We thank the reviewers for commenting that this proposal "*is likely to result in a paradigm shift in the dopamine transporter field.*" Still, the Committee felt that the proposal would benefit from specific revisions. The application has been revised throughout to address the reviewers' concerns and take into consideration new developments in the field. Only major changes are indicated by lines on the right. **CRITIQUE 1: Concern:** "*The approaches are..based on intriguing preliminary DA efflux data, but..it is not known whether the manipulations ..will alter the uptake functionality of DAT…"* **Response:** To address this concern fully, we now provide uptake data for cells expressing the Tac-hDAT construct, the hDAT construct with the N-terminal Lys (3 and 5) substituted to Asn, and for hDAT cells in which PIP_2 has been depleted with PAO. **Conc:** "*The innovative aspect of the "lever" concept..is diminished by earlier introduction …"* **Resp:** We regret the omission of Dr. Sitte's paper, as he is a friend and collaborator; it was an oversight. The innovation of our proposal stems from our ability to translate the "lever" concept into detailed functional mechanisms in a structural context that explain why, and to what extent, the DAT N-terminus regulates transporter function in a "lever-like" manner. We further aim to translate these discoveries into a mechanistic understanding of amphetamine (AMPH)-induced behavior. **Conc:** *"..it is stated that Ser 2 and 4 are thought..to regulate DA efflux..the references provided actually point to Ser7 or 12..Thus..Lys3 and 5 are possible sites of PIP2 interaction may need an alternative explanation."* **Resp:** Our new computational studies suggest close spatial proximity of Ser 7, 12, 13 to Lys 3, 5, 19, indicating why/how Ser7 and 12 may play a fundamental role, in addition to Ser 2 and 4. **Conc:** *"ideally, amphetamine uptake needs to be known."* **Resp:** We have now included measurements of both AMPH uptake and AMPH EC_{50} in terms of DA efflux. See below why we did not calculate the Km for AMPH. Due to space constraints, the responses to all other recommendations were either implemented in the proposal or addressed in response to reviewer #2. **CRITIQUE 2: Conc:** "*A major weakness of the application is the failure to place the proposed studies in the context of other recent, highly relevant studies by the applicant.*" **Resp:** Within the constraints of this 12-page application we now have done our best to place the proposed studies within the context of our work and that of others in the field. However, we do maintain the focus on DAT/PIP_2 interaction. We reason that it is crucial to first understand, at a detailed level addressed here, how this interaction occurs and is regulated. We then intend to extend the model with the other suggested players, such as syntaxin 1A and flotillin. **Conc:** *"it is unclear how the proposed experiments would demonstrate specific and direct PIP2-DAT interactions…"* **Resp:** This is now addressed. In addition to immunoprecipitation, GST-N-terminus liposome-pulldown experiments are included to demonstrate direct DAT/PIP_2 interactions. **Conc:** "*unclear why similar computational strategies were not proposed.."* **Resp:** We now propose such computational studies. **Conc:** *"Tac fragment..is fused to the N-terminus of the DAT. The rationale for using this particular construct (as opposed to..its N-myristoylation) is not articulated."* **Resp:** We reasoned that: a) N-terminal myristoyl groups are known to facilitate the targeting of proteins to cholesterol-rich domains in membranes and this could confound the interpretation of results; and b) The Tac fragment has been used successfully to modulate AMPH-induced 5HT efflux by the serotonin transporter[1]. **Conc:** *"Km for amphetamine uptake and DA efflux are not reported."* **Resp:** Km for AMPH uptake has been reported by other groups. However, we and others (personal communication) determined that at µM range concentrations, the lipophilicity of AMPH results in a large non-specific component (~75%) of total uptake, compromising the interpretation. Therefore, we determine for hDAT K/N and ^{Tac}hDAT the uptake of AMPH at a low concentration using HPLC, and the EC_{50} for AMPH-induced DA efflux by electrophysiological means, using a rapid perfusion apparatus. **Conc:** *"Thus no firm conclusions about PIP2 per se can be reached..especially in the absence of PIP2 rescue experiments. The time-tested way to selectively deplete PIP2 is through activation of a Galphaq-linked receptor, with rescue."* **Resp:** We now propose PIP_2 rescue experiments. As suggested, we now show that muscarinic M1 receptor (Gαq coupled) stimulation decreases DA efflux and that intracellular dialysis of PIP_2 rescues this decrease. In the previous submission, we proposed a chemical dimerization strategy for PIP_2 manipulation[2]. We thank the reviewer for pointing us to a new dimerization strategy [3] published after the submission of our proposal (06/04/2012); Dr. Hammond has provided us with the new reagents. **Conc:** *"The methodologies proposed to examine DAT-PIP2 interactions (figure 6) may need some reconsideration..Biochemical interaction of PIP2 and proteins is..typically quantified using ..GST fusion proteins rather than by immunoprecipitation."* **Resp:** We proposed GST-N-terminus liposome-pulldown experiments and provide preliminary data. Immunoprecipitation experiments are used here to evaluate changes in DAT/PIP_2 interaction in a cellular context. **Conc:** ***"****the Drosophila model..should be discussed in the context of a recent study by the applicant"s erstwhile collaborators (Pizzo et al., Mol Psychiatry 2012)."* **Resp:** Data in this paper, published after the submission of our proposal, strongly support our model, as is now discussed. **Conc:** *"the lack of discussion regarding potential issues related to assessing DA efflux from the cell body of fly DA neurons."* **Resp:** We have addressed this point in S.A. #3c. **Conc:** *"It is unclear if the proposed Aims overlap with..R56 DA013975."* **Resp:** No overlap: these R56-Bridge awards enable the gathering of additional data for a new R01 application, which is the case here. **CRITIQUE 3:** We are grateful for the kind comments and suggestions, which have been incorporated. We now propose metabolic phosphorylation of the N-terminus instead of using phosphospecific antibodies, in studies coordinated by Dr. Foster (see subcontract).

SPECIFIC AIMS

The dopamine (DA) transporter (DAT), a member of the neurotransmitter Na^+ symporters family, controls DA homeostasis and neurotransmission by the active reuptake of synaptically released DA. The DAT is the major molecular target responsible for the rewarding properties and abuse potential of amphetamine (AMPH) as well as cocaine. AMPH acts as a DAT substrate, promoting the reversal of DA transport, which results in DA efflux *via* DAT. This efflux leads to increased extracellular DA levels, thought to be important for the psychomotor stimulant properties of AMPHs[4]. The N-terminus of the DAT is a structural domain of critical importance for the ability of AMPH to cause this DA efflux[5-7]. Our preliminary data strongly suggest that **anchoring** the DAT N-terminus to the plasma membrane (achieved by its tethering to an additional transmembrane segment) impairs AMPH-induced DA efflux **without** affecting DA uptake. Therefore, we hypothesize that the functional role of the DAT N-terminus is dependent on the conformations it adopts through its different interactions with the plasma membrane and, as such, it determines different modes of DAT function (efflux versus uptake).

We have shown that DAT N-terminus phosphorylation at the five most distal Ser is required for DA efflux, but does not affect DA uptake[5]. We reasoned that this phosphorylation (a cellular process stimulated by AMPHs)[8] promotes DA efflux by disengaging DAT N-terminus from the plasma membrane, and by regulating N-terminal conformations. Thus, it is important to determine: a) the **nature** of the DAT interactions with the plasma membrane; and b) **how phosphorylation** of the N-terminus promotes outward (rather than inward) movement of DA. This will clarify how modifications of DAT induced by AMPH contribute to its actions.

Phosphatidylinositol-4,5-bisphosphate (PIP_2) is a key phospholipid primarily enriched at the inner leaflet of the plasma membrane[9]. Although PIP_2 is a main signaling molecule, it also acts as an essential cofactor to regulate protein function[9], including ion channels and ion transporters[10]. Our preliminary studies, including computational modeling and simulations, suggest an electrostatic mode of interaction of the DAT N-terminus with PIP_2, which constrains the mobility of the N-terminus by anchoring it to the plasma membrane. This interaction limits DA efflux but not uptake. These studies also suggest a role for this interaction in the N-terminus phosphorylation that dictates specific N-terminal conformations. Thus, our **mechanistic hypothesis** is that upon phosphorylation, the DAT N-terminus escapes the constraint of the electrostatic interaction with PIP_2, and that both events are required to enable the observed actions of AMPH. We will probe this mechanistic hypothesis according to the following **specific aims**:

S.A. #1. To determine the nature of the interaction between DAT N-terminus and PIP_2, and describe it in a structural context provided by computational modeling

a. To determine experimentally the interaction of DAT with PIP_2, and whether (and how) AMPH disrupts it.
b. To produce a molecular level description of the interaction between the DAT N-terminus and PIP_2, and its dynamic consequences, using computational simulations.

S.A. #2. To determine the role of N-terminus phosphorylation in regulating how DAT and PIP_2 interact, and its effect on DAT function

a. To identify whether PIP_2 manipulations regulate DAT function.
b. To identify the PIP_2 binding sites on the DAT.
c. To define the mechanism by which DAT N-terminus phosphorylation regulates DAT function.

As described in S.A. #3 below, the molecular discoveries of S.A. #1 and 2 will be evaluated mechanistically *in vivo,* using a behavioral model we developed in *Drosophila melanogaster*. In this system, we have established that AMPH-induced locomotion is a DA-associated and DAT-dependent behavior, which allows us to test whether the identified changes in the DAT N-terminus interactions regulate AMPH-induced behaviors. We established that deletion of *Drosophila* DAT (dDAT) in DA neurons of flies inhibits AMPH-induced locomotion (see below). Importantly, the ability of AMPH to cause locomotion in flies lacking dDAT **is restored** by the expression of the human DAT (hDAT) in DA neurons. This underlies our strategy to elucidate how posttranslational modifications and associations of N-terminus DAT with the plasma membrane determine AMPH-induced behaviors, in a timely and inexpensive manner. It also allows us to translate to DA neurons our molecular observations regarding how the N-terminus of **hDAT** dictates DA efflux. Thus, S.A. #3 shifts the focus to testing newly discovered molecular mechanisms of N-terminal interactions and AMPH actions in neurons and *in vivo*, using *Drosophila* as an animal model:

S.A. #3. To determine the role of DAT/PIP_2 interactions in AMPH-induced behaviors

a. To determine whether N-terminus conformations regulate AMPH-induced behaviors.
b. To establish the role of DAT/PIP_2 interactions in AMPH-induced locomotion.
c. To study AMPH-induced DA efflux in *Drosophila* DA neurons.

