AMPHETAMINE INDUCES ACCUMULATION OF THE NOREPINEPHRINE TRANSPORTER INTO RAB4- AND RAB11-POSITIVE COMPARTMENTS

By

Jessica Moore

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Aurelio Galli

Roger Colbran

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TABLE OF CONTENTS

FIGURES

ABBREVIATIONS

ADHD: attention deficit hyperactivity disorder

Akt: protein kinase B

AMPH: amphetamine

CNS: central nervous system

DA: dopamine

DAT: dopamine transporter

DN: dominant negative

ERC: endocytic recycling compartment

FIP2: Rab11 family interacting protein 2

ICQ: intensity correlation quotient

KO: knockout

LC: locus ceruleus

NE: norepinephrine

NET: norepinephrine transporter

PBS: phosphate buffered solution

PKC: protein kinase C

PNS: peripheral nervous system

SCG: superior cervical ganglion

SE: sorting endosome

SERT: serotonin transporter

SLC6: sodium- and chloride-dependent neurotransmitter transporter

TfR: transferring receptor

TM: transmembrane

CHAPTER I

INTRODUCTION

I-1 Neurotransmitter transporters

Precise neurotransmitter signaling requires a means of transmitter removal from the synapse, controlling the intensity and duration of the signal. Either enzymatic degradation or cellular uptake, or a combination of these, may remove transmitter from the extracellular space. As early neurotransmitter studies focused on acetylcholine, biologists first assumed that removal of all transmitters occurred similarly, via degradation by specific enzymes. However, Axelrod's experiments using radioactively labeled norepinephrine (NE) showed that tissues take up NE, leading to the hypothesis that reuptake by the releasing neuron contributes significantly to inactivation of transmitters other than acetylcholine (Hertting and Axelrod 1961). Additional experiments with other labeled transmitters indicated that inactivation of gamma-aminobutyric acid (GABA) and glutamate occurs through the same mechanism, further supporting this idea (Iversen LL 1971).

Molecular identification of the proteins responsible for uptake of each transmitter led to further evidence of the importance of their function, through the association of mutations in transporter genes with disease and the effects of engineered disruption of those genes. For example, a missense mutation in the gene for a glutamate transporter, excitatory amino acid transporter 2, identified in an amyotrophic lateral sclerosis patient, reduces its function by half, causing increased extracellular glutamate which likely contributes to the excitotoxic motor neuron degeneration of that disorder (Aoki M 1998, Trotti D 2001). Similarly, a coding polymorphism in the serotonin transporter (SERT),

identified in patients displaying symptoms of obsessive compulsive disorder plus Asperger's syndrome or anorexia nervosa, doubles its uptake function, which likely causes altered behavior by reducing serotonergic signaling (Ozaki N 2003, Kilic F 2003). The mutation in the norepinephrine transporter (NET, see section I-5) identified in patients with orthostatic intolerance provides yet another example, showing the necessity of proper transmitter signal termination (Shannon JR 2000).

The effects of inactivation of one of the transporter genes, as they occur in a controlled background, support a causative role of spontaneous mutations in transporter genes in human disease and allow direct examination of transporters' role in controlling transmitter concentrations. Knockout of any one of these transporters prolongs the extracellular lifetime of neurotransmitter; for example, mice lacking the DA transporter (DAT) clear DA 300 times slower than wildtype mice (Jones SR 1998). Altered transmitter dynamics affect behavior as well as vital functions. For example, mice lacking functional GABA transporter subtype 1 (GAT1) have disordered movement as well as activity patterns indicating increased anxiety (Chiu CS 2005). Mice lacking SERT display irregular colon motility, while disrupting the NET gene affects cardiovascular function (Chen JJ 2001, Keller NR 2004, also see section I-4).

The work described in this thesis investigates the effects of amphetamine (AMPH) on NET's subcellular distribution, particularly the specific compartment in which NET accumulates following AMPH exposure. The remainder of this introduction therefore describes NET in more detail, as well as AMPH's effects on NET function and localization and on physiological functions that result from effects on NET.

I-2 Norepinephrine transporter function and structure

Expression cloning identified the norepinephrine transporter (NET) as the protein responsible for reuptake of norepinephrine (NE) (Pacholczyk T 1991), already determined to be the major means of NE clearance (Iversen 1967; Iversen 1975). Cloning also revealed NET's amino acid sequence, allowing prediction of its topology (Pacholczyk T 1991). NET contains 12 transmembrane (TM) spanning helices, intracellular amino and carboxy termini, and a large glycosylated extracellular loop between the third and fourth TM domains, as do all members of the sodium and chloride-dependent cotransporter family SLC6, which includes the DAT, SERT, and GAT1 (Fig. 1A) (Torres GE 2003; Chen NH 2004). Finally, NET likely functions as a dimer, as monomers of hNET with distinct antigenicity can be co-immunoprecipitated (Kocabas AM 2003). Further, a NET mutant that fails to traffic to the cell surface prevents wildtype transporter from reaching the surface, indicating that NET forms imers prior to cell-surface delivery (Hahn MK 2003).

These transporters move substrate intracellularly by coupling transport to the diffusion of Na^+ ions down their concentration gradients. Radioactive uptake studies led to the proposal of the alternating access model, in which binding of one Na^+ ion to a NET facing the extracellular space (with its internal gate closed) facilitates binding of one molecule of NE, which induces a change to an inward-facing conformation of NET (with its outer gate closed) (Fig. 2A-B) (Jardetzky O 1966). NET then releases the ions and NE, which allows NET to return to its outward-facing conformation. Current recordings from cells heterologously expressing NET, however, demonstrated far greater currents than predicted by the stoichiometry of the alternating access model, suggesting that NET also functions as an NE-gated channel (Fig. 2C) (Galli A 1996). Further description of NET structure followed the determination of the crystal structure of a homologous

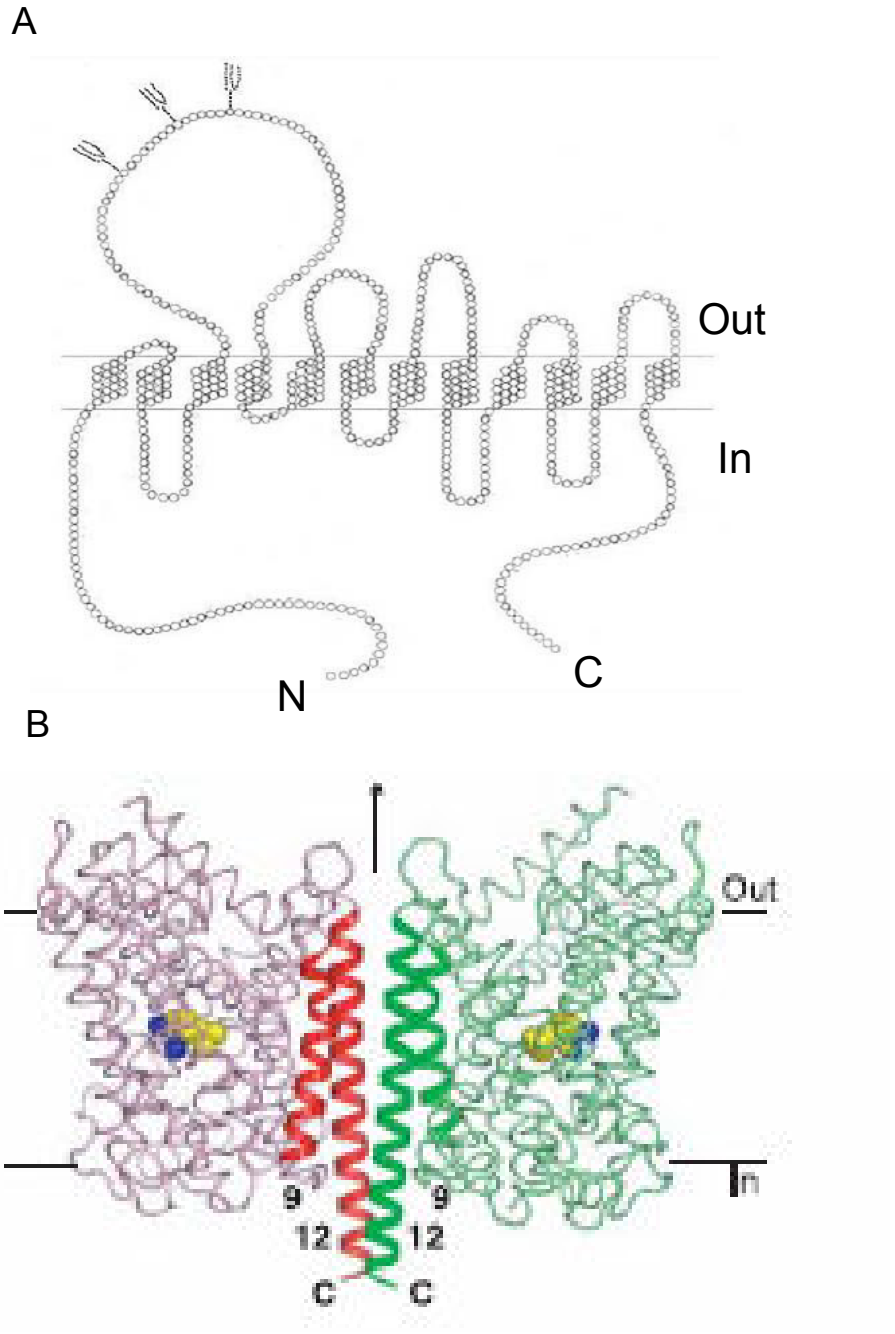


Figure 1. Norepinephrine transporter structure. A, NET topology. Each circle represents an amino acid. Letters indicate N and C termini. Pitchfork shapes indicate sites of glycosylation. From Pacholczyk et. al. 1991. **B,** Crystal structure of the *Aquifex aeolicus* leucine transporter, a NET homolog. Red and green indicate distinct monomers. Numbers indicate the position of transmembrane domains within the protein sequence. Yellow and blue space-filling structures represent bound substrate and ions, respectively. From Yamashita et. al. 2005.

