Cardiac norepinephrine transporter protein expression is inversely correlated to chamber norepinephrine content

Erica A. Wehrwein,1 Lindsay M. Parker,1 Anna A. Wright,1 John M. Spitsbergen,2 Martin Novotny,3 Dagmar Babankova,4 Greg M. Swain,3 Beth A. Habecker,4 and David L. Kreulen1

1Department of Physiology, Michigan State University, East Lansing; 2Department of Biological Sciences, Western Michigan University, Kalamazoo; and 3Department of Chemistry and Neuroscience Program, Michigan State University, East Lansing, Michigan; and 4Department of Physiology and Pharmacology, Oregon Health and Sciences University, Portland, Oregon

Submitted 14 March 2008; accepted in final form 11 June 2008

NOREPINEPHRINE (NE) released from cardiac sympathetic nerve terminals is removed from the neuroeffector junction by the neuronal NE transporter (NET), which belongs to the large gene family of Na+/Cl−-dependent neurotransmitter transporters (8, 9, 40). NET is in sympathetic nerve fibers of the heart (4, 15, 33) and is enriched in nerve varicosities (43, 44). NET in sympathetic fibers is critical for removal of extracellular NE in the heart, and reuptake is reduced in sympathetically denervated animals (4, 18, 23). There are numerous studies that indicate the presence of NET in the heart and altered reuptake in the diseased heart (12, 13, 15, 17, 33). NE reuptake in the whole heart is highly efficient, and it is estimated that ∼90% of released NE is cleared by NET (14, 15). There is approximately a 20-to-1 ratio of released NE to NE “spillover,” in which only ∼1 out of 20 molecules of released NE is lost to the plasma (28). There are regional differences in NE reuptake among chambers of the heart and within a single heart chamber (5, 10), but it is not known whether this is related to chamber-specific differences in NET.

The majority of cardiac sympathetic axons and nerve terminals originate in the cell bodies of the bilateral stellate ganglia. Bilateral stellate ganglionectomy in the rat reduces cardiac NE content by 89–100% (41), suggesting that >90% of sympathetic innervation arises from the stellates. In the rat, all heart chambers receive bilateral innervation from the right and left stellate ganglia; however, the majority of the right ventricular innervation arises in the left stellate ganglion, and there is substantial ipsilateral innervation to the atrial appendages (41). The cardiac targets of stellate axons are the sinoatrial node, the conducting system, cardiomyocytes, and coronary vessels (49); however, there are cell bodies in the stellate complex that are noncardiac (1, 37, 49). It is unknown whether NET protein expression is different in the right vs. left stellate ganglia, but NET mRNA is expressed at higher levels in the right stellate ganglion (32).

Although NET is found in sympathetic fibers along with tyrosine hydroxylase (TH), it is not known whether the amount of NET protein is proportional to the NE content of the heart chambers. The atria are highly innervated by the sympathetic nervous system (25) and contain high levels of NE per gram of heart tissue (39); therefore it is likely that the atria contain more NET than the less-densely innervated ventricles. This idea is supported by the work of Lee et al. (31), which showed a positive relationship between NE content and NET, such that NET binding sites are reduced when NE is depleted with reserpine, while NET binding is increased when NE levels are raised by treatment with monoamine oxidase inhibitors. This is true for other transmitter systems as well. Acetylcholine release influences the uptake of choline by neurons, such that an increase in cholinergic activity and acetylcholine release is coupled to an increase in choline uptake and vice versa (24). Furthermore, NET knockout mice have reduced total heart NE (26). Taken together, we hypothesized that a positive correlation would exist between NET and NE with high levels of NET corresponding to the high NE content in the atria. The purpose of this study was to assess expression of NET protein in stellate ganglia and heart chambers, and determine whether cardiac NET protein expression was positively correlated to cardiac NE content.