RESEARCH STRATEGY

a) Significance: Amphetamine (AMPH) abuse represents a major societal problem, placing a burden on the individual due to its profound medical and psychological complications including dependence and death[11]. AMPH's addictive properties are mediated through elevation of extracellular dopamine (DA) by inducing DA efflux *via* the DA transporter (DAT) and inhibition of DA reuptake[4, 12]. DA plays a major role in the regulation of cognitive and behavioral functions. Abnormalities in the DA system have been implicated in a number of psychiatric and neurological disorders, including drug addiction. DAT, the protein responsible for DA reuptake at the plasma membrane, is the major molecular target of AMPH and cocaine[4]. Gene knockout experiments in multiple organisms, including *Drosophila melanogaster*[13](see Approach section), point to DAT as the main target for the locomotor stimulatory effects of AMPH[14].

It is known that AMPHs stimulate the activity of conventional kinases[15-18], resulting in phosphorylation of multiple serine residues in the amino-terminus (N-terminus) of DAT[8]. We demonstrated that AMPH-induced, DAT-mediated DA efflux is supported, at least in part, by the ability of AMPH to cause activation of Ca^{2+}/calmodulin-dependent protein kinases II (CaMKII), and subsequent phosphorylation of DAT N-terminus serines[6]. Prevention of phosphorylation of the N-terminus of DAT, by truncation or targeted mutations, dramatically inhibits the ability of AMPH to induce both DA efflux[5] and, in *Drosophila*, hyperlocomotion[13]. However, how this phosphorylation translates in increased DA efflux and locomotion, remains unclear.

It has been shown that disrupting DAT membrane raft localization inhibits AMPH-induced DA efflux[19], and N-terminus phosphorylation[20]. We showed that localization of DAT in membrane raft is mediated, at least in part, by the raft-associated protein Flotillin-1(Flot1). Importantly, in *Drosophila*, Flot1 is required for AMPH-induced hyperlocomotion[13]. Thus, these membrane microdomains might coalesce the molecular elements regulating AMPH-induced DAT N-terminal phosphorylation, DA efflux, and behaviors.

Our experimental and computational experiments suggest phosphatidylinositol-4,5-bisphosphate (PIP_2) as a regulator of AMPH-induced DA efflux and associated behaviors. PIP_2 is a phospholipid mainly concentrated at the inner leaflet of the plasma membrane[9]. PIP_2 is enriched in lipid raft domains[21], and is an essential cofactor regulating protein recruitment and function[22]. Among those proteins is the DAT-associated protein syntaxin 1[23-25]. We found that the DAT N-terminus is engaged in electrostatic interactions with PIP_2, which points to how PIP_2 can participate in coordinating the complex molecular events underlying the AMPH actions. Yet, we cannot exclude PIP_2 binding to other DAT domains. However, to obtain an understanding at the detailed molecular level, we focus this application on the direct interaction between DAT N-terminus and PIP_2. This will generate a basis for further evaluating the importance of PIP_2 for DAT membrane localization (e.g. enriched lipid domains) and/or its association with syntaxin 1 for AMPH actions. The generalizable mechanism of the DAT/PIP_2 interaction and the ability to link this mechanism to behavioral aspects of AMPH (insufficiently understood mechanistically) underscore the significance we perceive for the proposed studies.

We hypothesize that PIP_2 serves as a membrane docking phospholipid that anchors to the plasma membrane a specific DAT domain (DAT N-terminus) to regulate DAT function. The negatively charged heads of PIP_2 are associated with positively charged Lys/Arg on the N-terminus (**Fig. 1a**). In this mode, the transporter has normal uptake, is "reluctant" to efflux DA, but is permissive to N-terminal phosphorylation. The electrostatic association between DAT and PIP_2 is weakened by AMPH-induced DAT N-terminal phosphorylation that shields the positively charged residues causing the N-terminus to uncouple from PIP_2 (**Fig. 1b**). In this status, DAT is in a "willing" mode, permissive for DA efflux and AMPH behaviors (**Fig. 1b**). Depletion of PIP_2 by pharmacologic or molecular means (**Fig. 1c**) disrupts DAT/PIP_2 interactions. In this conformation, the transporter is "unwilling" to be **phosphorylated and to efflux DA**. This mode impairs the ability of AMPH to cause DA efflux, without altering DA reuptake.

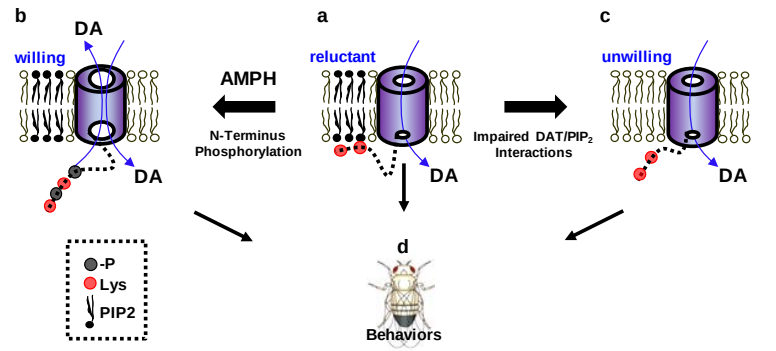

Fig. 1. Model of AMPH-induced DA efflux: (a) Reluctant: N-terminus DAT interacts electrostatically by positively charged amino acids with PIP_2 at the plasma membrane. This state is permissive for N-terminus phosphorylation and capable of DA uptake. **(b) Willing:** AMPH promotes DAT N-terminus phosphorylation (adding negative charges) which causes changes in N-terminus conformation. This state allows for DA efflux and uptake. **(c) Unwilling:** disruption of DAT/PIP_2 interactions impairs phosphorylation. This state is permissive of DA uptake but not efflux. **(d) *Drosophila*** *in vivo* model.

We will test these hypotheses *in vitro* in heterologous expression systems and DA neurons. *In vivo*, we developed a new paradigm (*Drosophila melanogaster)* to examine the mechanistic determinants identified *in vitro* in the context of DAT-mediated behavioral effects of AMPH (**Fig. 1d**).

(b) Innovation: In the last decade, it became clear that in order to pharmacologically target the AMPH actions,

it is essential to understand how **to precisely manipulate DAT to prevent DA efflux, without disrupting DAT-mediated DA transport.** In this proposal, we will elucidate how a DAT structural domain (the N-terminus) dictates different aspects of the transport cycle. The computational modeling will provide a molecular structural context for the experimental results, and therefore, we will understand how to precisely target the N-terminus interactions with PIP_2 to decrease the ability of AMPH to cause DA efflux without altering DA uptake. The innovative nature of the combined experimental and computational approach is further enhanced by the introduction of a behavioral model. Thus, the research plan we present evaluates the significance of the detailed molecular discoveries by translation to *in vivo* mechanisms using *Drosophila* as a behavioral model for AMPH actions. Because many mechanisms of synaptic neurotransmission are shared between *Drosophila* and humans, flies are used here as a powerful model system for systematically examining the behavioral consequences of our molecular manipulations in a time and cost effective manner.

Defining the signaling molecules and DAT post-translational modifications supporting DA efflux may also help for a better understanding of the etiology of DA-related neuropsychiatric disorders, such as attention deficit hyperactivity disorder (ADHD) and autism. Recently, we determined that DAT coding variants associated with ADHD[26, 27] and *de novo* mutations associated with autism (manuscript in preparation) display anomalous DA efflux (ADE). We demonstrated that ADE is supported by N-terminus phosphorylation[27]. Thus, understanding PIP_2 regulation of N-terminus phosphorylation may also help to explain the anomalous behavior of these DAT variants leading to new insights for the treatment of these devastating neuropsychiatric disorders.

Approach: Due to space limitations we are forced to minimize the description of routine methodologies. However, we feel that this proposal will benefit from a detailed description of the amperometric techniques.

Amperometric measurements of DA efflux in single cells: In these experiments (**Fig. 2**), the membrane potential of the cell (heterologous systems (CHO cells) expressing hDAT (**hDAT cells**)) is not under voltage clamp, but rather physiologically dictated. A carbon fiber electrode (5 µm) is juxtaposed to the plasma membrane, held at +700 mV (a potential greater than the oxidation potential of DA), and used to measure DA efflux through oxidation reactions (**Fig. 2**). Cells are incubated in a solution containing DA (1 µM). DA is preloaded into the cells (**Fig. 2**, red circles) by active transport mediated by hDAT. The preloading is not required in neurons (see below) since they have endogenous DA. The amperometric electrode measures DA efflux as electrical currents (black trace) as a result of the oxidation of DA molecules. The DA efflux is induced by AMPH (**Fig. 2**, black circles). By convention, oxidation current is plotted positive. Therefore, an upward deflection in the amperometric currents corresponds to an outward flux of DA.

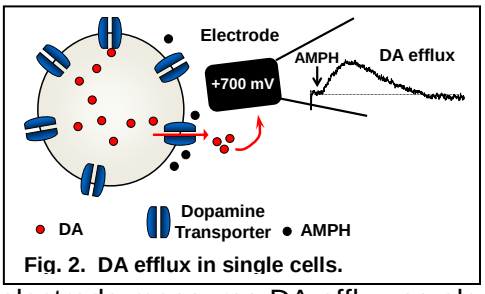

Fig. 2. DA efflux in single cells.

Concurrent Measurements of Transporter Currents and DA Efflux in a single cell: It is now possible to simultaneously record neurotransmitter efflux and transporter-mediated currents from a single cell by combining amperometry with the whole cell patch clamp (**APC**) technique[5] (**Fig. 3**). As above, the amperometric electrode, touching the cell membrane, measures DA levels by oxidation/reduction reactions. The innovation here is that the whole cell patch electrode controls the voltage of the cell, as well the intracellular ionic composition, including DA, while recording hDAT ionic current (movement of ions mediated by hDAT)[5] (**Fig. 3**). At the same time, we monitor DA efflux with the amperometric electrode[5]. With this technique we can measure amperometric currents obtained at different voltages, and plot them against the voltage (mV) applied to the cell by the patch pipette, to determine the voltage dependence of DA efflux[5]. To understand how PIP_2 regulates DAT function and DA efflux, we have to be able to perfuse the intracellular milieu to control ionic composition (e.g. Na^+) and to deliver compounds (e.g. PIP_2) while recording DA efflux.

