BJP BRITISH JOURNAL OF PHARMACOLOGY

BPS BRITISH PHARMACOLOGICAL SOCIETY

British Journal of Pharmacology (2010), 159, 797–807

0007-1188/10
www.brjpharmacol.org

THEMED SECTION: IMAGING IN PHARMACOLOGY

RESEARCH PAPER

Dynamic monitoring of NET activity in mature murine sympathetic terminals using a fluorescent substrate

Lauren K Parker, Julia A Shanks, James AG Kennard and Keith L Brain

Department of Pharmacology, University of Oxford, Oxford, UK

Background and purpose: To validate a fluorescence approach for monitoring norepinephrine transporter (NET) transport rate in mature sympathetic terminals, and to determine how prejunctional muscarinic receptors affect NET rate.
Experimental approach: Confocal imaging of a fluorescent NET substrate [neurotransmitter transporter uptake assay (NTUA)] as it accumulates in the mature sympathetic nerve terminals of the mouse isolated vas deferens. Fluorescence recovery after photobleaching (FRAP), enhanced green fluorescence protein (EGFP)-transgenic mice and contraction studies were also used.
Key results: NTUA fluorescence accumulated linearly in nerve terminals, an effect that was prevented with NET inhibition with desipramine (1 μM). Such accumulation was reversed by amphetamine (10 μM), which is known to reverse the direction of transport of NET substrates. NTUA labelling was not present in cholinergic terminals (identified using EGFP fluorescence expressed in transgenic mice under a choline acetyltransferase promoter). FRAP experiments, altered nerve terminal distribution with reserpine pretreatment and co-imaging in terminals filled with a cytoplasmic marker (Alexa 594 dextran) indicated that the NTUA labelling was largely confined to vesicles within varicosities; vesicular exchange between varicosities was rare. The rate of NTUA accumulation was slower in the presence of the muscarinic agonist carbachol (10 μM) demonstrating muscarinic inhibition of NET rate.
Conclusions and implications: A straightforward protocol now exists to monitor NET transport rate at the level of the single nerve terminal varicosity, providing a useful tool to understand the physiology of NET regulation, the action of NET inhibitors on mature sympathetic terminals, dynamic vesicular tracking and to identify sympathetic terminals from mixed terminal populations in living organs.
British Journal of Pharmacology (2010) **159,** 797–807; doi:10.1111/j.1476-5381.2009.00574.x; published online 5 February 2010

This article is part of a themed section on Imaging in Pharmacology. To view the editorial for this themed section visit http://dx.doi.org/10.1111/j.1476-5381.2010.00685.x

Keywords: NET; sympathetic; confocal; fluorescence; cholinergic receptors; noradrenaline

Abbreviations: ChAT, choline acetyltransferase; DAT, dopamine transporter; EGFP, enhanced green fluorescence protein; FRAP, fluorescence recovery after photobleaching; NET, norepinephrine transporter; NTUA, neurotransmitter transporter uptake assay; PSS, physiological salt solution; ROI, region of interest; SERT, serotonin transporter; VMAT, vesicular monoamine transporter

Introduction

The extracellular concentration of the transmitter noradrenaline, the primary transmitter released from sympathetic postganglionic nerve terminals, depends dynamically on the rate of its release and its rate of clearance. While much is known of the regulation of noradrenaline release by exocytosis, its clearance is harder to study. Classically, there are two uptake systems for peripheral noradrenaline: neuronal uptake-1, attributed to norepinephrine transporter (NET) (SLC6A2) and non-neuronal uptake-2 (predominantly SLC22A3).

The pharmacology of NET inhibitors is a fertile and complex field. Many of the inhibitors are also dopamine transporter (DAT) and serotonin transporter (SERT) inhibitors (particularly the tricyclic antidepressants, including

Correspondence: Keith L Brain, Department of Pharmacology, University of Oxford, Mansfield Road, Oxford OX1 3QT, UK. E-mail: keith.brain@pharm.ox.ac.uk

Lauren K Parker and Julia A Shanks contributed equally.
Received 18 July 2009; revised 26 September 2009; accepted 13 October 2009

desipramine), and are drugs of abuse (e.g. amphetamine and cocaine). NET polymorphisms have been associated with orthostatic intolerance (Shannon *et al*., 2000) and hypertension in people with type 2 diabetes (Ksiazek *et al*., 2006), yet it is their promiscuous response to drugs that makes them most interesting. Despite the importance of NET there are no methods currently available for measuring NET rate independently of noradrenaline exocytosis at the level of an individual nerve terminal in a physiological system.

In an attempt to assess NET transporter function we investigated the effect of a commercially available assay [neurotransmitter transporter uptake assay (NTUA)], which has been validated to monitor NET transport rate in cell culture systems (Jørgensen *et al*., 2008). We sought to determine whether the fluorescent NET substrate in this kit could be used to quantify relative changes in transporter activity in mature sympathetic nerve terminals, and whether this uptake was specific for noradrenergic over cholinergic terminals. To test the usefulness of this probe for investigating drug action, we investigated the muscarinic regulation of NET transporter rate. Muscarinic receptor activation is known to inhibit NET transport rate, in a variety of cell lines, acting through a PKC-dependent pathway that modifies NET surface expression (Apparsundaram *et al*., 1998a,b). However, whether such regulation occurs in nerve terminals is unknown.

Methods

Isolating the mouse vas deferens

Vasa deferentia were removed from 8–12 week old Balb/c mice (Harlan, UK), which had been killed by cervical fracture. Experiments were in accordance with the European Communities Council Directives (86/609/EEC of 24 November 1986). The vasa deferentia were dissected from the animal and placed in a standard physiological salt solution (PSS) containing (mM): NaCl 118.4, NaHCO3 25.0, NaH2PO4 1.13, CaCl2 1.8, KCl 4.7, MgCl2 1.3 and glucose 11.1. Loosely adherent connective tissue was cleared under a dissecting microscope.

NTUA assay imaging

Each vas deferens was cut longitudinally through the lumen with small iris scissors to create a flat sheet of tissue. This was pinned, serosal side up, onto a Silgard-covered superfusion bath. This bath was transferred onto the stage of a Leica SP2 upright confocal microscope (Leica Microsystems, Wetzlar, Germany), where the tissue was continuously superfused with warmed (32–34°C) PSS at a rate of 1.65 mL·min^{-1} into a bath volume of about 1.5 mL.

The NTUA is described by the manufacturer as containing two compounds (http://www.moleculardevices.com/pages/reagents/neurotransmitter_kit.html): a fluorescent NET substrate and a proprietary masking dye that quenches extracellular fluorescence. Throughout this manuscript, 'NTUA fluorescence' is used to describe any fluorescent signal that arises following exposure to this kit, as we cannot be entirely certain of which compound causes this signal.

In pilot experiments, a blindly chosen field within the tissue was observed under the confocal microscope while a 1:10 NTUA solution was added. In many experiments, fluorescent structures reminiscent of nerve terminal varicosities were identified within such blindly chosen fields, but in other experiments no such terminals were observed within the initial field of view because we had chosen a focal plane too deep in the tissue for the confocal microscope to image or the local innervation density was low. Therefore, in order to identify noradrenergic terminals prior to kinetic measurements, for all later experiments, we exposed the tissue to a 1:100 NTUA solution on the microscope stage for 30 min, then washed in NTUA-free PSS for a further 30 min before imaging. This produced dim images showing nerve terminals and allowed a field of view containing several terminals to be chosen for subsequent kinetic imaging. The NTUA concentrations used here are much lower than the undiluted concentration recommended by the manufacturer for imaging cell cultures. However, these lower concentrations allowed a longer period to monitor changes in transporter rate, which allows time for drug changes within such thick tissues, and is more economical under conditions of continuous indicator superfusion.