MATERIALS AND METHODS

Animals

Adult male Sprague-Dawley rats (8 wk old, 250–275 g; Charles River, Portage, MI) were used. All animal experiments were per-
formed in accordance with the “Guide for the Care and Use of Laboratory Animals” (National Research Council) and were approved by the Animal Use and Care Committee of Michigan State University. Rats were anesthetized with a lethal dose of pentobarbital sodium (65 mg/kg ip; Sigma-Aldrich, St. Louis, MO) followed by thoracotomy. The dissected heart chambers were frozen immediately by contact with dry ice and stored at −80°C until further processing.

6-Hydroxydopamine Denervation Procedure

The neurotoxin 6-hydroxydopamine hydrochloride (6-OHDA; St. Louis, MO) was used as a NET substrate (29, 42, 48) to selectively impair sympathetic nerve terminals by depleting NE. Animals were randomly divided into control (untreated) and denervated groups. 6-OHDA was prepared in a mixture of 0.9% (154 mM) sodium chloride and 0.5% (28.4 mM) ascorbic acid solution fresh before each administration and kept protected from light. A subcutaneous injection of 6-OHDA (250 mg/kg) was administered to the loose skin between the shoulder blades with three consecutive injections during 1 wk on days 1, 3, and 5. On day 7, 2 days after the final dose, the animals were killed. Age- and sex-matched control animals were untreated.

Immunohistochemistry

Whole mount atria immunolocalization. After the hearts were removed, the left and right atria were dissected and rinsed to remove blood. Each atrium was fixed as a whole mount using Zamboni’s fixative (47) for 20 min at room temperature. Fixed atria were washed in PBS and incubated for 24 h (4°C) with primary antibody in PBS containing 1% BSA and 0.3% Triton X-100. Antibodies used were rabbit anti-NET 411 (43) (courtesy of R. D. Blakely, Vanderbilt University), mouse anti-TH (1:250) (Chemicon, Temecula, CA), and rabbit anti-TH (1:500) (Chemicon). After 24 h, atria were washed in PBS (3 × 5 min) followed by incubation for 2 h (25°C) in secondary antibody (goat anti-mouse IgG conjugated to Alexa Fluor 488; Molecular Probes, Carlsbad, CA) 1:1,000 dilution in PBS containing BSA (1%) and Triton (0.3%) and goat anti-rabbit IgG conjugated to Alexa Fluor 594 (Molecular Probes) 1:1,000 dilution in PBS containing BSA (1%) and Triton (0.3%). Atria were washed 3 × 5 min in PBS and were mounted on slides and viewed using a confocal microscope (model LSM 510; Zeiss). Filter settings for Alexa Fluor 488 were pass 505–530 and Alexa Fluor 594 were band pass 570–615. Samples were optically sectioned in 2-μm slices and then digitally reconstructed to attain a projection encompassing multiple layers of the tissue.

Ganglia immunohistochemistry. Stellate ganglia were fixed in 10% neutral buffered formalin for 2 h and then transferred to 70% ethanol, routinely processed, embedded in paraffin, and sectioned on a rotary microtome at 5 μm. Standard avidin-biotin complex staining steps were performed at room temperature. The polyclonal primary antibody NET 48411 was used (1:250 in normal antibody diluent; Scytek, Logan, UT). Biotinylated goat anti-rabbit IgG H+L (Vector, Burlingame CA) in normal antibody diluent 1:200 was applied for 30 min followed by application of Vectastain Elite ABC Reagent (Vector) for 30 min. Slides were developed using Nova Red Peroxidase substrate kit (Vector) for 15 min. Sections of right and left stellate ganglia from an animal were mounted on the same slide for a direct comparison under the same experimental conditions. Images were viewed using standard brightfield microscopy (model BX60; Olympus, Center Valley, PA). NET staining in ganglia was quantified by assessing all individual neuron cell bodies in a single section of stellate ganglia using NIH Image J Version 1.37 software. The nucleus did not stain positive for NET and was not included in the determination of staining intensity. The arbitrary intensity of NET staining was determined on color-inverted images by using the straight line measurement tool and drawing a line in a region defined by the user to contain cytoplasmic NET staining in every cell in a section from each ganglion. The mean intensity values in arbitrary units for individual neurons were then averaged to determine total NET staining intensity per ganglia. Staining intensity of right stellate ganglia was compared with left stellate ganglia using a paired t-test.