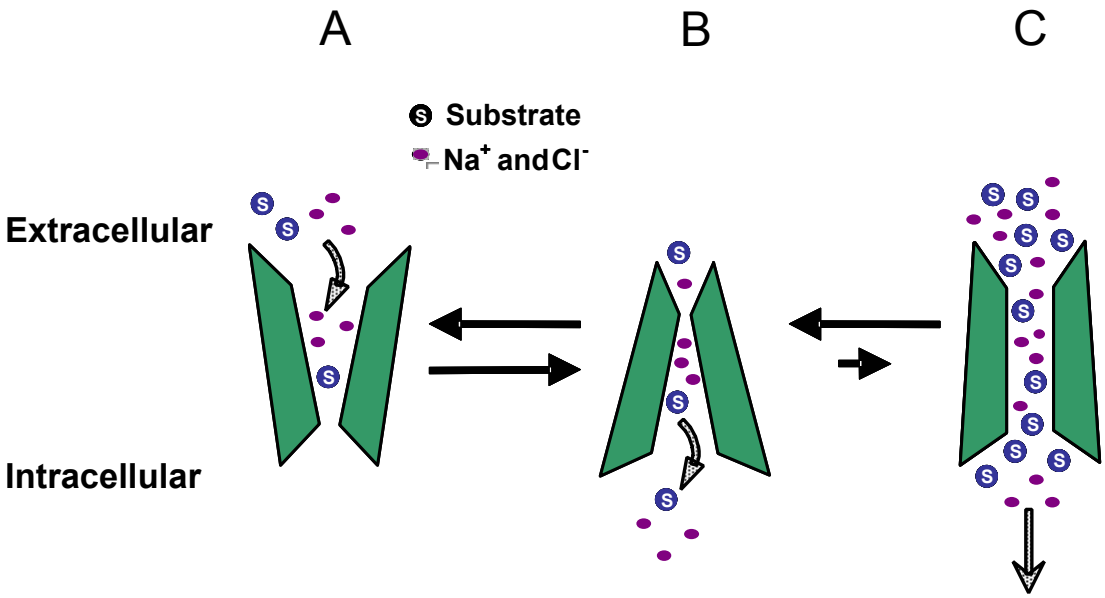


Figure 2. Mechanism of NE transport by NET. A-B. Alternating access model. **A** indicates the outward-facing conformation, which allows ion and substrate from the extracellular space to bind, causing a change in conformation to inward-facing (**B**), where ions and substrates are released into the cytoplasm. **C** indicates the channel mode of the transporter, which allows ions and substrate to diffuse through the channel. The length of the arrows signifies the probability of changing between modes. Courtesy of Kris Kahlig, Vanderbilt University.

bacterial leucine transporter, which identified the binding sites for substrate and Na^+, as well as the internal and external gates (Fig. 1B) (Yamashita A 2005).

I-3 Sites of NET expression

Comprehensive understanding of AMPH's effects via NET necessitates description of NET's expression pattern throughout the body, as systemically administered AMPH would reach all of these. Noradrenergic neurons in both the central nervous system (CNS) and the periphery, as well as the adrenal medulla, express NET protein, and functional and pharmacological evidence point toward expression in the lung and placenta as well (Schroeter S 2000; Eisenhofer G 2001). The CNS NE system includes two major noradrenergic cell groups, the locus ceruleus (LC) of the dorsorostral pons and lateral tegmental nuclei (LTN) of the medulla. The LC projects via the dorsal noradrenergic bundle throughout the cortex, as well as to the hippocampus and ventral tegmental area (VTA) of the midbrain, and the LTN project via the ventral noradrenergic bundle to the hypothalamus and thalamus. Both nuclei innervate the thalamus, amygdala, cerebellum, and spinal cord. Peripheral noradrenergic nuclei originate in the sympathetic ganglia, located paravertebrally, prevertebrally, or previscerally (adjacent to target organs), and project to nearly every organ of the body, including the heart, blood vessels, trachea and bronchi, liver, smooth muscle of the gastrointestinal tract, and urinary and reproductive organs.

I-4 Genetic manipulation of NET

NET knockout (NET KO) mice have increased basal extracellular NE levels due to slowed NE clearance ((Xu F 2000)). This genetic manipulation also alters DA homeostasis; extracellular DA levels in the striatum decrease, leading to supersensitivity

of the D_2 and D_3 types of DA receptors (Xu F 2000). Lack of uptake of DA by NET may contribute to these effects on the DA system; NET's affinity for DA is greater than that for NE, and a NET-selective inhibitor increases extracellular DA in the prefrontal cortex (PFC) (Carboni E 1990; Di Chiara G 1992; Giros B 1994; Gu H 1994). Uptake studies in NET KO mice provide more direct evidence for this explanation: cortical and striatal synaptosomes from NET KO mice show reduced uptake of [^{3}H] DA, and a DAT-selective blocker does not further reduce uptake in the PFC (Morón JA 2002). These alterations have diverse behavioral effects related to many psychiatric and neurological conditions. NET knockout (NET KO) mice respond in the forced-swim and tail suspension tests similarly to wildtype (WT) mice treated with antidepressants (Xu F 2000). These mice also have reduced locomotor activity in novel environments, lowered seizure threshold and seizure severity, and increased pain threshold and opioid analgesia in the warm-water tail flick test (Bohn LM 2000; Ahern TH 2006; Mitchell HA 2006). Such behavioral results support NET's involvement in regulation of mood and motivation, attention, overall brain excitability, and pain sensation. Disruption of NET also alters cardiovascular function and regulation of energy homeostasis. NET KO mice display tachycardia and hypertension during activity and weigh less than WT mice (Xu F 2000; Keller NR 2004).

I-5 Human disorders related to NET and AMPH

AMPH use causes improved mood or euphoria, suppression of appetite, accelerated heart rate, increased blood pressure, and improved concentration. Though AMPH targets DAT and SERT as well as NET, these effects likely at least partially result from AMPH's actions on NET, as related symptoms characterize several human disorders associated with variation in the NET gene or treated by NET-specific drugs. For

example, the persistent low mood and negative emotions of depression improve with NET inhibitors, such as desipramine, reboxetine, and venlafaxine.

Polymorphisms in the NET gene may cause some cases of depression; the T182C variant in the NET promoter associates with depression in a Japanese as well as a Korean population (Inoue K 2004; Ryu SH 2004), and a study including the effects of negative life events showed an increased effect of this polymorphism and demonstrated an effect of another 5' upstream variant, G1287C, on risk for depression for which other groups had failed to show association (Owen D 1999; Sun N 2008). A refined approach, subdividing the population according to specific symptoms, found association of T182C with recurrent depression and of A3081T with increased appetite (Hahn MK 2008). Genetic variation in NET may also affect appetite and eating behavior when altered in the absence of depression; another variant upstream of the NET gene associates with anorexia nervosa (Urwin RE 2002), and variation in this region contributes to differences in response to a DAT/ NET blocker for obesity (Spraggs CF 2005).

Though these and other examples of association evidence suggest NET's involvement in a variety of disorders, only one coding variant yet exists that demonstrates a direct, simple relationship between NET and a specific disorder. The variant of NET in which a proline replaces the more common alanine at position 457 causes orthostatic intolerance, a type of dysautonomia characterized by lightheadedness, syncope, and postural tachycardia (Shannon JR 2000). This variant functions as a dominant-negative, preventing cell-surface delivery of wildtype transporter (Hahn MK 2003). Altered NET function in the periphery also likely contributes to misregulation of blood pressure; patients with essential hypertension show increased NE spillover from the heart and kidney (Esler M 1988). Further, several separate coding variants identified in patients with either hypertension or hypotension show altered uptake function (Halushka MK 1999 ; Hahn MK 2005). The specific norepinephrine reuptake inhibitors atomoxetine

and venlafaxine cause slight increases in blood pressure (Mayer AF 2006; Rynn MA 2007).

These drugs, especially atomoxetine, treat symptoms of attention deficit hyperactivity disorder (ADHD). Eight to nine percent of US children 8-15 years old meet the DSM-IV criteria for ADHD: six or more symptoms of inattention (e.g., does not appear to listen when spoken to directly, often loses things needed for important tasks, etc.) or six or more symptoms of hyperactivity/impulsivity (e.g., fidgeting or squirming, difficulty being quiet, interrupting others often, etc.) to a disruptive and inappropriate degree (Froehlich TE 2007, CDC website). Atomoxetine's therapeutic value may lie in its effects on DA signaling; it increases extracellular DA as well as NE in the PFC, indicating that excessive DA reuptake by NET may underlie ADHD symptoms (Bymaster FP 2002). Variation in the NET gene may also contribute directly to the disorder itself; several studies have shown association between NET polymorphisms and ADHD (Bobb AJ 2005; Kim CH 2008; Kim JW 2008), though many others have failed to show association (Xu X 2005; Cho SC 2008).

I-6 AMPH as therapy and abused drug

Treatment of ADHD, narcolepsy, and, historically, obesity includes AMPH, which targets NET, as well as DAT and SERT. Common medical opinion holds that its action, as well as that of another psychostimulant, methylphenidate (MPH), on DAT contributes most to their efficacy for ADHD; nonetheless, atomoxetine's effectiveness strongly suggests that NET plays a significant role. Additionally, genetic evidence supports NET's involvement in the mechanism of psychostimulant ADHD treatment, as a variant in the NET gene associates with response to MPH (Yang L 2004). AMPH treats the excessive daytime sleepiness (EDS) of narcolepsy, but does not alleviate its other symptoms, cataplexy

(temporary muscle weakness) and rapid eye movement sleep disturbances. AMPH promotes wakefulness in all individuals, but narcoleptics require very high doses to prevent EDS (Mitler MM 1994). Its efficacy in alleviating this symptom likely requires action on DAT in addition to NET; the very similar stimulant methamphetamine does not increase wakefulness in DAT KO mice (Wisor JP 2001). AMPH's appetite reduction effect led to its use to induce weight loss in obese patients, but its hypertensive side effects and propensity for abuse put it out of favor.

Use of the psychostimulants AMPH and cocaine often leads to abuse and addiction, as they act on DAT to increase extracellular DA in the nucleus accumbens (NAcc), which current addiction theory holds as the basis of reward. However, NET may also play an important role in the abuse potential of psychostimulants. The NAcc, as well as other dopaminergic areas in the reward circuit, receives considerable noradrenergic input. Further, specific NET blockers have reduced cocaine consumption by addicts and reduced the subjective intense positive effects ('high') of AMPH in recent studies, and specific NET gene variants associate with these AMPH effects (Szerman N 2005; Dlugos A 2007; Sofuoglu M 2008).

I-7 AMPH's actions on NET

I-7.1 AMPH-induced efflux

AMPH modulates both NET's transport function and its subcellular localization, and each effect increases extracellular concentrations of substrate. Early pharmacological investigations on the effects of AMPH showed that it inhibits [^{3}H]-NE uptake into and induces release of previously accumulated [^{3}H]-NE from the brain *in vivo* and into brain tissue in vitro (Glowinski J 1965; Häggendal J 1967; Stein L 1969; Ziance RJ 1972). Sequencing of the NET gene allowed later investigations to show, by heterologous

expression, that these effects occur due to direct actions of AMPH on NET (Wall SC 1995; Burnette WB 1996). The facilitated exchange diffusion model proposed that AMPH, by acting as a substrate, increased the number of inward-facing transporters, which allowed binding of internal substrate and subsequent reverse transport (Fischer JF 1979).

