as well as on the prevailing level of activation of the probed network.

Keywords

Microdialysis; Analytes; 3-Mercaptopropionic acid (3-MPA); γ -Aminobutyric acid (GABA); Glutamic acid (glutamate); ECoG; Steady-state; Model; Paradoxical; Theory

1. Introduction

The convulsant 3-mercaptopropionic acid (3-MPA) is a competitive inhibitor of glutamic acid decarboxylase (GAD), the enzyme that converts glutamic acid to γ -aminobutyric acid (GABA) [1,2]. 3-Mercaptopropionic acid increases glutamic acid and decreases GABA concentrations in the brain [3-5]. A steady-state chemical model of generalized seizures using systemically administered 3-MPA developed in our lab [5,6] allows probing of certain aspects of the temporal behavior of seizures and of their severity, which is not possible with “bolus models” because of the inherent unsteadiness of their concentrations. However, since the seizures are partial (focal) in 70% of adult-onset epilepsy [7], the development of a steady-state chemical model of partial or “focal” epileptiform activity through the infusion of 3-MPA to discrete brain regions seems more relevant because of its greater clinical translatability.

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Disclosures

The authors have no conflict of interest to report.

The results of this study draw attention to a paradoxical unexpected increase in GABA concentration in the hippocampus and striatum in the presence of 3-MPA and also to the dependency of the effects of inhibition of GAD on the model of ictogenesis and on the prevailing level of network activation.

2. Methods

2.1. Microdialysis

In vivo microdialysis [8,9] allows for the sampling of analytes with good spatial and temporal resolution. The probe is flushed with artificial cerebral spinal fluid (aCSF), and small molecular weight analytes in the brain's extracellular space diffuse down their concentration gradient across the probe membrane where the sample is collected. Microdialysis probes can also be utilized to deliver compounds to tissue [9-11]. In this study, 10-mM 3-MPA was perfused directly through the microdialysis probe into the striatum and hippocampus of both anesthetized and awake and freely moving animals.

2.2. Chemicals

Monobasic sodium phosphate, dibasic sodium phosphate, sodium chloride, potassium chloride, magnesium chloride, calcium chloride, disodium ethylenediaminetetraacetate (Na_2EDTA), 85% *o*-phosphoric acid, and 0.3- μm alumina powder were purchased from Fisher Scientific (Pittsburgh, PA). Levo-aspartic acid, L-glutamic acid, L-arginine, L-alanine, γ -aminobutyric acid, DL-2-aminoadipic acid, 3-mercaptopropionic acid, ammonium acetate, 1-octanesulfonic acid, HPLC-grade methanol, and HPLC-grade tetrahydrofuran were purchased from Sigma-Aldrich (St. Louis, MO). Triple-distilled mercury was obtained from Bethlehem Apparatus Company (Hellertown, PA). Ketamine HCl was obtained from Fort Dodge Animal Health (Fort Dodge, IA). Xylazine was obtained from Lloyd Laboratories (Shenandoah, IA). Acepromazine was obtained from Boehringer Ingelheim Vetmedica, Inc. (St. Joseph, MO). All solutions in water were prepared with 18.2-M Ω distilled, deionized water (Labconco, Kansas City, MO).

2.3. Animals

Male Wistar rats from Charles River (Charles River Laboratories, Wilmington, MA) weighing between 300 and 450 g were used. The animals were kept on a 12-hour light/dark cycle and had free access to food and water prior to surgery. During survival experiments, the animals were given free access to food and water. The research described follows the principles stated in the Guide for the Care and Use of Laboratory Animals, NIH publication 86-23, 1996 edition and was approved by the University of Kansas Research Committee.

2.4. Surgical procedures

2.4.1. Animal and instrumentation preparation—Experimental animals were initially anesthetized by inhalation of isoflurane followed by an i.p. injection of a ketamine (67.5 mg/kg)/xylazine (3.4 mg/kg)/acepromazine (0.67 mg/kg) mixture. The animals were closely monitored during all procedures. Booster doses of one-fourth or one-half of the original dose of ketamine were used as needed to maintain adequate anesthesia. The animal's body temperature was maintained at approximately 37 °C using a Homeothermic Blanket Control Unit (Harvard Apparatus, Holliston, MA).