Tissue NE Content

Capillary electrophoresis with electrochemical detection (CE-EC) using a boron-doped diamond electrode was employed for NE determination in the heart chambers. The CE-EC system, electrochemical detection cell, and electrode fabrication are described elsewhere (11, 38). The protocol is similar to that described previously (39).

[^H]nisoxetine Binding

Preparation of cardiac membranes. Frozen heart chambers were pulverized on dry ice and then transferred to a mortar and pestle that had been prechilled with liquid nitrogen. Tissue was further processed by grinding and then suspended in the appropriate amount of homogenization buffer without detergent to keep membranes intact (50 mM Tris, pH 7.4, 120 mM NaCl, 5 mM KC). The suspended tissue in solution was transferred to an ice-cold 10 ml glass hand-held homogenizer for 10 strokes of further gentle processing in ice. For ventricular tissue, the homogenate was centrifuged (Sorvall RC 5B Plus) at 700 g for 10 min at 4°C to remove nuclei and cellular debris, and atrial membranes were used without centrifugation. Samples were spun at 40,000 g for 30 min after which the supernatant was discarded, and the pellet was resuspended in an additional 4 ml of buffer. A second identical spin was performed, the supernatant discarded, and the pellet stored at −80°C until use.

Binding assay. NET protein expression in cardiac membranes from individual heart chambers was estimated from maximum binding capacity (Bmax) values of full saturating binding curves using[^H]nisoxetine (Perkin-Elmer, Waltham, MA) in a manner similar to that described previously (33, 52). Frozen membrane pellets were resuspended in ice-cold incubation buffer (50 mM Tris, pH 7.4, 300 mM NaCl, 5 mM KC) on ice just prior to use. The resuspended membranes were loaded in quadruplicate into 96-well reaction plates and aliquots were used in parallel for a Bradford protein assay to determine protein concentration. Samples were assessed for total and background binding. Full saturating binding curves were run in duplicate using 0.37–50 nM[^H]nisoxetine; additional duplicate wells with 1.5 mM desipramine were used to determine nonspecific binding. Samples were incubated on a shaker for a minimum of 4 h at 0°C and then filtered through glass fiber filters presoaked in 0.5% polyethyleneimine using a 96-well Filtermate cell harvester (Packard Biosciences, Shelton, CT). Standard scintillation counting was performed using Ecolite scintillation fluid (ICN Biomedicals, Irvine CA).

NET Western Blotting and Antibody Verification

Methods for NET Western blotting and antibody verification are available at the AJP-Regulatory, Integrative and Comparative Physiology website in the online supplement.

Data Analysis

Data are presented as means ± SE for the number of animals. Statistical significance was assessed by a Student’s t-test or one-way ANOVA, where appropriate, using Prism 4.0 software (GraphPad Software, San Diego, CA). Data were statistically significant if P < 0.05.

RESULTS

NET in Sympathetic Fibers

Immunohistochemical localization using confocal microscopy was performed to assess the cellular site of NET protein
in the heart. In whole mount preparations of the atria, NET immunoreactivity was colocalized in nerve fibers with TH immunoreactivity (Fig. 1).

Chamber NE Content

The atria had a greater amount of NE per gram of tissue than the ventricles. NE values from heart chambers are similar to previously reported values (39): right atrium (RA), 2.387 μg/g tissue; left atrium (LA), 1.597 μg/g tissue; right ventricle (RV), 0.713 μg/g tissue; and left ventricle (LV), 0.3567 μg/g tissue. NE content of the RA was significantly higher than all other chambers (P < 0.05), while the LA was significantly higher than RV and LV (P < 0.05). NE tissue content per chamber was RV = LV (Fig. 2).

