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Ca²⁺ Dependent Surface Trafficking of Norepinephrine Transporters Depends on Threonine 30 and Ca²⁺ Calmodulin Kinases

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Abstract

The antidepressant-sensitive norepinephrine (NE) transporter (NET) inactivates NE released during central and peripheral neuronal activity by transport into presynaptic cells. Altered NE clearance due to dysfunction of NET has been associated with the development of mental illness and cardiovascular diseases. NET activity *in vivo* is influenced by stress, neuronal activity, hormones and drugs. We investigated the mechanisms of Ca²⁺ regulation of NET and found that Ca²⁺ influenced both V_{max} and K_m for NE transport into cortical synaptosomes. Changes in extracellular Ca²⁺ triggered rapid and bidirectional surface trafficking of NET expressed in cultured cells. Deletion of residues 28–47 in the NET NH₂-terminus abolished the Ca²⁺ effect on surface trafficking. Mutagenesis studies identified Thr30 in this region as the essential residue for both Ca²⁺-dependent phosphorylation and trafficking of NET. Depolarization of excitable cells increased surface NET in a Thr30 dependent manner. A proteomic analysis, RNA interference, and pharmacological inhibition supported roles of CaMKI and CaMKII in Ca²⁺-modulated NE transport and NET trafficking. Depolarization of primary noradrenergic neurons in culture with elevated K⁺ increased NET surface expression in a process that required external Ca²⁺ and depended on CaMK activity. Hippocampal NE clearance *in vivo* was also stimulated by depolarization, and inhibitors of CaMK signaling prevented this stimulation. In summary, Ca²⁺

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AUTHOR CONTRIBUTIONS: US conceived the idea for the project, conducted uptake assays

Chemical compounds: desipramine (PubChem CID: 2995); EGTA (PubChem CID: 6207); PMSF (PubChem CID: 4784); thapsigargin (PubChem CID: 446378); BIM (PubChem CID: 2396); W7 (PubChem CID: 124887); KN93 (PubChem CID: 5312122); STO609 (PubChem CID: 16760660); BAPTA/AM (PubChem CID: 2293)

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signaling influenced surface trafficking of NET through a CaMK-dependent mechanism requiring Thr30.

Keywords

Calcium; Calcium Calmodulin Kinase I; Calcium Calmodulin Kinase II; Norepinephrine transporter; trafficking; NE clearance

INTRODUCTION

The neurotransmitter norepinephrine (NE) regulates cardiovascular physiology in the peripheral nervous system (PNS), and modulates autonomic, cognitive, and emotional behaviors in the central nervous system (CNS). The major mechanism for NE inactivation in both the PNS and CNS is reuptake of released NE by presynaptic NE transporters (NET) (Iversen, 1974). NET is a member of the Na⁺ and Cl⁻ dependent monoamine transporter family (SLC6A) (Pacholczyk *et al.*, 1991). Dysfunction of NE clearance or altered NET density has been associated with attention deficit, depression, and cardiovascular disorders (Esler *et al.*, 1981; Hadley *et al.*, 1995; Klimek *et al.*, 1997; Merlet *et al.*, 1992; Shannon *et al.*, 2000). NET is the target of psychoactive agents including cocaine and amphetamines, tricyclic antidepressants such as desipramine (DMI), and NE selective reuptake inhibitors, currently prescribed for the treatment of mood, anxiety and attention-deficit disorders.

NET activity is tightly controlled *in vivo*. Surface trafficking of NET between cytoplasmic compartments and plasma membranes is an important mechanism controlling NET activity. Electron micrographic studies show that most NET proteins predominantly reside within the cytoplasm of NE axons in the brain (Miner *et al.*, 2003; Schroeter *et al.*, 2000). Chronic stress increases NET localization at the plasma membrane without altering NET protein expression or detectable morphological changes in axon terminals (Miner *et al.*, 2006). Surface expression of NET is likely coordinated with neuronal activities and NE release from synaptic vesicles. NET localizes at the sites of activity dependent vesicle recycling, co-localizes with synaptic markers such as syntaxin1A, vesicular monoamine transporter 2 (VMAT2), and synaptophysin in primary cultured neurons (Savchenko *et al.*, 2003; Schroeter *et al.*, 2000), and engages in functional interaction with syntaxin 1A (Sung *et al.*, 2003). Transporter trafficking appears to involve diverse cellular compartments. NET is found associated with noradrenaline-containing secretory granules/large dense core vesicles (LDCV) in PC12 cells (Kippenberger AG, 1999) and also found in early or recycling endosomes in sympathetic neurons and the cortex of brains (Leitner, 1999; Matthies *et al.*, 2009; Matthies *et al.*, 2010).

Endogenous hormones activating G protein coupled or tyrosine kinase receptors, psychotropic drugs and depolarization modulate the activity and surface expression of NET (Apparsundaram *et al.*, 1998; Kantor *et al.*, 2001; Mandela and Ordway, 2006; Mannangatti *et al.*, 2011; Savchenko *et al.*, 2003; Sumners and Raizada, 1986). Although Ca²⁺ plays a critical role at the center of multiple signaling pathways, the mechanisms through which it regulates NET activity remain to be fully elucidated. Protein kinase C (PKC), Ca²⁺ calmodulin kinases (CaMK), p38 mitogen activated protein kinase (p38 MAPK), myosin

light chain kinase (MLCK), protein phosphatases, and syntaxin 1A have been implicated in Ca^{2+} dependent signaling to regulate monoamine transporters (Apparsundaram *et al.*, 2001; Kantor and Gnegy, 1998; Kantor *et al.*, 1999; Mannangatti *et al.*, 2011; Sung and Blakely, 2007; Turetta *et al.*, 2002; Uchida *et al.*, 1998; Uchikawa *et al.*, 1995; Yura *et al.*, 1996). In this study, we explored molecular mechanisms for Ca^{2+} regulation of NET and found that Ca^{2+} regulates NET activities by inducing rapid surface trafficking. We identified Thr30 of NET and CaMKs as essential players in this regulation.

MATERIALS AND METHODS

Antibodies, siRNA, and cDNA constructs

We used anti-hemagglutinin (HA) antibody (3F10) conjugated with peroxidase (Roche, Mannheim, Germany), anti-CaMKI (Santa Cruz Biotechnology, Santa Cruz, CA), anti-CaMKII δ (Santa Cruz Biotechnology), anti-transferrin receptor (Zymed, South San Francisco, CA), and anti-NET antibody (NET17-1, Mab Technologies, Atlanta, GA) for immunoblots. Rabbit polyclonal NET antibodies 43408 raised against an epitope at the extracellular loop of NET (Savchenko *et al.*, 2003) and monoclonal anti- β tubulin (Sigma, St. Louis, MO) antibodies were used for immunohistochemistry. siRNAs for CaMKI and CaMKII δ (SMARTpool™) were purchased from Dharmacon (Lafayette, CO). cDNA constructs for human NET and its mutants (NET 28–47, NET T30A, NET T30E) were N-terminally tagged by inserting HA-tag (YPYDVPDYA) between the first and second amino acids.

Cell culture, transfection, stable cell lines, and preparation of cortical synaptosomes

Cell culture for CHO, CAD, and PC12 cells was performed as described elsewhere (Mandela and Ordway, 2006; Sung *et al.*, 2003). CHO-NET and CHO-NET T30A cells were generated by stably transfecting HA-NET or HA-NET T30A in pcDNA5/FRT (Invitrogen, Carlsbad, CA) into CHO-Flip-In cells (Invitrogen). The stable clones were selected using hygromycin B (500 $\mu\text{g}/\text{ml}$, Invitrogen) and maintained in Ham's F12/10% fetal bovine serum (FBS)/L-Glutamine/penicillin/streptomycin (L-Glu/pen/strep) supplemented with Zeocin 100 $\mu\text{g}/\text{ml}$ (Invitrogen). CAD-NET and CAD-NET 28–47 cells were generated by stably transfecting HA-NET or HA-NET 28–47 in pcDNA3 into CAD cells and maintained in DMEM/F12/ 8% FBS/ L-Glu/ pen/ strep/200 $\mu\text{g}/\text{ml}$ of G418 (Mediatech, Herndon, VA). Transfection was performed by using TransIT-LT1 transfection reagent (Mirus, Madison, WI). All cells were plated on poly-D-lysine coated plates and incubated for 48 hours for cDNA transfection and for 48 to 72 hours for siRNA transfection prior to assays. Synaptosomal preparations were performed as described previously (Mannangatti. *et al.*, 2015; Sung *et al.*, 2003). Briefly, brain cortex was dissected from mice (C57BL/6, male) and homogenized in 10 mM HEPES, 0.32 M sucrose, pH 7.4 using a Teflon pestle/ homogenizer. Homogenates were centrifuged at 1,000 g for 5 minutes (min) at 4°C, and then the supernatant re-centrifuged at 16,000 g for 20 min at 4°C. The pellets were collected as synaptosomes.