The following describes our standard imaging protocol. To allow for some focal plane drift over the course of the experiment, 10-slice stacks of images were acquired (1 μm between slices) every 2 min; a 40× 0.8 NA dipping objective was used, with excitation wavelength 458 nm and emission band 490–570 nm. After three such control stacks, the tissue was superfused with a 1:10 NTUA solution for 12 min, followed by a 45 min period in which the preparation was returned to PSS only. The relative transporter rate was determined by measuring the change in the fluorescent signal over time ($\Delta F/\Delta t$) in the presence of 1:10 NTUA, relative to the fluorescent signal in the control period (Fo). By normalizing with respect to the control period (1:100) we can measure a change in the pump rate between the control period and the test period (1:10 dilution).

EGFP-ChAT mice

Ex-breeder EGFP-ChAT mice, were generously donated by P. Bolam (MRC Neuroanatomical Unit, Oxford, UK). They were originally supplied by The Jackson Laboratory (Bar Harbor, ME, USA), strain B6.Cg-Tg(RP23-268L19-EGFP)2Mik/J, stock number 007902.

Imaging was performed as described above except that the excitation wavelength for enhanced green fluorescence protein (EGFP) was 496 nm, with a common emission band of 510–590 nm. The suboptimal excitation wavelength for EGFP excitation was chosen because the otherwise-preferable 488 nm wavelength excited NTUA. Cross-talk was further reduced by using line-by-line switching of the excitation wavelength using the microscope's acousto-optical tunable filter. Under these conditions, spectral de-convolution of the images was not required.

Nerve terminal dextran loading

Each vas deferens was transferred to an organ bath; the cut prostatic end of each vas deferens was secured in a glass micropipette containing 0.25 mg·μL^{-1} of the 10 kDa dextran conjugate of Alexa 594 (Invitrogen, Renfrew, UK), using a protocol similar to that previously described for loading

dextran-conjugated Ca^{2+} indicators (Brain and Bennett, 1997). The preparations were left in contact with the dye for 9 h, removed from the micropipette and then left for a further 3 h to remove any extracellular non-specific diffusion of the dye, all at room temperature.

Image analysis
Image analysis was performed with Image SXM version 1.88 (from http://www.liv.ac.uk/~sdb/ImageSXM/) using custom-written macros. For NTUA measurements a region of interest (ROI) was drawn around a short terminal segment (15–25 μm in length; 4–6 varicosities) from the first control image stack. For subsequent image stacks, the slice on which this segment was in focus was identified, the ROI restored and automatically fitted to the terminal (using a algorithm based on highest local fluorescent signal), then the mean fluorescent signal from this ROI was measured. For photobleaching experiments, the ROI was drawn around single varicosities.

Contraction
Isometric recordings were made from whole vasa deferentia suspended longitudinally in 5 mL organ baths, attached to force transducers coupled to a PowerLab A/D converter and recording system (ADInstruments, Hastings, UK). Platinum ring electrodes were used to elicit contractions with trains of 10 stimuli at 10 Hz (pulse width 0.5 ms; 90 V) applied every 5 min. Preparations were allowed to equilibrate for 1 h under a resting tension of 9.8 mN before beginning the experiment. Drugs were added directly to the organ baths and were removed by flushing with multiple bath changes of PSS over 10 s. Contraction amplitude and recovery kinetics were calculated using Chart v5.5.3 (ADInstruments). The relation time constant (τ) was calculated by determining the time taken for the force to fall from 90% to 33% (i.e. to fall by 1/e) of its peak amplitude. Relative changes in this time course were measured in a pair-wise manner, taking the ratio of τ in the presence of the drug (NTUA or desipramine; last three recordings) to τ in the immediately preceding three recordings.

Drug preparation
The NTUA kit was purchased from MDS Analytical Technologies (catalogue R8173; Wokingham, Berkshire, UK) and diluted with PSS (10 mL per unit) to make a stock solution. This was divided into 1 mL aliquots which were then frozen (–20°C) for subsequent use. On the day of the experiments, the stock was diluted 1:10 or 1:100 (as indicated) with PSS. Desipramine, amphetamine, cyclopentolate and carbachol were stored as stock 10 mM solutions in water; hydrocortisone and reserpine was stored at 10 mM in dimethyl sulfoxide. All drugs went through no more than one freeze-thaw cycle. The molecular target nomenclature conforms to BJP's *Guide to Receptors and Channels* (Alexander *et al*., 2008).

Statistics
When comparing NTUA fluorescence rate of change, the normality of the control distribution was checked with a Kolmogorov–Smirnov test; if normally distributed, then an unpaired Student's *t*-test was used. An F-test was used to determine whether the variance within each set was similar – if not, Welch's correction was applied. If the control distribution was not normally distributed, then a Mann–Whitney test was used. All statistical analysis was with GraphPad Prism (GraphPad Software, San Diego, CA, USA). Throughout the text, np refers to the number of preparations (vasa deferentia); nt is the number of terminals.

Results

NTUA labelling
To determine whether NET transporter rate could be monitored optically, the sympathetically innervated mouse vas deferens was incubated in NTUA (1:100) for 30 min, then returned to PSS for 30 min. After this, a dense network of varicose terminals, typical of varicose nerve terminals, could be seen running among the smooth muscle cells (Figure 1A; *nt* = 30; *np* = 3). Upon adding 1:10 NTUA, the fluorescent signal in these structures increased steadily and additional cellular labelling (which may have been in smooth muscles cells or other cells types) was also identified. Returning the solution to bathing in PSS for 40 min resulted in a great reduction in the cellular labelling, while the fluorescence remained within the varicose terminals. The fluorescent signal was measured in individual nerve terminal branches over time (Figure 1B). During the period of NTUA (1:10) exposure, the fluorescence increased linearly (*nt* = 30; *np* = 3).

To determine if the additional cellular labelling was due to uptake-2 into smooth muscle or other cells, the tissue was pre-incubated with hydrocortisone (30 μM); one of the many actions of hydrocortisone is to inhibit uptake-2 (Moura *et al*., 1990). However, subjectively there was no change in the additional cellular labelling. There was also no change in the rate of accumulation of NTUA into the nerve terminals (*nt* = 16; *np* = 3; control mean = 23.9 ± 2.6%·min^{-1}; hydrocortisone mean = 17.6 ± 1.8%·min^{-1}; $P > 0.05$).

Specificity for NET
To determine whether the increased fluorescence upon NTUA exposure was due to the activity of NET, tissues were pre-incubated for 6 min in desipramine (1 μM). NTUA (1:10) did not accumulate in the terminals: following washout with PSS the fluorescence was not significantly greater than zero with respect to the control period (Figure 2A,B; *nt* = 16; *np* = 3). These findings are consistent with uptake and clearance of the NTUA through NET into nerve terminals.