Recent evidence, such as the differing ion dependence of uptake and efflux of AMPH, questions this model (Pifl C 1999). Electrophysiological studies of DAT, which likely responds to AMPH in a very similar fashion to NET, also support a different mechanism for AMPH-induced efflux. As this effect depends on intracellular Na^+, AMPH-induced inward Na^+ current may cause increased outward transport of substrate (Khoshbouei H 2003). Further, AMPH-induced efflux seems to involve Ca^{2+}/calmodulin-dependent protein kinase II (CaMKII), as AMPH increases intracellular Ca^{2+} and inhibiting this kinase or mutating DAT to prevent its interaction with CaMKII reduces efflux (Gnegy ME 2004; Fog JU 2006). This effect of AMPH may involve yet another component, as single-transporter recordings show AMPH-induced bursts of DA efflux, suggesting that AMPH may induce channel-like activity of DAT (Kahlig KM 2005).

I-7.2 AMPH-induced transporter internalization

The work described in this thesis focuses on AMPH-induced NET trafficking, or net movement of NET within the cell to a distribution distinct from that observed in basal conditions. AMPH causes a decrease in the proportion of NET molecules on the plasma membrane within one hour of application, as shown by cell surface biotinylation (Dipace C 2007). Active transporters likely constitute the population removed from the surface, as the magnitude of single-transporter DAT currents does not change upon AMPH exposure (Kahlig KM 2004). This may result from their proximity to AMPH molecules transported into the cell; the failure of uptake-impaired DAT to traffic in response to AMPH, combined with the ability of intracellular AMPH to cause DAT internalization,

suggests that transport of AMPH must precede trafficking (Kahlig KM 2006). AMPH may reduce numbers of NET on the cell surface by decreasing rates of return of recycling NET to the plasma membrane, by increasing rates of endocytosis, or both. AMPH effects on NET trafficking may mimic those on DAT, which probably involve faster internalization, as dominant negative (DN) dynamin I reduces AMPH-induced reduction of uptake and cell surface expression (Saunders C 2000).

AMPH might accelerate NET internalization by inducing its ubiquitination, as a recent review suggests may occur for DAT (Miranda and Sorkin 2007). These authors hypothesize that amphetamine's activation of protein kinase C (PKC) would cause ubiqitination of the transporter. PKC induces DAT ubiquitination, as demonstrated by mass spectrometry, immunoblotting, and fluorescence resonance energy transfer (Miranda M 2005). Reducing protein levels of a specific E3 ligase, Nedd4-2, by RNA interference or mutation of specific lysine residues in DAT's N terminus decreases the ubiquitination of DAT caused by PKC activation, further strengthening this conclusion (Sorkina T 2006, Miranda M 2007). However, whether AMPH-induced DAT internalization requires PKC remains unclear. Neither mutation of DAT C-terminal residues required for PKC-induced trafficking nor inhibition of PKC activity affects AMPH-regulated DAT trafficking (Boudanova E 2008). Knockdown of PKC or use of a variety of PKC inhibitors rather than a single pharmacological agent would provide more conclusive results. AMPH might also cause ubiquitination of DAT and/or NET independently of PKC.

As inward transport of AMPH by NET likely precedes internalization of NET, it logically follows that AMPH would activate intracellular signaling pathways. Indeed, AMPH-induced NET internalization involves multiple kinases and NET-associated proteins. Studies on DAT revealed the importance of protein kinase B (Akt) for this trafficking effect of AMPH; constitutively active (CA) Akt reduced AMPH-induced decreases in DAT

surface expression (Garcia BG 2005). This likely occurs by overpowering the effect of AMPH on Akt activity; AMPH decreases Akt's ability to phosphorylate GSK3α in a dose-dependent manner (Wei Y 2007). This effect on DAT trafficking via Akt fits with other evidence that Akt activity supports basal cell surface DAT expression as part of the insulin signaling pathway (Carvelli L 2002; Garcia BG 2005). However, our group has observed insulin-induced reduction of NET plasma membrane expression, rather than the augmentation seen for DAT, by cell surface biotinylation (Sabrina Robertson and Heinrich Matthies, unpublished; Carvelli L 2002), though others using uptake assays have seen only alterations in NET transport capacity, and not the proportion of NET on the surface, in response to insulin (Apparsundaram S 2001). In either case, if AMPH-induced regulation of NET trafficking involves Akt, it would probably not affect NET cell surface expression in the same way as for DAT.

Akt's involvement in AMPH regulation of NET trafficking seems likely, as both AMPH-induced NET internalization and AMPH-induced inhibition of Akt via DAT require CaMKII (Dipace C 2007; Wei Y 2007). Either chelation of intracellular Ca^{2+} or pharmacological inhibition of CaMKII prevent prevent AMPH-stimulated NET trafficking (Dipace C 2007), and the same CaMKII inhibitor blocks the reduction in Akt activity caused by AMPH (Wei Y 2007). AMPH-induced NET internalization also involves an increase in the proportion of NET associated with the *t*-soluble *N*-ethylmaleimide-sensitive factor attachment receptor (SNARE) protein syntaxin1A, which also requires activation of CaMKII by AMPH (Dipace C 2007). Association with syntaxin1A may increase NET's probability of endocytosis, or even possibly mediate interaction with a member of the endocytosis machinery.

I-8 NET's residence in endosomes

Understanding of the mechanism by which AMPH causes internalization of NET necessitates identification of its intracellular destination; those proteins that regulate traffic into and out of such a site would likely be regulated by some component of the AMPH-induced signaling pathway. AMPH exposure probably enriches NET in the same membrane compartment(s) through which it constitutively recycles. We expect that NET recycles through a similar route to that of other SLC6 family members, which appear to move through the canonical endocytic recycling pathway, composed of the sorting endosome (SE) and the endocytic recycling compartment (ERC) (Fig. 3).

Much of the work elucidating the functions of Rab GTPases has compared the effects of overexpressing wildtype Rab proteins with that of overexpressing point mutants on recycling of transferrin (Tf) and its receptor (TfR). Dominant negative (DN) mutants either cannot bind guanine nucleotides or, like the Rab4 mutant we use, have lower affinity for GTP than for GDP (GDP-locked). Constitutively active mutants have decreased GTPase activity and thus remain bound to GTP. Constitutively active Rab5 increases the rate of TfR endocytosis, and the GDP-locked form inhibits TfR internalization, implying that Rab5 functions in the earliest step of recycling, directing traffic from the plasma membrane to the SE (Stenmark H 1994). Overexpression of wildtype Rab4 prevents release of iron from Tf, suggesting that Rab4 moves Tf out of acidic endosomes (van der Sluijs P 1992). Wildtype Rab4 increases the proportion of TfR on the cell surface and GDP-locked Rab4 inhibits recycling of transferrin; Rab4 probably transports cargo from the SE to the plasma membrane (van der Sluijs P 1992; McCaffrey MW 2001). Alternatively, cargo may sort from the SE to the ERC. As Rab11 localizes to the ERC and DN Rab11 inhibits Tf recycling, Rab11 likely regulates traffic from the ERC to the plasma membrane (Ullrich O 1996; Ren M 1998).

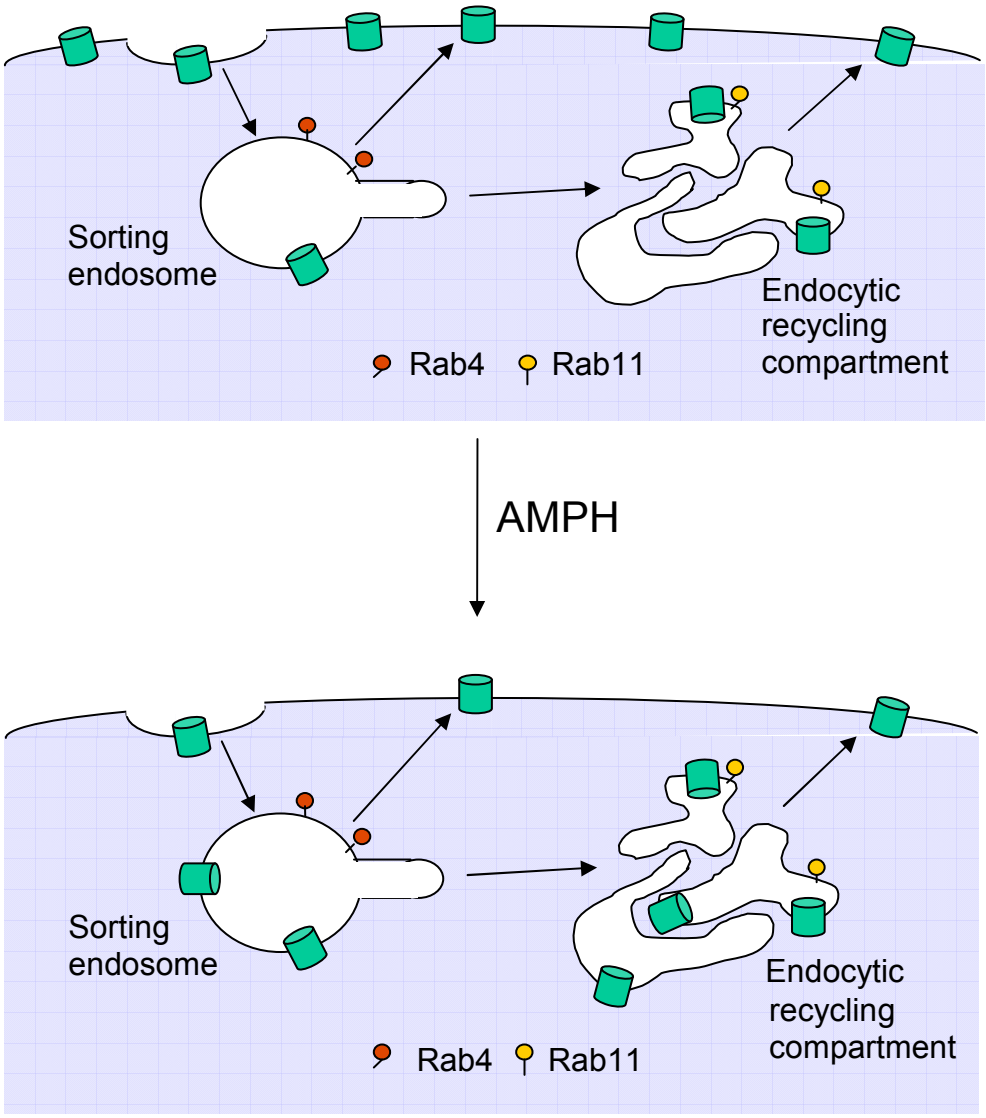


Figure 3. Model of AMPH-induced NET trafficking through the classical endocytic pathway. **A.** Basal conditions: NET (green cylinders) constutively recycles through the SE and ERC. Rab4 aids in trafficking from the SE to the plasma membrane and Rab11 aids in trafficking from the ERC to the surface. **B.** Following semi-prolonged (30-60 minute) AMPH exposure. An increased proportion of NETs on either or both endosomal populations due to faster endocytosis or slowed return to the plasma membrane leaves fewer NETs on the surface.