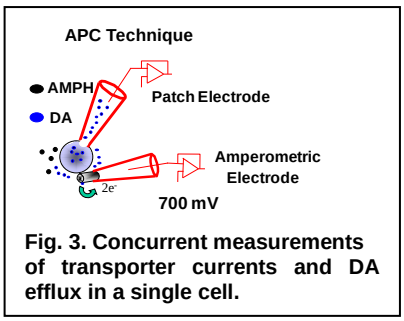

Fig. 3. Concurrent measurements of transporter currents and DA efflux in a single cell.

S.A. #1. To determine the nature of the interaction between DAT N-terminus and PIP_2, and describe it in a structural context provided by computational modeling

Rationale and Strategy: Our model of the process (Fig. 1) is that under physiological conditions DAT is "reluctant" to efflux DA because of the association of N-terminal DAT with the plasma membrane, and specifically with PIP_2. In this mode (Fig. 1a, reluctant), DAT has normal DA uptake. Our preliminary data suggest (see below) that "anchoring" the N-terminus to the plasma membrane impairs DA efflux without affecting DA uptake. We demonstrated that phosphorylation of the DAT N-terminus is required for AMPH to induce DA efflux[5, 6]. Thus, it is possible that AMPH-induced phosphorylation[8] uncouples the N-terminus from PIP_2, to support DA efflux (Fig. 1b, willing). This aim will decipher the nature of the interactions of DAT with PIP_2. We will determine whether the N-terminus interacts with PIP_2, and whether AMPH disrupts the

association between DAT and PIP_2. Computational approaches will interpret structurally the experimental results, predict PIP_2 interaction sites on the N-terminus, and evaluate the strength of these interactions.

1a. To determine experimentally the interaction of DAT with PIP_2, and whether (and how) AMPH disrupts it: We rationalized that the interaction of DAT with PIP_2 anchors the N-terminus to the plasma membrane. In addition, AMPH must disrupt DAT/PIP_2 association in order to promote DA efflux (Fig. 1a-b). To probe this hypothesis, we created a construct, termed ^{Tac}hDAT. This construct contains a single membrane-spanning interleukin 2-receptor subunit, Tac (T-cell activation) sequence[28]. The Tac fragment is fused to the N-terminus of hDAT (^{Tac}hDAT). The Tac fragment is inserted into the plasma membrane due to its hydrophobic core, therefore it anchors the DAT N-terminus to the plasma membrane and restricts its mobility. This strategy has been used in the past for the serotonin transporter[1]. We first examined whether ^{Tac}hDAT cells exhibit normal DA uptake. We demonstrate that in ^{Tac}hDAT cells DA uptake was not significantly different from that of hDAT cells (**Km:** hDAT = 1.76 ± 0.30 µM, ^{Tac}hDAT = 2.00 ± 0.33 µM; n=4; p≥ 0.6 by Student's t-test; **Vmax**: hDAT =9.6 ± 0.5 pmol/10^6 cells/min, ^{Tac}hDAT = 10.0 ± 0.36 pmol/10^6 cells/min; n=4; p≥ 0.5 by Student's t-test). These data demonstrate that anchoring the N-terminus did not impair DAT-mediated DA uptake, nor the affinity for the substrate DA. In addition, in ^{Tac}hDAT cells, uptake of AMPH (10 nM for 5 min) quantified by HPLC was not significantly different from that of hDAT cells (^{Tac}hDAT = 80 ± 16 % of hDAT; n=3-4; p≥0.5 by Student's t-test). The choice of a low concentration of AMPH is justified in the "Introduction to Application" (Critique #2).

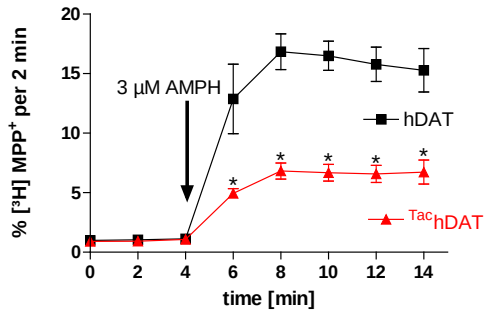

Fig. 4. Anchoring the N-terminus to the plasma membrane decreases substrate efflux: The cells were loaded with 0.1µM [³H]MPP⁺ for 20 min and superfused with AMPH (arrow). 2-min fractions were collected. n=4; *=p≤0.001; two-way ANOVA with Bonferroni post test.

Next, we determined whether the anchoring of the N-terminus to the plasma membrane diminishes AMPH-induced substrate efflux. hDAT and ^{Tac}hDAT cells were incubated with 1-[^{3}H]methyl-4-phenylpyridinium ([^{3}H]MPP$^+$, a DAT substrate commonly used to measure efflux). Then, cells were exposed to AMPH to induce substrate efflux, and 2 min fractions were collected (radioactive counts are expressed as percent of total counts). **Figure 4** shows that tethering DAT N-terminus to the plasma membrane reduced significantly the ability of AMPH to induce substrate efflux. Importantly, comparable results were obtained using amperometry on single cells as described in Fig. 2 (p≤0.01; hDAT versus ^{Tac}hDAT; n=5). Finally, with a rapid perfusion apparatus, we determined in hDAT and ^{Tac}hDAT cells the EC_{50} for AMPH by stimulating DA efflux with different AMPH concentrations (0.01 µM to 1 µM). The amplitude (peak) of the amperometric currents (see Fig. 2) was utilized to derive the EC_{50}. No differences were found, in terms of EC_{50}, between hDAT (0.49±0.09 µM; n=4) and ^{Tac}hDAT (0.48±0.05 µM; n=4) cells (p ≥ 0.9 by Student's t-test).

Here, we will adopt the APC technique (Fig. 3) to fully characterize what are the biophysical changes of ^{Tac}hDAT that alter AMPH-induced DA efflux without affecting DA uptake. The advantage of the APC technique is that it combines the whole cell patch clamp technique with amperometry (Fig. 3). We will define the molecular mechanisms underlying changes in DA efflux. It is possible that the decreased ability of AMPH to cause DA efflux in ^{Tac}hDAT cells is due to changes in the voltage dependence (how DAT senses the membrane voltage) of DA efflux. To test this, we will clamp with the whole cell electrode the hDAT and ^{Tac}hDAT cells at different voltages, while recording DA efflux with the amperometric electrode, to determine amperometric-voltage relationships[29]. It is also possible that anchoring the N-terminus to the plasma membrane regulates the affinity of DAT for intracellular and/or extracellular Na^+. Our technique permits to change intracellular Na^+ concentration (with the whole cell electrode) or extracellular Na^+ concentration (with the bath perfusion) while recording DA efflux to calculate Na^+ affinity (K_m) as in Khoshbouei *et al.* [29]. We will reveal how the N-terminus association with the plasma membrane regulates hDAT function (e.g. DA efflux).

Our model predicts that the relationship of DAT with PIP_2 is important for DA efflux. Thus, we hypothesized that DAT associates with PIP_2, that the N-terminus is the site of this association (Fig. 1a, and see below), and that AMPH requires disrupting DAT/PIP_2 association to cause DA efflux (Fig. 1b). Here, we will determine biochemically whether DAT interacts with PIP_2 at the N-terminus and whether AMPH decreases this interaction.

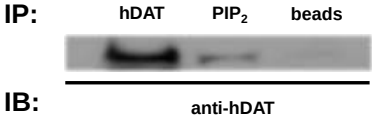

Fig. 5. hDAT immunoprecipitates with PIP_2: hDAT cells were IP with an anti-DAT or -PIP_2 antibody and probed (IB) for DAT. Beads support absence of non specific binding. (n=1, represent. of 2).

In **Figure 5,** we provide evidence supporting the association of DAT with PIP_2. We probed this by immunoprecipitating (**IP**) in hDAT cells either hDAT (anti-DAT antibody) or PIP_2 (anti-PIP_2 antibody) and then immunoblotting the immunoprecipitates for DAT (**IB**: anti-DAT). A representative immunoblot suggesting DAT/PIP_2 interaction is shown in **Figure 5**. As initial negative control experiments, we provide three lines of evidence: 1) In the absence of primary antibody against PIP_2 no signal (IB) was detected for DAT in the immunoprecipitates (Fig 5, beads); 2) in hDAT-null cells (cells not expressing hDAT) no signal (IB) was detected for hDAT in PIP_2

immunoprecipitates (data not shown; n=3); and 3) in mice, we were able to detect DAT in the PIP_2 immunoprecipitates from the striatum of WT animals, but not mice lacking (KO) DAT (data not shown; n=3). These data strongly suggest that PIP_2 associates with hDAT. To further demonstrate this interaction, these experiments will be repeated multiple times and analyzed with appropriate statistical methods[30]. Also, the complementary immunoprecipitation (immunoprecipitate DAT and immunoblot for PIP_2) will be performed.

What we are interested in here, however, is identifying the N-terminus as the region that is essential for the interaction of the DAT with PIP_2. We provide below functional, biochemical, and computational data suggesting that this interaction occurs, at least in part, at the 22 most distal N-terminus amino acids. We have created a mutant DAT in which the first 22 amino acids have been deleted (hDAT-del22)[5]. This region is essential for AMPH-induced phosphorylation[8]. We will test this construct for its interaction with PIP_2 by co-IP as in Fig. 5. Changes in interactions will be evaluated by immunoblot analysis. It is possible that the DAT/PIP_2 interaction is partially preserved in the DAT-del22. If so, we will progressively truncate more of the N-terminus to determine the critical region for hDAT/PIP_2 interaction. Once we have identified this critical region, smaller deletions and amino acids substitution (see S.A. #2) will define the residues mediating this interaction. These experiments will be coordinated with the computational analysis of Dr. Weinstein (see subcontract).

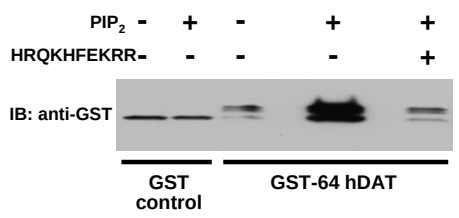

Fig. 6. hDAT N-terminus binds directly to PIP_2: GST-64 is enriched in liposome-pull downs with liposomes containing PIP_2 (+) as compared to liposomes lacking PIP_2 (-). Preincubation of liposome containing PIP_2 with the basic peptide (HRQKHFEKRR) inhibited pull down of GST-64. Pull down of GST control was not altered by the presence (+) of absence (-) of PIP_2 (n=1 representative of 3).