If the fluorescent component of NTUA is a NET substrate, then it may also be more rapidly removed from the terminals in the presence of amphetamine, as it has been argued to do for noradrenaline. So, after exposing the preparation to NTUA (1:10) as previously, each vas deferens was then perfused with 10 μM amphetamine in PSS. The rate of fall of the fluorescent signal in the terminals was then monitored (Figure 2C). In many cases, the fluorescence disappeared before the end of the 35 min washout period in amphetamine. In such cases, the fluorescence was measured until the terminals could no

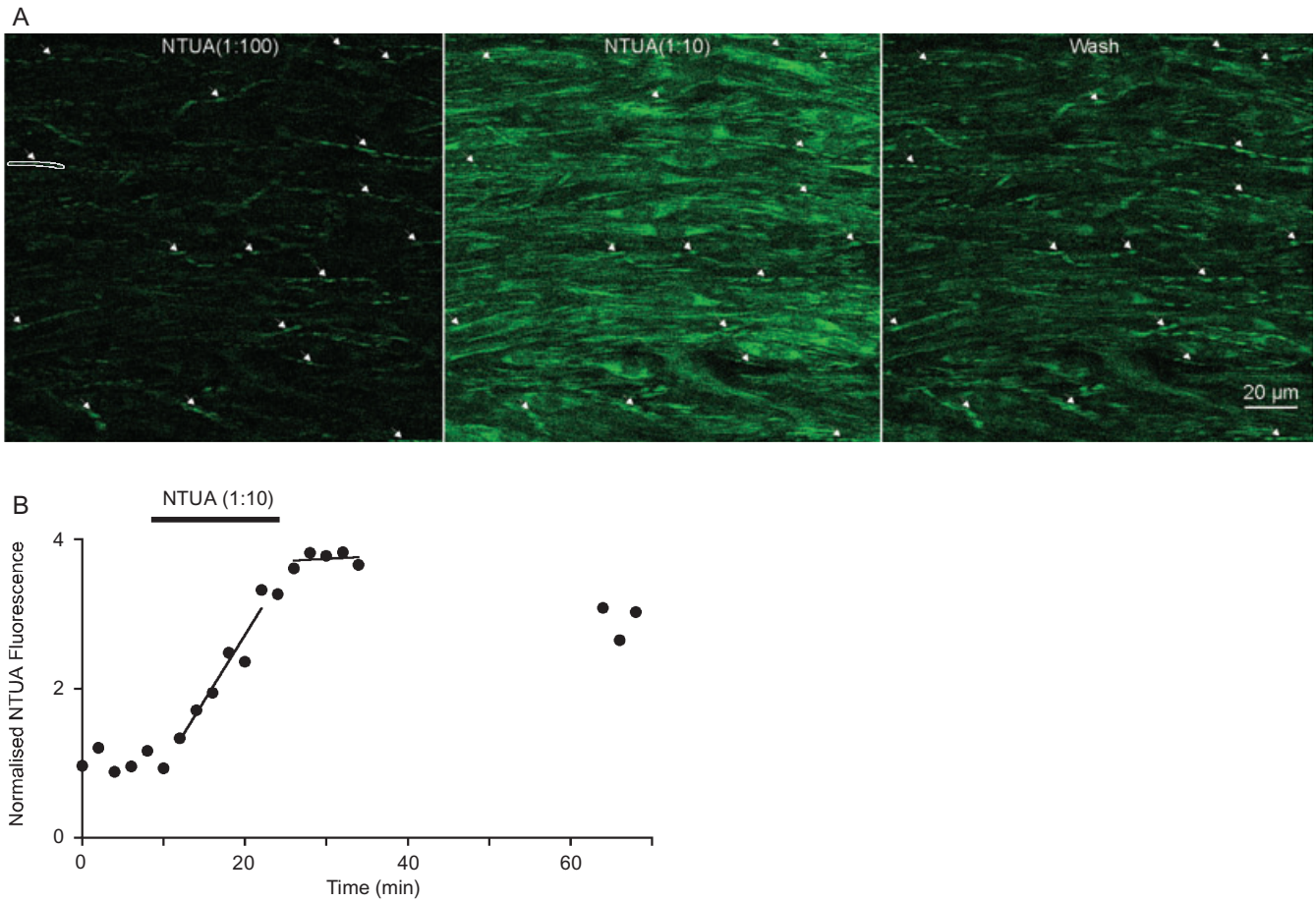

Figure 1 A series of images of the same portion of the mouse vas deferens during neurotransmitter transporter uptake assay (NTUA) exposure is shown. Following exposure to low-concentration NTUA (1:100), varicosities are faintly visible. At the end of NTUA (1:10) exposure, these varicosities were brighter, but there was also significant perinuclear staining in another cells type (probably smooth muscle cells). After returning the preparations to physiological saline (wash) for 30–40 min, the nerve terminal labelling remained, but the perinuclear staining was greatly reduced or abolished. Many nerve terminals (arrows) could be monitored throughout the recording period, although some were too close to non-terminal labelling and were not included in the analysis. The change in fluorescence in the nerve terminal outlined in A, normalized with respect to the mean signal in the control [NTUA (1:100)] period, increased linearly during NTUA (1:10) exposure (B), falling only slowly upon return to physiological saline.

longer be detected. The rate of fall of fluorescence was about five times faster in amphetamine than in the control PSS (Figure 2D; $P < 0.001$).

Relationship between noradrenergic and cholinergic terminals

If NTUA is a NET substrate, it should be taken into noradrenergic but not cholinergic terminals. To determine whether this was indeed the case, NTUA labelling was investigated in the vasa deferentia of mice that express EGFP under a choline acetyltransferase (ChAT) promoter (i.e. in cholinergic neurons). Prior to NTUA labelling, EGFP fluorescence was identified in many axon bundles running over the surface of the tissue, in some smaller bundles (where their profiles were smooth) and in a few cases in varicose terminals ($np = 3$). Following NTUA labelling, the fluorescence signal of the NTUA and EGFP could be clearly separated (as described in the Methods). NTUA labelling did not occur in any EGFP-labelled structures. In most fields, only NTUA labelling could be seen (Figure 3A). However, in nerve terminal bundles, EGFP–ChAT terminals were in close association with NTUA-positive terminals. A similar close association was present in the few examples where isolated EGFP–ChAT terminals (i.e. those not in bundles) could be identified. The density of NTUA terminals was much higher, by at least an order of magnitude, than EGFP–ChAT positive terminals.

Intraterminal location of NTUA labelling

NTUA appeared punctate, but it was unclear whether the labelling was confined to the varicosities or extended into the intervaricose axonal segments (which would suggest free diffusion in the cytoplasm). To investigate this, the 10 kDa dextran conjugate of Alexa 594 was orthogradely loaded into the sympathetic nerve terminals overnight; such vasa deferentia were then loaded with NTUA and the distribution of the two indicators compared (Figure 3B; $nt = 21$; $np = 2$). The Alexa 594 could be clearly seen in the varicosities and intervaricose axonal segments, consistent with its expected cytoplasmic distribution. However, the NTUA was present only in the varicosities.

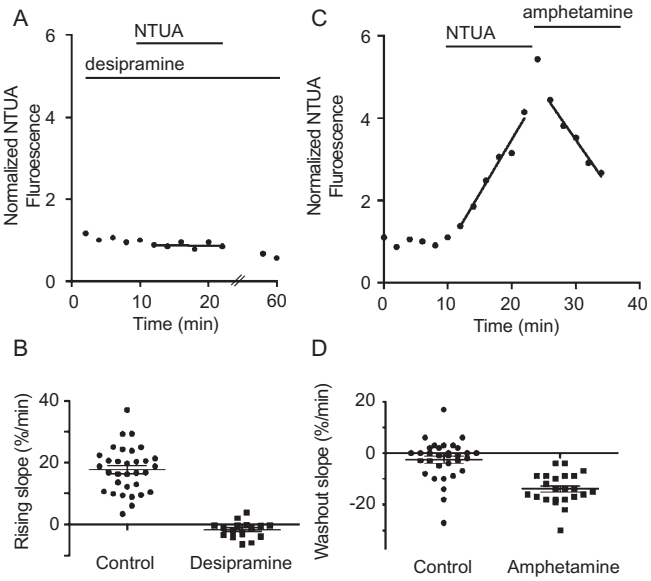

Figure 2 Pretreatment with desipramine (1 μM) prevents fluorescence accumulating in nerve terminals (A). The results of all experiments are summarized in B, for these experiments measuring the relative change in fluorescence at the end of the loading protocol compared with the control period [neurotransmitter transporter uptake assay (NTUA 1:100)]. Adding amphetamine after the NTUA (1:10) exposure causes a rapid loss of fluorescence in the nerve terminal (C), suggesting removal by reversal of norepinephrine transporter. The rate of loss of NTUA fluorescence in the nerve terminal after removal of extracellular NTUA (1:10), both in the presence and absence of amphetamine, is shown in D; each dot represents a different nerve terminal. The exposure protocol is identical to that shown in C.