Immunoisolation of organelles containing TfR from DAT-PC12 cells revealed the residence of DAT on the same compartments at steady state (Melikian HE 1999). This basal co-residence of DAT and TfR has also been demonstrated by confocal imaging of immunostained PAE DAT cells (Sorkina T 2005). AMPH does indeed enrich DAT in the endosomes through which it recycles, as AMPH treatment increases colocalization of DAT with both Rab5, a marker of the SE, and Rab11, a marker of the ERC (Sorkina T 2003). SERT likely traffics through the SE, as SERT and Rab4 physically associate upon exposure to high [5-HT], indicating their proximity even in basal conditions (Ahmed BA 2008; Ahmed BA JB 2008). GAT1 also seems to follow the canonical pathway; immunoisolated GAT1-containing vesicles include Rab5 and Rab11 (Deken SL 2003). That three closely related transporters all share the same recycling route all strongly support our hypothesis.

Previous work on NET, however, has been interpreted as evidence of NET's residence in synaptic vesicles (SVs), which does not preclude NET's recycling through endosomes. SVs may form from endosomes, as all SVs contain Rab5 and pharmacologic inhibition of endosome fusion depletes SVs (Fischer von Mollard G 1994; Rizzoli SO 2002). Evidence of NET trafficking through SVs comes from the similar fractionation pattern of NET and [^{3}H]NE on density gradients of PC12 cell lysates preloaded with [^{3}H]NE (Kippenberger AG 1999). This does not exclude the possibility that NET and [^{3}H]NE are in separate vesicles of similar size; the size of primary endocytic vesicles likely matches that of SVs. NET's differing distribution on sucrose density gradients from that of VMAT2, however, argues against NET's residence on SVs (Leitner B 1999). As results on this question conflict, clear conclusions remain elusive.

CHAPTER II

MATERIALS AND METHODS

II-1 Cell culture and transfection

CAD cells are catecholaminergic and do not endogenously express NET (Qi Y 1997). hNET was cloned into pcDNA3, stably transfected into the CAD background, and selected and maintained with 200 µg/mL G418.

Superior cervical ganglion (SCG) neurons were cultured according to Savchenko et. al. 2003; briefly, SCGs were dissected from 1-3 day old C57/Bl6 mice and dissociated for 30 min in 3 mg/mL collagenase/ 0.5 mg/mL trypsin (followed by termination with 10% FBS in DMEM). Cells were plated and incubated with 3% fetal bovine serum in UltraCulture medium containing NGF for 2 hrs at 37° C to allow fibroblasts to adhere. Supernatant medium (containing SCG cells) was centrifuged, resuspended in medium supplemented with FBS, and cultured on poly-D-lysine- and collagen-coated glass-bottom plates for 10-14 days before experiments (treating with 1 µM 5-fluoro-5-deoxyuridine after 24 hrs).

Rab11-FIP2 ΔC2-cherry and Rab4b(Q67L)-GFP are gifts of Dr. James Goldenring. Transient transfections were performed using Fugene 6 reagent (Roche) with 1 µg cDNA per 500,000 cells 24 hours before experiments. For double transfections, 0.5 µg cDNA of each construct was used.

II-2 Antibodies and other reagents

Monoclonal anti-hNET 17-1 (MAb Technologies, Atlanta, GA), monoclonal anti- Na^+/K^+ ATPase α subunit (Developmental Studies Hybridoma Bank), polyclonal anti-Rab4

(AbCam), and polyclonal anti-Rab11a (Zymed) were used at dilutions of 1:1000, 1:100, 1:1000, and 1:250-1:1000, respectively, for immunoblotting, with detection by enhanced chemiluminescence reaction. Monoclonal anti-mNET 05 (MAb Technologies, Atlanta, GA), polyclonal anti-Rab4 (AbCam) and polyclonal anti-Rab11a (gift of Dr. James Goldenring) as described above were used at dilutions of 1:500, 1:250, and 1:1000 for immunocytochemistry. Fluorescent secondary antibodies included highly cross-absorbed donkey anti-mouse or rabbit IgG (Molecular Probes). AMPH was obtained from Sigma (St. Louis, MO).

II-3 Cell surface biotinylation

Biotinylation experiments were performed on intact cells, allowing comparison of hNET surface expression (relative to total) across conditions, as described previously (Sung U 2003; Garcia BG 2005; Miranda M 2005). Briefly, CAD hNET cells were plated at a density of 1 x 10^6 per well in a six-well poly-(D-lysine) coated plate (Sigma). Cells were starved of serum 1 hour prior to treatment, and immediately following treatment were washed with cold PBS containing Ca^{2+}/Mg^{2+}. Cells were then incubated with 1.0 mg/mL sulfosuccinimidyl-2-(biotinamido)ethyl-1,3-dithiopropionate [NHS-SS-biotin] (Thermo Scientific, Rockford, IL) for 30 min, washed, quenched with 100 mM glycine, and extracted in lysis buffer (PBS Ca^{2+}/Mg^{2+}, 1% Triton 100-X, and 0.5 mM PMSF) (all on ice. Membranes and nuclei were removed by centrifugation, and lysates were incubated with immobilized streptavidin beads (ThermoScientific) for 1 hr at room temperature. Total fractions were reserved, beads were washed three times in lysis buffer, and bound proteins eluted with 2X sample buffer containing 2-mercaptoethanol. Proteins were then separated by SDS-PAGE and immunoblotted. For estimation of relative amounts of proteins, the exposed films of the immunoblots were scanned, and band intensities were measured with Scion Image (Scion Corporation, Frederick, MD).

II-4 Immunostaining

SCG neurons were serum starved for one hour in DMEM:F12 and treated with vehicle or AMPH for thirty minutes. Neurons were subsequently fixed with PBS Ca^{2+}/Mg^{2+},/4% paraformaldehyde, washed three times with PBS Ca^{2+}/Mg^{2+}, permeabilized and blocked with PBS Ca^{2+}/Mg^{2+}/4% bovine serum albumin (BSA)/0.15% Tween-20, and immunostained with the appropriate antibody dissolved in PBS 4% BSA/0.05% Tween-20. Primary antibodies were visualized with the appropriate covalently labeled secondary antibody. Immunofluorescence was imaged using a Perkin Elmer UltraView confocal with a Nikon Eclipse 2000-U microscope equipped with a 60X lens with an N.A. of 1.49. Image processing was performed using Image J and Adobe Photoshop.

II-5 mNET internalization analysis

Quantitation of mNET internalization analysis was achieved by first acquiring an intensity plot along a line through the center of each bouton image (using ImageJ). This line extended outside the limits of the bouton so that the lowest intensity, assigned a value of zero, corresponds to a pixel with no staining. Intensity values were normalized to the maximum value and the distance from the first peak to the last peak was divided into 20 bins. The average pixel intensity for each bin calculated, then averaged with the pixel intensity for that bin for each bouton image in the same condition.

II-6 Colocalization analysis

Only images in which there was no pixel saturation were analyzed. Background fluorescence was first subtracted in ImageJ by selecting an unstained area of each image (away from any neurons) and running the background subtraction plugin available at http://www.uhnresearch.ca/facilities/wcif/fdownload.html. The intensity correlation

quotient was then determined by running the ICA plugin for ImageJ developed by Tony Collins and Elise Stanley (Toronto Western Research Institute), also available at the above link. The ICQ indicates whether intensity of staining for two proteins varies in synchrony over space; an ICQ of +0.5 means that in any pixel with a certain intensity of staining for one protein, the intensity of staining for the other protein studied will be exactly the same, while an ICQ of 0 signifies no relation between the two staining patterns and an ICQ of –0.5 indicates an inverse relationship. Multiplying the difference of staining intensity at a particular pixel from the mean intensity for that protein across the analyzed space by the corresponding quantity for the other protein at the same pixel yields the product of the differences from the mean (PDM) for that pixel; plotting the PDMs for all pixels and dividing the number of pixels with positive PDM by the total number of pixels, then subtracting 0.5, yields the ICQ.

II-7 Density gradient separation

CAD hNET cells homogenized in PBS (cleared of nuclei by centrifugation at 1,000 x g for 10 minutes) were mixed with Opti-Prep to a final concentration of 13% and loaded into Sorvall Ultracrimp® tubes. Centrifuging at 60,000 rpm in a vertical 70V6 rotor in a Sorvall Ultra 80 produced a density gradient. 400 µL fractions were collected by displacement with mineral oil using a Popper Micromatic pipetting assembly.The refractive index of each fraction was measured using a Bausch & Lomb Abbé refractometer. The density was calculated from the refractive index by the formula: Density = 3.298 (refractive index) - 3.3967. Proteins in all fractions were separated by sodium dodecyl sulfate polyacrylamide gel electrophoresis and immunoblotted, and estimation of relative amounts of each per fraction were determined as described for biotinylation experiments. Relative amounts of a protein in each fraction were plotted as fractions relative to the total (sum of all fractions). Where multiple experiments are

averaged and plotted together, densities and band intensities of all fractions within bins of 0.005 g/mL are averaged and plotted as a single point.

CHAPTER III

RESULTS

III-1 AMPH leads to a decrease in surface NET expression

Dipace et. al. demonstrated the ability of 10 μM AMPH to induce a reduction in surface NET expression in CAD cells stably expressing hNET (Dipace C 2007). We sought to investigate this effect at a different time of exposure; the previous study included only results for 1, 5, 10, and 60 minute treatments and showed a significant decrease in surface hNET only at 60 minutes. We chose to examine internalization at 30 minutes of AMPH treatment because, for later experiments, we wanted to minimize the possibility of lysosomal degradation of the transporter, shown for the DAT upon prolonged PKC activation (Miranda M 2005). After treating, we incubated intact cells with biotin, allowing modification of all surface proteins and later collection by streptavidin. Figure 4A shows representative immunoblots of biotinylated and total fractions from CAD hNET cells treated with either vehicle (CTRL) or 10 μM AMPH for 30 minutes at 37° C (to allow membrane trafficking). The amount of hNET on the surface of cells treated with AMPH noticeably decreases in this experiment compared to CTRL, while levels of total hNET remain the same. Figure 4B contains quantitation of multiple experiments normalized to CTRL; the proportion of hNET on the surface relative to total significantly decreases by 81.3 ± 3.9 %.