The incision sites were prepared by shaving away as much hair as possible and then disinfecting with three alternate scrubs each of Prodine Scrub (0.75% aqueous iodine, Phoenix Pharmaceutical, Inc., St. Joseph, MO, USA) and a 70% (v:v) solution of aqueous ethanol. The incisions were closed with sutures or surgical staples. All solutions injected

into the animal were filtered using a disposable 0.2- μ m nylon syringe filter (Acrodisc filters, Fisher Scientific). Surgical tools, drapes, sutures, cannulas, and rinsing water were sterilized with ethylene oxide.

2.4.2. Brain implantation of electrodes, microdialysis guide cannula, and probe—Once fully anesthetized, animals were securely positioned in a stereotaxic apparatus with the incisor bar set at 3.3 mm from the interaural sagittal suture. Adventitious tissue covering the skull was removed using cotton swabs. A 1-mm diameter hole was drilled through the skull at the insertion site, and an intracerebral guide cannula (CMA Microdialysis, Inc., North Chelmsford, MA) was lowered into the cerebral cortex using a micromanipulator attached to the stereotaxic apparatus. The guide cannula was positioned 2 mm above the hippocampus and 4 mm above the striatum and then affixed to the skull with dental cement. The dummy probe in the guide cannula was then replaced with a specially designed probe with an internal Ag/AgCl working electrode (Applied Neuroscience, London, U.K.) for ECoG recordings. A 4-mm microdialysis probe with a polyarylethersulfone (PAES) membrane was used in the striatum (putamen) with the following coordinates: +0.2 mm (posterior), +3.2 mm (lateral), and -7.5 mm (ventral); and a 2-mm probe was used in the hippocampus (CA1) with the following coordinates: -3.3 mm (anterior), +1.7 mm (lateral), and -3.7 mm (ventral).

2.5. Experimental design

Following surgery, animals were allowed to recover for 4 h for anesthetized experiments and for 24 h for awake experiments, during which artificial cerebral spinal fluid (aCSF) for microdialysis (145-mM NaCl, 2.7-mM KCl, 1.0-mM MgCl₂, 1.2-mM CaCl₂, 0.45-mM NaH₂PO₄, and 2.33-mM Na₂HPO₄) was perfused through the probe at a rate of 1.0 μ L/min with a CMA/100 microinjection pump. Prior to dosing with 3-MPA, six samples of background aCSF were collected over 10-minute intervals. After aCSF background collection, a solution of 10-mM 3-MPA in aCSF was administered through the probe at a rate of 1.0 μ L/min for 50 min into the striatum and the hippocampus (CA1) of both awake and anesthetized rats. This dose and the rate of administration were selected to attain a 3-MPA concentration in the microperfused tissue equivalent to that achieved in the systemic steady-state model [5,6]. Once the 3-MPA administration was terminated, aCSF was perfused through the probe for the following 70 min of sampling. Samples were continuously collected over 5-minute intervals for 2 h. Control experiments were performed in both awake and anesthetized animals in both brain regions where aCSF alone was perfused through the probe.

2.6. Electrocorticograph (ECoG) recordings

Electrocorticograph recordings were started 60 min prior to 3-MPA dosing to establish a baseline. Electrocorticograph recordings were made using a SynAmps RT system (Compumedics Neuroscan, Charlotte, NC). Microdialysis probes with an internal Ag/AgCl working electrode (Figs. 4 and 5) were connected to the headbox, and Ag/AgCl wires were placed under the scalp as the reference and ground. The data were collected using SCAN software (Compumedics Neuroscan, Charlotte, NC). A 20,000-Hz sampling rate was reduced to a 200-Hz sampling rate by computing mean values of the signals within adjacent time windows comprising 100 samples.

2.7. Microdialysis sample analysis

2.7.1. 3-MPA analysis by HPLC-EC—A 2.5- μ L sample was injected onto an Agilent ZORBAX 3.5- μ m SB-C18 column (2.1 $\times$ 75 mm, Agilent Technologies, Santa Clara, CA) with a Phenomenex C18 guard cartridge for the analysis of 3-MPA in brain microdialysate.