To establish the direct interaction of DAT and PIP_2, we will generate a related series of purified recombinant GST-fused N-terminal DAT fragments of different amino acidic lengths[30], guided by our IP and computational studies. The lipid binding analysis of the GST-fusion proteins will be conducted using pure liposomes composed of mixed lipids (phosphatidylcholine either with or without PIP_2 as described in Varnai *et al*.[31]). The different GST fusion proteins will be added to the liposomes. The GST fusion proteins bound to liposomes will be pelleted (liposome-pull down) and the binding assessed by western blotting (anti-GST antibody). As negative controls for the DAT fusion proteins, we will use GST alone and DAT C-terminal GST fusion proteins. As an additional control, we will "sequester" PIP_2 by preincubating the liposomes with a basic peptide consisting of the sequence of the putative PIP_2 binding domain of the Kv7.2 channel (HRQKHFEKRR)[32] (see S.A. #2a). As a negative control for the liposomes, we will use liposome lacking PIP_2 or with added either phosphatidylinositol 4-phosphate (PI_4P) or phosphatidylinositol (PI) instead of PIP_2. As a positive control, we will use a PIP_2 sensor (PLC-δ1 PH domain GFP/6xHis-tagged)[31], and its binding to PIP_2 assessed with an anti-GFP antibody.

Figure 6 shows preliminary evidence that liposomes containing PIP_2 (**+**) strongly pull down GST fusion proteins comprising the first 64 N-terminal amino acids of hDAT (GST-64), as compared to liposomes lacking (**-**) PIP_2. We "sequester" PIP_2 by preincubating liposomes with the basic peptide HRQKHFEKRR[32]. This strongly decreased pull down of GST-64. Also, the presence (**+**) or absence (**-**) of PIP_2 did not alter pull down of GST alone. These data support a direct interaction between PIP_2 and the N-terminus.

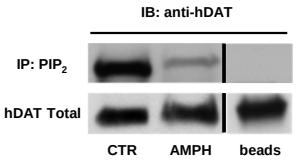

Fig. 7. AMPH decreases DAT/PIP_2 interaction: Top lane: hDAT cells were IP with an anti-PIP2 antibody and probe (IB) for hDAT. Bottom lane: total DAT is shown. Beads support absence of nonspecific binding. (n=1, representative of 2).

Our model predicts that AMPH, in order to cause DA efflux, must disrupt, at least in part, the interactions between DAT and PIP_2. Consistent with our model, **figure 7** shows that AMPH (10 µM for 10 min) decreases the amount of hDAT recovered in the PIP_2 immunoprecipitates. We will repeat these experiments and determine the time course of this AMPH action by exposing the cells to 10 µM AMPH for different time periods (1-10 min). In addition, dose response curves for AMPH (0.01-10 µM) will be generated using times of exposure in the dynamic range, as determined by the time course experiments. Since AMPH causes hDAT to disengage from PIP_2, we will elucidate the kinase involved in this phenomenon using pharmacological and molecular tools as described before[6, 27]. We will begin our studies with CaMKII, but later examine the role of PKC[5, 6, 16, 27].

1b. To produce a molecular level description of the interaction between the DAT N-terminus and PIP_2, and its dynamic consequences, using computational simulations: The computational analysis of the interaction between the DAT N-terminus and PIP_2–containing membranes will take advantage of a 3D molecular model of the N-terminus. This model was constructed in preliminary computational studies that are supported by another NIH grant devoted to structure prediction of loop regions in membrane proteins (DA015170-07 (H. Weinstein, PI)). The structural model of the N-terminus was obtained with a combination of protocols designed to predict the 3D folds of the wild type (WT) loop segment composed of residues 1-59. We used the structure-prediction tool Rosetta[33] combined with MD techniques for enhanced conformational sampling to predict and model the folding of the DAT N-terminus, in both WT and mutant states (see S.A. #2). **Figure 8a** shows the electrostatic potential (EP) isosurfaces

surrounding the molecular model of the WT N-terminal segment, highlighting the specific belt-like arrangement of the Lys/Arg (positive residues). By juxtaposing this structure to a model membrane, our simulation predicts that these Lys/Arg interact electrostatically with PIP_2, resulting in the segregation of PIP_2 near the N-terminus (**Fig. 8b**). These positive residues can play a critical role in DAT/PIP_2 interactions, as indicated by the relative strength of their interaction with PIP_2 (the color coding on the membrane shows the region to achieve the highest segregation of PIP_2 (blue), 2.5 times larger than the PIP_2 ambient concentration (yellow/green)).

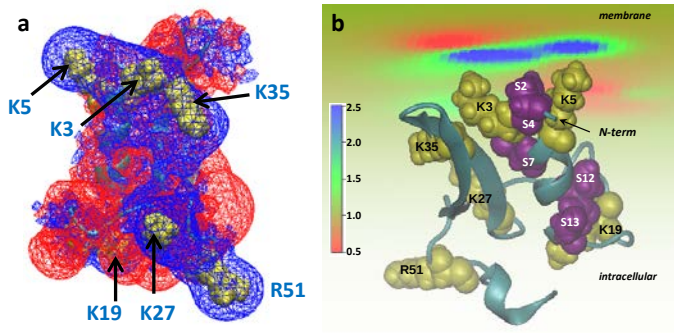

Fig. 8: Electrostatic interactions between DAT and PIP2. (**a**) EP isosurfaces (+1kt/e shown as *blue wireframes* and -1kt/e as *red wireframes*) derived from the predicted structure of the wild type N-terminus. (**b**) View from the intracellular side of the N-term (cartoon) adsorbing on the lipid membrane. For clarity, the orientation of the N-term in panel (b) was obtained by 180° rotation of the N-term configuration from the panel (a). The level of the PIP_2 segregation by the N-term is illustrated (as ratios of local and ambient lipid fraction values) in color code. The juxtapositioning of the positive residues (yellow) that attract PIP_2 electrostatically with the Ser residues (purple) is highlighted.

Our computational experiments for PIP_2/N-terminus interaction will use a series of protocols based on multi-scale quantitative methods we have described recently in great detail[25, 34, 35]. These quantitative methods have been very successful in determining the membrane interaction properties and their functional effects in a variety of proteins in the classes of GPCRs and neurotransmitter transporters [25, 34, 35]. These methods also revealed the effect of syntaxin 1 phosphorylation on PIP_2-syntaxin 1 interaction[25]. Briefly, the protocol uses a full-scale atomistic molecular dynamics (MD) simulations of the system, in combination with a mean-field-based coarse-grained (MFCG) description of the multi-component membrane. The MFCG predicts the steady state of the system consisting of a multi-component membrane and membrane-associated proteins by self-consistent minimization of the governing free energy function[34, 35]. To evaluate the interaction of DAT N-terminus with PIP_2–containing membranes, the calculation of PIP_2 sequestration (**Fig. 8b**) is iterated with atomistic MD simulations of the resulting model until (usually very rapid) convergence occurs. The subsequent atomistic MD simulations probe the conformational changes in the N-terminus in response to lipid rearrangements, and redistribute the lipid components accordingly. This yields an efficient sampling of both membrane remodeling (changes in lipid composition and deformations) and protein conformational dynamics. In these preliminary calculations, the model membrane is compositionally asymmetric to mimic the asymmetry in plasma membrane lipid compositions (5:45:50 PIP_2/POPE (phosphatidylethanolamine) / POPC(phosphatidylcholine) on the intracellular leaflet, and 30:70 sphingomyelin/POPC (but no PIP_2) on the extracellular leaflet[36]. We will evaluate by computational probing the effects of membrane composition and various degrees of PIP_2 depletion (all the way to 0%) on: **a) conformational stabilizations** (structural changes related to N-terminus interaction with the plasma membrane); and **b)** the **relative strength** of PIP_2 segregation around N-terminus (**Fig. 8b**). This will allow for rational design of N-terminus mutants for both computational and biochemical approaches in S. A. #2. These data will provide a comprehensive and quantitative molecular description of the manner in which the N-terminus DAT interacts with PIP_2 and how this interaction regulates the structural phenotypes (conformational energy) of the N-terminus.

Expected outcomes and interpretations and possible problems: In this aim, we are poised to identify the N-terminus as a structural domain directly interacting with PIP_2 by means of biochemistry, and define structurally this interaction by computation. We will clarify how the interaction of the N-terminus with the plasma membrane regulates the transporter cycle (e.g. changes in the voltage dependence of DA efflux, hDAT affinity for substrates, and/or for cotransported ions such as Na^+). We will also define the time and concentration required for AMPH to disrupt DAT/PIP_2 interaction. This is important, since we want to correlate the ability of AMPH to cause DA efflux (S.A. #2) with its ability to disrupt DAT/PIP_2 association, in a time dependent fashion. AMPH exposure also causes DAT trafficking, as has been previously described by our group[37, 38] and others[12]. Our preliminary data suggest that 10 min of AMPH exposure is sufficient to decrease robustly DAT/PIP_2 interaction (Fig. 7) as well as to stimulate efflux (Fig. 4). Our data show that AMPH ≤ 10 min does not induce hDAT trafficking **away** from the plasma membrane[37] (and data not shown). Therefore, it is unlikely that the decreased DAT/PIP_2 interaction caused by AMPH stems from a trafficking phenomenon. This indicates that AMPH has multiple modalities to alter DA homeostasis (efflux versus trafficking). Still, to control for possible effects of trafficking, we can determine AMPH-induced changes in DAT/PIP_2 interaction directly at the plasma membrane by means of biotinylated IP[30]. While the computational studies will stem from validated computational models and formalisms, we maintain an appropriate outlook on pitfalls. For example, the lack of structural models for DAT N-terminus has led us to establish a protocol that uses the structure-prediction tool

Rosetta[33] combined with MD techniques for enhanced conformational sampling to predict and model the folding of the DAT N-terminus. However, with ongoing support from other NIH grants (DA015170, Dr. Weinstein, PI) focused on loop prediction and validation, we will continue to probe these predicted structures with biophysical and biochemical methods, and will evaluate any changes in structural characteristics in relation to the attachment of the N-term segment to the rest of the DAT structure. Results from NMR measurements will also serve to identify any needed refinements. The rigorous sampling of configuration space of the N-terminal peptide on the membrane surface will be continuously probed with additional, multiple long-time all-atom MD simulations. As in our publications[25, 39], the computational studies will be integrated with the experimental results of this proposal.