Assay buffers, transport assays, and Ca²⁺ imaging

NE transport assays using synaptosomes (50–100 ng/reaction) or cells were performed as previously described (Sung *et al.*, 2003). Uptake assays were carried out in HEPES-buffered Krebs Ringer (KRH) (in mM: 120 NaCl, 4.7 KCl, 1.2 KH₂PO₄, 10 HEPES, 1.2 MgSO₄, 2.2 CaCl₂, pH 7.4,) with (in mM) 0.1 pargyline, 0.1 ascorbic acid, 0.1 troponin, and 1.8 mg/ml glucose, unless otherwise mentioned in Figure (Fig) legends. When Ca²⁺ free-KRH (KRH with 0.2 mM EGTA and without CaCl₂) was used, KRH for the parallel experiment contained 0.2 mM EGTA as well. Uptake assays were initiated by addition of [³H]-NE (1-[7,8-³H] noradrenaline, Amersham Pharmacia, Piscataway, NJ) at 50 nM final concentrations. For kinetics assays, 50, 100, 200 nM of [³H]-NE, 400, 600, 800, 1,000, 1,200, 1,500 nM of 20% [³H]-NE and 80% of unlabeled NE (Sigma) were used. Nonspecific uptake was defined using 1–10 μ M DMI. All assays were carried out at 37 °C for 10 min in triplicates. Ca²⁺ imaging was performed as described previously (Apparsundaram *et al.*, 2001). Briefly, cells were incubated in 0.5 mM Fura-2/acetoxymethyl ester (Fura-2/AM, Molecular Probes, Eugene, Oregon) in Hanks' balanced salt solution (HBSS, in mM; 10 HEPES, 140 NaCl, 2 KCl, 1 CaCl₂, 1 MgCl₂, and 5 D-glucose) for 60 min at 24–26°C, followed by two washes with HBSS. Cells were superfused with Ca²⁺ free-KRH and 0.1–1 μ M thapsigargin (an inhibitor of sarco/endoplasmic reticulum Ca²⁺-ATPases) for 10 min. The medium was replaced with KRH or Ca²⁺ free-KRH. [Ca²⁺]_i was measured in individual cells by dual-wavelength spectrofluorometry with a Nikon inverted microscope attached to a Compix Calcium Imaging System consisting of a charge-coupled device camera (Dage-MTI CCD-72; Michigan, IN) attached to an IBM compatible computer executing SIMCA C-Imaging software (Compix Inc., Mars, PA). Cells were exposed to excitation wavelengths of 340 and 380 nm every 2 s, and the emitted fluorescence was measured in real time at 510 nm. The ratio of emission at 340 and 380 nm excitation was used as an index of [Ca²⁺]_i.

Depletion of Ca²⁺, restoration of Ca²⁺, and depolarization of cells

For Ca²⁺ depletion, synaptosomes in KRH were divided into 2 aliquots, replaced with fresh KRH or Ca²⁺ free-KRH, and incubated at 37°C for 5 min prior to the addition of radio-labeled NE. For Ca²⁺ recovery, synaptosomes in Ca²⁺ free-KRH were transferred to fresh KRH or Ca²⁺ free-KRH, and incubated at 37°C for 5 min prior to the addition of radio-labeled NE. Ca²⁺ manipulation of cells was performed prior to surface biotinylation. Ca²⁺ depletion was carried out by incubating cells in complete medium with 10 mM EGTA/30 mM BAPTA/AM (CHO cells) or complete medium with 10 mM EGTA (CAD cells) for 1 min at 24–26°C or for 5–10 min at 37°C. Control cells were treated in the same way by using vehicle. Ca²⁺ recovery was carried out by incubating cells in Ca²⁺ free-KRH with 0.1–1 μ M thapsigargin for 10 min at 37° to deplete cytoplasmic Ca²⁺. Cells were washed once with Ca²⁺ free-KRH, replaced with KRH or Ca²⁺ free-KRH, and incubated for 1 min at 24–26°C to 5 min at 37°C prior to biotinylation. For depolarization experiments, cells in KRH were stimulated by incubating in KRH containing 80 mM KCl and reduced NaCl for 10 min at 37 °C.

Biotinylation, phosphorylation, and protein gel electrophoresis

Cell surface biotinylation was performed by labeling surface proteins with membrane impermeable and cleavable biotin (Ez-link NHS-sulfo-S-S-biotin, Pierce, Rockford, IL) (Sung *et al.*, 2003). Surface proteins were harvested from cell extracts using streptavidin coupled agarose matrix and eluted with protein sample buffer (pH 6.9, 6% SDS, 4 M urea, 125 mM Tris, 4 mM EDTA, 6 mM β -mercaptoethanol). For Ca^{2+} stimulated phosphorylation, CHO-NET and CHO-NET T30A cells were pre-incubated in phosphate free DMEM for 2 hours, and then incubated in phosphate free KBB (in mM: 25 NaHCO_3 , 125 NaCl, 5 KCl, 5 MgSO_4 , 10 glucose, pH7.3) with 1.5 mM CaCl_2 and carrier-free [^{32}P]-labeled orthophosphate (0.5 mCi/ml, Amersham) for 3 hours at 37°C. Cells were briefly rinsed with KBB with 0.2 mM EGTA and incubated in KBB/0.2 mM EGTA/carrier-free [^{32}P]-labeled orthophosphate (0.5 mCi/ml) for 15 min to deplete Ca^{2+} . Then, cells were incubated with vehicle or CaCl_2 (final concentration 2.2 mM) for 5 min at 24–26°C, washed with phosphate buffered saline (PBS)/0.5 mM PMSF and lysed in PBS/1% TRITON X 100/ in mM; 0.5 PMSF, 1 okadaic acid, 10 NaI, 1 Na orthovanadate, and 10 Na pyruvate. Extracts were centrifuged at 16,000 g for 20 min and incubated with IgG coupled Sepharose (Amersham) for 30 min. Supernatant was incubated with anti-HA agarose beads pre-blocked with non-labeled CHO cell lysates. Bound proteins to anti-HA matrix were analyzed by SDS/PAGE. Phosphorylated bands were captured using Phosphoimager (Typhoon 9400, Molecular Dynamics/GE Healthcare Life Sciences, Sunnyvale, CA) and analyzed using ImageQuant 5.2 (Molecular Dynamics). Protein electrophoresis, unless mentioned in the Figure legend, was performed using 10% SDS/PAGE. Exposed films of immunoblots were scanned using an Agfa Duoscan T1200. Captured images were processed in Adobe® Photoshop® and quantified using ImageJ 1.37i (NIH).

A proteomic analysis of NET-associated proteins

NET-associated proteins were purified by immunoprecipitation and analyzed by mass spectrometry (MS). For the purification of transporter associated protein complexes, we immunoprecipitated HA-tagged NET 28–47 from stably transfected CAD cells. CAD cells, a CNS catecholaminergic cell line (Qi *et al.*, 1997), express catecholaminergic markers including tyrosine hydroxylase and VMAT2 (Wang and Oxford, 2000) and produce catecholamines (Qi *et al.*, 1997), but do not express NET (Qi *et al.*, 1997). Although CAD cells stably expressing wildtype NET were able to be established in low passage number of culture, it was not possible to maintain the stable cells in expanded culture more than a few passages of cell divisions despite extensive efforts (data not shown). Attempts to purify NET proteins from the expanded culture stably expressing NET yielded only a small amount of the intact NET protein and 48–50 kDa of degradation products that reacted with anti-NET17-1 monoclonal antibodies (Mab Technologies) (data not shown). On the other hand, CAD cells supported stable expression of HA-tagged NET 28–47 in the expanded culture. CAD cells stably expressing HA-tagged NET 28–47 expressed relatively low amounts of NET 28–47 proteins (1.2 ± 0.2 pmol/mg membrane proteins) by nisoxetine binding assays and were able to transport 1.5 ± 0.1 pmol of NE/ 10^6 cells/min when measured at 50 nM NE. Immunoprecipitation of HA-tagged NET 28–47 was conducted from CAD stable cells, with negative control of mock immunoprecipitation from the same number of parental CAD cells. Immunoprecipitation was conducted both from total cell lysates and enriched membrane

