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QUANTIFICATION OF RESERVE POOL DOPAMINE IN METHIONINE SULFOXIDE REDUCTASE A NULL MICE

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Abstract—Methionine sulfoxide reductase A knockout (*MsrA*$^{-/-}$) mice, which serve as a potential model for neurodegeneration, suffer from increased oxidative stress and have previously been found to have chronically elevated brain dopamine (DA) content levels relative to control mice. Additionally, these high levels parallel the increased presynaptic DA release. In this study, fast-scan cyclic voltammetry (FSCV) at carbon-fiber microelectrodes was used to quantify striatal reserve pool DA in knockout mice and wild-type control mice. Reserve pool DA efflux, induced by amphetamine (AMPH), was measured in brain slices from knockout and wild type (WT) mice in the presence of α-methyl-p-tyrosine, a DA synthesis inhibitor. Additionally, the stimulated release of reserve pool DA, mobilized by cocaine (COC), was measured. Both efflux and stimulated release measurements were enhanced in slices from knockout mice, suggesting that these mice have greater reserve pool DA stores than wild-type and that these stores are effectively mobilized. Moreover, dopamine transporter (DAT) labeling data indicate that the difference in measured DA efflux was likely not caused by altered DAT protein expression. Additionally, slices from *MsrA*$^{-/-}$ and wild-type mice were equally responsive to increasing extracellular calcium concentrations, suggesting that potential differences in either calcium entry or intracellular calcium handling are not responsible for increased reserve pool DA release. Collectively, these results demonstrate that *MsrA*$^{-/-}$ knockout mice maintain a larger DA reserve pool than wild-type control mice, and that this pool is readily mobilized.

Key words: methionine sulfoxide reductase, reserve pool, dopamine, voltammetry, cocaine, amphetamine.

The abnormal regulation of dopamine (DA) has been associated with multiple neurodegenerative disease states (Bird and Iversen, 1974; Morgan et al., 1987), yet the role of DA reserve pool storage and mobilization in the pathophysiology of these conditions is now being revealed. Many of these conditions, such as Parkinson's disease, Huntington's disease, and Lou Gehrig's disease, have been associated with the increased production or reactive oxygen species, thereby enhancing the degree of cell oxidative stress in the brain (Patten et al., 2010; Cohen, 1983; Perez-Severiano et al., 2004).

Interestingly, elevated DA levels have also been associated with enhanced oxidative stress (Oien et al., 2008b). Indeed, one model of oxidative stress, methionine sulfoxide reductase A knockout (*MsrA*$^{-/-}$) mice, have been reported to have chronically high brain DA levels (Oien et al., 2008b). These mice lack the antioxidant enzyme *MsrA*, which is part of the Msr system. Methionine sulfoxide post-translational modifications can be reversed by the Msr system, which consists of *MsrA* (reduces *S* methionine sulfoxide enantiomer) and *MsrB* (reduces *R* methionine sulfoxide enantiomer) (Moskovitz, 2005). The *MsrA*$^{-/-}$ mouse is hypersensitive to oxidative stress, accumulates higher levels of carbonylated protein, and expresses brain pathologies associated with neurodegenerative diseases (Moskovitz et al., 2001; Pal et al., 2007). Recent studies have shown that these mice have abnormally high DA levels in the brain at the ages of 6 and 12 months, compared with wild type (WT) control mice. Additionally, these high levels parallel an increased presynaptic DA release when stimulated *in vitro* without drug treatments.

A possible mechanism for an increase in stimulated DA release in *MsrA*$^{-/-}$ mice involves the mobilization of reserve pool DA. In general, DA-containing vesicles are believed to be separated into three pools: the readily releasable pool (RRP), the recycling pool, and the reserve pool (Neves and Lagnado, 1999; Rizzoli and Betz, 2005). The RRP undergoes exocytosis on mild stimulation and is replenished by the mobilization of the recycling pool vesicles. The reserve pool, mobilized on prolonged periods of synaptic activity (Neves and Lagnado, 1999), is the largest pool consisting of 80–90% of the total vesicles (Rizzoli and Betz, 2005). Pharmacological manipulations, using a combination of alpha-methyl-p-tyrosine (aMPT) and either cocaine (COC) or amphetamine (AMPH; Venton et al., 2006; Ortiz et al., 2010), have been used to measure reserve pool DA quantitatively.