S.A. #2. To determine the role of N-terminus phosphorylation in regulating how DAT and PIP_2 interact, and its effect on DAT function

Rationale and Strategy: Among phosphoinositides, PIP_2 plays a fundamental role both as precursor of second messengers and as an essential cofactor that regulates protein function[9, 40-42]. Our computational and biochemical studies suggest that the DAT N-terminus interacts with PIP_2 (Fig. 6, 8). Here, we will determine whether this interaction regulates DAT function including DA efflux (Fig. 1a). In our model, the interaction of PIP_2 with DAT is also required for N-terminus phosphorylation. However, once this phosphorylation occurs upon AMPH exposure, the N-terminus disengages from PIP_2, becoming PIP_2-independent, and conferring to the DAT the ability to efflux DA. Thus, we will determine how essential the interaction of DAT with PIP_2 is for coordinating N-terminus phosphorylation (Fig. 1c). Importantly, we will determine the true role of N-terminus phosphorylation for DA efflux. We will learn how to selectively impair the ability of AMPH to cause DA efflux, without altering DA uptake. Finally, to evaluate and integrate this mechanistic hypothesis in a detailed structural context, we will employ the computational protocol described in S.A. #1b to provide experimentally testable predictions regarding the role of specific residues in the N-terminus (Lys/Arg) as well as N-terminus phosphorylation for DAT/PIP_2 interaction.

2a. To identify whether PIP_2 manipulations regulate DAT function: In S.A. #1, we provide preliminary evidence suggesting that PIP_2 interacts with DAT. In this sub aim, we will determine whether this interaction regulates transporter function. Our model predicts that the interaction between PIP_2 and DAT coordinates its phosphorylation. PIP_2 depletion would therefore inhibit AMPH-induced N-terminus phosphorylation and, as a consequence, DA efflux (Fig. 1c). We demonstrated that pharmacological depletion of PIP_2 with phenylarsine oxide (PAO, 20 µM for 20 min) which inhibits phosphatidylinositol 4-kinase, decreases the ability of AMPH (arrow) to cause DA efflux in hDAT cells **(Fig. 9a)**. The amperometric signals (DA efflux) are mediated by hDAT since they are sensitive to both cocaine and mazindol (DAT blockers), and absent in cells lacking hDAT (data not shown). **Figure 9b** shows quantitation of the amperometric currents (peak) after treatment with either vehicle (CTR) or PAO. This significant decrease in DA efflux is not caused by a reduction in DAT plasma membrane expression. Using biotinylation[27], we determined that PAO (20 µM for 20 min) did not decrease hDAT cell surface expression (n=3; vehicle versus PAO; p≥ 0.17 by Student's t test). In addition PAO did not affect [^{3}H]DA (50 nM) uptake (n=3; vehicle versus PAO; p≥ 0.12 by Student's t test). The depletion of cellular PIP_2 induced by PAO was confirmed by an ELISA assay. Lipids were extracted after treatment of hDAT cells with either vehicle or 20 µM PAO for 20 min. Standards for PIP_2 and lipid extracts were then incubated in parallel with a PIP_2 detector protein (PLC-δ1 PH domain). Following this incubation, the reacted mixtures were added to a PIP_2-coated plate for competitive binding, where a peroxidase-linked secondary detection reagent and colorimetric substrate is used to detect the complex PIP_2-(PIP_2 detector protein). At 20 min, PAO decreased the total levels of PIP_2 to 9.1±1.6 % of control conditions (n=4; p≤0.01 by Student's t-test). This, and other methodologies[43] will determine the effectiveness of the pharmacological and molecular manipulations of PIP_2 levels proposed below.

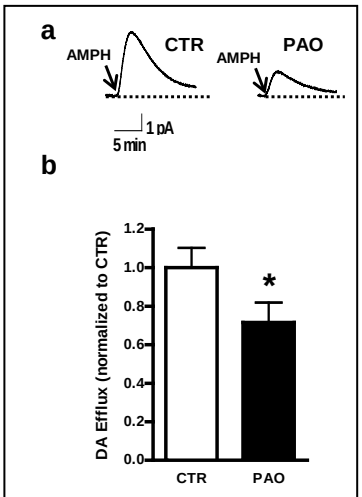

Fig. 9. PIP_2 depletion decreases AMPH-induced DA efflux: (a) Representative traces of AMPH-induced DA efflux after exposure to vehicle (CTR) or PAO. **(b)** Quantitation of amperometric peak currents for the different treatments normalized to vehicle control (*p<0.01, Student's t-test, n=10).

We will further characterize the PAO regulation of DA efflux and uptake by exploring different time of exposure and PAO concentrations (10-100 µM; 1-20 min). The DA efflux will be measured as in figures 4 and 9. Although our preliminary data strongly suggest that PAO does not decrease DA uptake as measured with a single DA concentration, changes in maximal velocity of uptake (V_{Max}) and DA affinity (K_m) will be evaluated. Once the effective time and concentration of PAO is determined, we will perform PIP_2 rescue experiments using the APC technique (Fig. 3). At the beginning of the experiment, the patch electrode containing PIP_2 (1-50 µM) will be held in cell attached. In this configuration, it

will not be able to dialyze the cytoplasm of the cell. Then, we will bath perfuse AMPH to record AMPH-induced DA efflux with the amperometric electrode (control). Subsequently, we will bath perfuse PAO (1-20 min) to deplete PIP_2 and, then, again AMPH, to measure the reduction in AMPH-induced DA efflux promoted by PAO. Finally, we will establish the whole cell configuration with the patch pipette. The electrode will now perfuse the cytoplasm of the cell with PIP_2 (1-10 min). Then, we will apply AMPH again. We expect the cytoplasmic dialysis of PIP_2 to rescue, at least in part, AMPH-induced DA efflux. The amount of AMPH-induced DA efflux rescued will be determined by quantifying the amperometric peak current, as in Fig. 9, and comparing it to both PAO and control conditions. Negative control experiments will be performed by omitting PIP_2 from the patch pipette.

As a proof of principle for the PIP_2 rescue experiments, we applied the APC technique to hDAT cells transfected with the muscarinic M1 acetylcholine receptors (mAChR). We reasoned that if activation of phospholipase C (PLC) by muscarinic stimulation suppresses DA efflux by PIP_2 depletion, then dialysis of PIP_2 into the cells with the whole cell pipette should blunt this modulation by providing an inexhaustible source of PIP_2[44, 45]. In the patch pipette, we used the short-chain water-soluble analog diC8-PIP_2 (2 μM) or vehicle[46]. At the beginning of the experiments the patch pipette was in cell attached, and therefore, not able to perfuse the cytoplasm of the cell. We recorded in control condition AMPH induced DA efflux. Then, we applied acetylcholine (100 μM) to the bath and the subsequent AMPH-evoked DA-efflux was significantly reduced to 54±12% of control conditions ($p \leq 0.05$; acetylcholine versus control; n=3). Finally, we established the whole cell configuration with the patch pipette to dialyze the cytoplasm of the cell with PIP_2. The cytoplasmic perfusion of PIP_2 rescued AMPH-induced DA efflux to 110±22% of control conditions ($p \geq 0.33$; dialyzed PIP_2 versus control; n=3). In contrast, following acetylcholine application, cytoplasmic perfusion of vehicle solution <u>without PIP_2</u> did not rescue DA efflux ($p \leq 0.008$; dialyzed vehicle versus control; n=3). These experiments demonstrate that receptor mediated depletion of PIP_2 inhibits DA efflux while dialysis of PIP_2 into the cell reverse this phenomenon. These data strongly suggest that PIP_2 regulates DA efflux and that we can deliver effectively PIP_2 inside the cell with the APC technique.

We also predict that DAT/PIP_2 interactions are required for AMPH to induce N-terminal phosphorylation (Fig. 1c). Direct interaction of PIP_2 with membrane proteins has been shown to regulate their phosphorylation[47]. We will pretreat hDAT cells with PAO (10-100 μM; 1-20 min) to deplete PIP_2 or vehicle followed by AMPH (10 μM; 5-10 min). Changes of AMPH-induced N-terminus phosphorylation caused by PAO will be evaluated by means of [^{32}P] incorporation. The first 21 amino acids are essential for AMPH-induced phosphorylation[8]. Therefore, as a negative control experiment, [^{32}P] incorporation will be determined in cells expressing hDAT-del22 construct. Dr. Foster, an expert in evaluating N-terminus phosphorylation by means of [^{32}P] incorporation, will perform these experiments (see subcontract). <u>The goal is to determine how DAT/PIP_2 interactions regulate N-terminus phosphorylation status.</u>

Molecular Manipulations: First, we will determine how augmented cytoplasmic PIP_2 levels alter DAT function. We will adopt the APC technique (Fig. 3). The whole cell electrode will be maintained in current clamp (the membrane potential of the cell is not under voltage clamp, but rather physiologically dictated). The electrode will be loaded with an intracellular solution containing DA[5] and either PIP_2 (1-50 μM) or vehicle[48]. After 1-10 min of intracellular perfusion with the whole cell electrode[27] (depending on the size of the tip and access resistance of the electrode), AMPH (1-10 μM) will be applied, and DA efflux recorded with the amperometric electrode[6, 27] and analyzed as in Fig. 9. Additionally, the whole cell electrode (under voltage clamp) can be used to obtain DA efflux at different voltages, to determine if intracellular PIP_2 delivery affects the voltage dependence of DA efflux.

