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The use of microwave irradiation for quantitative analysis of neurotransmitters in the mouse brain

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ABSTRACT

Background: Assessing neurotransmitter metabolism in the brain is essential in studying the effects of drugs, dietary modification and characterizing transgenic mouse models of human neurodegenerative diseases. Regional brain concentrations of parent neurotransmitters and related metabolites are informative and provide a snap shot of the steady-state levels. The choice in method of sacrificing mice may differ from one laboratory to another, and the technique in removal of brain may have limitations depending on speed in which tissue can be dissected and frozen to prevent post-mortem changes.

New methods: In order to better assess neurotransmitter metabolism in an effective and standardized manner we evaluated microwave irradiation as a method of sacrificing mice. Mice were sacrificed by CO_2 asphyxiation followed by cervical dislocation or microwave irradiation at 4 Kw for 1.1 s. Brain tissue was harvested into five regions and stored at −80 °C until analysis by either LC–MS/MS for acetylcholine, choline and GABA, or HPLC-EC for dopamine, serotonin and norepinephrine and related metabolites.

Results: The results of our study showed considerable differences in the levels of neurotransmitters between the two methods of sacrifice. Overall, the concentrations of neurotransmitters were higher in mice sacrificed by microwave irradiation, except for GABA, which was lower.

Comparison with existing method(s): Previous microwave irradiation studies employed presently outdated equipment and neurotransmitter analysis methods, and were not as comprehensive.

Conclusions: The combination of microwave irradiation with LC–MS/MS and HPLC-EC detection allows accurate and sensitive measurement of several neurotransmitter systems in discrete mouse brain regions.

1. Introduction

Neurotransmitters are a group of signaling molecules or chemical messengers that allow individual neurons to communicate. Various classes of neurotransmitters and neuropeptides exist and can be found distributed throughout the body. In brain tissue 4 major classes predominate, catecholamines, indoleamines, amino acids and cholinergic. Catechol- and indoleamines are often grouped as biogenic amines or monoamines and include dopamine (DA), norepinephrine (NE), and serotonin (5-HT). Primary and secondary defects in synthesis, transport or degradation of biogenic amines neurotransmitters are involved in pediatric neurotransmitter diseases and with other neuropsychiatric disorders such as, Alzheimer's disease, Parkinson's disease, major depression, schizophrenia, autism and Rett's syndrome (Francis, 2005; Hensler et al., 2013; Kurian et al., 2011; Pearl et al., 2007; Rodan et al., 2015). The amino acid neurotransmitter, Y-aminobutryic acid (GABA), is the most abundant inhibitory neurotransmitter within the central nervous system, and impaired GABAergic transmission is associated with epilepsy, anxiety disorders, schizophrenia and cognitive dysfunction in Down Syndrome (Contestabile et al., 2017; Uehara et al., 2015; Wong et al., 2003). The cholinergic excitatory neurotransmitter, acetylcholine (ACh) plays an important role in learning and memory. It has been shown to be altered in many neurodegenerative diseases such as Alzheimer's disease, vascular dementia and Parkinsonian dementia (Bartus et al., 1982; Bohnen et al., 2003; Wang et al., 2009). Due to the importance of these neurotransmitters in the central nervous system it is imperative that accurate quantitation methods are validated. Furthermore, quantitative measurements that provide a snap shot of steady-state levels of these parent neurotransmitters and their related metabolites in regional brain tissue are essential in studying the effects of drugs, dietary modification and in the characterization of transgenic mouse models of human diseases related to neurotransmitter function.