To investigate whether the NTUA substrate was taken into vesicles, the vesicular monoamine transporter (VMAT) inhibitor reserpine was used (Figure 4A). *In vitro* pretreatment with reserpine for 90 min did not significantly decrease the rate of uptake of NTUA (Figure 4B; 33 ± 4%·min^{-1} compared with 24 ± 3%·min^{-1} in the controls; *nt* = 28; *np* = 2; one sided *t*-test $P = 0.97$) but did increase the rate at which NTUA fluorescence was lost from the nerve terminals during the washout (Figure 4C; –10.1 ± 1.2%·min^{-1} compared with –2.6 ± 1.4%·min^{-1} in the controls; *nt* = 28; *np* = 2; $P < 0.01$). Additionally, after the washout period, the fluorescence was detected in the intervaricose segments (Figure 4A; comparing this with the punctate distribution seen under normal conditions in Figure 3B). The apparently cytoplasmic distribution and the more rapid clearance of NTUA fluorescence from the nerve terminal during the washout, suggest that the NTUA fluorescent substrate accumulates in vesicles through VMAT under normal conditions, which removes it from the cytoplasm and protects it from extrusion by NET.

To further investigate the intra-terminal distribution and movement of the NTUA, fluorescence recovery after photobleaching (FRAP) was used. In NTUA-labelled tissues, after a control stack of images, a small square area around one to two varicosities was photobleached by continued exposure to a mixture of high-intensity 458 nm and 476 nm light for 2 min (Figure 5A). On image stacks acquired immediately after such photobleaching, the targeted varicosities had almost no NTUA labelling visible. There was some fall in the fluorescence of nearby varicosities (on the same or different nerve terminal branches), a phenomenon attributed to scattering of the photobleaching illumination in this thick tissue. The time course of fluorescence recovery in the photobleached varicosity was measured, using nearby varicosities as a control for the effects of NTUA labelling loss over time (Figure 5B). The fluorescence recovered only very slowly (compared with the bleaching controls), with a time course (time to recover to 1/e) of 120 min (median of *np* = 6; range 107–250 min). In some experiments, the fluorescence appeared to recover in a stepwise manner, with the reappearance of small (diffraction limited) spots of bright fluorescence with the targeted varicosity (for an example, see Figure 5C). One FRAP experiment was performed in a tissue loaded with Alexa 594 dextran. In this tissue, the Alexa dextran 594 was photobleached with high intensity 594 nm excitation in addition to the shorter wavelengths (458 and 476 nm; Figure 5D). While the NTUA was locally bleached, the Alexa 594 fell in proportion to the signal in adjacent varicosities, consistent with the rapid recovery of fluorescence by diffusion of the 10 kDa dextran conjugate between the varicosities.

These observations are consistent with little cytoplasmic exchange of NTUA and only rare vesicular movement between varicosities.

Contraction

To assess the toxicity of the NTUA compound, and its effects on neurotransmitter release or uptake, contraction studies were performed. Under control conditions, short trains of stimuli of 10 pulses at 10 Hz induced a brief twitch which is predominantly noradrenergic (Cleary *et al*., 2003; Williams *et al*., 2007). NTUA (1:10) reduced the amplitude of contraction by 18.6 ± 5.9% (*np* = 7; $P < 0.05$; Figure 6A,B) but did not affect the time course of relaxation (1 ± 10% of the control; *np* = 7; $P = 0.97$; Figure 6C,D). The effect of the NTUA on neurogenic contraction was reversible on removing the compound.

To confirm that NET inhibition prolongs the neurogenic twitch, the effects of desipramine (1 μM) were determined. Desipramine slowed the relaxation following the twitch (time course of relaxation increased 26 ± 14%; *np* = 13; $P < 0.001$; Figure 6C,D). These findings suggest that the NTUA assay protocol used here does not significantly inhibit the uptake of noradrenaline following its release from nerve terminals, improving its usefulness as an assay method for endogenous NET activity. Desipramine (1 μM) also decreased the amplitude of the contraction (by 26 ± 4%; *np* = 13; $P < 0.001$; Figure 6B), an effect most readily attributable to postjunctional α_1-adrenoceptor antagonism (discussed next).

To determine whether NTUA (1:10) might block postjunctional α_1-adrenoceptors, the contractile response to exogenously applied noradrenaline (10 μM) in the presence of desipramine (100 nM), with and without NTUA (1:10), was assessed. NTUA (1:10) had no effect on the amplitude of contraction to exogenously applied noradrenaline (–1.4 ± 3.9%; *np* = 5; $P = 0.94$).

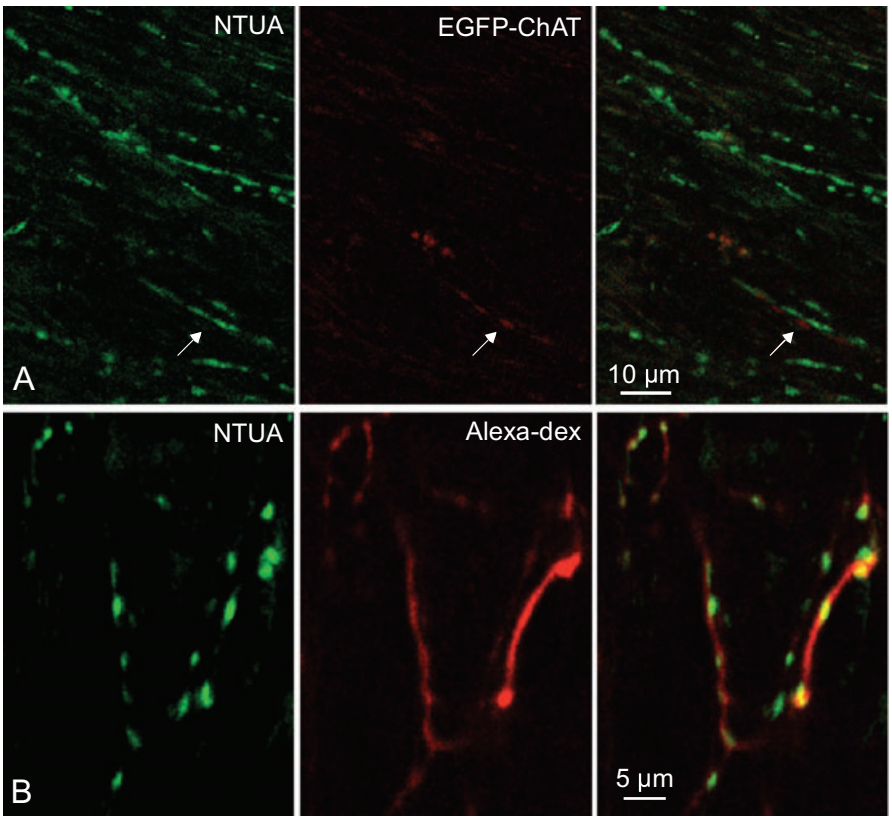

Figure 3 Neurotransmitter transporter uptake assay (NTUA) (1:10) labelling (green) in nerve terminals of the mouse vas deferens in transgenic mice expressing enhanced green fluorescence protein (EGFP) under the choline acetyltransferase (ChAT) promoter (A). Cholinergic terminals (EGFP positive; pseudo-coloured red) were very sparse (the only one present in this field is marked with an arrow), but commonly ran close to NTUA-labelled terminals. No NTUA labelling was found in EGFP-positive terminals. This suggests that the NTUA specifically labels sympathetic noradrenergic terminals. There is some cross-talk between the channels; no attempt at cross-correction has been made. In normal Balb/C mice, loading a proportion of the nerve terminals with Alexa 594 dextran (red) prior to NTUA (1:10) exposure shows that NTUA labelling (green) is confined to the varicosities and does not spread to the intervaricose nerve terminal segments (B).