We will further assess whether PIP_2 interacts electrostatically with DAT to regulate DAT function. We will perfuse cells through the whole cell electrode (as described above) with a basic peptide (positively charged at physiological pH) while recording DA efflux with the amperometric electrode. <u>It consists of the sequence of the putative PIP_2 binding domain of the Kv7.2 channel</u>[32]. This peptide interacts electrostatically with the polar head groups of PIP_2 to "sequester" PIP_2 in such a manner as to hinder its interaction with plasma membrane proteins[32]. It is targeted and tethered to the plasma membrane through a fatty acid moiety, in this case palmitic acid[32]. Specifically, we will use palmitoyl-H<u>R</u>QKHFE<u>K</u>RR (positively charged) to compete against DAT for the PIP_2 interactions[32]. In Fig. 6, we show that this peptide disrupts the direct interaction of DAT and PIP_2. As a control we will use palmitoyl-H<u>A</u>QKHFE<u>AAA</u>[32]. Our model predicts that the interaction between PIP_2 and DAT coordinates DAT <u>phosphorylation</u> (Fig. 1c). Thus, we expect palmitoyl-H<u>R</u>QKHFE<u>K</u>RR to inhibit AMPH-induced N-terminus phosphorylation and, as a consequence, DA efflux. In preliminary experiments, we determined that 3 μM of palmitoyl-HRQKHFEKRR (10 min of intracellular perfusion) was effective in reducing AMPH-induced (10 μM) DA efflux (**Fig. 10a**) with respect to the control peptide (**Fig. 10b**). These data underscore the importance of DAT/PIP_2 interactions in the regulation of DA efflux, and <u>point to the type of interaction, electrostatic in nature</u>. These experiments will be repeated (as in Fig. 10) using different concentrations of these peptides (0.1-10 μM[32]), as well as varying perfusion times. Using the APC technique, we will determine whether their ability to decrease AMPH-induced DA efflux is a consequence of changes in DAT affinity for AMPH (EC_{50} for DA efflux), the cotransported ions (e.g. Na^+), and/or the voltage dependence of

DAT function. Since these peptides are permeable to the plasma membrane[32], they can be utilized for [^{3}H]DA uptake experiments in hDAT cells. Cells will be exposed to these peptides to determine whether they selectively affect DA efflux (see Figs. 4, 9) without altering DA uptake. In parallel, we will run biotinylation experiments to evaluate whether these peptides alter DAT surface expression. We are not expecting these peptides to alter either the trafficking of DAT or DA uptake, since depletion of PIP_2 with PAO did not decrease DAT surface expression and DA uptake (see above). In addition, possible changes in the EC_{50} for AMPH-induced DA efflux will be determined. We will also use these peptides to demonstrate that AMPH requires DAT/PIP_2 interactions in order to induce N-terminus phosphorylation. hDAT cells will be preincubated with effective concentrations of these peptides (as established in the efflux studies) and AMPH-induced phosphorylation of the N-terminus hDAT evaluated by [^{32}P] incorporation, as described above.

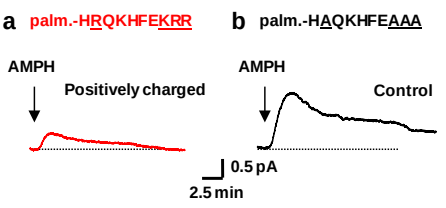

Fig. 10. "Sequestering" polar head groups of PIP_2 with a basic (positively charged) peptide impairs DA efflux: Representative traces of DA efflux recorded from cells perfused with the whole cell electrode, either with the positively charged peptide (red) **(a)**, or with the control peptide (black) **(b)**. Representative of n=2.

Finally, we will alter PIP_2 levels by utilizing a chemical dimerization strategy as described by Suh et al.[2]. This strategy alters PIP_2 levels independent of PLC regulation of IP_3 and Ca^{++} [2]. Cells will be co-transfected with two constructs. The first, the LDR construct, consists of a membrane-anchoring domain (Lyn_{11}) fused to an mTOR (**FRB**) domain and tagged by YFP. The second construct contains the FK506 binding protein (**FKBT**) domain fused to a 5' lipid phosphatase. This construct is fused to CFP. Cells will be exposed to an analog of rapamycin (AP20187, ARIAD) that binds both the **FKBT** and **FRB** domains, leading to the formation of the **FRB-FKBT** complex directly at the plasma membrane. The FKBT-coupled phosphatase is recruited to the membrane by the FRB domain upon rapamycin exposure, thereby decreasing PIP_2 levels. The colocalization between the YFP and CFP signals is used to verify formation of the **FRB-FKBT** complex. We have now obtained these constructs. Using this approach, we will study the role of PIP_2 in regulating hDAT function by determining changes in DA efflux and uptake as specified above. Recently, a new strategy for altering independently either PI_4P or PIP_2 has been published[3]. If the liposome-pulldown experiments (S.A. #1a) suggest that PI_4P can also bind to N-terminus DAT, this new strategy will be adopted (Dr. Hammond generously provided us with these new reagents)[3].

2b. To identify the PIP_2 binding sites on the DAT: Electrostatic interactions between PIP_2 and the target protein are thought to lead to changes in protein conformations and, as a consequence, their activity[9, 49]. Our data suggest that DAT interacts with PIP_2 (Figs. 5, 6) at the N-terminus (Figs. 6, 8). To date, no unanimous protein sequences have been described for the PIP_2 binding site. However, it is thought that PIP_2 will bind to positively charged residues (basic) such as Arg and Lys[9]. Figure 8 represents the computational simulation of N-terminal DAT and the spatial distribution of N-terminus Lys and Arg. Our simulation studies suggest that these positive amino acids (in particular Lys3, 5, and 19) might mediate DAT\PIP_2 interactions. Our preliminary data suggest that mutation of Lys3 and Lys5 to Asn (hDAT K/N) disrupts, at least in part, the interaction between hDAT and PIP_2 (**Fig. 11**). In these cells, we immunoprecipitated (**IP**) PIP_2 (top lane) and then immunoblotted the immunoprecipitates for DAT (**IB**: anti-DAT). In hDAT K/N cells the amount of DAT recovered in the PIP_2 immunoprecipitates is reduced. In the absence of primary antibody against PIP_2 no signal (IP: beads) was detected for DAT in the immunoprecipitates (middle row). The total DAT in the hDAT K/N cells was not decreased with respect to hDAT cells (bottom lane; total DAT). These data strongly suggest that the K/N mutation decreases DAT/PIP_2 interaction. This experiment will be repeated and the decreased association between DAT and PIP_2 determined by Western blot analysis. If we confirm that hDAT K/N exhibits only a partial reduction in DAT/PIP_2 association, we will further mutagenize the N-terminus. We will start by substituting with Asn the Lys (Lys 3,5,19) together, two at the time, and finally, one at a time. This is to identify their respective contribution to the DAT/PIP_2 interaction. However, if we find these Lys to be only partially involved in this interaction, we will substitute Lys27, Lys35, and Arg51 that we deem potentially important for this interaction (Fig. 8). When the Lys/Arg involved in DAT/PIP_2 association are fully defined by IP, we will determine their contribution to DAT/PIP_2 direct interaction in liposome-pulldown experiments, by substituting these residues with Asn in our GST-64 construct.

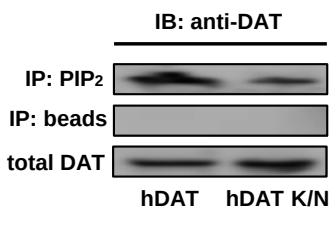

Fig. 11. N-terminus Lys mediated DAT/PIP2 interaction: Top: hDAT cells were IP with an anti-PIP2 antibody and probed (IB) for DAT. Middle: IP: beads support absence of non-specific binding. Bottom: total DAT proteins are shown. (n=1, representative of 2)

Computationally, we will evaluate the interaction of membrane models with the various N-terminus constructs (i.e. individual and/or combinations of Lys/Arg to Asn substitutions). We will first evaluate changes in conformational stabilizations (Fig. 8a), and then the relative strength of PIP_2 aggregation (Fig. 8b). We will combine the mean-field descriptions (MFCG) with extensive atomistic MD simulations to quantify the differences in the conformational

dynamics of the wild type and mutants N-termini on the membrane surface. This will allow us to assess the relative strengths of membrane binding for the various N-terminus constructs, in membrane with varying PIP_2 contents, through corresponding adsorption free energy calculations.

The question remains: which DAT functions are altered by disruption of the interaction between N-terminus and PIP_2? Answering this question will advance our understanding of how the structural domains of the DAT confer different DAT activities. Our model predicts that the interaction between PIP_2 and DAT coordinates N-terminus phosphorylation and DA efflux (Fig. 1c). Thus, disrupting this interaction should inhibit DA efflux.

As proof of principle, we have measured AMPH-induced DA efflux in cells expressing either hDAT or hDAT K/N. Our data suggest that AMPH has a decreased ability to cause DA efflux in hDAT K/N cells (**Fig. 12**; compare hDAT **(a)** to hDAT K/N **(b)**; n=1 representative of 2). Importantly, hDAT K/N plasma membrane expression, as measured by biotinylation, was not significantly different from hDAT (hDAT K/N was 120 ± 19% of hDAT; n=4-5; p≥ 0.4 Student's t-test). Consistently, no differences in DA uptake were found in hDAT K/N expressing cells with respect to hDAT cells (**Km**: hDAT = 1.7 ± 0.3 µM, hDAT K/N = 1.8 ± 0.3 µM; n=4-5; p≥ 0.8 by Student's t-test; **Vmax**: hDAT =9.6 ± 0.5 pmol/10^6 cells/min, hDAT K/N= 8.2 ± 0.6 pmol/10^6 cells/min; n=4-5; p≥ 0.09 by Student's t-test). In hDAT K/N cells, AMPH uptake (10 nM for 5 min) quantified by HPLC was also not significantly different from that of hDAT cells (hDAT K/N = 120 ± 20 % of hDAT; n=3-4; p≥0.2 by Student's t-test). As described above, we will confirm the reduced DA efflux for hDAT K/N (Fig. 12), and then mutate the three Lys (Lys 3, 5, 19) together, two at the time, and finally, one at a time to identify their respective relevance to DA efflux as measured as in figures 4 and 12. If we find these Lys to be only partially involved in DA efflux, we will further mutagenize in the N-terminus Lys27, 35 and Arg51 (see Fig. 8). In these mutants, we will also control for changes in [^{3}H]DA and AMPH uptake, protein cell surface expression, as well as relative affinity for DA (Km) and AMPH (EC_{50} for DA efflux).

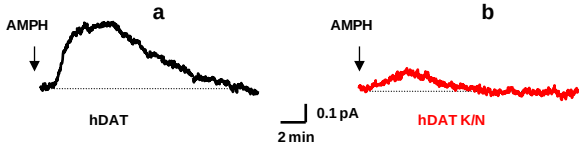

Fig. 12. The two most distal Lys of DAT N-terminus regulate AMPH-induced DA efflux: Representative traces of DA efflux recorded from hDAT **(a)**, or hDAT K/N **(b)** cells.

The goal is to generate hDAT N-terminus mutants (single or multiple Lys/Arg substitutions) that do not interact with PIP_2, and thus, **do not efflux DA**. We predict these mutants to be resistant to phosphorylation (Fig. 1c, "unwilling" to efflux DA). Thus, in these mutants, we will determine AMPH-induced N-terminus phosphorylation ([^{32}P] incorporation) and compare to that of hDAT (see subcontract Dr. Foster).