preparation of CAD cells. Total cells or membrane fractions were extracted in PBS/1% TRITON X100/0.5 mM PMSF pH 7.4 (PBS/TRITON) and incubated with IgG coupled Sepharose 6 (Amersham) at 4°C for 1 hour to reduce nonspecific binding during immunoprecipitation. Immunoprecipitation was performed by incubating the extracts with anti-HA-coupled agarose matrix for 1 hour at 4°C. Immunoprecipitated proteins were washed with extraction buffer and eluted with 0.2 M glycine. One aliquot (10% of eluted materials) was subjected to immunoblotting with anti-HA to ensure the purification of NET 28–47, one aliquot (30% of eluted materials) was subjected to silver staining to ensure the quality of co-immunoprecipitated proteins, and one aliquot (60% of eluted materials) was subjected to MS analysis as described below. The immunoprecipitated proteins were reduced with dithiothreitol, alkylated with iodoacetamide, and digested with trypsin. The digested proteins were analyzed by MS using on-line multidimensional microcapillary liquid chromatography (strong cation exchange and reverse phase) coupled nano ESI/MS/MS (electrospray tandem MS) using an ion-trap mass spectrometer (LCQ Deca XP; ThermoFinnigan, San Jose, CA) equipped with a nanoelectrospray source (James A. Hill Instrument Services, MA) as previously described (Sanders *et al.*, 2002). All spectra were searched against the mouse and human protein database from NCBI (the National Center for Biotechnology Information) using the SEQUEST-PVM algorithm (Sadygov *et al.*, 2002). The program Extractms2, developed and provided by Jimmy Eng and John R. Yates III (The Scripps Research Institute, La Jolla, CA), was used to generate the ASCII peak list and identify +1 or multiply charged precursor ions from the native mass spectrometry data file. The criteria for evaluating the SEQUEST search results are described previously (Link *et al.*, 1999). For multiply charged precursor ions ($z = +2$), an independent search was performed on both the +2 and +3 mass of the parent ion. A weighted scoring matrix was used to select the most likely charge state of multiply charged precursor ions (Link *et al.*, 1999; McAfee *et al.*, 2006). From the database search, fully tryptic peptide sequences with SEQUEST cross-correlation scores (C_n) ≥ 1.5 for +1 ions, ≥ 2 for +2 ions, and ≥ 2 for +3 ions were considered significant and used to create the list of identified proteins. The comparison and statistical analysis of the data between NET 28–47-immunoprecipitation and mock immunoprecipitation were performed using an analysis software BIGCAT (Bioinformatic Graphical Comparative Analysis Tools) (McAfee *et al.*, 2006). The protein identification p-score was generated by multiplying the individual peptide p-scores together and taking the natural log, and indicated the probability of the peptide hit accruing randomly (McAfee *et al.*, 2006). Accurate protein identification was defined as the match of 2 or more unique peptides from the same protein (Link *et al.*, 1999; Washburn *et al.*, 2001). Data from the NET 28–47 immunoprecipitation and from the negative control immunoprecipitation were compared. The MS-identified proteins were manually evaluated for functional description and annotation by searching the databases of NCBI and EMBL-EBI (the European Molecular Biology Laboratory-European Bioinformatics Institute). MS analysis was able to detect a number of peptides from NET (data not shown) and other cellular proteins including calmodulin, CaMKI, and CaMKII δ (Table 1).

Animals

Sprague-Dawley rats (150–200 g, Harlan, Indianapolis, IN) and male C57BL/6 mice (25–30 g, Harlan for synaptosomal preparations or Jackson Laboratory, Bar Harbor, ME, for *in vivo*

measurements of NE clearance) were group-housed under standard conditions, kept on a 12:12 hr (at the Vanderbilt University Health Science Center) or a 14:10 hr (the University of Texas Health Science Center) light/dark cycle. Animals were allowed ad libitum access to food and water. All experiments were done in accordance with protocols approved by the Institutional Animal Care and Use Committees at Vanderbilt University Health and the University of Texas Health Science Center at San Antonio, and were carried out in accordance with the Guide for the Care and Use of Laboratory Animals as adopted and promulgated by the National Institutes of Health. Every effort was made to minimize animal discomfort or suffering, and the number of animals used.

Primary neuronal culture and immunohistochemistry

Neurons from the sympathetic superior cervical ganglia (SCG) or brainstem were cultured as described previously (Savchenko *et al.*, 2003). SCG and brainstem were harvested from anesthetized (Nembutal, 50 mg/kg) rats aged postnatal day 1–3. Sympathetic neurons were cultured in UltraCulture medium (BioWhittaker, Walkersville, MD) supplemented with nerve growth factor (20 ng/ml; Sigma), 3% FBS, 2 mM L-glutamine, penicillin (100 units/ml), streptomycin (100 µg/ml) at 37°C in humidified 5% CO₂. Brainstem neurons were plated in basal medium (Gibco, Grand Island, NY), supplemented with B27 (Gibco, Grand Island, NY) and 0.5 mM glutamine. Neurons were cultured 9–14 days and used for drug treatment and NET trafficking into the plasma membrane was established by immunofluorescent staining. Neurons were incubated in either a 2.5 mM K⁺ solution (KRH) (in mM: HEPES 25, D-glucose 33, MgCl₂ 2, CaCl₂ 2, NaCl 120, KCl 2.5), a solution with a high concentration of K⁺ ions (40 mM or 90 mM K⁺ ions with reduced Na⁺ ions, respectively) for 5 min with or without pretreatment (10 min) of 1 µM KN93 (an inhibitor of CaMKI and CaMKII), or Ca²⁺-free KRH. Neurons were rinsed with cold PBS and incubated with 3% normal serum for 10 min to block nonspecific binding. Surface labeling of NET was performed by staining with rabbit polyclonal NET 43408 antibody followed by labeling with CY3-conjugated secondary antibody under nonpermeabilized conditions as described previously (Savchenko *et al.*, 2003). Subsequently, neurons were fixed with 3% paraformaldehyde in the presence of 0.2% NP-40 for 10 min and stained with mouse monoclonal β-tubulin antibodies, followed by incubation with anti-mouse secondary antibody conjugated with Alexa 488. Specimens from multiple fields were examined with a Zeiss LSM 510 Meta imaging system equipped with internal He/Ne and external Ar/Kr lasers. z-Series images were collected by optical sectioning at intervals of 1 µm. The brightness of images was optimized so that the maximal signal was not saturated and minimal signal was not as black. The correction of image brightness was equal in each experimental group: the pinhole size was adjusted so that light went through the pinhole but noise was not increased and the background was also adjusted by decreasing the gain in the photon detection system. Images were collected, averaged across 15–20 fields from replicated experiments (n=3), and pixel intensity calculated in original images. The double channel images were used for quantification analysis: the green channel images with β-tubulin immunoreactive neurons were updated for selection of axonal region to calculate the red channel images with NET immunoreactivity in the selected area such that the intensity of NET immunofluorescent signal was calculated in selected areas along axons and somata. Immunoreactivity for each treatment was evaluated by Metamorph 5.0 image program

(Molecular Devices, Sunnyvale, CA). Pseudo-colored images were converted from NET immunoreactive staining (red channel images only) by Metamorph image program for analysis of NET density on the surface membrane after each treatment compared to control.

Whole cell patch clamp recording of NET currents

Patch clamp experiments were performed using an amplifier Axopatch 200B with a low-pass filter set at 1 kHz. Quartz patch pipettes with 5–7 M Ω resistance were pulled by a programmable puller (model P-2000, Sutter Instruments, Novato, CA) and filled with the internal solution containing (in mM) 120 KCl, 2 MgCl₂, 10 HEPES, 2 MgATP, 30 dextrose, pH 7.35. SCG neurons were washed twice with a control solution containing (in mM) 130 NaCl, 5 CaCl₂, 0.5 MgCl₂, 1.3 KCl, 10 HEPES, 34 dextrose, pH 7.35 before experiments. NET-mediated current was defined as the current recorded under control condition minus the current recorded in presence of 5 μ M DMI. Neurons were clamped at –50 mV and the NET-mediated current was recorded by stepping the membrane voltage to –120 mV for 500 msec before and after a 2 s depolarizing step at –10 mV. NET-mediated current was studied under the control condition, in the presence of 200 μ M CdCl₂, and after preincubation with 5 μ M KN93 (15 min) or 2 μ M STO609 (an inhibitor of CaMKK) (20 min).

In vivo measurement of NET-mediated NE clearance

Mice were anesthetized by intraperitoneal (ip) injection (2 ml/kg body weight) of a mixture of chloralose (35 mg/kg) and urethane (350 mg/kg). A tube was inserted into the trachea to facilitate breathing and mice were then placed into a stereotaxic frame. Mice were supported by a raised foam platform to facilitate respiration. Body temperature was maintained at 36°C by a water-circulated heating pad (Seabrook, Cincinnati, OH). Carbon fiber electrodes were constructed using a single carbon fiber (30 μ m diameter; Specialty Materials, Lowell, MA), which was sealed inside fused silica tubing (Schott, North America, Elmsford, NY). High-speed chronoamperometric recordings were made using the FAST-12 system (Quanteon, Nicholasville, KY) as previously described (Montañez *et al.*, 2003). Stepped oxidation potentials of 100 msec pulses of +0.55 volts were applied to the carbon fiber electrode. Each pulse was separated by a 900 msec interval during which time the resting potential was maintained at 0.0 V. Voltage at the active electrode was applied with respect to a Ag/AgCl reference electrode positioned in the contralateral superficial cortex. Oxidation and reduction currents were digitally integrated during the last 80 msec of each 100 msec voltage pulse. The exposed tip of the carbon fiber (150 μ m in length) was coated with Nafion® (5% solution, Aldrich Chemical Co., Milwaukee, WI; 3–4 coats baked at 200°C for 3 min per coat) to provide a >500-fold selectivity of NE over ascorbic acid (250 μ M). Under these conditions, during *in vitro* calibration in phosphate-buffered saline (PBS, 100 mM, pH 7.4) the relationship between oxidation current and increasing concentration of NE (0 to 8 μ M) was linear ($r^2 = 0.9$). The detection limit for the measurement of NE was defined as the concentration that produced a response with a signal-to-noise ratio of 3 and in these experiments averaged 49 ± 5 nM ($n = 28$ electrodes). Only electrodes displaying a selectivity ratio for NE over ascorbic acid greater than 500:1 and a linear response ($r^2 = 0.9$) to NE were used. The electrochemical recording assembly consisted of a Nafion-coated, single carbon fiber electrode attached to a 4-barreled micropipette such that their tips were separated by ~200 μ m. Barrels were filled with NE (200 μ M), KN93 (400 μ M), STO609

(400 μ M), potassium chloride (KCl, 150 mM) or vehicle. For KN93 the vehicle was PBS and for STO609, PBS with 0.05% DMSO. For all solutions, ascorbic acid (2%) was added as an antioxidant and the pH adjusted to 7.3–7.4. All drugs were tested *in vitro* prior to use. None produced an electrochemical signal by themselves, nor did they influence the signal produced by NE *in vitro*. The electrode-micropipette recording assembly was lowered into the dentate gyrus (AP, -1.6 from bregma; ML, ± 0.5 from midline; DV, -2.0 from dura) (Montañez *et al.*, 2003). At the conclusion of each electrochemical recording experiment, an electrolytic lesion was made to mark the placement of the electrode tip. The brain was removed, rapidly frozen on powdered dry ice and stored at -80°C until they were sectioned for histological verification of electrode localization in the dentate gyrus. Since neither vehicle (PBS or 0.05% DMSO) affected NE clearance, data for both vehicle groups were pooled.