Other factors, such as calcium transport, may also influence the amplitude of stimulated DA release plots. Transient increases in intracellular calcium concentration trigger vesicular exocytosis (Nachshen and Sanchez-Armass, 1987; Kume-Kick and Rice, 1998) as well as the movement of RRP and reserve pool vesicles (Rose et al., 2002). Moreover, the increase in oxidative stress may result in calcium dysregulation. For example, the activity of calmodulin, a calcium regulatory protein that activates the plasma membrane calcium

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Abbreviations: aCSF, artificial cerebral spinal fluid; AMPH, amphetamine; aMPT, alpha-methyl-p-tyrosine; COC, cocaine; DA, dopamine; DAT, dopamine transporter; FSCV, fast-scan cyclic voltammetry; *MsrA*$^{-/-}$, methionine sulfoxide reductase A knockout; RRP, readily releasable pool; WT, wild type.

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ATPase (PMCA), diminishes because of oxidative post-translational modifications as tissues age (Michaelis et al., 1996). The oxidation of specific methionines in calmodulin results in about a 50% reduction of PMCA activation (Bartlett et al., 2003), thereby impairing the ability of cells to clear calcium from the cell (Palacios et al., 2004). Oxidized calmodulin can accumulate in brain tissues as a result of low antioxidant levels and it is speculated that oxidation of methionines on calmodulin may be acting as a molecular switch in calcium regulation, oxidative stress, and DA release (Chen et al., 2001; Bigelow and Squier, 2005).

To investigate possible mechanisms underlying elevated DA content and release found in *MsrA*$^{-/-}$ mice, fast-scan cyclic voltammetry (FSCV) at carbon-fiber microelectrodes was used to measure the mobilization and efflux of reserve pool DA in striatal brain slices from *MsrA*$^{-/-}$ mice and WT control mice (Oien et al., 2008b). We hypothesized that the DA reserve pool is enhanced in *MsrA*$^{-/-}$ mice compared with WT control mice. In order to measure reserve pool DA, slices were pre-treated with αMPT and then treated with either AMPH, to measure the efflux of reserve pool DA, or with COC, to measure the stimulated release of mobilized DA reserve pool vesicles. Collectively, our results suggest that reserve pool DA is more abundant in the *MsrA*$^{-/-}$ striatum and that the number of vesicles is greater compared with WT controls.

EXPERIMENTAL PROCEDURES

Animals

The *MsrA*$^{-/-}$ and WT control mice have been described previously (Moskovitz et al., 2001). All mice used in these experiments were fed *ad libitum*, housed with 12-h of light per day, and caged individually. All procedures and conditions of live mice, including euthanasia, were approved by the University of Kansas Institutional Animal Care and Use Committee. Every effort was made to minimize the number of animals used and animal suffering.

Brain slice preparation

Brain slices of 12-month-old *MsrA*$^{-/-}$ mice and age-matched WT control mice were prepared as previously described (Johnson et al., 2006). Brain slices were used as an experimental preparation because they allow for the direct measurement of locally evoked DA release and generally provide higher throughput than whole animal preparations. Mice were anesthetized by isoflurane inhalation and then decapitated. The brain was immediately removed and placed in ice cold artificial cerebrospinal fluid (aCSF) consisting of (mM): NaCl 126, KCl 2.5, NaH_2PO_4 1.2, $CaCl_2$ 2.4, $MgCl_2$ 1.2, $NaHCO_3$ 25, HEPES 20, and D-Glucose 11. The pH of the aCSF was adjusted to 7.4. The cerebellum was removed from the brain using a razor blade and the brain was then mounted on an aluminum block. A vibratome slicer (Leica, Wetzlar, Germany) was used to make 300 μm thick coronal slices. Each brain slice was equilibrated in the superfusion chamber which was maintained at 34 °C, through which aCSF flowed at a continuous rate of 2 mL/min, before obtaining measurements.

