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MEASUREMENT OF PLASMALEMMAL DOPAMINE TRANSPORT, VESICULAR DOPAMINE TRANSPORT, AND K⁺-STIMULATED DOPAMINE RELEASE IN FROZEN RAT BRAIN TISSUE

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Abstract

This report describes experiments designed to 1) establish the specificity of dopamine (DA) transporter (DAT)-mediated plasmalemmal DA transport, vesicular monoamine transporter-2 (VMAT-2)-mediated vesicular DA transport, and K⁺-stimulated DA release in samples prepared from frozen rat striata, and 2) characterize the time-course of the effects of freezing on these processes. The procedure described herein uses a simple method of freezing brain tissue (first cooling in ice-cold buffer and then freezing at –80 °C) that allows for the storage of rat striata followed by the assay of DA transport and K⁺-stimulated DA release using rotating disk electrode voltammetry. Plasmalemmal DA transport into samples prepared from frozen striata was blocked by the DAT inhibitor, cocaine, and vesicular DA transport was blocked by the VMAT-2 inhibitor, dihydrotetrabenazine. Additionally, K⁺-stimulated DA release was Ca²⁺-dependent. Freezing decreases DAT-mediated DA transport, VMAT-2-mediated DA transport, and K⁺-stimulated DA release. However activity is still measurable even after 3 weeks of storage. These results suggest that rat striata retain some DA transport and DA release activity even when stored frozen for a few weeks. Frozen storage of rat striata may thus be valuable for experiments requiring lengthy assays, the accumulation of material, or the transport of samples from one laboratory to another for analysis. These results may also be applicable to the study of frozen human brain tissue.

Keywords

Dopamine; Dopamine Transporter; Striata; Vesicular Monoamine Transporter-2

1. INTRODUCTION

There are several methods available for use in studying plasmalemmal neurotransmitter transport (Eshleman et al., 2001; Fowler et al., 1989; Haberland and Hetey, 1987; Nichols et al., 1989; Schwarcz, 1981; Stenstrom et al., 1985) or K⁺-stimulated neurotransmitter release (Drapeau, 1988; Fowler et al., 1989; Nichols et al., 1989) using frozen rat brain tissue. These tissue freezing methodologies have also been adapted to the study of human and baboon brain (Hardy et al., 1983; Mash et al., 2002). However, most of these rat brain tissue methods were

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developed to serve as models for procedures to study post-mortem human brain autopsy samples. As such, they often include post-mortem delays of several hours where the brain is left *in situ* (Fowler et al., 1989; Haberland and Hetey, 1987; Schwarcz, 1981). These methods may also include complex protocols involving freezing at -10°C followed by freezing in an acetone/dry ice mixture with subsequent storage in liquid nitrogen (Drapeau, 1988), or incubation with sucrose and dimethylsulfoxide followed by freezing at -25°C and then storage in liquid nitrogen (Haberland and Hetey, 1987).

To the best of our knowledge, none of these methods using frozen rat brain tissue has investigated the transport of neurotransmitters such as dopamine (DA) into synaptic vesicles. Vesicular transport is an important aspect of DA cycling and the vesicular monoamine transporter-2 (VMAT-2) is the sole neuronal element responsible for sequestering cytoplasmic DA. The VMAT-2 is thus an important regulator of DA neurotransmission and alterations in VMAT-2 function may change intra- and extra-neuronal DA levels and consequent postsynaptic events. VMAT-2-containing vesicles may be classified as either membrane-associated or cytoplasmic depending on whether they do or do not, respectively, co-fractionate with striatal synaptosomal membranes after osmotic lysis (Volz et al., 2009a; Volz et al., 2007).

The present work aimed to establish a simple freezing protocol that would permit the selective measurement of vesicular DA transport, plasmalemmal DA transport, and DA release in samples prepared from frozen rat striata using rotating disk electrode voltammetry. The goals were to 1) establish the specificity of DA transporter (DAT)-mediated plasmalemmal DA transport, VMAT-2-mediated vesicular DA transport, and K^{+} -stimulated DA release in samples prepared from frozen rat striata, and 2) characterize the time-course of the effects of freezing on these processes.

2. MATERIALS AND METHODS

Male Sprague-Dawley rats (300–360 g) were purchased from Charles River Laboratories (Raleigh, NC) and housed in a light- and temperature-controlled room with free access to food and water. Animal procedures were approved by the University of Utah Institutional Animal Care and Use Committee and were conducted in accordance with the National Institutes of Health Guidelines for the Care and Use of Laboratory Animals. After decapitation, the striata were rapidly dissected and placed in ice-cold buffer (126 mM NaCl, 4.8 mM KCl, 1.3 mM CaCl_2 , 16 mM sodium phosphate, 1.4 mM MgSO_4 , and 11 mM dextrose at pH 7.4) for the duration of the remaining dissections (total time in buffer ≤ 45 min). When all dissections were finished, the striata were then either used fresh or placed in ice-cold plastic micro-centrifuge tubes (with no buffer present) and stored at -80°C for 48 hours to 8 weeks as described in the figure legends. Frozen striata were thawed at 22°C for 20 min (again with no buffer present) prior to use.