To replicate this finding in neurons at the single-bouton level, we treated neurons cultured from mouse superior cervical ganglia with 10 μM AMPH for 30 minutes and immunostained for murine NET (mNET). Figure 5A shows representative confocal sections of boutons of neurons treated with either vehicle (CTRL) or AMPH.

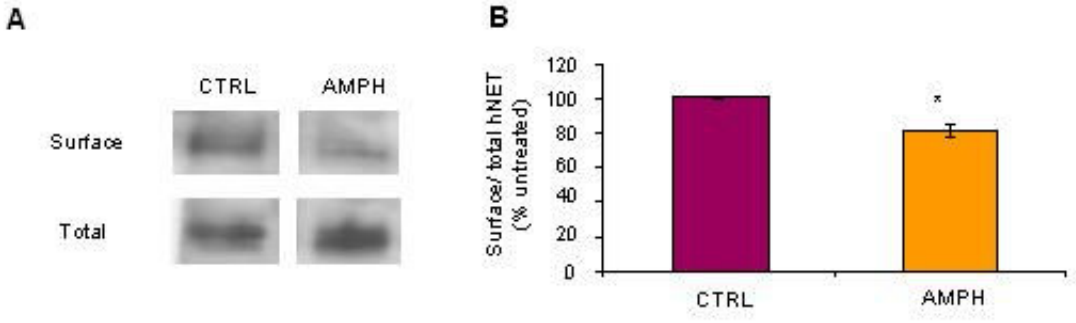


Figure 4. AMPH decreases surface hNET expression. A, representative immunoblots of biotinylated and total fractions from CAD hNET cells treated with 10 μM AMPH or vehicle (CTRL) for 30 min. All bands from same blot and exposure. **B,** Quantification of immunoblots from multiple experiments. Density of biotinylated samples was normalized to the density of the corresponding total sample and data are expressed as percentage of control. Bars indicate the mean ± S.E. and are compared to control by one-way ANOVA, * indicates $p < 0.05$; n=3.

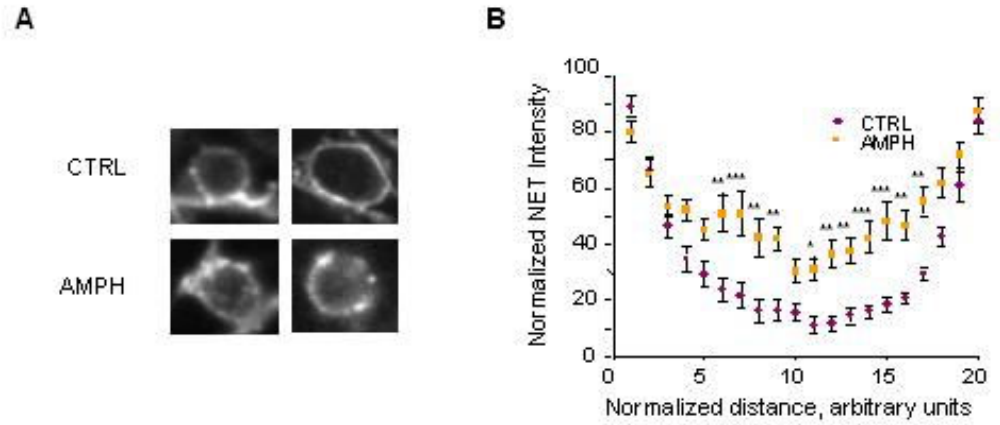


Figure 5. AMPH increases intracellular presence of NET in terminal boutons. A, representative images of confocal sections of boutons of SCG neurons treated with vehicle (CTRL) or 10 μM AMPH for 30 min and immunostained for mNET. **B,** Quantification of multiple images. Background subtracted pixel intensities were normalized to the peak intensity (assigned a value of 100) and averaged within corresponding bins ($1/20^{th}$ of peak-to-peak distance) for all images in each condition. Data are expressed as mean ± S.E.and conditions are compared by two-way ANOVA; *$p<0.05$, **$p<0.01$, ***$p<0.001$; n=9.

Qualitatively, more staining occurs inside the boutons in the AMPH-treated neurons. We quantitated multiple boutons (Fig. 5B) from pixel intensity plots along lines drawn through the center of each bouton. This line extended beyond the limits of the bouton into the extraneuronal space, so that the zero intensity value would represent no staining. We then normalized each plot to its peak intensity (100%), divided the peak-to peak distance into 20 bins, and calculated the mean pixel intensity of mNET staining for each bin. The intra-bouton staining is significantly higher in AMPH-treated neurons for 11 bins, indicating that mNET is internalized with AMPH treatment. This novel finding signifies that the AMPH-induced trafficking phenomenon is not limited to heterologous cells, and likely occurs in vivo.

As we intended to investigate the destination of internalized NET biochemically, we tested whether separation of CAD hNET cell extracts across a density gradient would illustrate this effect of AMPH. Representative immunoblots in Fig. 6A compare the distribution of hNET across the iodixanol (Optiprep®) gradient in cells treated with vehicle (CTRL) to that in cells treated with 10 μM AMPH for 30 minutes. Basally, fractions at lighter densities (1.02-1.05 g/mL) contain the greatest proportion of hNET. Upon treatment with AMPH, the distribution shifts so that higher density fractions (1.09 and 1.13 g/mL) contain the greatest proportion of hNET. Figure 6C plots the data for this representative experiment. We expected that those fractions hNET shifts away from upon AMPH exposure (those of lighter densities) would likely represent the plasma membrane, based on our other results (see Fig. 1 and 2). To determine the densities at which cell surface membrane migrate, we immunoblotted the same density gradient fractions in Fig. 6A and C for the Na^+/K^+ ATPase α subunit (Fig. 6 B and D). In both CTRL and AMPH treated cells, lighter density fractions (<1.05 g/mL) contain all Na^+/K^+ ATPase, indicating that these fractions do indeed represent the plasma membrane. That the low density hNET peak and the Na^+/K^+ ATPase peak do not correspond exactly

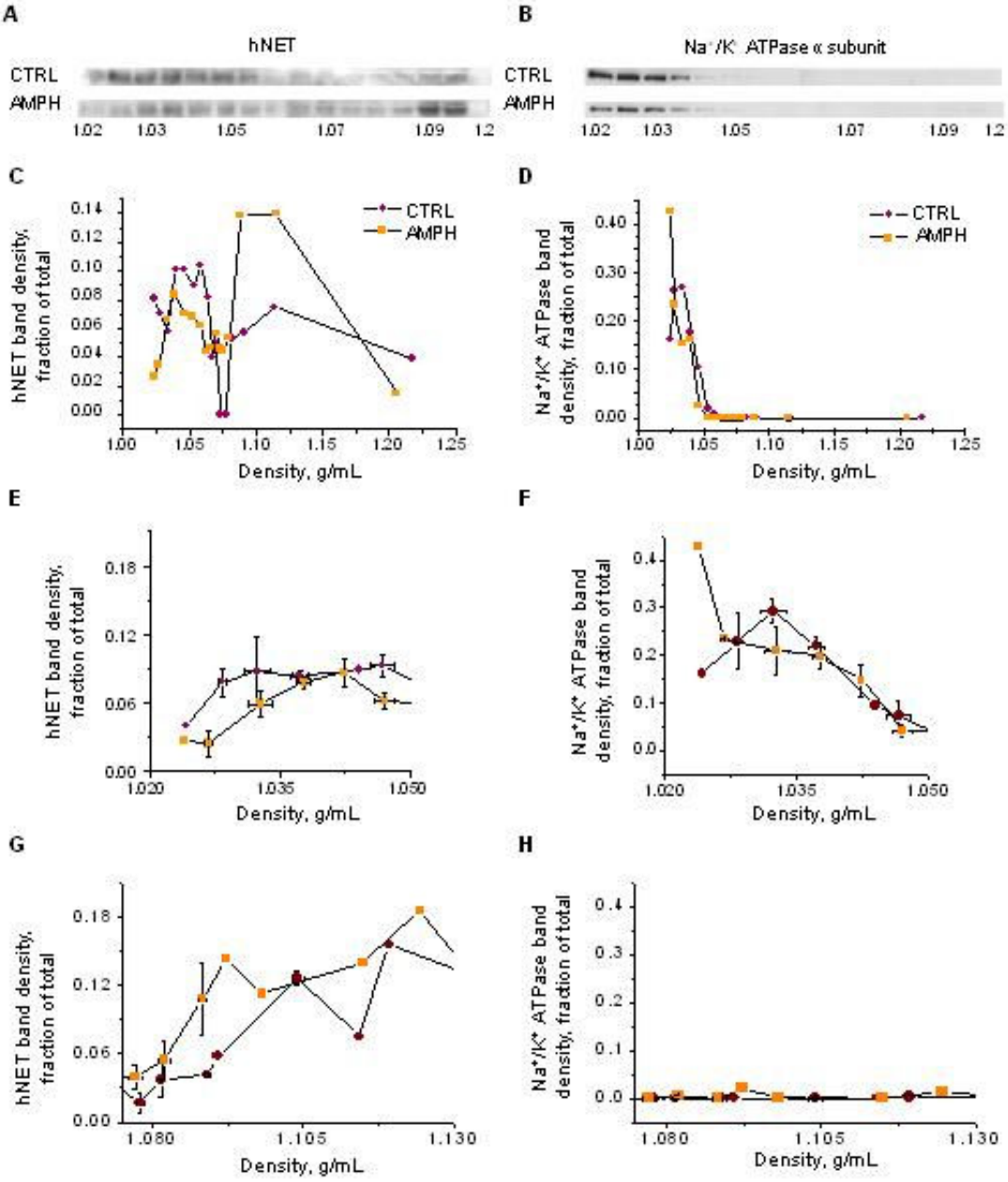
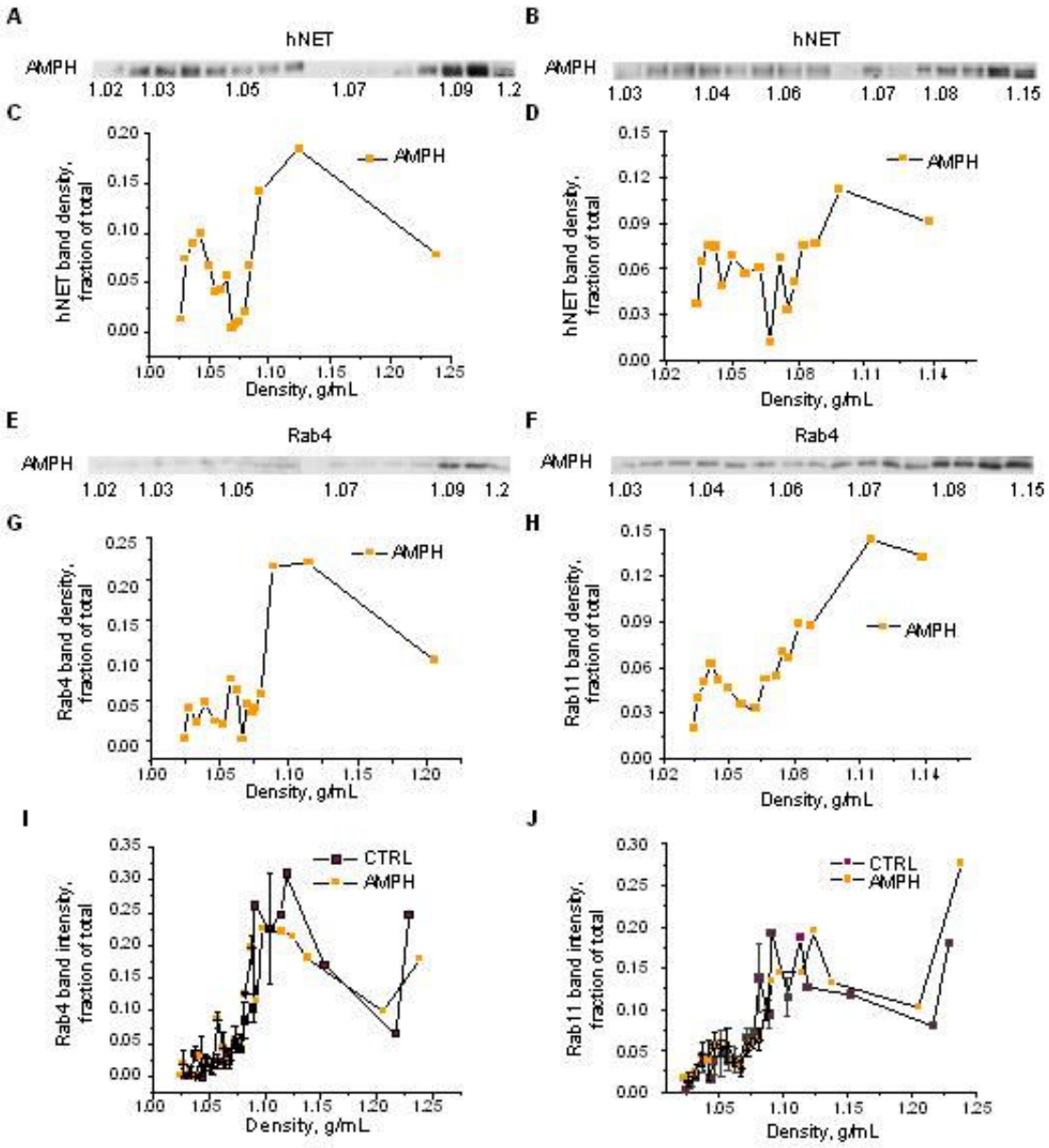


