Journal of
Alcoholism & Drug Dependence

Hiranita and Freyberg, J Alcohol Drug Depend 2016, 4:6
DOI: 10.4172/2329-6488.1000e136

Editorial Open Access

Importance of Substrate-Coupled Proton Antiport by the Vesicular Monoamine Transporter in the Actions of Amphetamines in *Drosophila* Brain

Takato Hiranita[1*] **and Zachary Freyberg**[2]

[1]*Division of Neurotoxicology, National Center for Toxicological Research (NCTR), U.S. Food and Drug Administration (FDA), AR 72079-9502, USA*

[2]*Department of Psychiatry, University of Pittsburgh, Pittsburgh, PA 15213, USA*

[*]**Corresponding author:** Hiranita T, Division of Neurotoxicology, National Center for Toxicological Research (NCTR), U.S. Food and Drug Administration (FDA), 3900 NCTR Rd, Jefferson, AR 72079, USA, Tel: 870-543-6823; Fax: 870-543-7745; E-mail: takato.hiranita@fda.hhs.gov

Received date: December 08, 2016; **Accepted date:** December 09, 2016; **Published date:** December 16, 2016

Editorial

Racemic α-methylphenethylamine (amphetamine) was discovered in 1910 [1] and its abuse potential has been well-documented [2-4]. Critical to the amphetamines' abuse potential is their ability to increase extracellular dopamine (DA) levels in nerve terminals of mesolimbic dopaminergic neurons [5-7]. This capacity to raise synaptic DA levels and the consequent *in vivo* behavioral effects of these drugs resemble the actions of other psychostimulants including cocaine and methylphenidate, albeit they likely result from different molecular mechanisms [5]. Unlike cocaine and methylphenidate, which are non-substrate inhibitors of the plasma membrane DA transporter (DAT), amphetamines are DAT substrates [5,8]. Following increases in cytoplasmic concentration via transport by the DAT, amphetamines are further concentrated within DA synaptic vesicles through actions of the vesicular monoamine transporter 2 (VMAT2). This leads to intraluminal alkalization and eventual redistribution of intraluminal DA into the cytoplasm, followed by subsequent efflux into the synaptic cleft by DAT [5]. Prevailing models of amphetamine action, including the weak base hypothesis, contend that amphetamine's property as a weak base (pKa=9.9) causes this intraluminal alkalization [5,9]. Since the vesicular pH gradient (ΔpH) is the primary driving force for vesicular DA uptake and retention, amphetamine-induced dissipation of this gradient is thought to be responsible for the redistribution of DA out of the vesicle lumen into the cytoplasm [10]. Nevertheless, the precise mechanisms underlying these discrete steps of amphetamine action remain poorly understood and the subject of continued debate. However, a recent study has provided further insights into these mechanisms [11]. Freyberg and colleagues demonstrated *in vivo* that substrate-coupled H+ antiport by VMAT is critical to the synaptic vesicle alkalization induced by amphetamines using novel genetic, pharmacological and optical approaches in *Drosophila* melanogaster and rodents [11]. Key points of this work include:

Essential role of Drosophila VMAT (dVMAT) in amphetamine action

An amphetamine-induced increase in *Drosophila* larval crawling velocity was five-fold lower in dVMAT null mutants compared to wildtype (WT) controls, suggesting that dVMAT is essential for amphetamine action *in vivo*. To examine the role of dVMAT specifically in DA neurons, dVMAT expression was selectively restored in DA neurons within a dVMAT null genetic background using the tyrosine hydroxylase (TH) promoter ("TH Rescue"). Feeding amphetamine to TH Rescue larvae increased their crawling velocity comparably to that of WT larvae. Thus, dVMAT expression specifically in DA neurons plays a critical role in mediating *in vivo* amphetamine-stimulated behavior in flies.