Abbreviations: DOPAC, 34-dihydroxyphenylacetic acid; 3-MT, 3-methoxytyramine; 5-HIAA, 5-hydroxyindoleacetic acid; ACh, acetylcholine; CB, cerebellum; Ch, choline; CX, cortex; DA, dopamine; HIP, hippocampus; HVA, homovanillic acid; MB, mid-brain; NE, norepinephrine; 5-HT, serotonin; STR, striatum; GABA, Y-aminobutryic acid

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Since the early 1970 s microwave irradiation has been employed to accurately quantitate brain concentrations of high energy compounds such as adenosine triphosphate (Schmidt et al., 1971). This technique rapidly heats the brain to approximately 85–95 °C and causes irreversible enzymatic inactivation within brain tissue, thereby preventing post-mortem metabolism of metabolites with high turnover rates (Bertrand et al., 1994; Blank et al., 1979; Guidotti et al., 1974; Schmidt et al., 1972). It has also been shown that microwave irradiation can simultaneously penetrate surface structures and deep brain areas thereby allowing for accurate regional measurements of brain metabolites (Schmidt et al., 1972). Furthermore, this technique preserves the architecture of the brain tissue allowing for regional dissection, which are informative and provide a snap shot of steady-state levels (Maruyama et al., 1980). Early studies investigated the use of microwave irradiation for the determination of ACh in rat brain tissue and concluded that when compared to decapitation, rats sacrificed by microwave irradiation permitted a more accurate determination of ACh concentrations (Butcher et al., 1976; Moroji et al., 1977). In addition, it has been determined that the concentration of ACh significantly varied by brain region (Schmidt et al., 1972). However, other investigators reported that measurements of ACh, GABA and other compounds obtained from rats that were microwaved were indistinguishable from those obtained by freeze-blowing (Guidotti et al., 1974). One study concluded that microwave irradiation was not essential when measuring NE, DA, 5-HT and 5-hydroxyindoleacetic acid (5-HIAA), a metabolite of 5-HT, but was required for accurate quantitation of 3,4-dihydroxyphenylacetic acid (DOPAC), a metabolite of DA (Moroji et al., 1977). In contrast, the same group later reported that microwave irradiation is necessary for precise quantitation of DA and its related metabolites (Moroji and Takahashi, 1978). Furthermore, another study suggested that mouse striatal DA is susceptible to post-mortem degradation however, NE is not at risk (Blank et al., 1979). These early studies using microwave irradiation to measure biogenic monoamines appear to be inconsistent. Table 1 outlines a general overview of the current literature in relation to this study; specifically to show the discrepancies and lack of information in the literature.

To our knowledge there are no reports in the literature that compare CO_2 asphyxiation, a very common method used to sacrifice mice, to microwave irradiation, or that employ reliable methods to accurately quantitate brain neurotransmitters. In order to better assess neurotransmitter metabolism in an effective and standardized manner we carefully evaluated focused microwave irradiation as a method of euthanizing mice to accurately determine whether post-mortem metabolism leads to aberrant results of five parent neurotransmitters and their metabolites in discrete regional brain tissue.

2. Materials and methods

2.1. Materials

Unless indicated, all chemicals used in this study were obtained from Sigma-Aldrich (St. Louis, MO, USA). Octyl sodium sulfate, heptafluorobutyric acid and formic acid were obtained from Fluka (Hampton, NH, USA). All stable isotope internal standards were purchased from CDN isotopes (Pointe-Claire, Quebec, Canada).

2.2. Chemical stability verification

For verification of chemical stability during microwave irradiation we implanted a 200 µl vial containing a standard solution of DA, 3-methoxytyramine (3-MT), DOPAC, homovanillic acid (HVA), 5-HT, 5-HIAA, GABA, ACh, and choline at a concentration of 0.25 µM, 1 µM, 5 µM and 10 µM in a hot dog to simulate a mouse. The hot dog was exposed to microwave irradiation at 4 kW for 1.1 s (Gerling Applied Engineering, Inc., model GA5013, Modesto, CA, USA). The standard solutions were analyzed before and after exposure to microwave irradiation. This experiment was repeated 4 times for each standard concentration.