Figure 6. AMPH decreases the proportion of NET that fractionates at low densities, partially overlapping with Na^+/K^+ ATPase. **A-B,** immunoblots of iodixanol density gradient fractions from CAD hNET cells treated with either vehicle (CTRL) or 10 μM AMPH for 30 min. **C-D,** plots of band densities of blots in A-B. **E-H,** mean band densities of multiple experiments plotted on expanded density axes; **E-F,** surface fractions, **G-H,** intracellular. **E and G,** hNET; **F and H,** Na^+/K^+ ATPase. n= 3.

may indicate that the two proteins reside in different subdomains of the plasma membrane, possibly a recycling domain and a domain from which proteins cannot leave the plasma membrane. Figures 3E-H plot mean distributions of hNET (Fig. 6E-F) and Na^+/K^+ ATPase (Fig. 6G-H) from several experiments, with the x axis expanded to show either the surface fractions (Fig. 6E and 6G) or the intracellular fractions (Fig. 6F and 6H). Figure 6E shows a trend towards a decrease in the proportion of hNET in the surface fractions, especially noticeable between 1.025 and 1.03 g/mL, and Figure 3F shows a trend towards an increase in the proportion of hNET in intracellular fractions. Na^+/K^+ ATPase does not shift to low densities with AMPH treatment across experiments. Thus, the shift in Fig. 6A-B represents a shift of hNET away from the cell surface and into an intracellular destination.

III-2 AMPH increases colocalization of NET with Rab4 and Rab11

To characterize the intracellular destination of hNET upon AMPH-induced internalization, we immunoblotted density gradient fractions from CAD hNET cells treated for 30 minutes with 10 μM AMPH for Rab4, a marker of the SE, and Rab11, a marker of the ERC. Figure 4 shows a representative experiment, in which hNET is enriched in higher density fractions of 1.09 and 1.13 g/mL (Fig. 7A, immunoblot; Fig. 7B, plot). These high density fractions also contain the greatest proportion of Rab4 (Fig. 7C, immunoblot; Fig. 7D, plot). Rab4's distribution does not change with AMPH treatment (Fig. 7E). Co-localization of hNET upon AMPH exposure with Rab4 in the same density gradient fractions suggests that AMPH treatment causes increased accumulation of hNET in the SE. High density fractions (1.1 and 1.15 g/mL) again contain the greatest proportion of hNET following AMPH exposure in a separate experiment (Fig. 7F, immunoblot, Fig. 7G, plot). Rab11 exists predominantly in high density fractions, with the greatest proportion



<u>Figure 7.</u> hNET cofractionates with Rab4 and Rab11 following AMPH treatment. CAD hNET cells were treated with vehicle or 10 μM AMPH for 30 min. A, C, E, G, representative experiment comparing iodixanol density gradient distribution of hNET with that of Rab4 in AMPH-treated cells. A and E, immunoblots; C and G, plots of band densities as fraction of total. A and C, hNET; E and G, Rab4. I, comparison of Rab4 distribution in vehicle-treated cells with that in AMPH-treated across multiple experiments (n=3). B, D, F, H, separate experiment comparing distribution of hNET with that of Rab11. B and F, immunoblots; D and H, plots of band densities as fraction of total. B and D, hNET; F and H, Rab11. J, comparison of Rab11 distribution in vehicle-treated cells with that in AMPH-treated across multiple experiments (n=3).

in those enriched with hNET by AMPH (Fig. 7H, immunoblot, Fig. 7I, plot). Rab11's distribution does not change with AMPH treatment (Fig. 7J). Similar to Rab4, co-localization of hNET with Rab11 in the same fractions suggests AMPH-induced accumulation of hNET in the ERC. In order to replicate these findings in neurons at the single bouton level, we treated SCG neurons with either vehicle or 10 μM AMPH for 30 minutes and immunostained for mNET and either Rab4 (Fig. 8A-B) or Rab11 (Fig. 8C-D). The example images in Figure 8A show no noticeable colocalization between mNET and Rab4 in the CTRL condition, and a small amount of overlap of staining after exposure of neurons to AMPH. We compiled the results of multiple experiments by calculating the intensity correlation quotient (ICQ) for each bouton and determining the mean (Fig. 8B) (Li et. al. 2004). In the CTRL condition, the ICQ of mNET and Rab4 staining is -0.01267 ± 0.02527 (n=12), indicating that the two patterns do not correlate, while upon AMPH treatment, the ICQ is 0.1015 ± 0.01584 (n=12 boutons, two experiments), meaning that mNET and Rab4 staining vary in partial synchrony (Fig. 8B). This result confirms that of our density gradient experiments, again suggesting AMPH-induced accumulation of NET in the SE.

We also applied this analysis to confocal images of boutons from SCG neurons immunostained for mNET and Rab11. The representative images in Fig. 8C show slight overlap of mNET and Rab11 staining in boutons of SCG neurons treated with vehicle (CTRL), and a much more noticeable colocalization upon AMPH exposure. Mean ICQ values from multiple images in each condition reveal the consistency of this pattern across experiments; the ICQ of mNET and Rab11 increases from 0.1043 ± 0.02476 (n=13) in control conditions to 0.2046 ± 0.02265 (n=13 boutons, two experiments) upon AMPH exposure (Fig. 8D).