A liquid chromatographic system with electrochemical detection was used for the detection of 3-MPA. The system consisted of a Shimadzu LC-20AD pump, a Rheodyne 9725i PEEK sample injector, and an LC-4C potentiostat (Bioanalytical Systems, West Lafayette, IN). The mobile phase was adapted from Stenken et al. and consisted of a phosphate buffer with methanol (90:10, v:v) [12]. Specifically, 25-mM NaH_2PO_4 and 0.5-mM Na_2EDTA were adjusted to pH 3.5 with 85% *o*-phosphoric acid.

Electrochemical detection was performed using a thin layer Au/Hg amalgam electrode. Preparation and use of the electrode have been described previously [13]. Briefly, a 3-mm Au electrode embedded in a PEEK block (Bioanalytical Systems, West Lafayette, IN) was polished with 15- μm , 6- μm , 3- μm , and 1- μm diamond polish (Bioanalytical Systems, West Lafayette, IN) followed by 0.3- μm alumina powder. Following polishing with diamond polish and alumina powder, the electrode was rinsed with methanol and water. Triple-distilled mercury was placed over the gold electrode and allowed to rest for 5 min. Excess mercury was then removed with the edge of a credit card, and the electrode sat overnight. Once the amalgamation process was complete, the electrode was placed into the electrochemical flow cell and was allowed to equilibrate with the mobile phase and to dissipate charging current.

The detection of 3-MPA was observed at +100 mV versus an Ag/AgCl reference electrode, and it occurred indirectly by the oxidation of mercury as the thiol passes over the electrode surface. The data were collected at 10 Hz and processed using a Chrom&Spec chromatography data system (Ampersand International, Beachwood, OH).

2.7.2. Amino acid neurotransmitter analysis by HPLC-fluorescence detection

—A liquid chromatographic system with fluorescence detection was used for the analysis of the amino acid neurotransmitters in the microdialysis samples. Two Shimadzu LC-10ADvp pumps, a 100- μL Shimadzu mixer, and a Rheodyne 9725i PEEK sample injector were connected to a Phenomenex Synergi 4- μm Hydro-RP column (150 $\times$ 2.0 mm, Phenomenex, Torrance, CA) with a Phenomenex C18 guard cartridge. The binary gradient was controlled by a Shimadzu SCL-10vp system controller. Mobile phase A consisted of 50-mM ammonium acetate whose pH was adjusted to 6.8 with glacial acetic acid. Five-percent tetrahydrofuran (THF) was added, making the final concentration 95% acetate:5% THF (v:v). Mobile phase B consisted of 100% methanol. The method was adapted from Shah et al. [14]. A Shimadzu 10AXL fluorescence detector was operated at an excitation wavelength of 442 nm and an emission wavelength of 490 nm [15]. The primary amines were derivatized with naphthalene-2,3-dicarboxyaldehyde [16]. For the analysis of the amino acid neurotransmitters, 2.5 μL of microdialysate was spiked with 1.5 μL of the internal standard (*D,L*-2-aminoadipic acid), 1.0 μL of 500-mM borate: 87-mM CN (100:20 v:v), and 1.0 μL of 3-mM NDA in an acetonitrile:water solution (50:50, v:v). Of that 6.0- μL sample, 5.0 μL was injected for analysis.

2.8. Statistical analysis

All statistical analyses were done using the paired *t*-test. Significant differences were assessed at values of $p < 0.05$ and $p < 0.01$ and were compared to a basal level of 100%. The error bars in the figures are expressed as mean $\pm$ SD.

3. Results

3.1. Preliminary experiments

Preliminary experiments were performed to determine the time required for the neurotransmitters to return to basal level in anesthetized animals (4 h) and awake,

unrestrained animals (24 h) after the surgical procedures. There was greater variability in the basal neurotransmitter levels in the awake rats than in the anesthetized rats, but it was not statistically significant. No significant changes occurred in amino acid neurotransmitter concentrations when aCSF was perfused through the probe in the striatum and hippocampus in both awake and anesthetized animals (data not shown).

3.2. 3-Mercaptopropionic acid analysis

The delivery of 10-mM 3-MPA into the striatum and hippocampus in awake and anesthetized animals was identical. 3-Mercaptopropionic acid was measured as a flux ($\mu\text{g}/\text{min}$) across the microdialysis membrane and into the brain. A constant delivery and steady-state concentrations of 3-MPA in the hippocampus and striatum (Fig. 1) were achieved during the 50-minute perfusion period. Clearance of 3-MPA from the probe was exponential.