Cholinergic regulation of NTUA labelling

To determine if NET transporter rate was affected by muscarinic receptor activation, the effect of the muscarinic agonist carbachol on NTUA rate was determined. Exposing the tissue to 10 µM carbachol for 6 min prior to, and during NTUA (1:10) application slowed the rate of uptake by 53% (from 25.3 ± 2.6%·min^{-1} in controls to 11.9 ± 0.9%·min^{-1} in carbachol-treated preparations; $P < 0.001$; $nt = 25$; $np = 4$, Figure 7). This effect was prevented in the presence of the muscarinic antagonist cyclopentolate (1 µM; 21.5 ± 2.4%·min^{-1}; $nt = 19$; $np = 3$; $P = 0.32$).

Discussion and conclusions

At the macroscopic level, I-123-MIBG imaging is an important tool for the identification of sympathetic innervation, although in the periphery it is more precisely a macroscopic measure of the mean local NET transporter rate (Glowniak *et al*., 1993). At the level of the single organ, noradrenaline uptake has been measured using the uptake of radioactive substrates, or the overflow of released noradrenaline escaping from the tissue, although this is also affected by the amount of transmitter released. Other fluorescent substrates have been used for assaying NET uptake in cultured cells, the most important of which is 4-(4-dimethylaminostyryl)-N-methylpyridinium (4ASP$^+$) (Schwartz *et al*., 2003; Mason *et al*., 2005). However, to date we have not been able to confidently use this compound to identify nerve terminals within intact tissues. So, we sought to explore the usefulness of the NTUA for the measurement of NET rate in mature terminals to understand the physiology and pharmacology of pump rate regulation, and as a tool to identify sympathetic terminals running within or on the surface of smooth muscle in living organs.

NTUA as a measure of changes in NET rate

NTUA uptake was prevented by the NET inhibitor desipramine, and assay loading was more rapidly removed in the presence of amphetamine, which acts to reverse the pump and hence remove substrates (like bretylium) from the nerve terminal (Ross and Gosztonyi, 1975). These observations imply that the NTUA uptake is NET dependant, a transporter densely expressed on noradrenergic terminals in the vas deferens (Schroeter *et al*., 2000), and that this transport can be reversed by amphetamine, as is the case for other NET substrates. It seems unlikely that this action could be explained by an action on DAT, as desipramine is about 4000 times more selective for NET over DAT (review by Baldessarini, 2001), nor

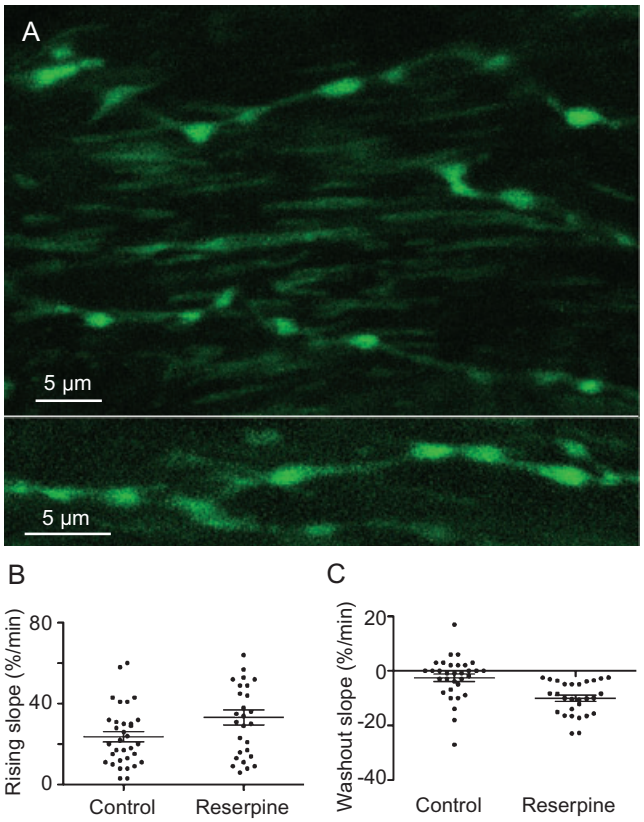

Figure 4 High magnification confocal images of nerve terminals filled with neurotransmitter transporter uptake assay (NTUA) in the presence of reserpine (1 µM) are shown (A). Two representative images from well-separated fields in the same preparation have been included and show that the intervaricose axonal segments fluoresce. After loading the terminals with NTUA (1:100), reserpine (1 µM) was applied; after 90 min, NTUA (1:10) was applied, as in Figure 2, for the measurement of uptake (B) and washout (C) rates.

on SERT, as there is no evidence for SERT activity in the vas deferens; for example, SERT inhibition with paroxetine does not increase the response to exogenously applied 5HT in rat vas deferens (Yaris *et al*., 2003).

In this work, we have shown that using a 1:100 NTUA solution allows the identification of noradrenergic terminals within the mouse vas deferens, an organ with a dense sympathetic innervation. The subsequent addition of 1:10 NTUA allows the rate of NET uptake to be monitored. Calibrating this value to an absolute rate would be complicated by the need to consider the illumination intensity and the detection sensitivity, which may vary between experiments. To avoid such complications, we have chosen to control for such variables by normalizing the rate of change of fluorescence in 1:10 NTUA to the basal fluorescence following exposure to 1:100 NTUA (in the same terminals under the same recording conditions). This means that the relative change in NET rate (compared with the conditions during exposure to the 1:10 solution) can be measured, but not the absolute uptake rate.

NTUA for the identification of sympathetic terminals

The bright, stable fluorescent signal from the nerve terminal varicosities penetrating deep into the tissue, with fluorescence absent from cholinergic terminals, makes this assay a useful tool for identifying noradrenergic sympathetic terminals from among a mixed population of nerve terminals. For example, for studies of Ca^{2+} in autonomic nerve terminals (Brain and Bennett, 1997; Brain *et al*., 2001; Brain and Cunnane, 2008) this assay could now be used to determine the nerve terminal type, because the excitation wavelength (458 nm optimum) is separate from that used for most Ca^{2+} indicators (488 nm). It should also be useful when imaging smooth muscle Ca^{2+} regulation during neurotransmission (Brain *et al*., 2002; Lamont *et al*., 2003) in order to locate noradrenergic nerve terminals. We have not yet confirmed an absence of NTUA uptake into sensory nerves, the other major nerve terminal population present in smooth muscle organs, although such uptake seems unlikely given the amphetamine and desipramine sensitivity (hence NET sensitivity) of all nerve terminal labelling.

