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All Aglow About Presynaptic Receptor Regulation of Neurotransmitter Transporters

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Running Title: D2 Regulation of Dopamine Transporters

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ABSTRACT

Mounting evidence supports the idea that neurotransmitter transporters are subject to many forms of posttranslational regulation typically associated with receptors and ion channels, including receptor and kinase-mediated changes in transporter phosphorylation, cell surface trafficking and/or catalytic activation. Although hints of this regulation can be achieved with traditional radiolabeled substrate flux techniques, higher resolution methods are needed that can localize transporter function *in situ* as well as permit real-time monitoring of transport function without confounds associated with coincident receptor activation. The elegant study by *Bolan et al.* capitalizes on the fluorescent properties of a recently introduced substrate for the dopamine (DA) transporter (DAT) termed ASP+, to illuminate a pertusis-toxin sensitive, ERK1/2-dependent pathway by which presynaptic DA D_2 receptors (D_2Rs) regulate DATs.

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Many examples exist where conceptual advances in science follow shortly after the introduction of technological improvements that permit greater resolution of phenomena in space and time. Two classic examples of this are the visualization of synaptic vesicles using electron microscopy and the detection of single channel states via patch clamp techniques. Both vesicles and channels were predicted by more indirect methods, but the direct, higher resolution methods allowed the fields of synaptic transmission and channel biophysics to quiet the ever-present quibbling over alternative models and move on to mechanistic studies. A similar transition may now be at hand with the study of neurotransmitter transporter regulation, where technical advances are aiding a paradigm shift away from transporters as constitutive “sinks” but rather as highly regulated targets of intracellular signaling pathways and associated proteins supporting presynaptic plasticity.

Plasma membrane neurotransmitter transporters, such as the cocaine-sensitive dopamine (DA) transporter (DAT, SLC6A3), are understood to dictate presynaptic clearance of extracellular neurotransmitter after its release(Gether et al., 2006). DATs, like many transporters, are typically assayed by monitoring the uptake of their radiolabeled, endogenous substrate. This approach, though quite sensitive, only monitors substrate *accumulation*, the end-result of transport, and as such gives little information about the temporal dynamics of the transport process. Additionally, radiotracer flux studies provide little by way of spatial information such as the subcellular sites of transporter function. Finally, because presynaptic receptors exist that also interact with the transported substrate (termed presynaptic autoreceptors), natural substrate flux studies can be confounded by coincident receptor stimulation. Because of this issue, past

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studies of DAT functional regulation by D_2 DA receptors (D_2Rs) have relied on interpretations from assays conducted with and without D_2R antagonists(Cass and Gerhardt, 1994; Meiergerd et al., 1993), by following surrogate measures for DA transporter function(Mayfield and Zahniser, 2001), or using assays from normal or D2R-deficient brain preparations(Dickinson et al., 1999). Bolan and coworkers have taken advantage of the fluorescent properties of a novel DAT substrate, ASP+ (4-(4-(dimethylamino)styrl)-*N*-methylpyridinium), to bypass these limitations: they provide clear evidence in culture models that presynaptic DRs can rapidly elevate the surface expression and DA clearance capacity of DATs. The high density of DATs in circuits supporting normal motor function and reward and DAT involvement in the action of both therapeutic and addictive psychostimulants indicate that these findings are of practical significance for both basic and translational scientists.

ASP^+ is a fluorescent analogue of MPP+ (1-methyl-4-phenylpyridinium), a well-known neurotoxin transported by monoamine transporters(Javitch et al., 1985). ASP+ was actually first implemented as a substrate for organic cation transporters (OCTs) (Mehrens et al., 2000), perhaps the more prevalent mechanism by which the molecule enters cells for access to mitochondria (ASP+ is typically sold as a mitochondrial probe). However, ASP^+ also passes all the conventional tests for a bona fide biogenic amine transporter substrate: it's accumulation in DAT or norepinephrine transporter (NET, SLC6A2) transfected cells is significantly above that seen in nontransfected cells, is Na^+- and Cl- dependent, cocaine-sensitive, and it competes with the natural substrates for accumulation(Schwartz et al., 2003). ASP+ greatly increases in fluorescence upon binding to protein, as occurs when a specific target is bound at the cell surface or after