The initial velocities of DAT-mediated plasmalemmal DA transport into striatal suspensions prepared from the striata, and VMAT-2 mediated vesicular DA transport into cytoplasmic and membrane-associated vesicles isolated from the striata, were measured using rotating disk electrode voltammetry as described previously (Volz et al., 2007; Volz et al., 2009b; Volz et al., 2006). The only exception to these published procedures was that the striatal suspensions prepared from frozen striata did not settle sufficiently by gravity and were instead centrifuged ($22,000 \times g$ for 15 min at 0°C) prior to being washed. The initial velocities, magnitudes, and durations of K^{+} -stimulated DA release from striatal suspensions prepared from the striata were also measured using rotating disk electrode voltammetry as described previously (Volz et al., 2009a). Statistical comparisons of the results were done using a one-way ANOVA with a Tukey post-test, and were considered significantly different when $p < 0.05$.

3. RESULTS

Before characterizing the time-course of the effects of freezing, the specificity of DA transport and DA release were first established. Plasmalemmal DA transport into striatal suspensions prepared from frozen striata was inhibited by the DAT inhibitor, cocaine (Fig. 1A). Similarly, vesicular DA transport into cytoplasmic vesicles isolated from frozen striata was inhibited by the VMAT-2 inhibitor, dihydrotetrabenazine (Fig. 1B). However, there was no detectable DA transport into membrane-associated vesicles isolated from frozen striata (data not shown) suggesting that VMAT-2 DA transport assays in these vesicles require fresh tissue. Finally, K^+ -stimulated DA release from striatal suspensions prepared from frozen striata was Ca^{+2} -dependent (Fig. 1C).

Freezing rat striata at $-80^{\circ}C$ for 48 hours to 8 weeks decreased the initial velocities of plasmalemmal DA transport into striatal suspensions (Fig. 2A). Plasmalemmal DA transport velocities were decreased by 35% after 48 hours at $-80^{\circ}C$ and by 55–60% after 1, 3, or 8 weeks. The initial velocities of vesicular DA transport into cytoplasmic vesicles isolated from rat striata were also decreased by freezing at $-80^{\circ}C$ (Fig. 2B). Vesicular DA transport velocities were decreased by 35% after 48 hours at $-80^{\circ}C$ and by 62–65% after 1, 3, or 8 weeks.

As seen with DA transport, DA release from striatal suspensions was also decreased by freezing at $-80^{\circ}C$. The initial velocities of K^+ -stimulated DA release were decreased by 24% after 48 hours, by 65% after 1 week, and by 72% after 3 weeks (Fig. 3A). Likewise, the magnitudes of K^+ -stimulated DA release were decreased by 36% after 48 hours, by 67% after 1 week, and by 73% after 3 weeks (Fig. 3B). DA release was not detectable after the striata had been frozen 8 weeks. Freezing did not alter the duration of DA release (fresh = 7.1 ± 0.9 s, 48 hrs = 7 ± 1 s, 1 week = 6.0 ± 0.5 s, 3 weeks = 7 ± 1 s, 8 weeks = none detectable).

4. DISCUSSION

The first experimental goal was to establish the specificity of plasmalemmal and vesicular DA transport, as well as K^+ -stimulated DA release, using samples prepared from frozen rat striata. This has already been done in fresh rat striatal tissue (McElvain and Schenk, 1992; Volz et al., 2007; Volz et al., 2006). As shown in Fig. 1, plasmalemmal DA transport into, and K^+ -stimulated DA release from, striatal suspensions prepared from rat striata frozen at $-80^{\circ}C$ were both detectable by rotating disk electrode voltammetry. Vesicular DA transport into cytoplasmic vesicles isolated from frozen striata was also detectable. However, there was no detectable vesicular DA transport into membrane-associated vesicles isolated from frozen striata (data not shown). In addition to the previously reported differences in DA transport kinetics and pharmacological regulation (Farnsworth et al., 2009; Volz et al., 2007), this disparity further underscores the dissimilarity between cytoplasmic and membrane-associated vesicles.

Plasmalemmal DA transport into striatal suspensions prepared from frozen rat striata was blocked by the DAT inhibitor, cocaine (Fig. 1A). Similarly, vesicular DA transport into cytoplasmic vesicles isolated from frozen striata was blocked by the VMAT-2 inhibitor, dihydrotetrabenazine (Fig. 1B). This suggests that the plasmalemmal and vesicular DA transport measured in samples prepared from frozen striata are mediated by the DAT and VMAT-2, respectively, as is seen with fresh tissue (Volz et al., 2007; Volz et al., 2006). K^+ -stimulated DA release from striatal suspensions prepared from frozen striata was Ca^{+2} -dependent (Fig. 1C), which suggests that the release represents exocytotic vesicular DA release as is seen with fresh tissue (McElvain and Schenk, 1992). Thus, the specificity of both plasmalemmal and vesicular DA transport, as well as K^+ -stimulated DA release, reported here

with frozen tissue successfully reproduces that reported previously for fresh tissue (McElvain and Schenk, 1992; Volz et al., 2007; Volz et al., 2006).