3.3. Striatum amino acid neurotransmitters

Fig. 2 shows the absolute changes in GABA and glutamate concentration (top panel) during the administration of 10-mM 3-MPA through the microdialysis probe into the striatum of anesthetized rats. Changes in the ratio of glutamate/GABA in these animals are presented in the bottom panel (Fig. 2). There was a 15-fold increase in glutamate in anesthetized rats, which was significantly different from basal levels ($p < 0.01$ and $p < 0.05$). A 5-fold increase in GABA in anesthetized rats was also significantly different from basal levels ($p < 0.05$). Fig. 3 shows the absolute changes in the concentrations of glutamate and GABA (top panel) and in their ratio (bottom panel) in the striatum of awake rats microperfused with 10-mM 3-MPA. A 250-fold increase in glutamate and a 50-fold increase in GABA, both statistically significant ($p < 0.01$ and $p < 0.05$), were measured for various time points.

3.4. Hippocampus amino acid neurotransmitters

Fig. 4 shows the increases in the concentrations of both glutamate and GABA (top panel) and in their ratio (glutamate/GABA) (bottom panel) in the hippocampus of anesthetized rats during microperfusion with 3-MPA. The significant ($p < 0.01$ and $p < 0.05$) 7-fold increase in glutamate was associated with a 2-fold increase in GABA which was only significantly different from basal levels ($p < 0.05$) at one time point. Fig. 5 (top panel) displays the absolute changes in hippocampal concentration in glutamate and GABA in awake rats microperfused with 3-MPA and their ratio (bottom panel). A 25-fold increase in glutamate and a 5-fold increase in GABA, both statistically significant ($p < 0.05$) at several time points, were observed.

3.5. ECoG recordings

The perfusion of 3-MPA triggered bursts of rhythmical “notched” slow wave complexes at 15–25 Hz lasted 1–2 s from tissue in the immediate vicinity of the probe (Fig. 6). Only 1/5 experts that visually analyzed this activity classified it as epileptiform.

4. Discussion

When endowed with electrical recording capabilities, microdialysis allows for the comprehensive profiling of electrochemical neuronal activity at the minicolumn/column scale. Quantification of changes in GABA and glutamate concentrations as a function of state (nonseizure vs. seizure and anesthetized vs. awake/unrestrained rats) under steady-state 3-MPA concentrations yields interesting and unexpected results in largely intact animals.

Unlike the systemic administration of 3-MPA [5,6], its microperfusion into the hippocampus and striatum increased GABA concentrations. Several mechanisms may be invoked to explain the elevations in GABA in this model:

1. Glutamate is actively transported into hippocampal GABAergic terminals and decarboxylated into GABA [17]. In the presence of considerable glutamate concentration elevations, as documented in these experiments, GABA synthesis may increase, even in the presence of 3-MPA. Glutamic acid decarboxylase (GAD) has two isoforms: GAD65 present in vesicles and GAD67 present in cytosol. 3-Mercaptopropionic acid selectively depresses GABA synthesis at synaptic vesicles and upregulates the cytosolic isoform [18]; disruption of the gene coding for GAD65 in mice does not alter the amplitude of miniature inhibitory postsynaptic currents [19], an index of GABA release into the synaptic cleft. This finding suggests that profound inactivation of GAD65 is compensated by increases in GAD67 activity;
2. Increases in postsynaptic glutamate stimulate GABA release [20-22], an innate mechanism that may counteract hyperexcitability [17]. Being ubiquitous in the brain, the two mechanisms described above would also operate with systemic 3-MPA infusions; therefore, they alone do not explain the increases in GABA when 3-MPA is microperfused into the hippocampus and striatum. What additional factor(s) can then explain the elevations in GABA? Maintenance of glutamatergic neuronal activity requires a continuous supply of glucose [23]. Up to 85% of astrocytic glucose consumption during brain activation (generalized seizures with systemic 3-MPA are an extreme form of activation) is triggered by the demand for glycolytically derived energy for Na⁺-dependent accumulation of glutamate and its amidation to glutamine [24]. That GABA decreases with systemically-administered 3-MPA may be explained by the marked increase in glucose utilization during generalized tonic-clonic seizures in animals to whom no energy substrate was supplied during relatively prolonged (2–3 h) experiments [5,6]; the heightened glucose consumption in the context of no exogenous supply restricted glutamate synthesis which, in turn, constrained that of GABA in those experiments [5,6];
3. Another plausible explanation for the elevation in GABA relies on the “inhibitory surround” phenomenon [25]; tissue abutting an experimentally induced “focus” is hyperpolarized [26]. Since GABA is elevated in the “inhibitory surround”, it may diffuse from this “surround” to the epileptogenic tissue immediately around the probe, accounting for the increase in its concentration in the microperfusion experiments.