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transport, though external quenchers are useful with some applications. Tracking ASP^+ can be accomplished on single cells with confocal microscopy and allows the real-time measurement of binding and transport(Schwartz et al., 2003; Schwartz et al., 2006)., demonstrating (in the case of NETs) that binding rates exceeding transport rates by 100X(Schwartz et al., 2005). Quenching experiments reveal that ASP^+ binds deep within the transporter, a property expected from localization of a intramembranous substrate binding site in the crystallized structure of a bacterial homolog, $LeuT_{Aa}$ (Yamashita et al., 2005). The combination of a fluorescent substrate with a GFP-tagged transporter opens the field to a wide variety of imaging techniques for studies of transporter biophysics and regulation. These approaches include confocal microscopy, flow cytometry, two-photon excitation (TPE) microscopy, fluorescence lifetime imaging microscopy (FLIM), total internal reflection (TIRF) microscopy, fluorescence correlation spectroscopy (FCS), and fluorescence recover after photobleaching (FRAP). ASP^+ has been combined with GFP-tagged NET to reveal astonishing details unavailable with radiotracer flux approaches(Schwartz et al., 2005) including the stoichiometry of substrate interactions ascertained with functional proteins *in situ*. Fluorescence anisotropy measurements also indicate two populations of ASP^+ with distinct rotational movements: a rapid binding phase localized to the cell surface and a relatively slow uptake phase II that represents mobile ASP^+. These populations are easily distinguished with ASP^+ monitoring, and spatial patterning, temperature dependence, affinity constants, and transport rates can all be assessed with this method. ASP^+-based technology also permits analysis of populations of cells and it is thus amenable to the automated, multi-well protocols typical of drug discovery paradigms(Mason et al., 2005).

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Previous studies in both heterologous preparations and native tissues have provided evidence that D2Rs increase DAT activity and/or surface expression through a Gi/Go dependent mechanism(Mayfield and Zahniser, 2001). Because D_2Rs are present both pre- and postsynaptically, and thus heterologous models have provided important evidence that D2R regulation can be cell autonomous, likely mediated entirely presynaptically. Bolan and coworkers also remind us that D_2Rs come in alternatively spliced forms, a D_{2L}R and a D_{2S}R with the D_{2S}R being presynaptic. If a presynaptic pathway for D_2R feedback control of DAT exists, then the D_{2S}R must be able to regulate DAT activity. Indeed, when Bolan and colleagues coexpress DAT with D_{2S}R and DAT, DAT activity, as monitored by the time-dependent accumulation of ASP^+, is increased with application of D_2R agonists quinpirole and PD128907. The effects of quinpirole and PD128907 on DAT are blocked by the D2 antagonist eticlopride and by pertussis toxin pretreatment, consistent with the activation of a D_2R-triggered, Gi/Go linked pathway leading to rapid upregulation in DAT activity. Further studies identify activation of ERK1/2 as a requisite step in DAT upregulation, but a step independent of sequences of the DAT NH2 terminus previously linked to regulatory phosphorylation. A rapid increase in DAT activity triggered by D_{2S}R activation could arise from catalytic activation, increased surface trafficking or both. Surface trafficking of transporters can be sensitively evaluated using flow cytometry(Savchenko et al., 2003), based on detection of native or engineered surface epitopes with epitope-specific antibodies. Bolan et al. use flow cytometry to show that D_{2S}R activation coincides with a commensurate change in DAT surface expression, findings validated with a biochemical approach (biotinylation). Finally, coimmunoprecipitation and bioluminescence resonance energy

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transfer (BRET) techniques are implemented to provide evidence that DAT and D_{2S}Rs are physically proximal, possibly enhancing opportunities for regulatory interactions.

The studies by Bolan and coworkers bring us closer to an understanding of biogenic amine transporters as components of presynaptic plasticity than we have ever been. The authors suggest that presynaptic D_{2S}R sense DA released through vesicular mechanisms and positively enhance DAT surface expression to insure DAT clearance. Although this group replicates their results in a neuronal cell line, a critical next step will be demonstrating this interaction in native neuronal membranes. Here the ASP^+ technique would appear potentially useful since, for example, NET activity can be visualized in superior cervical ganglia neurons}(Schwartz et al., 2003), with imaging feasible down to the level of single varicosities(Mason et al., 2005). Eliminating transport by other ASP^+ pathways such as the OCTs and defining specificity through the use of DAT knockout mouse preparations will likely be essential in these more complex preparations. Bolan and colleagues identify ERK1/2 as targets for D_{2S}R activation in the regulation of DAT, but though there is support for this pathway in native preparations(Moron et al., 2003), one wonders whether this pathway is the whole answer for synaptic preparations. In the current report, a DAT trafficking pathway sensitive to ERK1/2 activation is proposed to support increases in DAT activity. Whether ERK1/2 engages accelerated relocation of intracellular DATs or a slowing of DAT endocytosis needs to be defined as well as the molecular effectors to bring about these changes. Finally, whether the ERK1/2 pathway is a common one linking other presynaptic receptors and transporters(Blakely et al., 2005) is now of great interest. The ASP+ approach could be implemented, for example, to study the possibility of α2 adrenergic

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regulation of NET. ASP+ is inefficiently transported by the serotonin transporter (SERT) but other fluorescent substrates have been developed that show promise for studies SERT regulation (Mason J et al., SFN Abstract 531.5, 2007). Regardless, the studies of Bolan et al. represent a shining example of closing the gap between what is merely suspected and what can be directly observed to advance us to mechanisms of transporter regulation.

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