Selective labelling of DA terminals with FFN206 as a fluorescent dVMAT substrate

An *ex vivo* whole fly brain preparation with the TH Rescue genotype was used to optically monitor dopaminergic vesicle contents using multiphoton microscopy. The fluorescent VMAT2-specific substrate, FFN206 [12], selectively labeled presynaptic DA nerve terminals throughout the brain. Specificity of dVMAT-dependent FFN206 labeling was confirmed following acute treatment with VMAT2 inhibitors including reserpine or the novel dihydrotetrabenazine derivative, (+)-CYY477 which completely blocked labeling. As a control, KCl-induced depolarization caused a rapid decrease in fluorescence due to exocytic release of FFN206 concentrated in the vesicle lumen. Amphetamine also induced FFN206 destaining in a manner that was insensitive to blockade of voltage-gated Na+ channels (tetrodotoxin) or nicotinic cholinergic receptors (tubocurarine), suggesting that the FFN206 redistribution was due to local action at the terminals. Taken together, these results indicate that FFN206 can function as a fluorescent dVMAT substrate and surrogate of vesicular DA content that selectively labels DA terminals.

Characterization of dVMAT-pHluorin, a vesicular pH biosensor

A novel genetically-encoded fluorescent reporter of intraluminal pH, dVMAT-pHluorin, was characterized in order to measure changes in the intraluminal pH of monoaminergic vesicles. The expression pattern of TH promoter-driven dVMAT-pHluorin appeared identical to the FFN206's punctate staining pattern seen in TH Rescue brains, suggesting that dVMAT-pHluorin expression was properly expressed at presynaptic DA nerve terminals. KCl-induced depolarization enhanced dVMAT-pHluorin fluorescence by shifting the pH biosensor moiety from the acidic vesicle environment to the neutral extracellular space following vesicle exocytosis.

Essential roles of Drosophila DAT (dDAT) and dVMAT in the amphetamine-induced alkalization of DA vesicles

To examine the phenomenon of vesicular DA redistribution further *in vivo*, Freyberg and colleagues selectively expressed the dVMAT-pHluorin biosensor in presynaptic DA nerve terminals to monitor amphetamine-induced changes in the pH of DA synaptic vesicles in a whole *Drosophila* brain. Both amphetamine and methamphetamine

caused intraluminal alkalization of DA vesicles in a concentration-dependent manner. In contrast, treatment with methylphenidate, a non-substrate DAT inhibitor that is also a lipophilic weak base (pKa=8.8) [13], had no effect on intraluminal vesicular pH. Thus, a weak base is insufficient on its own to cause intraluminal alkalization of DA vesicles. The role of dDAT in transporting amphetamines into cells was further examined by expressing dVMAT-pHluorin selectively on a dDAT null mutant background [14] to assess whether amphetamine-induced alkalization results from amphetamines' passive diffusion across the plasma membrane or via dDAT-dependent import. Vesicular alkalization induced by amphetamine or methamphetamine was not observed in the dDAT null mutant background. Similarly, pretreatment with the VMAT inhibitors reserpine or (+)-CYY477 blocked amphetamine-induced alkalization. Thus, these data suggest dependency on both dDAT and dVMAT for amphetamine-induced intraluminal alkalization. In contrast, alkalization was still observed following treatment with the lipophilic weak base chloroquine, even in the absence of dDAT expression or acute dVMAT blockade by reserpine or (+)-CYY477. This result demonstrates that, unlike amphetamines, chloroquine causes intraluminal alkalization independently of DAT and VMAT via lipophilic diffusion. Taken together, these results suggest that, in contrast to the earlier weak base hypothesis [9], amphetamines do not behave like classic lipophilic weak bases (e.g., chloroquine [9]). Rather, they require the actions of both plasma membrane DAT and VMAT acting in tandem to cause vesicular alkalization.