2.3. Animals

All experiments involving mice were performed in accordance with protocols approved by the Institutional Animal Care and Use Committee at Baylor Scott and White Research Institute. Five month old male C57BL/6 *J* mice (n = 30) were obtained from Jackson Laboratory (Sacramento, CA, USA). Mice were housed two to six per cage in a temperature-controlled enriched environment, maintained on a 12 h light dark cycle, and were allowed food and water *ad libitum*. At 6 months of age half of the mice (n = 15) were sacrificed by CO_2 asphyxiation followed by cervical dislocation. The other subset of mice (n = 15) were sacrificed by focused microwave irradiation at 4 kW for 1.1 s (Gerling Applies Engineering, model GA5013, Modesto, CA, USA). This unit has a unique applicator system that directs a large amount of focused irradiation in a short amount of time to the head of the mice secured in a plastic restraint device. Immediately following euthanasia the brain was harvested, and dissected into five regions (striatum, hippocampus, cortex, cerebellum, and mid-brain). Tissues were stored at −80 °C until time of analysis. On average the complete process takes less than five minutes per mouse.

2.4. Metabolite analysis

Brain tissue was deproteinized with 4 volumes (cortex, cerebellum, and mid-brain) or 9 volumes (striatum, hippocampus, and frontal cortex) of ice-cold 0.1 M perchloric acid (PCA) containing 0.1 M diethylenetriaminepentaacetic acid (DETAPAC) and 1 M dithioerythritol (DTE). Samples were immediately centrifuged at 11,000 *g* for 15 min at a temperature of 8 °C.

The biogenic amines, DA and 5-HT, and their metabolites (3-MT, DOPAC, HVA, and 5-HIAA) were analyzed using HPLC (Shimadzu SIL-20AC/LC-20AD, Columbia, MD, USA) with electrochemical detection. The metabolites were eluted on a reverse-phase Phenomenex Gemini 5µ C18 250 x 3.0 mm column (Torrance, CA, USA) maintained at 35 °C with a flow rate of 0.350 ml/min. The mobile phase was composed of 50 mM potassium dihydrogen phosphate, 1 mM octyl sodium sulfate, 54 µM ethylenediaminetetraacetic acid (EDTA), and 14% methanol, and adjusted to pH = 2.65 with 85% phosphoric acid. Detection of the compounds was performed on an ESA Microdialysis cell 5014B and ESA Guard cell 5020 (Waltham, MA, USA), with cell potentials set at $E_1 = -10$ mV, $E_2 = +400$ mV, and guard cell = + 600 mV. Amine stock standards were prepared in 0.1 M hydrochloric acid at a concentration of 1 mM and stored at −80 °C. Calibration standards were diluted with ice-cold 0.1 M PCA containing 0.1 M DETAPAC and 1 M DTE to a final concentration of 1 µM. 10 µl of PCA extract was injected straight onto the HPLC using a Dynamax-Rainin refrigerated autosampler (model A1-200, Emeryville, CA, USA). The analytical measurement range (AMR) of the assay is 0.01–10 µM for all analytes except 5-HT and DA. 5-HT and DA AMR is 0.02-10 µM. The coefficient of variation for the lower limit of quantitation, intra- and inter- day precision are all below 20%. Intra-day precision was evaluated by repeat analysis of bi-level matrix matched quality control material 5 runs in a single day. Inter-day precision was evaluated by repeated analysis of bi-level matrix matched quality control materials on 20 different days. Detailed validation data provided in the supplementary material (Table S1).

Norepinephrine was analyzed using HPLC (Shimadzu SIL-20AC/LC-20AD, Columbia, MD, USA) equipped with electrochemical detection. NE was eluted on a reverse-phase Phenomenex Gemini C18, 5 µm, 250 x 3 mm column (Torrance, CA, USA). The column was maintained at 35 °C with a flow rate of 0.5 ml/min. The mobile phase was composed of 75 mM sodium dihydrogen phosphate, 1.5 mM sodium dodecyl sulfate, 25 µl EDTA, 5% methanol, 20% acetonitrile and adjusted to pH = 5.6 with 85% phosphoric acid. Detection of NE was performed on