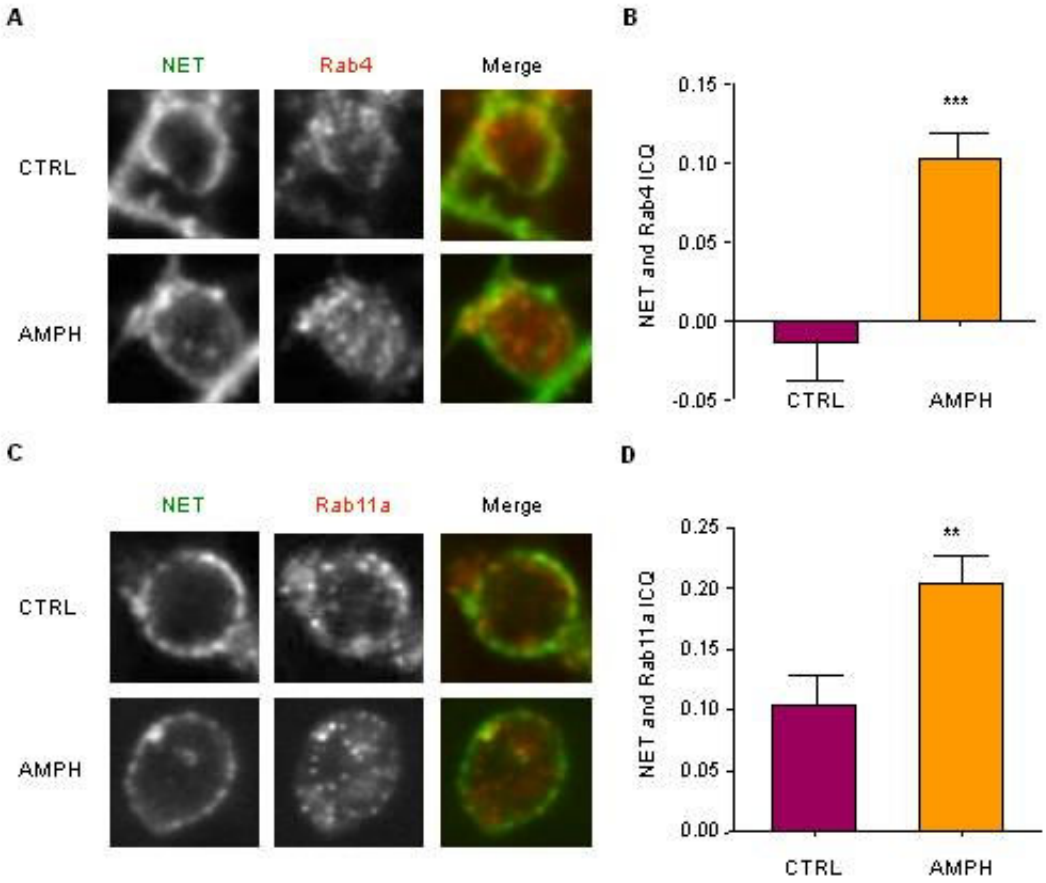


Fig. 8 AMPH increases colocalization of hNET with Rab4 and Rab11. A and C, confocal sections of SCG boutons treated with either vehicle (CTRL) or 10 μM AMPH for 30 min. **A,** Immunostaining for mNET and Rab4; **C,** mNET and Rab11. **B and D,** Plots of intensity correlation quotients (ICQ) in each condition. **B,** ICQ of NET and Rab4, n= 12, 2 experiments, **D,** ICQ of NET and Rab11, n= 13, 2 experiments.

III-3 NET trafficking involves Rab4 and Rab11 function

We investigated not only the sites in which NET accumulates upon AMPH treatment, but also the functional roles Rab4 and Rab11 play in AMPH-regulated NET trafficking. Rab proteins do not simply identify endosomal populations, but also facilitate the delivery of vesicles to destination compartments (Zerial M 2001). Rab4 controls traffic from the SE to the plasma membrane (van der Sluijs P 1992). Because NET accumulates in Rab4-positive sites with AMPH treatment (Fig. 7A-D and 8A-B), we suspected that NET may be returned to the cell surface through this Rab4-regulated pathway. To address this question, we disrupted Rab4 function by transiently transfecting either a GDP_locked (dominant negative) form of the protein, Rab4 S22N (DN Rab4), or empty vector into CAD hNET cells and comparing AMPH-induced internalization by cell-surface biotinylation. As shown in the representative immunoblots in Fig. 9A, the proportion of hNET in the surface fraction relative to that in the total decreases visibly with both 10- and 30-minute exposures to 10 μM AMPH (compared to vehicle treatment) in vector-transfected cells, while hNET surface expression remains the same following either exposure time in cells transfected with DN Rab4. Quantitation of multiple experiments in Figure 9B demonstrate that AMPH induces noticeable internalization at both time points (88 ± 2.5 % at 10 minutes and 84 ± 3.5 % at 30 minutes) in vector-transfected cells, and there is no internalization after AMPH exposure in DN Rab4-transfected cells. This result implies that AMPH-regulated NET trafficking does indeed involve Rab4.

Traffic from the ERC to the plasma membrane requires Rab11, and NET also accumulates in Rab11-positive sites (Fig. 7F-I, 8C-D), so we hypothesized that AMPH-regulated NET trafficking involves Rab11 (Ren M 1998; Trischler M 1999). Our approach to test this again involved disrupting Rab11 function and measuring AMPH-induced internalization. We blocked Rab11 function by transfecting with a dominant negative form of Rab11 family interacting protein 2 (Rab11 FIP2) lacking the C2 domain

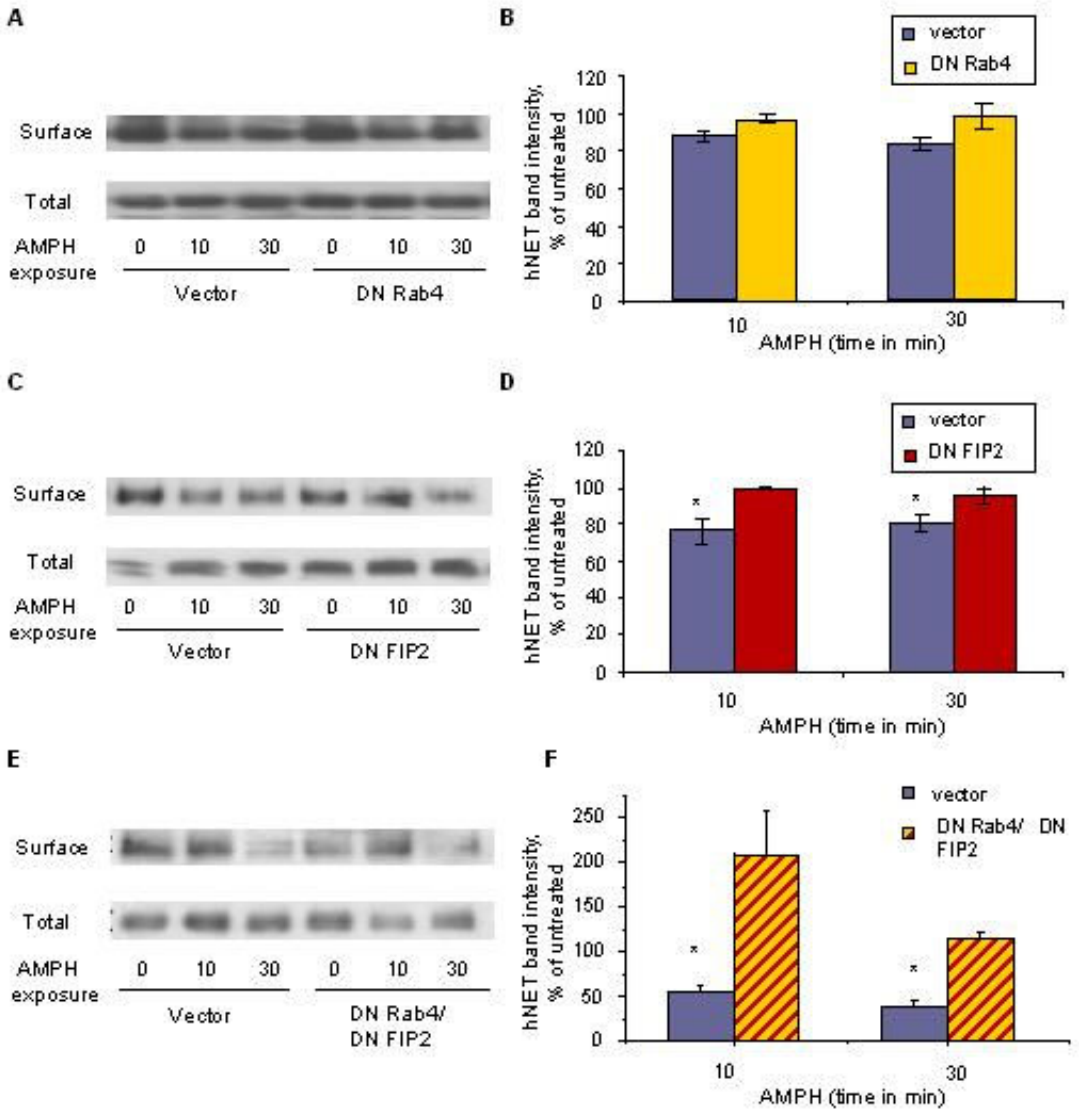


Figure 9. Disrupting function of Rab4 or Rab11 prevents AMPH-induced NET internalization. A-B, CAD hNET cells transfected with DN Rab4 were treated with vehicle (0) or 10 μM AMPH for the indicated times and subjected to cell-surface biotinylation. **A,** representative immunoblots of surface and total fractions. **B,** quantification of multiple experiments. **C-D,** CAD hNET cells transfected with DN FIP2 treated as previous. **C,** representative immunoblots of surface and total fractions. **D,** quantification of multiple experiments. **E-F,** CAD hNET cells transfected with both DN Rab4 and DN FIP2. **E,** representative immunoblots of surface and total fractions. **F,** quantification of multiple experiments. * indicates $p<0.05$ compared with control. n=3 for all experiments.

(DN FIP2), which severely disrupts plasma membrane recycling (Hales CM 2002). Translocation of Rab11 FIP2 to the plasma membrane from the ERC requires the C2 domain, which binds phosphatidylinositol-(3,4,5)-triphosphate (PIP_3) and phosphatidic acid (Lindsay AJ 2004). Rab11 FIP2 (both full-length and the DN truncation) interacts with both Rab11 and the motor protein myosin Vb, possibly acting as an adaptor between the two (Hales CM 2001). The proportion of hNET on the surface of vector-transfected CAD hNET cells noticeably decreases upon 10- or 30-minute AMPH treatment compared to vehicle-treated controls, while the level of surface hNET is unaltered by AMPH in cells transfected with DN FIP2 (Fig. 9C). These data are consistent across multiple experiments; significant AMPH-induced internalization occurs in vector-transfected cells (76.3± 6.7 % at 10 m and 80.74± 4.6 % at 30 m), while we observe no effect of AMPH on surface levels of hNET in DN FIP2-transfected cells (Fig. 9D). DN FIP2's ability to block AMPH-induced internalization likely means that AMPH-regulated NET trafficking also entails Rab11 function.

Our results suggest that reducing the function of either of these Rabs may increase return of NET to the plasma membrane through the route involving the other Rab. In the case of DN Rab4, blocking traffic from the SE to the surface might increase traffic to the ERC, increasing the amount of NET residing there. This could then drive NET through the Rab11-regulated pathway to the plasma membrane, perhaps by exceeding the capacity of the ERC to store NET. Therefore, we examined whether blocking function of both Rab4 and Rab11 simultaneously would have a different effect on AMPH-induced NET internalization from that of blocking either alone. Contrary to our expectation, surface hNET expression is unchanged upon AMPH treatment in CAD hNET cells double-transfected with both DN Rab4 and DN FIP2, though there appears to be a trend toward an increase at 10 min, while surface hNET expression in vector-transfected cells decreases at both timepoints (54.99± 7.69 % at 10 min and 38.55± 6.29 % at 30 min)

(Fig. 6E, immunoblots, 6F, quantitation of multiple experiments). As this result mimics that of blocking either Rab4 or Rab11 function alone, the effects of blocking Rab4 and Rab11 function on AMPH-induced NET internalization appear independent of one another.