Statistical analyses

Data were analyzed using GraphPad Prism 6 or 7 (Graphpad Software Inc., San Diego, CA) and expressed as mean $\pm$ SEM of at least 3 independent experiments. Details of statistical analyses are described in Figure legends.

RESULTS

Ca^{2+} regulates NE transport

We found NE transport activity to be Ca^{2+} dependent. NE transport into mouse cortical synaptosomes, prepared in HEPES-buffered KRH containing a physiological 2.2 mM Ca^{2+} concentration, was decreased to $40 \pm 7\%$ by a 5 min incubation in Ca^{2+} -free KRH (Fig. 1A, left). Ca^{2+} dependent changes in NET activity were reversible. When synaptosomes in Ca^{2+} free-KRH were transferred to KRH, NE transport increased by $223 \pm 16\%$ (Fig. 1A, right). Analysis of NE transport kinetics revealed that the activity change consisted of an increase in maximal uptake velocity (V_{max}) by $55 \pm 13\%$ and a decrease in affinity (K_{m}) by $43 \pm 3\%$ by Ca^{2+} (Fig. 1B).

Ca^{2+} regulates surface trafficking of NET

Changes in the V_{max} for transport can result from increased or decreased transporter surface expression. Examination of NET surface abundance in synaptosomes is problematic due to the low expression level. However, in CHO cells transfected with NET, we found that changes in NET surface expression can be readily measured. Previously we showed that increasing extracellular Ca^{2+} led to an increase in NE transport in CHO cells (Sung and Blakely, 2007). Cytoplasmic Ca^{2+} levels within these cells responded to changes in extracellular Ca^{2+} (Fig. 2A). When CHO cells, preloaded with Fura-2 and incubated in Ca^{2+} free-KRH, were supplied with Ca^{2+} to an external concentration of 2.2 mM, a transient increase in cytoplasmic Ca^{2+} was observed. Surface expression of NET in CHO cells decreased upon removal of Ca^{2+} and increased when Ca^{2+} was restored (Fig. 2B). Surface expression of NET was monitored by labeling surface NET with membrane impermeable biotin, followed by purifying biotinylated proteins and immunoblotting for detection of NET. Quantification of immuno-blots is summarized in the bar graphs shown in Fig. 2B. The decrease in surface NET when Ca^{2+} was removed and the increase when it was restored

were essentially complete within 1 min and sustained for at least 5 min. As controls, surface levels of transferrin receptors were monitored under the same Ca^{2+} manipulating conditions (Fig. 2C). Neither Ca^{2+} depletion ($p = 0.15$) nor Ca^{2+} replacement ($p = 0.18$) resulted in consistent trafficking responses of transferrin receptors.

Thr30 in the NET NH₂ terminus is required for Ca^{2+} triggered surface trafficking

The NH₂-terminus of NET has been implicated in regulation of trafficking and is responsible for Ca^{2+} -dependent interaction with syntaxin 1A, a protein that participates in NET surface trafficking (Sung *et al.*, 2003; Sung and Blakely, 2007). The NET NH₂ terminus diverges from those of other biogenic amine transporters (Fig. 3A). To identify the site controlling responses to Ca^{2+} , we empirically created a series of N-cytoplasmic domain mutants including NET 28–47 (Dipace *et al.*, 2006). NET 28–47 expressed on the cell surface at levels comparable to that of wildtype NET ($106 \pm 18\%$ relative to wildtype NET, $n=3$). Unlike wildtype NET, surface expression of NET 28–47 failed to respond to either Ca^{2+} depletion or Ca^{2+} addition (Fig. 3B left). The deleted region contains Thr30 (Fig. 3A), a potential site of phosphorylation by serine/threonine kinases (Mannangatti *et al.*, 2011), which is unique to NET among biogenic amine transporters, is conserved among NETs from different species, and is known to be important for cocaine-triggered changes in NET surface density (Mannangatti *et al.*, 2011). We examined the region encompassed by NET amino acids 28–47 with respect to potential sites of phosphorylation. Mutation of Thr30 to either alanine (NET T30A; Fig. 3B Middle) or glutamate (NET T30E; Fig. 3B Right) eliminated the effect of Ca^{2+} depletion or restoration on NET surface expression. NET T30A and NET T30E exhibited normal transporter protein surface expression ($117 \pm 19\%$ for NET T30A and $107 \pm 12\%$ for NET T30E relative to wildtype NET, $n= 3$ and 4, respectively), and NE transport activities (data not shown) as wildtype NET. In addition to Thr30, NET contains several additional serine and threonine residues as potential phosphorylation sites including Thr58, Thr238, Ser259, Ser502, Ser579, Thr580, and Ser583. Each of these positions was mutated and all of the mutants retained sensitivity to Ca^{2+} manipulations (data not shown).

Although NET 28–47, NET T30A and NET T30E were insensitive to changes in external Ca^{2+} , these mutants appeared to respond differently to Ca^{2+} from intracellular sources (Fig. 3C). Although NET T30A did not respond to Ca^{2+} depletion by EGTA (Fig. 3C Left, Ca^{2+} to no Ca^{2+}), the mutant retracted from the surface by Ca^{2+} depletion in Ca^{2+} free-KRH containing 0.1 μM thapsigargin, an inhibitor of sarco/endoplasmic reticulum Ca^{2+} -ATPases, and stayed intracellularly even after Ca^{2+} supplement (Fig. 3C Right, no Ca^{2+} to Ca^{2+}). NET T30E appeared to stay constitutively at the surface (Fig. 3C Left and Right).

The effect of Ca^{2+} on NET surface expression in CHO cells coincided with an increase in NET phosphorylation (Fig. 3D). CHO cells stably expressing NET or NET T30A at the same genomic locus (see Materials and Methods) and equally active for NE transport (data not shown) were metabolically labeled with radioactive inorganic phosphate (^{32}Pi), depleted of Ca^{2+} and then stimulated by Ca^{2+} for 5 min prior to cell lysis and immunoprecipitation of NET. Immunoblotting of aliquots of the immunoprecipitated proteins for detecting NET demonstrated similar expression levels for wildtype NET and NET T30A (Fig. 3D, left).

Autoradiography of the same immunoprecipitated proteins indicated that a band co-migrating with NET at ~90 kDa was phosphorylated in response to Ca^{2+} addition to cells expressing wildtype NET, but not NET T30A (Fig. 3D, right, lower arrow). Also in the immunoprecipitate were phosphoproteins migrating at 150–200 kDa (Fig. 3D upper arrow) that did not co-migrate with NET immuno-reactivity. Parallel immunoprecipitations from NET T30A-transfected cell lysates contained neither the phosphorylated transporter nor co-immunoprecipitated 150–200 kDa proteins. This raises the possibility that Ca^{2+} dependent NET phosphorylation recruited interactions with other phosphoproteins.

High K^{+} -induced depolarization has been reported to enhance NE transport in PC12 pheochromocytoma cells (Mandela and Ordway, 2006). In our study evaluating trafficking of transiently expressed HA-tagged NET, high K^{+} (80 mM KCl) increased the level of NET on the surface of PC12 cells (Fig. 4 bars on left). On the other hand, surface level of NET T30A did not increase (Fig. 4 center bars), and surface level of NET T30E actually decreased by high K^{+} (Fig. 4 bars on right).

CaMKI and CaMKII are involved in Ca^{2+} regulation of NET

We evaluated influences of Ca^{2+} activated kinases on NE transport in synaptosomes. Treatment of synaptosomes with bisindolylmaleimide (BIM, a PKC inhibitor) or KN93 (an inhibitor of multifunctional CaMK) did not show any apparent effect on NE transport, while W7 (a calmodulin antagonist) inhibited NE transport (Fig. 5A). However, pre-incubation of synaptosomes with KN93 changed the processes of Ca^{2+} dependent NE transport (Fig. 5B, C). KN93 attenuated the reduction in NE transport that occurs in response to Ca^{2+} removal (i.e. under vehicle conditions, NE transport was reduced by $60 \pm 7\%$, but by only $39 \pm 6\%$ in the presence of KN93) (Fig. 5B). Under conditions of Ca^{2+} addition, NE uptake increased by $125 \pm 34\%$ under vehicle conditions, compared to $209 \pm 51\%$ with KN93 (i.e. compared to the synaptosomes in Ca^{2+} free-KRH with vehicle equal to $100 \pm 14\%$) (Fig. 5C). Interestingly, under Ca^{2+} free conditions NE uptake was increased by $111 \pm 31\%$ in the presence of KN93 compared to synaptosomes in Ca^{2+} free-KRH in the presence of vehicle ($100 \pm 14\%$) (compare white bars in Fig. 5C). On the other hand, BIM had no effect on NE transport in response to Ca^{2+} removal or addition (Fig. 5D, E).