DA release in brain slices

Carbon-fiber microelectrodes were fabricated using a single 7 μm diameter carbon-fiber (Goodfellow Cambridge Ltd, Huntingdon, UK) that was aspirated through a glass capillary tube (1.2 mm outer diameter, 0.68 mm inner diameter, 20 mm long, A-M Systems, Inc, Carlsborg, WA, USA), and was pulled using a heated coil puller (Narishige International USA, Inc., East Meadow, NY, USA; Kraft et al., 2009). The carbon-fiber was trimmed to about 25 μM and further insulated using with epoxy resin (EPON resin 815 C, EPIKURE 3234 curing agent, Miller-Stephenson, Danbury, CT, USA), and then cured at 100 °C for 1 h. The electrodes were backfilled with 0.5 M potassium acetate to provide an electrical connection between the carbon fiber and an inserted silver wire.

A triangular waveform was applied to the carbon-fiber electrode starting at −0.4 V, increasing to +1.0 V, and then scanning back down to −0.4 V. For stimulated release measurements, a scan rate of 300 V/s and an update rate of 60 cyclic voltammograms (CVs) per second were used. For measurements of AMPH-induced DA efflux, an update rate of five CVs was used to limit the memory space occupied by the file. A headstage amplifier (UNC Chemistry Department Electronics Design Facility, Chapel Hill, NC, USA) was interfaced with a computer through a breakout box and custom software provided by R.M. Wightman and M.L.A.V. Heien (University of North Carolina, Chapel Hill, NC, USA). A Ag/AgCl reference electrode consisted of a chorided silver wire. The carbon fiber microelectrode was inserted 100 μm into the dorsolateral caudate–putamen region of the striatum between the prongs of a bipolar stimulation electrode (Plastics One, Roanoke, VA, USA), which was separated by a distance of 200 μm. A single pulse at 60 Hz was applied to the brain slice and the current was then measured at the peak oxidation potential for DA (about +0.6 V vs. Ag/AgCl reference electrode). Working electrodes were calibrated with DA standards of known concentration in a flow cell before and after each use. The average of pre- and post-calibration measurements was used as the calibration factor. Drugs were introduced into the brain slice superfusion chamber by opening a three-way valve that allowed the desired solution to flow by gravity feed.

For AMPH-induced DA efflux experiments, brain slices were stimulated with single electrical stimulus pulses until the magnitude of evoked DA release was consistent between measurements. Brain slices were then treated with 50 μM aMPT. During this treatment, stimulated DA release was measured every 5 min. Once DA release diminished, 20 μM AMPH was added to the aCSF/aMPT solution and a 25 min duration file was collected.

To measure electrically evoked DA release from mobilized reserve pool vesicles, the same procedure for pre-treatment with aMPT was used. However, slices were treated with 20 μM COC after the stimulated release was diminished. During this treatment, electrically-evoked release was measured every 5 min.

Sensitivity of DA release to extracellular calcium

To measure changes in the sensitivity to calcium in *MsrA*$^{-/-}$ mice, DA release evoked by a single electrical stimulus pulse was first measured in slices with aCSF that contained 2.4 mM calcium. Next, aCSF containing 0 mM calcium was introduced into the superfusion chamber. Once DA release disappeared, brain slices were cumulatively treated with aCSF containing 0.6, 1.2, 1.8, and 2.4 mM calcium. Slices were treated with each concentration for 15 min, with files collected every five 5 min using a single pulse stimulation. Values obtained at the 15-min point were used in Fig. 4.

Immunoblotting

Methionine sulfoxide reductase A knockout- and WT-brains were dissected from post-mortem mice. Each striatal lysate was placed in a glass tube and homogenized with teflon in 50 mM HEPES buffer pH 7.4 plus protease inhibitors (Roche) at 4 °C. Homogenized extract was centrifuged for 20 min to separate the membrane fraction. Buffer containing 50 mM HEPES, 0.62% CHAPS, and 150 mM NaCl was added to each membrane fraction and agitated for 30 min. Each fraction was centrifuged for 20 min. Protein concentration of the supernatant was determined by BCA Protein Assay Kit (Pierce). Equal protein amounts (20 μg) of each soluble membrane fraction