After establishing the specificity of DA transport and DA release, the second experimental goal was to investigate the time-course of the effects of freezing on these processes. Plasmalemmal DA transport velocities measured by rotating disk electrode voltammetry were decreased after 48 hours at -80°C and further decreased after 1 week with no additional decrease at 3 or 8 weeks (Fig. 2A). This is consistent with similar reported decreases in [^3H]DA, [^{14}C]DA, [^3H]norepinephrine, [^{14}C]acetylcholine, [^3H]serotonin, and [^3H]glutamate transport into samples prepared from frozen rat striata, rat cerebral cortex, and rat nucleus accumbens (Eshleman et al., 2001; Fowler et al., 1989; Haberland and Hetey, 1987; Nichols et al., 1989; Schwarcz, 1981; Stenstrom et al., 1985).

Like plasmalemmal DA transport velocities, the vesicular DA transport velocities into cytoplasmic vesicles measured by rotating disk electrode voltammetry were decreased after 48 hours at -80°C and further decreased after 1 week with no additional decrease at 3 or 8 weeks (Fig. 2B). To the best of our knowledge, this is the first report of measuring vesicular DA transport in samples isolated from frozen rat brain tissue.

The effects of freezing on K^+ -stimulated DA release had a different time course from plasmalemmal and vesicular DA transport. Both the velocity and magnitude of DA release were decreased after 48 hours at -80°C , with further decreases occurring after 1 and 3 weeks (Fig. 3). There was no detectable DA release after 8 weeks at -80°C . There is some disagreement in the scientific literature regarding the effects of freezing on neurotransmitter release. Consistent with the effects shown in Fig. 3, K^+ -stimulated [^3H]norepinephrine and [^{14}C]acetylcholine release from rat cerebral cortex synaptosomes are both decreased by freezing (Nichols et al., 1989). However, freezing rat striatal synaptosomes has also been reported to not significantly alter K^+ -stimulated [^3H]DA release (Drapeau, 1988). Lastly, freezing rat cerebral cortex samples has been reported to decrease 40 mM K^+ -stimulated [^3H]serotonin release (i.e., the K^+ concentration used in the present work) while increasing 25 mM K^+ -stimulated [^3H]serotonin release (Fowler et al., 1989). It is possible that differences in the tissue preservation techniques and the experimental methodologies used account for these disparities.

In conclusion, this report presented a simple method of freezing brain tissue (first cooling in ice-cold buffer and then freezing at -80°C) that allows for the storage of rat striata followed by the assay of DA transport and K^+ -stimulated DA release activity using rotating disk electrode voltammetry. The specificity of both plasmalemmal and vesicular DA transport, as well as K^+ -stimulated DA release, in frozen tissue successfully reproduces that reported for fresh tissue (McElvain and Schenk, 1992; Volz et al., 2007; Volz et al., 2006). While freezing rat striata at -80°C decreases both DA transport and DA release, activity is still measurable even after 3 weeks of storage. All of these results suggest that rat striata retain DA transport and DA release activity when stored frozen for a few weeks. Frozen storage of rat striata may thus be valuable for experiments requiring lengthy assays, the accumulation of material, or the transport of samples from one laboratory to another for analysis. These results may also be applicable to the study of frozen human brain tissue.

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Fig. 1.

Plasmalemmal (Panel A) and vesicular (i.e., into cytoplasmic vesicles) (Panel B) DA transport into samples prepared from striata frozen for 1 week at -80°C are inhibited by the DAT inhibitor cocaine and the VMAT-2 inhibitor dihydrotetrabenazine (DHTBZ), respectively, while K^{+} -stimulated DA release (Panel C) is Ca^{+2} -dependent. Panels A and B each depict representative DA concentration versus time clearance profiles of exogenously applied DA (shown by the arrow). DA transport in the clearance profiles is indicated by a downward sloping line as DA is transported into synaptosomes or vesicles where the rotating disk electrode cannot detect it. The cocaine and dihydrotetrabenazine concentrations were $100\text{ }\mu\text{M}$ and 60 nM , respectively. Panel C depicts representative DA concentration versus time release profiles

induced by exogenously applied 40 mM K⁺ (shown by the arrow) in the presence and absence 1.3 mM Ca⁺². DA release is indicated by an upward sloping line.

Fig. 2.

Freezing striatal tissue at -80 °C for various times (48 hours to 8 weeks) decreases the initial velocities of both DAT-mediated plasmalemmal DA transport into striatal suspensions (Panel A) and VMAT-2-mediated vesicular DA transport into cytoplasmic vesicles (Panel B). Each column represents the mean $\pm$ SEM of four separate measurements. A single asterisk indicates a statistical difference compared with all other groups while two asterisks indicate a statistical difference compared with both fresh tissue and tissue frozen for 48 hours.

Fig. 3.

Freezing striatal tissue at -80 °C for various times (48 hours to 8 weeks) decreases the initial velocities (Panel A) and magnitudes (Panel B) of K⁺-stimulated DA release from striatal suspensions. Each column represents the mean $\pm$ SEM of four separate measurements. A single asterisk indicates a statistical difference compared with all other groups. ND = no detectable DA release.