Substrate-coupled H+ antiport by dVMAT alkalizes vesicles

Though the above data suggest that amphetamines require transporters for cellular and vesicular entry, since these drugs are weak bases [9], it has been hypothesized that they are transported into the vesicle lumen predominantly in a neutral state, whereupon they bind intraluminal H+ from the acidic vesicle lumen to eventually dissipate the DA vesicle ΔpH [5]. Complicating this model is the understanding that dVMAT is not only a monoamine transporter, but also behaves as a substrate: H+ antiporter. Therefore, for every substrate transported across dVMAT into the vesicle lumen, two intraluminal protons are transported into the cytoplasm [15]. This model was validated by showing that VMAT substrates other than amphetamines, including both DA and FFN206, were capable of alkalizing the DA vesicle lumen. Nevertheless, the majority of dVMAT and mammalian VMAT2 substrates are also weak bases, making it difficult to distinguish the relative contributions of these substrates' weak base properties from those of VMAT-mediated substrate-coupled H+ antiport. Thus, to disentangle these two aspects of VMAT substrate action and specifically focus on the contribution of substrate-coupled H+ antiport to intraluminal alkalization, the dual DAT and VMAT substrate 1-methyl-4-phenylpyridinium (MPP+) was used because it cannot buffer luminal H+ due to the fixed positive charge of its methyl-pyridine nitrogen. Consistent with the substrate-coupled H+-antiport model, MPP+ treatment induced alkalization in presynaptic DA nerve terminals. These findings indicate that transport-dependent H+ antiport by VMAT is sufficient to decrease intraluminal H+ concentration and alkalize the vesicle lumen without a need for H+ buffering. Moreover, MPP+ caused FFN206 destaining in DA terminals of TH Rescue brains, suggesting that the alkalization was sufficient to cause redistribution of intraluminal DA vesicle content. To test whether at least some of these effects were due to vesicular exocytosis, MPP+ was rapidly applied to brains co-expressing TH-driven dVMAT-pHluorin and genetically-encoded tetanus toxin light chain (TeTxLC), a blocker of exocytosis [16]. While the positive control, KCl-induced alkalization, was sensitive to TeTxLC expression, MPP+- induced alkalization was insensitive. These data suggest that MPP+-induced alkalization works primarily via a VMAT-dependent mechanism rather than via exocytosis. Further, the results suggest that the intrinsic substrate-coupled H+ antiport function of VMAT is a critical mechanism underlying amphetamine-induced alkalization.

Preclinical efficacy and specificity of VMAT2 inhibitors as treatment for amphetamine abuse using a rat model

The VMAT2 inhibitor (+)-CYY477 dose-dependently produced insurmountable antagonism of d-methamphetamine self-administration in rats trained to self-administer cocaine. Importantly, the d-methamphetamine antagonist effects were specific because cocaine self-administration and food-reinforced responding were insensitive to d-methamphetamine-sensitive doses of (+)-CYY477. The effects of (+)-CYY477 were not idiosyncratic because other VMAT2 inhibitors have been independently reported in other recent studies to block d-methamphetamine self-administration in rats despite their relative low affinity to VMAT2 [17-23] (Table 1). The behavioral specificity of (+)-CYY477 was superior to that of the prototype VMAT2 inhibitor (±)-tetrabenazine which was equipotent in decreasing self-administration of d-methamphetamine and food-reinforced responding [18]. Further, (+)-CYY477 had the highest affinity for VMAT2 among those with d-methamphetamine antagonist effects (Table 1). Consequently, (+)-CYY477 may be an excellent tool to dissect VMAT2-specific effects without the confound of simultaneous actions on DAT because of the drug's considerably higher selectivity to VMAT2 (Ki=7.18 nM) over DAT (Ki>100,000 nM) [11]. Such a high affinity of (+)-CYY477 for VMAT2 would be preferential for clinical use since maximal VMAT2 binding *in vivo* would help overcome the limited blood-brain barrier permeability typical for VMAT2 inhibitors. Ultimately, these properties may render (+)-CYY477 a potential treatment for amphetamine abuse, given the centrality of VMAT function to amphetamine's actions in the central nervous system.