Intraterminal location of the NTUA fluorescence

The punctate pattern of NTUA fluorescence, limited to nerve terminal varicosities, and very slow recovery of fluorescence during the FRAP experiments both suggest that the compound is tightly constrained or bound within the varicosities. The most likely location of the indicator is the neurotransmitter vesicles, as most NET substrates are also VMAT substrates. More directly, in the presence of reserpine, which binds to and inhibits the VMAT-2 found in sympathetic postganglionic neurons (for review, see Varoqui and Erickson, 1997), the NTUA fluorescence was distributed more diffusely in the nerve terminal cytoplasm and was more susceptible to removal from the terminals during the washout period. The distribution of fluorescence throughout the cytoplasm is reminiscent of the Falck–Hillarp type fluorescence seen in terminal from reserpinized animals acutely re-exposed to catecholamines (Hamberger *et al*., 1964), which was also argued to be an effect of inhibition of vesicular uptake. The absence of an effect of reserpine on NTUA uptake into the terminals is analogous to the finding that noradrenaline uptake is, at least initially, similarly unaffected (Kopin *et al*., 1962). The faster loss of fluorescence during the washout is consistent with the cytoplasmic location of the NTUA fluorescent substrate, making it more susceptible to loss, probably by reverse transport through NET. It should be noted that while acute reserpine treatment does not deplete the terminals of noradrenaline, it does inhibit vesicular uptake (Iversen, 1967). If the NTUA fluorescent substrate is sequestered in vesicles, then the faster decline in fluorescence observed in the presence of amphetamine can only be explained if amphetamine also removes it from vesicles; such removal is likely to occur because amphetamine reverses VMAT (Pifl *et al*., 1995).

The FRAP experiments suggest that there is little exchange of neurotransmitter vesicles between varicosities over about an hour. Such tight sequestration also implies that the unit of independent measurement of NET rate, monitored by the rate of increase of fluorescence, could be as small as the single nerve terminal varicosity.

Functional effects of NTUA exposure

It was possible that the NTUA (1:10) might compete with noradrenaline for NET uptake. If such competition was

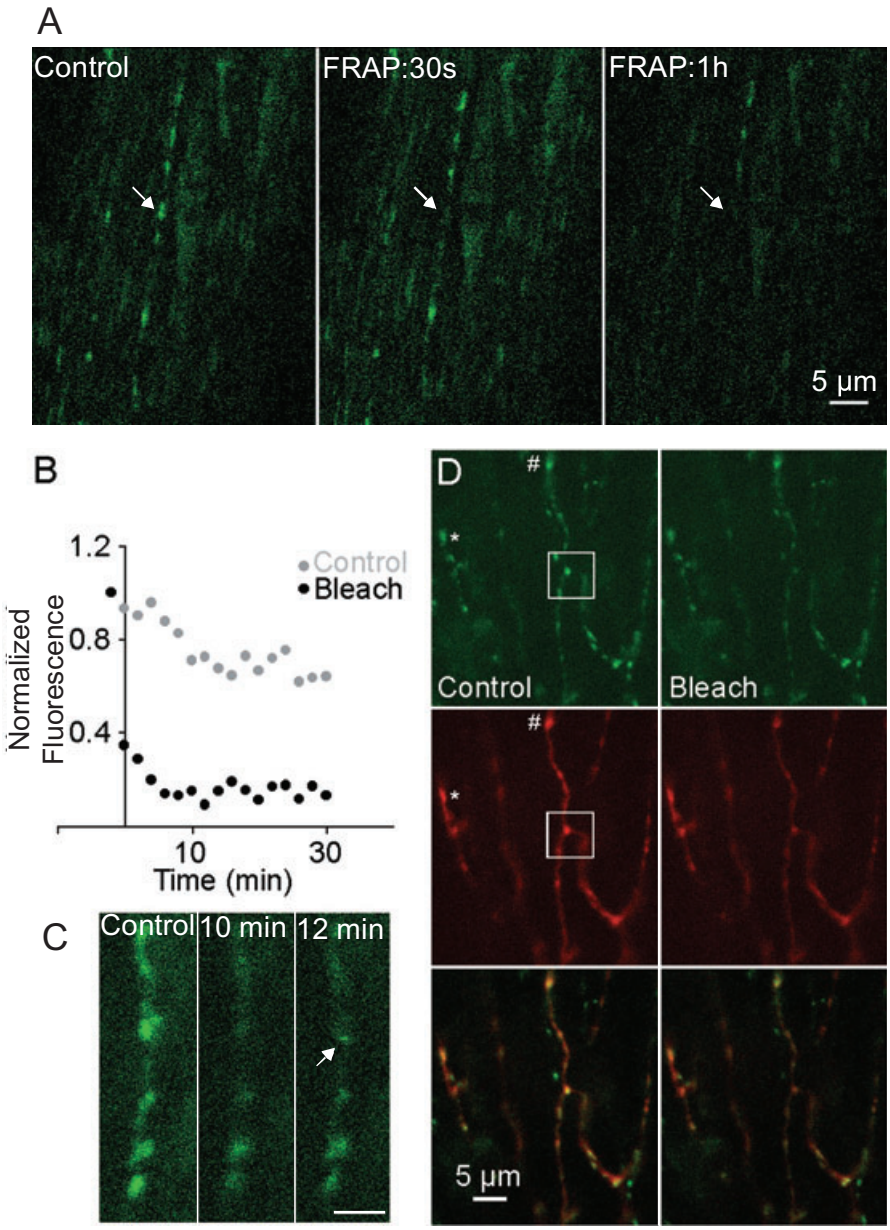

Figure 5 Fluorescence recovery after photobleaching (FRAP) the neurotransmitter transporter uptake assay (NTUA)-labelled terminal at the arrow caused a persistent loss of fluorescence (A) which only slowly recovered with respect to control varicosities on the same terminals (B). In C, three frames from another such experiment are shown, with photobleaching at $t = 0$ min. Between the recordings at $t = 10$ and 12 min punctate, fluorescence returned in a diffraction-limited spot within the target varicosity (arrow); this fluorescence persisted throughout the rest of the experiment. Such FRAP experiments were also carried out in nerve terminals filled with Alexa 594 dextran (D). In such cases, the varicosity in the photobleached region (boxed) lost its NTUA labelling, but the Alexa 594 labelling was lost throughout the entire terminal that crossed this target region. For example, note that the varicosity indicated with a # has a significant fall in Alexa 594 (red) labelling but not NTUA labelling; this varicosity is on the nerve terminal that crosses the target region. However, the varicosity at * does not lose labelling on either channel. These findings suggest that the FRAP protocol does not disrupt the nerve terminal (as Alexa 594 can diffuse to the target region through the nerve terminals to be bleached) and confirms that even a 10 kDa cytoplasmically located molecule can readily move between varicosities.

important for the clearance of exocytosed noradrenaline, then it should slow the time taken for relaxation following a brief twitch, a hypothesis confirmed with the NET inhibitor desipramine (which slowed the relaxation by 26%). However, at the highest concentration used in the imaging experiments, NTUA (1:10) had no effect on this measure, implying that it did not significantly inhibit noradrenaline uptake by NET.