CHAPTER IV

DISCUSSION

IV-1 AMPH-induced NET internalization in boutons

AMPH exposure has been shown to cause a decrease in cell surface expression of NET; however, this effect has only been investigated in heterologous cells in a single report (Dipace C 2007). AMPH-induced internalization of DAT, however, has been studied in greater detail (Saunders C 2000; Kahlig KM 2004; Garcia BG 2005; Gnegy ME 2004; Johnson LA 2005a; Johnson LA 2005b; Kahlig KM 2006; Wei Y 2007; Boudanova E 2008). Further, a single, high dose of AMPH induces a significant reduction of DA uptake in striatal synaptosomes when administered one hour prior to preparation, indicating the physiological relevance of this phenomenon (Fleckenstein AE 1999). We reveal the first evidence of AMPH-induced redistribution of NET away from the plasma membrane in noradrenergic neurons, implying that this effect on NET contributes to AMPH's ability to increase extracellular NE in vivo. Further, our demonstration that AMPH leads to internalization of NET in boutons implies that AMPH transport capacity would decrease at sites of NE release, affecting extracellular NE faster than would AMPH-induced trafficking at the soma.

IV-2 Basal mNET distribution in neurons

Our mNET immunostaining results, showing most NET on the surface of terminal boutons in basal, untreated conditions, contrast earlier ultrastructural findings in the rat prefrontal cortex using the polyclonal anti-mNET antibody 43411 (Miner LH 2003; Miner LH 2006). Many factors could contribute to this difference; most importantly, we

included Ca^{2+} in our perfusion solutions, while animals in the immuno-electron microscopy studies were perfused with PBS lacking Ca^{2+} prior to sectioning and staining. We have found that incubation of SCG neurons in PBS lacking Ca^{2+} leads to an increase in intracellular staining by the monoclonal mNET antibody (H. Matthies, unpublished results). This observation appears to conflict with the AMPH-induced increases in intracellular Ca^{2+} associate with NET internalization (Dipace C 2007). Zero-Ca^{2+}-induced NET internalization would not likely involve decreased CaMKII activity, as inhibition of CaMKII prevents AMPH-induced NET internalization and CaMKII activity supporting both basal NET surface expression and leading to NET internalization seems improbable (Dipace C 2007). Decreased intracellular Ca^{2+} might inhibit Akt independently of CaMKII, through some other kinase or phosphatase.

Additionally, the epitopes for the two antibodies differ: the monoclonal antibody we use recognizes residues 5-17, while the polyclonal antibody recognizes residues 585-607 (Matthies *in press*, Schroeter S 2000). NET's C terminus may be phosphorylated or bound to some interacting protein while it resides on the plasma membrane, preventing detection by 43411. A difference in NET surface expression between noradrenergic projections to the cortex and SCG neurons does not likely account for the discrepancy, as we can alter NET's cellular distribution by Ca^{2+} manipulation.

IV-3 Similarity to other SLC6 transporters

The intracellular destination of NET internalized upon AMPH exposure was previously undescribed. In this report we provide evidence, both by biochemical and imaging methods, that AMPH increases NET's accumulation in Rab4- and Rab11-positive sites (Fig. 7-8), which may represent the SE and ERC. Our demonstration of NET's colocalization with Rab4 after substrate (AMPH) exposure is consistent with the finding

that the serotonin transporter colocalizes with Rab4 after exposure to high concentrations of serotonin (Ahmed BA 2008). Our findings also agree with those for AMPH regulation of DAT trafficking: that AMPH increases its colocalization with a different SE marker, Rab5, and with Rab11 (Sorkina T 2005). This similarity in trafficking routes among members of the SLC6 gene family points to a possible common signaling pathway activated by substrate influx that supports net movement of these related transporters to endosomes. Stronger support might come from future immunoisolation studies, which could show that NET resides on the same membranes as Rab4 and Rab11. Additionally, future investigation might compare the distribution of NET in AMPH-treated cells to that of other markers to show that Rab4-positive membranes represent the SE and Rab11-positive fractions represent the ERC.

We also show that some NET fractionates at high densities, overlapping with Rab4- and Rab11-containing fractions, even in the basal state (Fig. 6 and 7). Further, we find spatially synchronous colocalization of NET with Rab11 in resting conditions, though of a lesser degree than in AMPH-treated neurons (Fig. 8). These results support our proposal that AMPH alters rates of trafficking into and/or out of the same compartments through which NET constitutively recycles. That these recycling compartments appear to contain Rab4 and Rab11, indicating the classical endosomal pathway, also agrees with previous findings on DAT, SERT, and GAT1 (Melikian HE 1999; Deken SL 2003; Sorkina T 2005; Ahmed BA 2008).

IV-4 Possible relation to AMPH-induced signaling

The signaling pathway involved in AMPH-induced trafficking of DAT and NET has been partially elucidated. Increased intracellular Ca^{2+} and activation of the kinases CaMKII and Akt are all part of the cascade leading to decreased surface expression of

transporter, but how these steps cause either faster internalization or slower return of transporter to the surface is unknown (Dipace C 2007; Wei Y 2007). By providing evidence for NET's increased residence in Rab4- and Rab11-positive compartments, we suggest a future direction for study of this regulation. Rab4 and Rab11 themselves may be targets of these kinases or intermediate players yet to be identified, as we have shown their function is involved in AMPH-induced NET internalization (Fig. 9). Alternatively or in addition, as the Rab4- and Rab11-positive compartments likely represent the SE and ERC, respectively, other proteins known to regulate traffic in and out of these sites may be involved in AMPH's effects on NET trafficking.

Future work may lead to a complete signaling cascade including the GTPase activating protein (GAP) or guanine nucleotide exchange factor immediately upstream of a Rab, possibly Rab4 or Rab11. A similar story has been detailed for the SLC2 member glucose transporter 4 (GLUT4), which facilitates inward diffusion of glucose and traffics to the cell surface upon insulin stimulation. Insulin signaling activates Akt, which phosphorylates Akt substrate of 160 kDa (AS160) (Cross DA 1995; Kane S 2002). Phosphorylation of AS160 inhibits its GAP activity for substrate Rabs including Rab8, Rab10, and Rab14, which are all involved in trafficking GLUT4 to the cell surface (Ishikura S 2007; Sano H 2007). Thus, Akt activation by insulin, through AS160, increases the level of these Rabs' activity, which speeds traffic of GLUT4 to the cell surface (Sano H 2003).

IV-5 NET reserve pool hypothesis

NET may return to the plasma membrane directly from either of these two compartments, and the rates of return differ considerably: recycling through only the SE requires approximately two minutes (Dunn KW 1989; Mayor S 1993), while traffic

through both the SE and ERC takes about ten minutes (Mayor S 1993). That NET may reside in two distinct locations inside the cell upon AMPH treatment suggests that there may be two separate means of regulation of return to the surface. The ERC may provide a reserve pool of NET, either in endosomes themselves or in post-endosomal vesicles, while NET could move directly from the SE to the plasma membrane.

A NET storage pool would echo the vesicular trafficking pattern of GLUT4, which recycles first through the SE and ERC, before GLUT4 storage vesicles (GSV) form from the ERC (Fischer Y 1997; Kessler A 2000; Foster LJ 2001; Zeigerer A 2002). Insulin may preferentially stimulate traffic from GSV to the cell surface, though it also speeds movement of GLUT4 through the endosomal pathway to the GSV (Becker C 2001; Foster LJ 2001; Zeigerer A 2002). Insulin-regulated traffic of GLUT4 both from the ERC to GSV and from GSV to the membrane involves Rab11; as AMPH-regulated NET traffic also involves Rab11, the GTPase could serve a similar dual function if the NET reserve pool were separate from the ERC (Zeigerer A 2002).

IV-6 Effect of blocking Rab function on AMPH-induced NET internalization

Our original model would predict that transfection of DN Rab4 or DN FIP2 would reduce the rate of return of NET to the cell surface, thereby enhancing the internalization effect of AMPH. We find the opposite, that transfection of these constructs blocks internalization of NET in response to AMPH treatment, increasing the proportion of NET on the plasma membrane relative to that in AMPH-treated vector-transfected cells. Though we based our model on the consensus view of Rab4 and Rab11 function in endocytic recycling, our results parallel previous observations of the effect of transfection of a DN Rab11 construct on carbachol-induced M4 muscarinic acetylcholine receptor internalization (Volpicelli LA 2002; Reiner C 2008). In these studies, the GDP-locked

form of Rab11a prevented carbachol-induced accumulation of M4 in the ERC and reduced the carbachol-induced decrease in M4 surface expression.

The authors of the latter study, as well as others working on GLUT4, have suggested that Rab11 may act in endocytosis of membrane proteins, though neither these investigators nor others have tested this directly (Uhlig M 2005; Reiner C 2008). In any case, our model appears too simple to explain our data; either Rab4 and Rab11 have more complex functions than those we supposed, or an additional GTPase may aid in return of NET to the plasma membrane. Further, our overexpression of only the DN constructs in a background of variable levels of NET expression may have minimized the effects of blocking Rab function, which may be more pronounced if NET were overexpressed as well. Our concern was only whether the function of these small proteins contributes to AMPH-induced NET internalization, and future studies may investigate the mechanism by which Rab4 and Rab11 regulate NET trafficking.

IV-7 Conclusions

In summary, we have shown that AMPH induces an accumulation of NET intracellularly, including in Rab4- and Rab11-positive sites, and that the function of these GTPases is involved in this AMPH-regulated trafficking effect. Treatment of SCG neurons with AMPH causes a redistribution of NET that increases its intracellular presence in terminal boutons. AMPH exposure increases the proportion of NET in high-density fractions which also contain the greatest proportions of Rab4 and Rab11, and increases the proportion of NET colocalized with these markers of the SE and ERC, respectively, as determined by the ICA quantitative method. Finally, disrupting the function of Rab4 and Rab11, either separately or in combination, blocks AMPH-induced NET internalization.

These findings support our hypothesis that AMPH increases NET's presence in endosomal compartments.

IV-8 Significance

Better understanding of AMPH's regulation of NET subcellular distribution may enhance insight into AMPH's effectiveness as a therapy as well as its undesired effects. More detailed investigation of the molecules involved in the AMPH-induced reduction in NET surface expression might also reveal possible sites of misregulation leading to disorders thought to involve NET, such as depression, hypertension, and ADHD. If future work identifies a unique effector in the AMPH-induced signaling pathway that causes NET internalization via endosomes, this could even lead to development of alternative drugs that selectively induce this effect without affecting DAT trafficking or transmitter efflux through either transporter. Such novel therapies might benefit ADHD patients and abusers of amphetamines.

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