We performed immunoprecipitations of HA-tagged NET 28–47, the deletion mutant that was constitutively expressed on the cell surface despite changes in extracellular Ca^{2+} (Fig. 3B, C), from stably transfected CAD cell extracts. We conducted a proteomic analysis of immunoprecipitated protein complexes by MS (rationale of using NET 28–47, not wildtype NET, for proteomic analysis is given in Materials and Methods). Calmodulin, CaMKI, and CaMKII δ in protein complexes associated with NET 28–47 were detected (Table 1). In contrast, only one peptide of calmodulin and none of CaMKI or CaMKII δ were detected in mock immunoprecipitation from parental CAD cell extracts. This further raised the possibility that CaMKI and CaMKII might participate in Ca^{2+} dependent NET regulation.

We evaluated roles of CaMKI and CaMKII in Ca^{2+} dependent NET trafficking in cell culture using the inhibitors KN93, an equipotent blocker of CaMKI and CaMKII (Hook and Means, 2001), and STO609, which inhibits CaMK kinase (CaMKK), an enzyme required for CaMKI activation (Fig. 6). KN93 and STO609 blocked both the reduction and elevation of

surface NET resulting from Ca^{2+} -depletion and restoration, respectively, in CHO cells (Fig. 6A). These findings support the involvement of CaMKI and possibly CaMKII in the response of NET surface localization to changes in extracellular Ca^{2+} in cultured cells.

RNA interference further supported roles of CaMK in Ca^{2+} regulation of NET. Transfection of CAD cells stably expressing wildtype NET with siRNA against CaMKI or CaMKII δ down-regulated expression of the targeted kinase without affecting total expression of either the 60 kDa (immature) or 90 kDa (mature) forms of NET (Fig. 6B). Suppression of CaMKII δ , but not CaMKI, decreased NE transport (Fig. 6C). As with pharmacological inhibition of CaMK and CaMKK (Fig. 6A), transfection of siRNA targeting CaMKI or CaMKII δ completely or partially prevented the decrease in NET surface expression that followed Ca^{2+} removal (Fig. 6D). In mock-transfected CAD cells, Ca^{2+} depletion dramatically decreased surface NET expression. However, in both CaMKI siRNA- and CaMKII δ siRNA-transfected cells, Ca^{2+} depletion had no significant effect on NET surface expression.

Ca^{2+} supports NET surface trafficking and activity in primary noradrenergic neurons

We previously generated a NET ectodomain antibody (43408) to measure surface NET expression (Savchenko *et al.*, 2003). Using this antibody in the absence of detergents, we tested the effect of Ca^{2+} on NET surface expression in primary brainstem neurons. Differences in NET distribution in the plasma membrane were evaluated in control conditions and after depolarization of the plasma membrane with 40 mM or 90 mM K^+ in KRH solution, in Ca^{2+} -free KRH or in the presence of KN93. Pseudo-colored pictures of brainstem neurons with the highest density (in red) and the lowest density (in violet) of NET in the plasma membrane are shown in Fig. 7 (A–K). In medium containing 1.3 mM Ca^{2+} and 2.5 mM K^+ , there was only minimal staining, which was largely restricted to the cell soma, when the NET 43408 epitope was low at the surface (Fig. 7A). When cells were depolarized with 40 mM or 90 mM K^+ for 5 min, surface NET labeled with 43408 antibodies increased both in somata and in neurites (Fig. 7B, E; arrows point to surface NET in neurites). The effect of high extracellular K^+ likely depends on Ca^{2+} , because elevated K^+ did not increase surface NET in Ca^{2+} -free medium (Fig. 7C, F). Preincubation of neuronal cultures with KN93 blocked the high K^+ -induced increase of surface NET, suggesting that the effect was dependent on CaMK activity (Fig. 7D, G). Quantitative measurement of these changes was obtained by capturing pixel intensities from neurites stained with the 43408 antibody (Fig. 7H) relative to co-labeling with anti- β tubulin (Fig. 7I, merged in 7J). The results demonstrate a significant increase in surface NET in 90 mM K^+ that required the presence of extracellular Ca^{2+} and was blocked by KN93 (Fig. 7K). We also observed K^+ and CaMK dependent changes in surface expression of NET epitopes in SCG neurons (data not shown), which was similar to that in brainstem neurons.

Depolarization increased NET activity in SCG neurons

NE transport is accompanied by ionic currents that are readily detected by whole cell patch clamping (Galli *et al.*, 1995). Using this technique, individual neurons were clamped at -50 mV and NET currents were elicited in a 500 msec step to -120 mV, with NET currents defined by desipramine (DMI) subtraction. This step generated DMI-sensitive inward

transient and leak currents, indicative of NET surface expression (Fig. 7L). When neurons were depolarized for 2 s to -10 mV to elicit Ca^{2+} entry via voltage-sensitive Ca^{2+} channels prior to the -120 mV test pulse, we recorded an increase in DMI-sensitive currents by 80% relative to the response prior to depolarization (Fig. 7L). Cd^{2+} , which blocks voltage-dependent Ca^{2+} channels, prevented the increase in NET currents (Fig. 7M). In addition, pre-incubation of KN93 and STO609 also prevented the increase in NET current (Fig. 7N), and STO609 actually decreased the NET current below baseline in the absence of depolarization.

CaMKs support basal and depolarization elicited NET activity *in vivo*

To ascertain whether the processes elaborated for Ca^{2+} -dependent surface trafficking of NET with *in vitro* models apply *in vivo* at noradrenergic synapses, we investigated clearance time (T80, the time it takes for the NE signal to decay by 80% of peak amplitude) in dentate gyrus of the anesthetized mouse. We previously demonstrated that NET-positive fibers are enriched in this region (Schroeter *et al.*, 2000) and that DMI-sensitive (Daws *et al.*, unpublished data) and nomifensine-sensitive (Cass *et al.*, 1995; Lin *et al.*, 1997; Lin *et al.*, 1993) NE clearance can be selectively monitored in this region using chronoamperometric techniques (Robertson *et al.*, 2010). As shown in Fig. 8A, NE clearance time was relatively constant when pulses of NE were applied at five min intervals over a 40-min period following pretreatment with vehicle (see Materials and Methods). In contrast, pretreatment with KN93 or STO609 prolonged NE clearance time, effects that were maximal 20 min following application of drug and returning to baseline 40 min following drug administration. Fig. 8C shows representative tracings for NE clearance before and 20 min after injection of either vehicle, KN93 or STO609. Injection of high K^{+} prior to pulse applications of NE resulted in a significant and rapid enhancement of NE clearance (reduced T80) that remained elevated for at least 30 min after injection (Fig. 8B). The effect of high K^{+} was blocked by pretreatment with either KN93 or STO609. Interestingly, with KN93 we observed a more pronounced reduction in NE clearance time than without K^{+} (compare KN93 effects in Fig. 8A and 8B), suggesting possibly a greater role of CaMKII pathways with depolarization. Regardless, these studies provide evidence for the participation of CaMKs in support of both resting and depolarization elicited NE clearance *in vivo*.

DISCUSSION

Neurotransmitter uptake via plasma membrane transporters is highly regulated, with catalytic function and transporter trafficking sensitive to regulation by G-proteins and tyrosine kinase-coupled receptor activation linked to Ca^{2+} signaling. How this regulation integrates with the demands imposed by Ca^{2+} -dependent vesicular NE release is largely unexplored, although both peripheral and central NE neurons demonstrate depolarization-triggered elevation in surface NET (Savchenko *et al.*, 2003) and depolarizing stimuli increase NE transport activity in PC12 cells (Mandela and Ordway, 2006). Prior studies have shown that functional activity of the SLC6 transporter family, including NET, are sensitive to changes in Ca^{2+} concentration in external media or disruption of intracellular Ca^{2+} concentrations (Uchida *et al.*, 1998; Uchikawa *et al.*, 1995; Yura *et al.*, 1996). We sought to determine how changes in Ca^{2+} signaling support constitutive NET function as well as the

transporter's response to depolarizing conditions. We first tried to synchronize cellular Ca^{2+} signaling by manipulating external Ca^{2+} in synaptosomes and in established cell cultures (Figs. 1, 2). This regimen is known to increase cytoplasmic Ca^{2+} to 0.5 - 1 μM in both non-neuronal and excitable cells (Fagan *et al.*, 2000; Fagan *et al.*, 1996). We found that Ca^{2+} sustains constitutive NE transport through rapid and bidirectional surface trafficking of NET proteins mediated by both CaMKI and CaMKII. Surface trafficking responses to either Ca^{2+} addition or Ca^{2+} removal depended critically on residue Thr30 located in the NH_2 terminus of NET, a site also required for Ca^{2+} elicited transporter phosphorylation. We demonstrated parallel changes in NET surface expression and function in neurons in response to depolarization-elicited Ca^{2+} influx and obtained evidence for CaMKs in NET function both *in vitro* and *in vivo* (Figs. 4, 7, 8), suggesting that Ca^{2+} and CaMK-dependent trafficking mechanisms are linked to demands imposed by changes in noradrenergic neuron excitability and NE release.