There was a reversible inhibition of twitch contraction observed with NTUA (1:10; by 19%). It may be that the compound, acting as a false transmitter, displaces noradrenaline from the neurotransmitter vesicles and hence decreases noradrenaline release following nerve stimulation. However, this inhibition was more rapidly reversible on removing the NTUA (1:10) than was the loss of fluorescence from the nerve terminal varicosities, which argues against this hypothesis. It

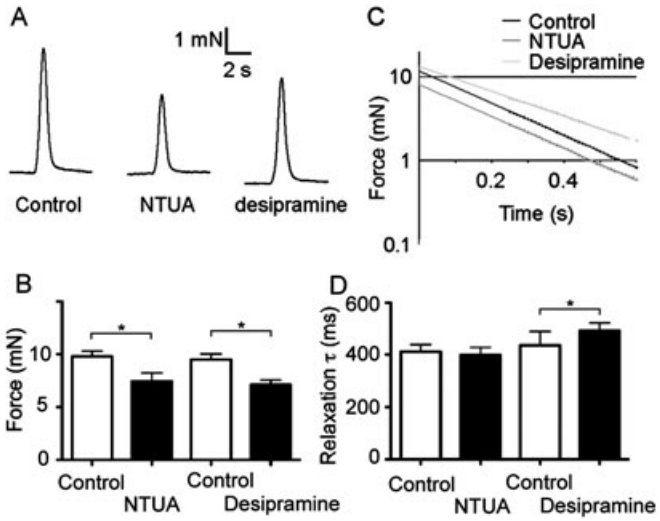

Figure 6 Field-stimulation-induced contraction of the mouse vas deferens (10 pulses at 10 Hz) is reduced in amplitude by neurotransmitter transporter uptake assay (NTUA) (1:10) in a reversible manner (A). A similar reduction in amplitude is observed with the norepinephrine transporter (NET) inhibitor desipramine (1 μM). These effects are summarized over all experiments in B. The rate of relaxation of the smooth muscle following the end of the field stimulation is slower in presence of desipramine, consistent with NET inhibition, but not NTUA (C). The change in the relaxation time constant (τ) is summarized in D.

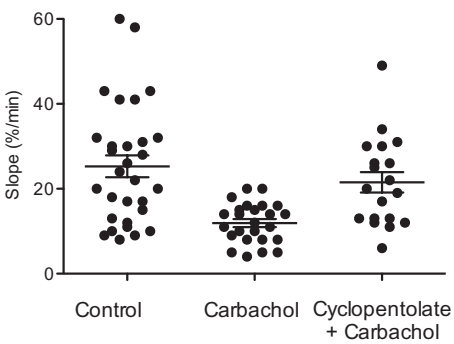

Figure 7 Neurotransmitter transporter uptake assay (NTUA) uptake is slowed in the presence of the muscarinic agonist carbachol (10 μM). This effect is prevented in the presence of the muscarinic receptor antagonist cyclopentolate (10 μM). The rate of NTUA accumulation was measured using the same protocol described in Figure 1B.

may be that there is an unidentified effect of NTUA (1:10) on either the nerve terminals or the smooth muscle that partially inhibits the neurogenic twitch contraction. Postjunctional antagonism of α_1-adrenoceptors seemed a likely hypothesis, an effect that can also explain the desipramine-induced inhibition (1 μM) of contraction; desipramine (at 1 μM, but not 100 nM) inhibits α_1-adrenoceptors, hence reducing neurogenic contraction in the rat vas deferens (Bradley and Doggrell, 1985). However, NTUA (1:10) did not affect the contractile response to exogenous NAd, arguing against α1adreoceptor-mediated antagonism.

Cholinergic innervation of the mouse vas deferens

The cholinergic innervation of the vas deferens is sparse in the longitudinal layer (Kaleczyc, 1998; Kihara, 1998; Kihara *et al*., 1998) but sufficient to generate a contraction following long trains of stimuli if noradrenergic and purinergic contraction is abolished (with prazosin and α,β-methylene ATP) and Ach breakdown is inhibited with neostigmine (Kaschube and Zetler, 1989; Cuprian *et al*., 2005). However, the finding that cholinergic terminals, when present, were often closely associated with catecholaminergic terminals (previously suggested on morphological grounds in the guinea pig vas deferens using electron microscopy; Gosling and Dixon, 1972) gives an anatomical basis for the observed α_2-adrenoceptor-mediated cross-inhibition from noradrenergic to cholinergic terminals (Cuprian-Beltechi *et al*., 2009). Where present, this intimate association between cholinergic and catecholaminergic terminals might also be of functional relevance for the activation of the lower-affinity nicotinic receptors on these sympathetic nerve terminals (Brain *et al*., 2001).

Most of the cholinergic terminals in the outer longitudinal layer of the guinea pig vas deferens are in large bundles, with fine nerves being sparse. The EGFP–ChAT labelling we observe suggests a similar pattern in the mouse. It may be that Ach is released *in passing* from preterminal axons in nerve bundles, and that acetylcholinesterase inhibition allows this normally ineffective ACh release to cause smooth muscle contraction.

Muscarinic inhibition of NET rate

NET rate is acutely regulated by neuronal activity (Sung and Blakely, 2007), nitrosylation of NET by NO (Kaye *et al*., 2000), endothelin-1 (Backs *et al*., 2005) and following the activation of muscarinic receptors through a phorbol ester-sensitive (PKC-dependent) mechanism (Apparsundaram *et al*., 1998a,b). The PKC-dependence of the muscarinic pathway is consistent with the presence of a known PKC-dependent regulator site on the transporter (Jayanthi *et al*., 2006). In the vas deferens, noradrenaline uptake is inhibited by okadaic acid (Bauman *et al*., 2000), attributed to a requirement for a phosphatase to stabilize NET surface expression, and botulinum toxin, due to a loss of NET surface expression (Sung *et al*., 2003). We now show that the muscarinic inhibition, to date determined only in cell lines, also occurs in mature nerve terminals. This muscarinic inhibition of noradrenaline uptake would allow a cooperative action of ACh with noradrenaline where both cholinergic and noradrenergic innervations are significant, of which the most important site is arguably the sinoatrial node.

Conclusions

An optical method now exists to monitor NET transport rate at the level of the single nerve terminal varicosity, although the non-specific tissue labelling that remains complicates the analysis and forces the use of high-resolution microscopy. This experimental approach can be used to understand NET regulation, an approach we have started by showing a muscarinic inhibition of transporter rate in mature sympathetic terminals, and the action of drugs on mature sympathetic terminals. We have also shown the first measurements of lateral vesicle movement between autonomic varicosities,

with such limited exchange suggesting tight function compartmentalization of varicosities. In the future, such fluorescent NET and VMAT substrates will also be useful tools to study the vast reserve pool of vesicles in sympathetic terminals – a buffer for local and circulating catecholamines.

Acknowledgements

This work was supported by a Wellcome Trust research fellowship to KLB (074128); JK is supported by a BHF studentship (FS/07/041).

Conflicts of interest

None. We have no commercial interest or association with MDS Analytical Technologies, and no commercial interest in the NTUA.

References

Alexander SP, Mathie A, Peters JA (2008). Guide to receptors and channels (GRAC), 3rd edition. *Br J Pharmacol* **153** (Suppl. 2): S1–S209.

Apparsundaram S, Galli A, DeFelice LJ, Hartzell HC, Blakely RD (1998a). Acute regulation of norepinephrine transport: I. protein kinase C-linked muscarinic receptors influence transport capacity and transporter density in SK-N-SH cells. *J Pharmacol Exp Ther* **287**: 733–743.

Apparsundaram S, Schroeter S, Giovanetti E, Blakely RD (1998b). Acute regulation of norepinephrine transport: II. PKC-modulated surface expression of human norepinephrine transporter proteins. *J Pharmacol Exp Ther* **287**: 744–751.

Backs J, Bresch E, Lutz M, Kristen AV, Haass M (2005). Endothelin-1 inhibits the neuronal norepinephrine transporter in hearts of male rats. *Cardiovasc Res* **67**: 283–290.

Baldessarini RJ (2001). Drugs and the treatment of psychiatric disorders. In: Hardman JG, Limbird LE (eds). *Goodman and Gilman's the Pharmacological Basis of Therapeutics*, 10th edn. McGraw-Hill: New York, pp. 447–483.

Bauman AL, Apparsundaram S, Ramamoorthy S, Wadzinski BE, Vaughan RA, Blakely RD (2000). Cocaine and antidepressant-sensitive biogenic amine transporters exist in regulated complexes with protein phosphatase 2A. *J Neurosci* **20**: 7571–7578.