Compound	VMAT2 ([3H]dihydrotetrazenazine)
(+)-CYY477	7.18 ± 1.14 [11]
(±)-Tetrabenazine	13 ± 1 [25]
Lobelane	2,040 ± 640 [26] 970 ± 190 [17]
UKCP-110	2,660 ± 366 [17]
GZ-793A	8,290 ± 2,790 [27]
meso-transdiene	9,880 ± 2,220 [28]
d-methamphetamine	80,100 ± 19,500 [26] No inhibition at 100 μM [29]
d-Amphetamine	No inhibition at 100 μM [29]
Cocaine	No inhibition at 100 μM [30]

Table 1: Inhibition by various compounds of specific binding to mammalian VMAT2 (Ki Value, nM).

Values listed are represented as the means ± SEMs (95% confidence limits).

These series of studies by Freyberg and colleagues [11] have demonstrated the importance of VMAT-mediated substrate-coupled H +antiport for the *in vivo* actions of amphetamines as well as the preclinical efficacy and specificity of a novel class of antagonists for d-methamphetamine self-administration. Ultimately, these findings suggest the potential for the development of new, highly specific VMAT2 inhibitors to be used as amphetamine-specific antagonists *in vivo*. The findings are especially relevant clinically since there is a lack of FDA-approved medications to treat abuse of amphetamine-type psychostimulants or substrates for DAT and there are only few, if any, candidate compounds that show preclinical efficacy as amphetamine antagonists at present (e.g., JHW 007 and AHN 2-005 [2]). Moreover, in the face of a growing epidemic of newer psychostimulants including 'bath salts' (which include methylone and mephedrone), the capacity of these drugs to act through DAT and VMAT [24] makes the development of such treatments even more socially relevant.

Acknowledgments

The present work was supported by the Division of Neurotoxicology/NCTR/U.S. FDA (Dr. Merle G. Paule). The information in this article is not a formal dissemination of information by the FDA and does not represent agency position or policy. The author thanks Drs. Merle G. Paule, Noriko Nakamura, Jaclyn R. Daniels, and Amy K. Goodwin at NCTR for comments during the preparation of the manuscript.