Interestingly, the sequence between 28–47 of NET is divergent from the corresponding NH_2 -terminal regions of SERT, GAT1 and DAT. However, Thr30 is conserved in mammalian NETs, suggesting that this site may support a NET-specific mechanism linking changes in NET activity to levels of intracellular Ca^{2+} (Fig. 3). The Thr30A mutation in NET blocked trafficking linked to Ca^{2+} and recovery of both phosphorylated NET T30A and associated phosphoproteins, suggesting a linkage of Thr30-dependent phosphorylation and NET trafficking. Possibly, Ca^{2+} elevations phosphorylate NET at Thr30 and consequently recruit other phosphoproteins, which in turn impact NET trafficking and localization at the surface. Phosphorylation of NET at Thr258/Ser259 has been reported in association with Ca^{2+} -independent, PKC ϵ -linked internalization of NET (Jayanthi *et al.*, 2006) with no effect on transporter insertion or recycling. In contrast, we found that Thr30-dependent phosphorylation correlated with Ca^{2+} -dependent increases in NET surface density, possibly arising from elevated transporter insertion rates. Using two ectodomain antibodies derived from different species, but targeted to the same epitope, we obtained evidence that depolarization-elicited NET surface elevations arise from directed insertion (Savchenko and Blakely, unpublished findings). Multiple phosphorylation events may modulate NET surface trafficking, possibly at different stages. Interestingly, NET T30A responded to thapsigargin differently from wildtype NET and other NET mutants (Fig. 3C). Ca^{2+} regulation may involve many different steps of NET trafficking including translocation between intracellular vesicles, as well as insertion into and retrieval from the plasma membrane. NET is known to be present in multiple intracellular compartments, including LDCV, rab4-positive early endosomes, and rab11-positive recycling endosomes (Kippenberger, 1999; Leitner, 1999; Matthies *et al.*, 2009; Matthies *et al.*, 2010). NET and NET T30A may have different sensitivity to Ca^{2+} mobilization from intracellular Ca^{2+} stores due to differences in trafficking between these intracellular compartments.

The NET NH_2 terminal domain is known to be important for interactions with other cellular proteins. The SNARE protein syntaxin 1A engages in Ca^{2+} -dependent interactions with residues 2–42 of NET in a manner sensitive to PKC activation but insensitive to inhibition of CaMK (Sung *et al.*, 2003; Sung and Blakely, 2007). Syntaxin 1A is known to dictate NET surface trafficking and also to interact with NET to modulate transporter function (Sung *et al.*, 2003). However, the present study indicates that Ca^{2+} /CaMK modulates surface

trafficking of NET independently of regulatory pathways involving PKC and syntaxin 1A (Fig. 5). The NH₂-terminus of NET also interacts with 14-3-3 proteins (Sung *et al.*, 2005) and protein phosphatase 2A (PP2A) (Sung *et al.*, 2005). CaMKII is known to interact constitutively with the DAT COOH terminus, and more weakly with the NET COOH terminus (Fog *et al.*, 2006), to regulate DA efflux via DAT, a process involved with phosphorylation of residues in the DAT NH₂ terminus. It would be interesting to examine whether NET assembles with a signaling complex including CaMK and PP2A/14-3-3 in support of NET trafficking. Ca²⁺-dependent association of phosphorylated NET with other phosphoproteins (Fig. 3D) suggest that, in addition to CaMKI and CaMKII, other cellular proteins may be involved in Ca²⁺-dependent surface trafficking of NET. Future studies may clarify Ca²⁺-dependent interactions as downstream targets of CaMKI and CaMKII for surface trafficking of NET.

Our data showing contributions of both CaMKI and CaMKII to basal, external Ca²⁺-triggered, and depolarization-elicited changes in NET trafficking (Figs. 6–8) reveal distinct but overlapping roles of CaMK isoforms in Ca²⁺-dependent NET surface trafficking. It is possible that CaMKI and CaMKII may regulate constitutive surface trafficking and contribute further to the events of acute mobilization of Ca²⁺. Ca²⁺ signaling has diverse temporal courses, amplitude, and locations within subcellular compartments, depending on whether Ca²⁺ influx is invoked through voltage gated Ca²⁺ channels or whether signaling cascades are triggered by presynaptic receptors (Berridge, 2006; Lysakowski *et al.*, 1999; Zucker and Regehr, 2002). Regulatory mechanisms involving both CaMKI and CaMKII may provide flexibility for modulating NET in response to various signaling states of neurons at rest and under conditions of neuronal excitation.

Our studies show that Ca²⁺ regulates NET surface expression through CaMKI and CaMKII. How do our findings relate to the control of NE transport under physiological conditions in neurons? Subsequent to our studies in a heterologous model, we demonstrated that Ca²⁺ is a critical mediator of both basal and depolarization-triggered NET trafficking in transfected and primary neuronal cultures (Figs. 4, 7). Additionally, we obtained evidence that CaMKs sustain basal NET function and are required for depolarization-elicited elevations in NE clearance time *in vivo* (Fig. 8). Our findings suggest that CaMKI and CaMKII-dependent trafficking processes establish quantitatively appropriate levels of NE uptake at rest and under conditions of neuronal excitation. As Ca²⁺ is an essential second messenger for excitation-coupled vesicular NE release, release and uptake of NE may likely coordinate through the actions of CaMKI and CaMKII to effect the fusion of NE vesicles in parallel with the fusion of NET vesicles (Nichols *et al.*, 1990; Schweitzer *et al.*, 1995). Interestingly, evidence derived from adrenal gland preparations and PC12 cells suggests that NET may reside on NE secretory vesicles (Kippenberger, 1999), though this remains to be tested in neurons.

In addition to Ca²⁺ channel-dependent mechanisms that support vesicular fusion, intracellular Ca²⁺ signaling can also be initiated by receptor stimulation. Endoplasmic reticulum-like structures and Ca²⁺-induced Ca²⁺ signaling are known to exist in presynaptic terminals (Bouchard *et al.*, 2003; Verkhratsky, 2005) and inositol trisphosphate (IP3) receptor inhibition reduces NET activity (Amano *et al.*, 2006). Thus, changes in presynaptic

Ca^{2+} and CaMK linked pathways may also be engaged as a consequence of presynaptic receptor stimulation and Ca^{2+} mobilization from intracellular Ca^{2+} stores. An example may be the movement of NETs triggered by angiotensin II in hindbrain noradrenergic neurons (Savchenko *et al.*, 2003; Sumners and Raizada, 1986).

Finally, the pathway we examined in the present studies may be important for understanding how psychotropic drugs modulate NET. Amphetamine alters activity of NET and DAT via mechanisms requiring Ca^{2+} , CaMK, and voltage-dependent Ca^{2+} channels (Kantor *et al.*, 1999; Kantor *et al.*, 2001; Kantor *et al.*, 2004). Amphetamine triggers a rise in intracellular Ca^{2+} in NET expressing cells and CaMK activation supports subsequent amphetamine-induced NET trafficking (Dipace *et al.*, 2006), suggesting that the psychostimulant may manipulate pathways established for the regulated trafficking of NET. Furthermore, a recent study reported roles of the NET Thr30 residue in cocaine sensitization and conditioned place preference in mice (Mannangatti. *et al.*, 2015).

Our studies raise the possibility that alterations in CaMK signaling pathways account for the findings that NET surface trafficking is increased in noradrenergic neurons in animals subjected to chronic stress (Miner *et al.*, 2006). Dysregulation of these pathways may thereby support neuropsychiatric syndromes linked to altered NE signaling including anxiety, depression, post-traumatic stress disorder and attention-deficit disorder.

Finally, several findings from the present study also indicate the likely involvement of a trafficking-independent mode of NET regulation supported by Ca^{2+} /CaMK pathways. In our study, increased Ca^{2+} reduced the K_m and increased V_{max} for NE uptake in synaptosomes, as well as increased NET surface expression in CHO cells. These data indicate that Ca^{2+} elicits changes in substrate recognition or permeation, consistent with previous reports (Apparsundaram *et al.*, 2001; Mandela and Ordway, 2006). NET can also be catalytically activated in SK-N-SH cells by Ca^{2+} -dependent, p38 MAP kinase linked pathways (Apparsundaram *et al.*, 2001). Mandela and Ordway (2006) found that NET in PC12 cells can be stimulated by depolarization in a KN93 sensitive manner, findings the authors ascribe to a trafficking-independent process. Future studies should be able to further dissect contributions of catalytic activation of receptor-induced regulation of NET as well as depolarization elicited elevations in NET activity.