2c. To define the mechanism by which DAT N-terminus phosphorylation regulates DAT function: The N-terminal Ser (Ser2, Ser4, Ser7, Ser12, and Ser13), all reside in close spatial proximity with positively charged Lys (Fig. 8). Importantly, phosphorylation of N-terminus Ser is shown to regulate DA efflux[5, 6, 27]. Based on our cobmputational data, we believe that N-terminal Ser phosphorylation, by adding negative charges, might reduce and drastically shield the electrostatic interactions between the DAT N-terminus and PIP_2. Although novel as a mechanism regulating transporter function, the electrostatic interaction between phosphorylated Ser and positively charged Lys has been observed for other plasma membrane proteins[50, 51].

Biochemistry: First, we will establish whether N-terminus phosphorylation decreases hDAT/PIP_2 interactions both by IP and liposome-pulldown experiments (Figs. 6, 11). In the IP experiments, we will pseudophosphorylate hDAT by mutating the five most distal N-terminus Ser to Asp, either all together (hDAT S/D), one at the time, or in combination. The IPs will be performed as in Fig. 11. In encouraging preliminary experiments, we determined that in hDAT S/D cells the amount of DAT recovered in the PIP_2 immunoprecipitates is reduced to 30 ± 10% of hDAT cells (n=2). The hDAT S/D cells have normal DA uptake and DAT surface expression[5]. Liposome-pulldown experiments (Fig. 6), starting with our GST-64 construct, will determine whether phosphorylation disrupts the direct interaction of the N-terminus with PIP_2. We will substitute the N-terminus Ser with Asp (as above) and compare the results with those obtained with WT N-terminus.

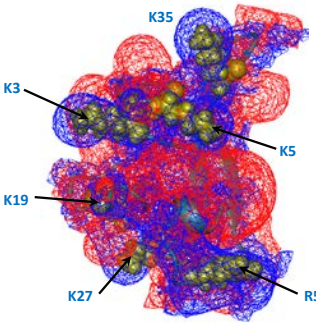

Fig 13: S7D-S12D mutation disrupts the iso-surface of positive electrostatic potential (EP). EP isosurfaces (+1kt/e shown as *blue wireframes* and -1kt/e as *red wireframes*) derived from the predicted structure of the S7D-S12D double mutant N-terminus (compare to Fig. 8).

Computation: To evaluate this mechanistic hypothesis in a detailed structural context, we will employ the full multi-scale computational protocol with a specific focus on the five most distal Ser (Ser2, Ser4, Ser7, Ser12, Ser13). These Ser will be substituted to Asp (and/or Ala) all together, one at the time, or in combinations. As proof of principle, we substituted both Ser7 and Ser12 to Asp (S7D-S12D). In **Figure 13**, we show how these S/D mutations modify the specific alignment of the Lys and Arg residues present in the WT N-terminus (**compare to Fig.13 to Fig. 8a**). Thus, pseudo-phosphorylation, by inducing conformational rearrangement, can disrupt the continuity of the calculated iso-surface of positive EP. Our hypothesis, supported by preliminary evidence, is

that this surface is the most likely to interact with the PIP_2-rich membrane (Fig. 8b). Thus, we hypothesize that the reactivity changes produced by S/D mutations will reduce the electrostatic interactions between the DAT N-terminus and PIP_2. This hypothesis will be tested by calculating the relative strength of the **interaction energies** of PIP_2 with our N-terminus mutants, as described in S. A. #1b. In contrast, S/A mutations should result in minimal changes in the electrostatic properties compared to the WT construct, and thus have no effect on the membrane interactions. These data will inform and be integrated with the biochemical studies above.

Physiology: In our model, AMPH in order to cause DA efflux requires to disrupt DAT/PIP2 interaction by N-terminus phosphorylation. We will determine if preventing N-terminal phosphorylation, by substituting the N-terminus Ser identified above to Ala, impairs the ability of AMPH to disrupt DAT/PIP_2 interaction as measured by IP (see Fig. 7). We also predict that once N-terminus phosphorylation occurs, DA efflux does not require DAT/PIP_2 interactions. Thus, we will substitute (in a hDAT K/N background with decreased PIP_2 binding and DA efflux) the N-terminal Ser with Asp (individually or in combination). We expect these substitutions to bypass PIP_2 requirements and to rescue AMPH-induced DA efflux. Yet, an important question remains: must the phosphorylated N-terminus disengage from the plasma membrane to cause DA efflux (Fig. 1b)? We will generate constructs in a ^{Tac}hDAT background where the five most distal N-terminal Ser are mutated to Asp (^{Tac}hDAT S/D) and determine AMPH-induced DA efflux. Since the N-terminus of ^{Tac}hDAT is tethered to the plasma membrane, we are expecting ^{Tac}hDAT S/D cells to have impaired DA efflux.

Expected outcomes and interpretations and possible problems: We will pinpoint the N-terminus residues interacting with PIP_2 and how phosphorylation decreases the strength of their interacting energies with the plasma membrane. We will determine how important DAT/PIP_2 interaction is for coordinating N-terminus phosphorylation (Fig. 1c). We will understand the true role of N-terminus phosphorylation for DA efflux, and how to selectively impair the ability of AMPH to cause DA efflux, without altering DA uptake. We predict that this is achievable by impairing (either pharmacologically or molecularly) DAT/PIP_2 interaction, which we believe is required for N-terminus phosphorylation. Thus, we anticipate hDAT mutants with reduced PIP_2 interactions (e.g. hDAT K/N) to be resistant to the ability of AMPH to cause N-terminus phosphorylation and, as a consequence, DA efflux. We also predict that once N-terminus phosphorylation occurs, DAT/PIP_2 interaction is no longer required for DA efflux. Thus, we will rescue DA efflux in hDAT K/N by pseudophosphorylating the N-terminus Ser. In contrast, pseudophosphorylation will fail to rescue DA efflux in the ^{Tac}hDAT background, due to its inability to free the N-terminus from the membrane.

It is evident to us that our predictions have to reconcile with the possibility that these manipulations cause trafficking of DAT, as well as differences in protein expression and, as such, alter DA efflux/uptake. We would like to point out that hDAT K/N (see above) and hDAT S/D[5] have normal uptake, cell surface expression, and DA affinity. In particular, hDAT K/N has normal AMPH uptake, suggesting that hDAT K/N impaired DA efflux is not a result of impaired AMPH uptake. Still, we have different approaches to reconcile DAT trafficking changes in our functional experiments. For example, we can monitor DAT cell surface expression by confocal imaging and/or biotinylation[37] and then utilize these data to normalize functional measurements such as uptake and efflux. To account for possible differences in AMPH affinity, AMPH EC_{50} for DA efflux will be evaluated in the different mutants, and if we determine specific differences, the AMPH concentrations will be corrected accordingly. In our liposome-pulldown experiments, we also expect Lys to Asn mutation as well as Ser to Asp to impair the direct interaction between DAT N-terminus and PIP_2. Importantly, we believe that that inhibiting AMPH-induced N-terminus phosphorylation by Ser to Ala substitutions (S. A. #2c) will inhibit the ability of AMPH to disrupt DAT/PIP_2 interactions. The computational analysis will identify the residues with the most prominent effects on: a) the specific alignment of the Lys and Arg residues present in the WT N-terminus; and b) on the relative strength of the interaction energies of PIP_2 with the N-terminus. This will be utilized in **synergy** with the proposed *in vitro* experiments. For the potential computational problems (see S.A. #1).

S.A. #3. To determine the role of DAT/PIP_2 interactions in AMPH-induced behaviors

Rationale and Strategy: For the study of fundamental cellular processes, including DA efflux, heterologous cell-based systems permit flexibility and efficiency. Nonetheless, in this aim we will translate our molecular discoveries to neurons and to behavioral studies. Thus, we created two new complementary models: a) *Drosophila* DA neurons, where we recorded DA efflux for the first time, and; b) *Drosophila melanogaster,* in which expression of DAT mutants can be manipulated in DA neurons to determine the importance of DAT/PIP_2 interactions and N-terminus phosphorylation for a behavioral assay, namely locomotion. Locomotion is an elemental behavior regulated by DA across species, including *Drosophila*[13, 52, 53]. DA modulation of motor circuits has been reported for every animal phylum in which it has been investigated[54]. Thus, using genetic approaches, flies offer a powerful model for elucidating the importance of DA efflux in AMPH-induced locomotion. This system allows the examination of the behavioral consequences (locomotion) of molecular manipulations rapidly and cost effectively. In addition, single DA neurons from transgenic flies will be isolated to determine whether molecular manipulations regulating AMPH behaviors affect AMPH-induced DA efflux.

3a. To determine whether N-terminus conformations regulate AMPH-induced behaviors: In S.A. #1a, we established that "anchoring" the N-terminus to the plasma membrane inhibits AMPH-induced DA efflux. Here, we will determine the behavioral consequences of this inhibition by constraining N-terminus DAT, *in vivo*, directly in DA neurons in *Drosophila*.

In *Drosophila*, locomotion requires functional DA neurotransmission[52, 55]. For determining changes in locomotion, **adult** *Drosophila* males were fed sucrose (5 mM) containing either AMPH (1 mM) or vehicle (CTR). Locomotion was measured by beam crossing detection over a 60 minute period (data binned in 5 min intervals; only indicated time points are shown for clarity). In wild-type (WT) *Drosophila*, AMPH significantly stimulates locomotion (**Fig. 14**) (WT AMPH, closed squares vs. WT CTR, open squares). Remarkably, in males lacking *d*DAT (dDAT *fumin; dDAT*^{fmn})[56], AMPH did not significantly increase locomotion (**Fig. 14**) (*dDAT*^{fmn} AMPH, closed triangles vs. *dDAT*^{fmn} CTR, open triangles). In flies treated with vehicle only, we did not measure any significant difference between WT and *dDAT*^{fm} (**Fig. 14**) (WT CTR, open squares vs. *dDAT*^{fmn} CTR, open triangles; p≥0.05). These data strongly support adult *Drosophila* as an animal model in which functional DAT is required for AMPH-induced locomotion (see also Pizzo *et al.* [13]).

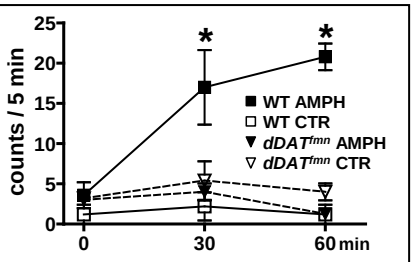

Fig. 14. AMPH induces a DAT-dependent increase in locomotion: *Drosophila* males were fed either vehicle or AMPH. AMPH enhances locomotion in WT but not in *dDAT*^{fmn}. (*p<0.01, two-way ANOVA. Bonferroni's test).