References

1. Barger G, Dale H (1910) Chemical structure and sympathomimetic action of amines. J Physiol 41: 19-59.
2. Hiranita T (2014) Preclinical efficacy of N-substituted benztropine analogs as antagonists of methamphetamine self-administration in rats. J Pharmacol Exp Ther 348: 174-191.
3. Hiranita T, Soto PL, Tanda G, Kopajtic TA, Katz JL (2013) Stimulants as specific inducers of dopamine-independent sigma agonist self-administration in rats. J Pharmacol Exp Ther 38: 605-615.
4. Hiranita T (2006) Suppression of methamphetamine-seeking behavior by nicotinic agonists. Proc Natl Acad Sci U S A 103: 8523-8527.
5. Sulzer D (2011) How addictive drugs disrupt presynaptic dopamine neurotransmission. Neuron 69: 628-649.
6. Garcés-Ramírez L (2011) Sigma receptor agonists: Receptor binding and effects on mesolimbic dopamine neurotransmission assessed by microdialysis in rats. Biol Psychiatry 69: 208-217.
7. Kohut SJ (2014) Preference for distinct functional conformations of the dopamine transporter alters the relationship between subjective effects of cocaine and stimulation of mesolimbic dopamine. Biol Psychiatry 76: 802-809.
8. Gowrishankar R, Hahn MK, Blakely RD (2014) Good riddance to dopamine: roles for the dopamine transporter in synaptic function and dopamine-associated brain disorders. Neurochem Int 73: 42-48.
9. Sulzer D, Rayport S (1990) Amphetamine and other psychostimulants reduce pH gradients in midbrain dopaminergic neurons and chromaffin granules: a mechanism of action. Neuron 5: 797-808.
10. Sulzer D (2005) Mechanisms of neurotransmitter release by amphetamines: a review. Prog Neurobiol 75: 406-433.
11. Freyberg Z (2016) Mechanisms of amphetamine action illuminated through optical monitoring of dopamine synaptic vesicles in *Drosophila* brain. Nat Commun 7: 10652.
12. Hu G, Henke A, Karpowicz RJ, Sonders MS, Farrimond F, et al. (2013) New Fluorescent Substrate Enables Quantitative and High-Throughput Examination of Vesicular Monoamine Transporter 2 (VMAT2). ACS Chem Biol 8: 1947-1954.
13. Marchei E (2010) Correlation between methylphenidate and ritalinic acid concentrations in oral fluid and plasma. Clin Chem 56: 585-592.
14. Kume K (2005) Dopamine is a regulator of arousal in the fruit fly. J Neurosci 25: 7377-7384.
15. Knoth J, Zallakian M, Njus D (1981) Stoichiometry of H+-linked dopamine transport in chromaffin granule ghosts. Biochemistry 20: 6625-6629.
16. Sweeney ST (1995) Targeted expression of tetanus toxin light chain in *Drosophila* specifically eliminates synaptic transmission and causes behavioral defects. Neuron 14: 341-351.
17. Beckmann JS (2010) The novel pyrrolidine nor-lobelane analog UKCP-110 [cis-2,5-di-(2-phenethyl)-pyrrolidine hydrochloride] inhibits VMAT2 function, methamphetamine-evoked dopamine release, and methamphetamine self-administration in rats. J Pharmacol Exp Ther 335: 841-851.
18. Meyer AC (2011) Tetrabenazine inhibition of monoamine uptake and methamphetamine behavioral effects: locomotor activity, drug discrimination and self-administration. Neuropharmacology 61: 849-856.
19. Horton DB (2011) meso-Transdiene analogs inhibit vesicular monoamine transporter-2 function and methamphetamine-evoked dopamine release. J Pharmacol Exp Ther 336: 940-951.
20. Neugebauer NM (2007) Lobelane decreases methamphetamine self-administration in rats. Eur J Pharmacol 571: 33-38.
21. Beckmann JS (2012) The effect of a novel VMAT2 inhibitor, GZ-793A, on methamphetamine reward in rats. Psychopharmacology (Berl) 220: 395-403.
22. Wilmouth CE (2013) Oral administration of GZ-793A, a VMAT2 inhibitor, decreases methamphetamine self-administration in rats. Pharmacol Biochem Behav 112: 29-33.
23. Hiranita T (2015) Preclinical Efficacy of Novel Vesicular Monoamine Transporter 2 Inhibitors as Antagonists of d-Methamphetamine Self-Administration in Rats. J Alcohol Drug Depend 3.
24. Anneken JH, Angoa-Perez M, Kuhn DM (2015) 3,4-Methylenedioxypyrovalerone prevents while methylone enhances methamphetamine-induced damage to dopamine nerve endings: beta-ketoamphetamine modulation of neurotoxicity by the dopamine transporter. J Neurochem 133: 211-222.
25. Nickell JR (2014) The vesicular monoamine transporter-2: an important pharmacological target for the discovery of novel therapeutics to treat methamphetamine abuse. Adv Pharmacol 69: 71-106.
26. Nickell JR (2010) Lobelane inhibits methamphetamine-evoked dopamine release via inhibition of the vesicular monoamine transporter-2. J Pharmacol Exp Ther 332: 612-621.
27. Horton DB (2011) Novel N-1,2-dihydroxypropyl analogs of lobelane inhibit vesicular monoamine transporter-2 function and methamphetamine-evoked dopamine release. J Pharmacol Exp Ther 339: 286-297.
28. Zheng G (2005) Defunctionalized lobeline analogues: structure-activity of novel ligands for the vesicular monoamine transporter. J Med Chem 48: 5551-5560.
29. Schwartz K, A Weizman, M Rehavi (2006) The effect of psychostimulants on [3H]dopamine uptake and release in rat brain synaptic vesicles. J Neural Transm 113: 1347-1352.
30. Partilla JS (2006) Interaction of amphetamines and related compounds at the vesicular monoamine transporter. J Pharmacol Exp Ther 319: 237-246.