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Abbreviations used

AP

anterior-posterior

BAPTA/AM

1,2-Bis(2-aminophenoxy)ethane-N,N,N',N'-tetraacetic acid tetrakis(acetoxymethyl ester)

BIM

bisindolylmaleimide

Ca²⁺-free KRH (Krebs-Ringer Bicarbonate)

KRH without Ca²⁺ and with 0.2 mM EGTA (ethylene glycol-bis(β-aminoethyl ether)-N,N,N',N'-tetraacetic acid)

CaMK

Ca²⁺/calmodulin-dependent protein kinase

CaMKK

CaMK kinase

CHO

Chinese hamster ovary

CNS

the central nervous system

DAT

dopamine transporter

DMI

desipramine

DV

dorsal ventral

FBS

fetal bovine serum

HA

hemagglutinin

HBSS

Hank's balanced salt solution

IP3

inositol 1,4,5-trisphosphate

K_m

affinity

KRH

HEPES ((4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid)-buffered KRH solution

LDCV

large dense core vesicles

MAPK

mitogen activated protein kinase

ML

medial lateral

MS

mass spectrometry

min

minute

NE

norepinephrine

NET

NE transporter

PBS

phosphate buffered saline

Pi

inorganic phosphate

PKC

protein kinase C

PMSF

phenylmethane sulfonyl fluoride

PNS

peripheral nervous system

PP2A

protein phosphatase 2A

SCG

superior cervical ganglia

SERT

serotonin transporter

STO6097-Oxo-7*H*-benzimidazo[2,1-*a*]benz[de]isoquinoline-3-carboxylic acid**TG**

thapsigargin

Veh

vehicle

VMAT

vesicular monoamine transporters

V_{max}

maximal uptake velocity

W7*N*-(6-Aminoethyl)-5-chloro-1-naphthalenesulfonamide**References**

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Highlights

- Ca^{2+} signaling influences NE clearance. Changes of external Ca^{2+} and neuronal depolarization increase surface NET proteins.
- Mutagenesis of Thr30 and inhibition of CaMKI/CaMKII affected Ca^{2+} dependent surface trafficking of NET.
- Our data indicate that Ca^{2+} modulates NET trafficking in a Thr30-dependent manner and via CaMKI/CaMKII mediated pathways.
- The mechanism revealed in this study may play a central role in NET regulation.

Figure 1.

Ca²⁺ dependent NE transport in cortical synaptosomes. NE transport assays were conducted with NE at 50 nM as described in Materials and Methods. Data are from 4–6 independent experiments and analyzed by a t-test. **A.** Ca²⁺ enhanced NE transport in a reversible manner. Left: Ca²⁺ depletion reduced NE transport ($p < 0.05$). Synaptosomes in KRH were replaced with KRH or Ca²⁺ free-KRH prior to NE transport assays. Right: Ca²⁺ increases NE transport ($p < 0.05$). Synaptosomes in Ca²⁺ free-KRH were transferred to KRH or Ca²⁺ free-KRH prior to NE transport assay. **B.** Ca²⁺ increased V_{max} and reduced K_m of NE transport. Analysis of NE transport kinetics of cortical synaptosomes was performed in KRH or Ca²⁺ free-KRH. Left: Ca²⁺ increased V_{max} ($p < 0.05$). Right: Ca²⁺ decreased K_m ($p < 0.05$).

Figure 2.

Ca^{2+} -dependent surface trafficking of NET in transiently transfected CHO cells. **A.** Ca^{2+} imaging for monitoring cytoplasmic Ca^{2+} in CHO cells. Cells, preloaded with Fura-2/AM, were superfused with Ca^{2+} free-KRH and 0.1 μM thapsigargin for 10 min. The medium was replaced with KRH or Ca^{2+} free-KRH. Replacement with medium containing Ca^{2+} induced immediate influx of Ca^{2+} , followed by gradual decline. Data represent averaged traces for the response of recordings from 27 individual cells. Standard error bars are omitted for clarity but represented no more than 5% of the normalized ratio values. **B.** Surface level of HA-NET was evaluated in CHO cells by surface biotinylation and immunoblotting with anti-HA. Band densities are averages from 4 independent experiments and analyzed by a t-test. Asterisk indicates statistically significant difference ($p < 0.05$). Left: Cells in complete media were supplemented with either vehicle or 10 mM EGTA/30 mM BAPTA/AM. Ca^{2+} depletion reduced surface NET within a minute. Right: Cytoplasmic Ca^{2+} was depleted by incubating cells in Ca^{2+} free-KRH with 0.1 μM thapsigargin. Cells were replaced with KRH

or Ca^{2+} free-KRH. Ca^{2+} increased NET at the surface within a minute. **C.** Neither Ca^{2+} depletion (5 min) nor Ca^{2+} replacement (1 min) resulted in statistically consistent trafficking responses of transferrin receptors. Changes in the surface level of transferrin receptors were evaluated with anti-transferrin receptors, using the same method for NET as described in **B**. Band densities are averages from 3 independent experiments and analyzed by a t-test.

Figure 3.

NH₂ domain of NET is crucial for Ca²⁺ dependent surface trafficking of NET. **A.** NH₂ cytoplasmic domains of NET (human, mouse, and rat), human DAT, GAT1, and SERT are compared. Alignment was performed using DNASTAR MegAlign 4.0.3 by clustal method. Conserved amino acids are shown in black background. The sequence between 28 to 47 amino acids in NET is marked by a line. NET contains 3 threonines in the NH₂ domain, as indicated by asterisks. **B.** NET 28–47, NET T30A, and NET T30E did not respond to Ca²⁺ depletion or Ca²⁺ recovery in external media. CHO cells were transiently transfected with HA-tagged NET 28–47, NET T30A, or NET T30E. External Ca²⁺ was manipulated to achieve Ca²⁺ depletion (5 min) and Ca²⁺ recovery (1 min) as described for wildtype NET in Fig. 2. Surface NET mutants were detected by surface biotinylation and immunoblotting with

anti-HA. Band densities are averages from 3–5 independent experiments. **C.** Comparison of surface trafficking of NET, NET 28–47, NET T30A, and NET T30E in response to changes in Ca^{2+} . Left: Responses of NET and mutants to Ca^{2+} depletion. Asterisk indicates statistically different surface levels between the presence and absence of Ca^{2+} ($p < 0.05$), and # indicates statistical difference of surface levels between NET in Ca^{2+} free KRH and NET mutants in Ca^{2+} free KRH ($p < 0.05$). Each bar represents the averaged band density of 3–6 independent experiments (one-way ANOVA followed by Tukey's multiple comparison; $p < 0.05$). Right: Responses of NET and mutants to Ca^{2+} increase. Asterisk indicates statistical difference of surface levels between the presence and absence of Ca^{2+} ($p < 0.05$), and # indicates statistical difference of surface levels between NET in KRH and NET T30A in KRH ($p < 0.05$). Each bar represents the averaged band density of 3–6 independent experiments (one-way ANOVA followed by Bonferroni's multiple comparison test; $p < 0.05$). **D.** Ca^{2+} phosphorylated NET and induced interactions with high molecular weight phosphorylated proteins. In contrast, Ca^{2+} did not induce phosphorylation of NET T30A and interactions with other phosphorylated proteins. CHO cells stably expressing HA-tagged NET or NET T30A cells were metabolically labeled with inorganic [^{32}P]-phosphate and depleted of Ca^{2+} in Ca^{2+} free-KRH. Cells were subjected to a 5 min-stimulation with Ca^{2+} by adding CaCl_2 to a final concentration of 2.2 mM or vehicle (the same volume of Ca^{2+} free-KRH), prior to cell lysis and immunoprecipitation. The immunoprecipitated proteins were divided into 2 aliquots, and analyzed by two parallel 3–12% linear gradient gels, one for immunoblotting with anti-NET (Left) and the other for image processing in phosphoimager (Right). Representative data from 3 experiments are shown.

Figure 4.

Thr30 is important for depolarization-elicited surface trafficking of NET in PC12 cells. PC12 cells were transiently transfected with HA-tagged NET, NET T30A, and NET T30E. Cells were stimulated with KRH containing 80 mM KCl for 10 min at 37°C prior to surface biotinylation and immunoblotting with anti-HA. Depolarization increased surface NET ($p < 0.05$). NET T30A did not change and NET T30E reduced ($p < 0.05$) surface levels in response to high K^+ . # indicates statistical difference between the surface levels of NET and mutants in depolarized cells ($p < 0.05$). Data were from three independent experiments (one-way ANOVA followed by Tukey's multiple comparison test; $p < 0.05$).

Figure 5.

CaMKs regulate Ca^{2+} modulation of NE transport in cortical synaptosomes. Data are from 6 independent experiments, and analyzed by a one-way ANOVA followed by Tukey's multiple comparison test. **A.** Inhibition of NE transport by inhibitors of PKC (BIM), CaMK (KN93), and calmodulin (W7). Synaptosomes were incubated with vehicle, 1 μ M BIM, 5 μ M KN93, or 10 μ M W7 in KRH for 15 min at 37°C. Asterisk indicates statistically different NE transport between synaptosomes treated with vehicle or drugs ($p < 0.05$). **B.** KN93 attenuates Ca^{2+} depletion-induced down-regulation of NE transport. Synaptosomes were pre-incubated with vehicle or KN93 (5 μ M) in KRH for 15 min, re-suspended in KRH, divided into 2 groups, and then transferred to either KRH or Ca^{2+} free-KRH prior to NE transport assay. Asterisk indicated statistically different NE transport between KRH and Ca^{2+} free-KRH ($p < 0.05$), and # indicates statistically different NE transport between vehicle and KN93 treated synaptosomes in Ca^{2+} free-KRH ($p < 0.05$). **C.** KN93 attenuates Ca^{2+} -induced increases in NE transport. Synaptosomes were pre-incubated with vehicle or KN93 in KRH, then washed with Ca^{2+} free-KRH for Ca^{2+} depletion, and transferred to either KRH or Ca^{2+} free-KRH prior to NE transport assay. Asterisk indicates statistically different NE transport

between KRH and Ca^{2+} free-KRH ($p < 0.05$), and # indicates the statistical difference in NE transport between vehicle and KN93 treated synaptosomes in Ca^{2+} free-KRH ($p < 0.05$). **D–E.** BIM did not show any effect on Ca^{2+} depletion-induced decrease or Ca^{2+} -induced increase of NE transport. **D:** Synaptosomes were pre-incubated with vehicle or BIM, suspended in KRH, divided into 2 groups, and transferred to either KRH or Ca^{2+} free-KRH prior to NE transport assay. Asterisk indicates statistically different NE transport in KRH or Ca^{2+} free-KRH ($p < 0.05$). **E:** Synaptosomes were pre-incubated with vehicle or BIM, suspended in Ca^{2+} free-KRH, divided into 2 groups, and transferred to either Ca^{2+} free-KRH or KRH prior to NE transport assay. Asterisk indicates statistically different NE transport in KRH or Ca^{2+} free-KRH ($p < 0.05$).