Several steps were taken to verify that the results were not due to technical errors or equipment malfunction. The retention times for amino acids for unknown samples were compared to standards prior to analysis on a daily basis, and amino acids were analyzed under basal conditions, where aCSF was perfused through the microdialysis probe instead of 3-MPA, and correctness of equipment calibration was ascertained for each experiment.

γ -Aminobutyric acid's pharmacokinetics and pharmacodynamics are dependent on the model, level of activation of the network, and also possibly on the spatial scale at which they are studied. For example, perfusion of hippocampal slices with 3.5-mM 3-MPA alone did not substantially alter the content of GABA nor sufficiently enhanced network excitability, suggesting that GABAergic inhibition may only be weakened in the presence of exogenous network activation [27], which the microperfusion steady-state model lacked.

The lack of emergence of unequivocal epileptiform activity or of seizures in the presence of disinhibition given the reduction in the GABA/glutamate ratio is of interest and deserves

further investigation. If this finding (no ictogenesis in a “milieu” where there is disinhibition, but both GABA and glutamate are above normal concentration) is reproduced in other studies, it raises the possibility that under certain conditions, changes in absolute GABA and glutamate concentration may be more critical to ictogenesis than changes in the GABA/glutamate ratio.

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Fig. 1. 3-MPA (in $\mu\text{g}/\text{min}$) delivered to (A) the striatum in anesthetized rats ($n = 8$), (B) the hippocampus in anesthetized rats ($n = 5$), (C) the striatum in awake rats ($n = 5$), and (D) the hippocampus in awake rats ($n = 5$). Bars represent the SD of concentration changes.

Fig. 2. Changes in the absolute concentration of glutamate and GABA (top panel) and in their ratio (glutamate/GABA) (bottom panel) in the striatum of anesthetized rats ($n = 14$) during (time $t = 0$ through $t = 50$ min) microperfusion of 10-mM 3-MPA. Bars represent the SD of concentration changes. Asterisks (*) indicate the points in time at which concentration changes were statistically significant.

Fig. 3. Changes in the absolute concentration of glutamate and GABA (top panel) and in their ratio (glutamate/GABA) (bottom panel) in the striatum of awake rats ($n = 5$) during (time $t = 0$ through $t = 50$ min) microperfusion of 10-mM 3-MPA. Bars represent the SD of concentration changes. Asterisks (*) indicate the points in time at which concentration changes were statistically significant.

Fig. 4.

Changes in the absolute concentration of glutamate and GABA (top panel) and in their ratio (glutamate/GABA) (bottom panel) in the hippocampus of anesthetized rats ($n = 9$) during (time $t = 0$ through $t = 50$ min) microperfusion of 10-mM 3-MPA. Bars represent the SD of concentration changes. Asterisks (*) indicate the points in time at which concentration changes were statistically significant.

Fig. 5. Changes in the absolute concentration of glutamate and GABA (top panel) and in their ratio (glutamate/GABA) (bottom panel) in the hippocampus of awake rats ($n = 5$) during (time $t = 0$ through $t = 50$ min) microperfusion of 10-mM 3-MPA. Bars represent the SD of concentration changes. Asterisks (*) indicate the points in time at which concentration changes were statistically significant.

Fig. 6.

A) Background ECoG sampling before dosing 3-MPA. (1) Signals from the microdialysis probe electrode; (2 and 3) signals from silver wires placed in close proximity to the microdialysis probe. B) ECoG sampling 28 min after the start of 10-mM3-MPA administration. (1) Signals from microdialysis probe electrode; (2 and 3) signals from silver wires placed in close proximity to the microdialysis probe.