To further support this behavioral model, we rescued the lack of response to AMPH treatment in the KO flies by using the Gal4/UAS system to express a single copy of hDAT (UAS-hDAT) in a *dDAT*^{fmn} mutant background, selectively in DA neurons[57]. This system has two parts: the Gal4 gene, encoding the yeast transcription activator protein Gal4, and the upstream activation sequence (UAS), a minimal promoter region, to which Gal4 specifically binds to activate the transcription of the gene of interest (hDAT constructs). We developed flies where the Gal4 expression is driven by the tyrosine hydroxylase (TH) promoter (TH-GAL4), driving the expression of Gal4 specifically in DA neurons (in flies octopamine, the analog of norepinephrine does not require TH for synthesis). The gene of interest (hDAT constructs) is inserted into an attB donor plasmid with a UAS site[58]. This approach allows for irreversible integration of the gene of interest into the identical genomic locus *via* an integrase (phiC31) through the integrated phage attachment site *attP* site (the recipient site in the *Drosophila* genome). This leads to the expression of comparable levels of mRNA for the transgenes (e.g. hDAT). These transgenics are generated with no need for mapping of the insertion site[58].

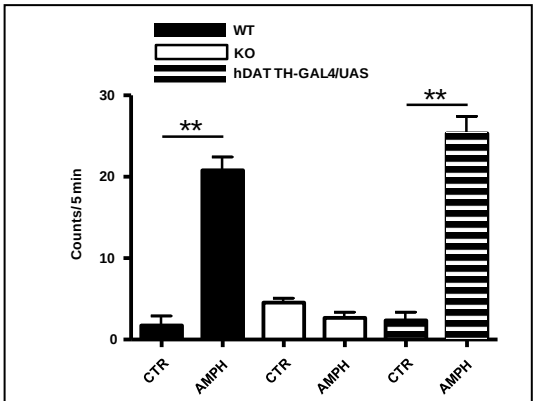

Fig. 15. AMPH-induced locomotion in *Drosophila* requires the DAT. hDAT rescues the response after 60 minutes of AMPH in *dDAT*^{fmn} (KO) (n=11; **p<0.001 versus respective control; one-way ANOVA; Bonferroni test).

Using this system, we rescued AMPH-induced locomotion in the *dDAT*^{fmn} mutant background by expressing hDAT specifically in DA neurons (**Fig. 15**). In WT, AMPH for 60 min significantly increased locomotion with respect to vehicle (AMPH vs. CTR, black bars), but had no significant effect on *dDAT*^{fmn} mutants (AMPH vs. CTR, white bars). Expression of hDAT (or dDAT, data not shown) in DA neurons, using the TH-GAL4/UAS system in a *dDAT*^{fmn} background (hDAT TH-GAL4/UAS) was able to rescue the mutant phenotype: AMPH significantly increased locomotion (AMPH vs. CTR, striped bars). This increase was comparable to that observed in WT. These data support DAT being necessary for the behavioral responses to AMPH and validate this model to study how changes in hDAT/PIP_2 interactions affect AMPH-induced behaviors.

First, we propose to use the TH-Gal4/UAS system to characterize the role of N-terminus interactions in AMPH-induced locomotion. Our preliminary data suggest that ^{Tac}hDAT has impaired AMPH-induced DA efflux (Fig. 4). In a *dDAT*^{fmn} background, we will generate flies expressing the transgene ^{Tac}hDAT in DA neurons using the Gal4/UAS system. Using these flies, we will determine the role of N-terminal constraints in AMPH-induced locomotion. We expect animals expressing ^{Tac}hDAT to have reduced AMPH-induced locomotion.

We have demonstrated that phosphorylation of the DAT N-terminus at the five most distal Ser is essential for AMPH-induced DA efflux[5, 6]. Recently, it has been shown in *Drosophila* **larvae** that N-terminus DAT phosphorylation is required for AMPH-induced behaviors[13]. To understand the true importance of N-terminus phosphorylation for AMPH-induced locomotor behaviors **in adult** *Drosophila*, we will generate flies expressing the transgenes hDAT S/D or hDAT S/A in DA neurons. We will generate benchmarks for the transgenes hDAT S/D or hDAT S/A to be used to interpret the experiments proposed below. We expect hDAT S/A flies to have reduced AMPH-induced locomotion due to diminished DA efflux[5]. In contrast, we anticipate that the hDAT S/D flies will respond to AMPH similarly to the hDAT flies (AMPH causes N-terminus phosphorylation).

An important question remains to be answered: is N-terminal phosphorylation ***per se*** sufficient to support

AMPH-induced locomotion? Our hypothesis is that in order to cause DA efflux, the DAT N-terminus, in addition to phosphorylation, needs to disengage from the plasma membrane. We will generate transgenic flies where the five most distal N-terminal Ser of ^{Tac}hDAT (the N-terminus is anchored to the membrane) are mutated to Asp (^{Tac}hDAT S/D). Since the N-terminus of ^{Tac}hDAT is tethered to the plasma membrane, we are expecting ^{Tac}hDAT S/D flies to have impaired AMPH-induced locomotion similar to that of ^{Tac}hDAT flies.

3b. To establish the role of DAT/PIP_2 interactions in AMPH-induced locomotion: In S.A. #2b, we will uncover which Lys/Arg in the N-terminus are responsible for coordinating both PIP_2 binding and N-terminus phosphorylation. Here, we will establish the role of these residues for AMPH-induced locomotion. In a *dDAT^{fmn}* (**KO**) background, we will generate transgenic flies guided by the results obtained in S.A. #2b. We will start by expressing hDAT K/N (Lys3 and Lys5 substituted with Asn) in DA neurons since this Lys substitution was effective in reducing DA efflux (Fig. 12) without altering DA uptake or surface expression. In addition, the effective Lys/Arg substitutions determined in S.A. #2b will also be evaluated. Our model (Fig. 1) predicts that the interaction of the DAT N-terminus with PIP_2 is required for DA efflux. Thus, we expect the hDAT K/N flies to have impaired AMPH-induced locomotion. We are encouraged by recent preliminary data indicating that flies expressing hDAT K/N in a *dDAT^{fmn}* **heterozygous** background (one copy of dDAT is still present) have reduced AMPH-induced locomotion with respect to flies expressing hDAT in the same background (hDAT K/N 63 ± 9% of hDAT; n=5; $p \leq 0.04$ by Student's t test). These data demonstrate that an hDAT construct with reduced AMPH-induced DA efflux (hDAT K/N Fig. 12) decreases AMPH-induced locomotion when introduced *in vivo.* Since we believe that once DAT N-terminus phosphorylation occurs, DA efflux becomes PIP_2-independent, we will rescue the reduced AMPH-induced locomotion of hDAT K/N flies by generating transgenic flies expressing hDAT K/N in which the N-terminal Ser have been changed to Asp. We expect the Ser substitution in a hDAT K/N background to bypass PIP_2 requirements and to rescue AMPH-induced locomotion. We also foresee the opportunity to substitute these Ser one at a time and or in combination.

3c. To study AMPH-induced DA efflux in *Drosophila* DA neurons: To understand whether changes in AMPH-induced behaviors of our transgenic flies are supported by altered AMPH-induced DA efflux, we will study DA efflux in the cell bodies of *Drosophila* DA neurons. This preparation was chosen because: a) it is virtually impossible to record DAT-mediated DA efflux from the nerve endings of DA neurons; b) we show (see below) that it allows for recordings of DA efflux; c) in the cell bodies of DA neurons DAT-mediated ion conductances[59], DA efflux[60], and uptake[61] have been reported; and d) strong evidence support somatodendritic release of DA in brain regions where cell bodies of DA neuron reside[61-63]. This indicates the cell body expresses the cellular machinery required to synthesize and transport DA[60].

We will measure DA efflux from DA neurons of *Drosophila* strains with a clearly distinct locomotor phenotype. We will determine whether these phenotypes are associated with altered AMPH-induced DA efflux. Brains of flies expressing a fluorescent marker (mCherry) and our hDAT constructs specifically in DA neurons (the expression is driven by the TH-GAL4/UAS system) will be dissociated and cultured[57]. In these neurons (**Fig. 16a**), we have succeeded in measuring AMPH-induced DA efflux without loading DA, thereby assessing release of endogenous DA (**Fig. 16b**). This DA efflux is absent in neurons obtained from *dDAT^{fmn}* (KO) flies (data not shown, n=3), and is blocked by cocaine (**Fig. 16b**), confirming that the efflux is hDAT-dependent. We will measure AMPH-induced DA efflux from cultured DA neurons of our *Drosophila* transgenics to integrate the efflux results with the AMPH-induced behaviors. For example, if we find that flies expressing hDAT K/N do not exhibit an increase in locomotion when exposed to AMPH, we will then assay DA efflux in single neurons.

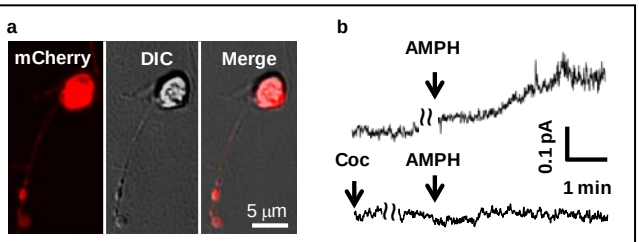

Fig. 16. AMPH-induced DA efflux in *Drosophila* neurons expressing hDAT: (a) Confocal imaging of *Drosophila* DA neurons expressing mCherry and hDAT using the TH-GAL4/UAS system. **(b)** In these neurons, AMPH causes hDAT-mediated DA efflux (top). This DA efflux is blocked by preincubation with cocaine (Coc, bottom).

Expected outcomes and interpretations and possible problems: Here, we will unearth the importance of DA efflux for AMPH-induced locomotion and how DAT/PIP_2 interaction regulates this behavior. From flies expressing the ^{Tac}hDAT S/D, we will also learn if phosphorylation *per se* is sufficient to cause AMPH-induced locomotion. As suggested by our preliminary data, we expect hDAT K/N flies to have decreased AMPH-induced locomotion. Yet, flies expressing hDAT constructs with the most effective N-terminus substitutions (Lys/Arg) impairing DAT/PIP_2 interactions will be generated and evaluated for locomotion. Although our methodology (TH-GAL4/UAS) leads to the expression of comparable levels of mRNA for the relevant transgenes (hDAT constructs), these transgenes can also be under the control of the dDAT promoter by generating dDAT-GAL4/UAS flies (experiments in progress). These flies will have expression of our hDAT constructs comparable to that of endogenous dDAT, and at the appropriate developmental time.

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