Figure 6.

CaMKI and CaMKII regulate Ca²⁺ dependent surface trafficking of NET. Bar graphs are quantification of data from 3–5 independent experiments (one-way ANOVA followed by Tukey's multiple comparison test; $p < 0.05$). **A.** Pharmacological inhibition with KN93 and STO609 demonstrates CaMK dependent NET trafficking in CHO cells. CHO cells, transiently transfected with HA-NET, were incubated with vehicle, KN93 (5 μ M), or STO609 (5 μ M) in complete media for 30 min at 37°C prior to Ca²⁺ manipulation in external media. Surface proteins were analyzed using surface biotinylation and immunoblotting with anti-HA. Incubation of cells with KN93 or STO609 blocked Ca²⁺ depletion-induced decreases in surface NET (Left) or Ca²⁺-induced increases in surface NET (Right). Asterisk indicates statistical difference of surface levels of NET in KRH and Ca²⁺ free KRH ($p < 0.05$). **B–D.** RNA interference of CaMKI and CaMKII indicates CaMKI and CaMKII dependent NE transport and surface trafficking in CAD-NET stable cells. CAD-NET cells were mock-

transfected (control) or transiently transfected with siRNA of CaMKI or siRNA of CaMKII δ . **B.** Transfection of CaMKI siRNA or CaMKII δ siRNA reduced protein expression of kinases, but did not influence NET expression. NET in total lysates showed 60 kDa immature and 90 kDa mature forms. **C.** NE transport after transfection of siRNA of CaMKI or CaMKII δ . Cells were incubated in KRH with vehicle or KN93 (5 μ M) at 37°C for 20 min prior to NE transport assay in the same buffer. KN93 inhibited NE transport in transfected CAD-NET cells (* with $p < 0.05$). NE transport in cells transfected with CaMKII δ siRNA was reduced compared to NE transport in mock-transfected cells (# with $p < 0.05$). **D.** siRNA of CaMKI and CaMKII δ inhibited Ca²⁺ dependent surface trafficking of NET. CAD-NET cells were incubated in complete media without or with 10 mM EGTA for 5 min at 37°C prior to surface biotinylation and immunoblotting. Mock-transfected CAD-NET cells exhibited Ca²⁺ dependent changes in surface NET level (* with $p < 0.05$). CaMKI siRNA and CaMKII δ siRNA prevented Ca²⁺ dependent surface trafficking of NET. Right panel shows quantification of surface NET.

Figure 7.

Depolarization increases surface level and activity of NET in primary noradrenergic neuronal culture in a Ca^{2+} and CaMK dependent manner. **A–K.** Surface NET was detected with 43408 extracellular epitope antibody in nonpermeabilized brainstem neurons. Surface NET labeling was sparse in 2.5mM K^+ medium (**A**), but became more evident as K^+ in medium was increased to 40 mM (**B**) and 90 mM (**E**), with the most notable change being an increase in neurite labeling (arrows). These changes in surface labeling were blunted in Ca^{2+} -free medium (no Ca^{2+} /EGTA; **C** and **F**) or when K^+ elevations were conducted in the presence of KN93 (**D**, **G**). To quantify these changes, we identified neurites co-labeled for NET (**H**) and β -tubulin (**I**, overlap in **J**) and measured average pixel intensity across 15–20 fields, averaging data from 3 independent replicate experiments (**K**). Depolarization resulted in a significant increase in average pixel density (* $p < 0.05$) that was lost when stimulation

was conducted in Ca^{2+} -free medium or in the presence of KN93 (one-way ANOVA followed by Bonferroni multiple comparisons test; $p < 0.05$). Scale bar = 20 μM . **L–N.** CaMKI and CaMKII support depolarization-dependent NET activities in sympathetic noradrenergic neurons. **L.** top: Voltage-step protocol used for whole cell patch clamp recordings of NET currents from single SCG neurons. Bottom: Presence of NET-mediated current was defined by incubation in the presence or absence of 5 μM DMI (I_{basal}). DMI-sensitive NET currents increased after a 2 s depolarizing voltage step at -10 mV prior to the test pulse. **M.** Time-dependence of the increase in NET currents following return to -50 mV holding potential, prior to the -120 mV test pulse. Data were obtained from the induced NET-mediated current after DMI subtraction and normalized for NET current elicited prior to the 50 mV depolarizing pulse (I_{basal}) ($n = 3$). **N.** Impact of Ca^{2+} channels and CaMK on stimulation of NET-mediated currents. NET currents were elicited as described in Panel **L** in control conditions, in the presence of 200 μM CdCl_2 , or after 15 min preincubation with KN93 or 20 min preincubation with STO609. Data were normalized to the current recorded in control conditions before prepulse stimulation (I_{basal}). The effect of prepulse depolarization was then compared for each condition with the stimulation elicited under control conditions (paired Student's t -test; $n = 3$). Asterisk indicates $p < 0.05$ and # denotes $p < 0.01$.

Figure 8.

CaMKI and CaMKII dependent NE clearance *in vivo*. **A.** CaMK regulates NE clearance in the dentate gyrus of hippocampus. NE was pressure-ejected into the dentate gyrus until reproducible signals were obtained. This is represented by the first point at time equals -2 min. At time equals 0 min, KN93 (50 pmol, filled squares), STO609 (50 pmol, filled circles) or an equivalent volume of vehicle (open circles) was pressure-ejected into the dentate gyrus. NE was applied again 2 min later, and at 5 min intervals thereafter. Baseline clearance rate (T80) of pulse applied NE was stable for the duration of recordings (40 min) under vehicle conditions. Compared to vehicle, KN93 and STO609 significantly increased the time (T80) for NE to clear from extracellular fluid in the dentate gyrus ($F_{2,216} = 11.95$, $p < 0.005$). **B.** Depolarization elicits a CaMK-sensitive decrease in NE clearance time. NE was pressure-ejected into the dentate gyrus until reproducible signals were obtained. This is represented by the first point at time equals -2 min. A depolarizing stimulus, high potassium (KCl 150 mM, 125 nl, i.e. 19 nmol), was locally applied at time equals 0 min. NE was applied again 2 min later, and at 5 min intervals thereafter. Depolarization reduced NE clearance time compared to non-depolarized controls (compare open circles in panel A and B), $F_{1,171} = 11.63$, $p < 0.002$). KN93 or STO609 given after KCl prevented depolarization-induced enhancement of NE clearance and subsequently inhibited clearance of exogenously applied NE from dentate gyrus ($F_{2,153} = 33.54$, $p < 0.001$). Data in **A** and **B** are mean $\pm$ SEM. * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$ compared to control (2-way repeated measures ANOVA with Bonferroni post-hoc comparisons). Asterisks above the symbols refer to KN93 and below the symbols to STO609. **C.** Representative oxidation currents, converted to a micromolar value using a calibration factor for NE determined *in vitro*, produced by pressure-ejection of NE (4 ± 1 pmol) into the dentate gyrus, before (open circles) or 20 min

following (closed circles) locally applied vehicle, KN93 or STO609, under non-depolarized conditions.

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MS analysis identified calmodulin, CaMKI, and CaMKII in NET 28–47 associated protein complexes. Protein p score represents the probability of the peptide hit occurring randomly. The cross correlation value and the precursor peptide ion charge state are shown.

Table 1

Identified proteins by MS (Database ID)	Protein p- score	Identified peptide sequences (amino acid number in the protein)	The cross correlation value (charge)
CaMKI (AK089742)	-4.85	R.FTCEQALQHPWIAGDTALDK.N (265–284)	3.0107 (+3)
		K.NIHQSVSEQIK.K (285–295)	2.4018 (+3)
		K.YLHDLGIVHR.D (131–140)	2.3398 (+3)
		K.NIHQSVSEQIK.K (285–295)	1.8394 (+2)
CaMKII δ (Q13557)	-8.66	K.ICDPGLTAFEPEALGNLVEGMDFHR.F (372–396)	4.4600 (+3)
		R.DLKPENLLASK.L (136–147)	2.6336 (+2)
		R.SGSPTVPIKPPCIPNGK.E (470–486)	2.2973 (+2)
		R.SGSPTVPIKPPCIPNGK.E (470–486)	2.2527 (+3)
Calmodulin (P02593)	-10.67	K.EAFSLFDKDGDTTTE.K (14–30)	3.5485 (+2)
		R.EADIDGQGQVNYEEFVQMMTAK.-(127–148)	3.5163 (+3)
		R.EADIDGQGQVNYEEFVQMMTAK.-(127–148)	3.1279 (+2)
		R.SLGQNPTAEALQDMINEVDADGNGTIDE-PEFLTM.M (38–71)	2.8537 (+2)
		K.DGNGYISAAELR.HK (95–106)	1.9256 (+2)