Bradley L, Doggrell SA (1985). Modification by desipramine of the effects of alpha-adrenoceptor antagonists on the contractile responses of the trisected rat vas deferens. *Gen Pharmacol* **16**: 475–482.

Brain KL, Bennett MR (1997). Calcium in sympathetic varicosities of mouse vas deferens during facilitation, augmentation and autoinhibition. *J Physiol* **502**: 521–536.

Brain KL, Cunnane TC (2008). Bretylium abolishes neurotransmitter release without necessarily abolishing the nerve terminal action potential in sympathetic terminals. *Br J Pharmacol* **153**: 831–839.

Brain KL, Trout SJ, Jackson VM, Dass N, Cunnane TC (2001). Nicotine induces calcium spikes in single nerve terminal varicosities: a role for intracellular calcium stores. *Neuroscience* **106**: 395–403.

Brain KL, Jackson VM, Trout SJ, Cunnane TC (2002). Intermittent ATP release from nerve terminals elicits focal smooth muscle Ca^{2+} transients in mouse vas deferens. *J Physiol* **541**: 849–862.

Cleary L, Vandeputte C, Docherty JR (2003). Investigation of postjunctional α_1- and α_2-adrenoceptor subtypes in vas deferens from wild-type and $\alpha_{2A/D}$-adrenoceptor knockout mice. *Br J Pharmacol* **138**: 1069–1076.

Cuprian AM, Solanki P, Jackson MV, Cunnane TC (2005). Cholinergic innervation of the mouse isolated vas deferens. *Br J Pharmacol* **146**: 927–934.

Cuprian-Beltechi AM, Solanki P, Teramoto N, Cunnane TC (2009). High spatial resolution studies of muscarinic neuroeffector junctions in mouse isolated vas deferens. *Neuroscience* **162**: 1366–1376.

Glowniak JV, Kilty JE, Amara SG, Hoffman BJ, Turner FE (1993). Evaluation of metaiodobenzylguanidine uptake by the norepinephrine, dopamine and serotonin transporters. *J Nucl Med* **34**: 1140–1146.

Gosling JA, Dixon JS (1972). Differences in the manner of autonomic innervation of the muscle layers of the guinea-pig ductus deferens. *J Anat* **112**: 81–91.

Hamberger B, Malmfors T, Norberg KA, Sachs C (1964). Uptake and accumulation of catecholamines in peripheral adrenergic neurons of reserpinized animals, studied with a histochemical method. *Biochem Pharmacol* **13**: 841–844.

Iversen LL (1967). *The Uptake and Storage of Noradrenaline in Sympathetic Nerves*. Cambridge University Press: London.

Jayanthi LD, Annamalai B, Samuvel DJ, Gether U, Ramamoorthy S (2006). Phosphorylation of the norepinephrine transporter at threonine 258 and serine 259 is linked to protein kinase C-mediated transporter internalization. *J Biol Chem* **281**: 23326–23340.

Jørgensen S, Nielsen EO, Peters D, Dyhring T (2008). Validation of a fluorescence-based high-throughput assay for the measurement of neurotransmitter transporter uptake activity. *J Neurosci Methods* **169**: 168–176.

Kaleczyc J (1998). Origin and neurochemical characteristics of nerve fibres supplying the mammalian vas deferens. *Microsc Res Tech* **42**: 409–422.

Kaschube M, Zetler G (1989). Noradrenergic, purinergic, and cholinergic transmission in the mouse vas deferens: influence of field-stimulation parameters, reserpinization, 6-hydroxydopamine and 4aminopyridine. *J Neural Transm* **76**: 39–53.

Kaye DM, Gruskin S, Smith AI, Esler MD (2000). Nitric oxide mediated modulation of norepinephrine transport: identification of a potential target for S-nitrosylation. *Br J Pharmacol* **130**: 1060–1064.

Kihara K (1998). Introduction to innervation of the vas deferens. *Microsc Res Tech* **42**: 387–389.

Kihara K, Sato K, Oshima H (1998). Sympathetic efferent pathways projecting to the vas deferens. *Microsc Res Tech* **42**: 398–408.

Kopin IJ, Hertting G, Gordon EK (1962). Fate of norepinephrine-H3 in the isolated perfused rat heart. *J Pharmacol Exp Ther* **138**: 34–40.

Ksiazek P, Buraczynska K, Buraczynska M (2006). Norepinephrine transporter gene (NET) polymorphism in patients with type 2 diabetes. *Kidney Blood Press Res* **29**: 338–343.

Lamont C, Vainorius E, Wier WG (2003). Purinergic and adrenergic Ca^{2+} transients during neurogenic contractions of rat mesenteric small arteries. *J Physiol* **549**: 801–808.

Mason JN, Farmer H, Tomlinson ID, Schwartz JW, Savchenko V, DeFelice LJ *et al.* (2005). Novel fluorescence-based approaches for the study of biogenic amine transporter localization, activity, and regulation. *J Neurosci Methods* **143**: 3–25.

Moura D, Azevedo I, Guimaraes S (1990). Differential distribution in, and release from, sympathetic nerve endings of endogenous noradrenaline and recently incorporated catecholamines. *Naunyn Schmiedebergs Arch Pharmacol* **342**: 153–159.

Pifl C, Drobny H, Reither H, Hornykiewicz O, Singer EA (1995). Mechanism of the dopamine-releasing actions of amphetamine and cocaine: plasmalemmal dopamine transporter versus vesicular monoamine transporter. *Mol Pharmacol* **47**: 368–373.

Ross SB, Gosztonyi T (1975). On the mechanism of the accumulation of 3H-bretylium in peripheral sympathetic nerves. *Naunyn Schmiedebergs Arch Pharmacol* **288**: 283–293.

Schroeter S, Apparsundaram S, Wiley RG, Miner LH, Sesack SR, Blakely

RD (2000). Immunolocalization of the cocaine-and antidepressant-sensitive l-norepinephrine transporter. *J Comp Neurol* **420**: 211–232.

Schwartz JW, Blakely RD, DeFelice LJ (2003). Binding and transport in norepinephrine transporters. Real-time, spatially resolved analysis in single cells using a fluorescent substrate. *J Biol Chem* **278**: 9768–9777.

Shannon JR, Flattem NL, Jordan J, Jacob G, Black BK, Biaggioni I *et al*. (2000). Orthostatic intolerance and tachycardia associated with norepinephrine-transporter deficiency. *N Engl J Med* **342**: 541–549.

Sung U, Apparsundaram S, Galli A, Kahlig KM, Savchenko V, Schroeter S *et al*. (2003). A regulated interaction of syntaxin 1A with the antidepressant-sensitive norepinephrine transporter establishes catecholamine clearance capacity. *J Neurosci* **23**: 1697–1709.

Sung U, Blakely RD (2007). Calcium-dependent interactions of the human norepinephrine transporter with syntaxin 1A. *Mol Cell Neurosci* **34**: 251–260.

Varoqui H, Erickson JD (1997). Vesicular neurotransmitter transporters. Potential sites for the regulation of synaptic function. *Mol Neurobiol* **15**: 165–191.

Williams DJ, Brain KL, Cunnane TC (2007). The effect of epibatidine on spontaneous and evoked neurotransmitter release in the mouse and guinea pig isolated vas deferens. *Br J Pharmacol* **150**: 906–912.

Yaris E, Kesim M, Kadioglu M, Kalyoncu NI, Ulku C, Ozyavuz R (2003). The effects of paroxetine on rat isolated vas deferens. *Pharmacol Res* **48**: 335–345.