Stellate Ganglia Immunohistochemistry

NET immunoreactivity was present in all visible neuron cell bodies in sections of left and right stellate ganglia (n = 4 animals, Fig. 3, A and B). At high magnification, NET immunoreactivity was visible throughout the cytoplasm of neurons of the bilateral stellate ganglia with some membrane localization discernable (Fig. 3C). There was no difference in the intensity of the NET immunoreactivity between right and left stellate ganglia (n = 518 left stellate neurons, n = 596 right stellate neurons, Fig. 3E).

Fig. 1. Norepinephrine (NE) transporter (NET) immunoreactivity localized to sympathetic nerve fibers in atria. Fixed atria from normal rats were stained using NET 411 or tyrosine hydroyxylase (TH) primary antibodies with fluorescent secondary antibodies as described in MATERIALS AND METHODS. Samples were viewed as a whole mount using confocal microscopy. A: NET staining (red) is shown in nerve fibers throughout the atria. B: TH staining (green) in sympathetic nerve fibers in the atria. C: NET and TH are colocalized (yellow) confirming the presence of NET in sympathetic fibers in the atrium. Scale bar: 50 μm.

Fig. 2. NE content is greater in the atria than the ventricles. Capillary electrophoresis with electrochemical detection using a boron-doped diamond electrode was employed for NE determination in homogenized heart chambers. The amount of NE is highest in the right atrium (RA) and lowest in the left ventricle (LV), and the atria have more NE per gram of tissue than do the ventricles. NE content is expressed in μg NE/g tissue ± 95% confidence interval (n = 5 for each chamber). RV, right ventricle; LA, left atrium; *P < 0.05 vs. RA, &P < 0.05 vs. LA.

Relationship of Total NET Protein Determined by Antagonist Binding to Tissue NE Content

Cardiac membranes from all heart chambers were used in saturation binding assays with [3H]nisoxetine to estimate the B_max of NET binding sites per chamber. A representative specific binding curve (total binding minus nonspecific binding determined by addition of despiramine) is shown (Fig. 4A). B_max values obtained from each of four chambers from eight hearts were averaged to get mean B_max per chamber. There were significantly more NET binding sites in the LV than in any other heart chamber (Fig. 4B; RA, 221.8 ± 46.4; LA, 315 ± 91.25; RV, 418.2 ± 68.76; and LV, 768.2 ± 128.1 fmol binding/mg tissue). There was a significant negative correlation between NE content and NET binding (Fig. 4C, P = 0.04, r² = 0.922). In other words, the NE content is greatest in the atria where the NET binding is the lowest, and NE content is the least in the ventricles where NET binding is the highest. Although there was unequal distribution of NET in different parts of the heart, there were no corresponding differences in NET immunoreactivity in the right vs. left stellate ganglia (Fig. 3).

NE Depletion by 6-Hydroxydopamine

To assess functional relevance of high NET expression in the ventricles, we used the neurotoxic NET substrate, 6-OHDA, to deplete NE. The atria were less affected by treatment than the ventricles (Fig. 5). In the RA and LA, NE content was reduced by 68.5% (n = 5, P < 0.05) and 61.3% (n = 5, P < 0.05), respectively. In the RV (n = 5, P < 0.0001) and LV (n = 5, P < 0.0001), NE content was reduced below the limit of detection (RV, ≥ 93.4%; LV, ≥ 89.8%).

NET Western Blotting and Antibody Verification

We also attempted to quantify cardiac and stellate ganglion NET protein using a commercially available antibody. These data are available at the AJP-Regulatory, Integrative and Comparative Physiology website in the online supplement.
ganglion and project to the sweat glands and rib periosteum. Efferent neurons are found in loose clusters at the core of the ganglia that are NET-negative in the adult (16). These cholinergic cell bodies that were NET negative. (1). Presumably ganglion sections in this study did not contain cholinergic cell bodies that were NET negative.

**NET in Stellate Ganglia**

With an interest in the regional pattern of NET protein in the heart, we first aimed to determine whether there was a sidedness to NET protein amount in the stellate ganglia, which are believed to be the site of NET protein synthesis in cardiac sympathetic nerves. There was no difference in NET protein expression between the right and left stellate, even though there is higher NET mRNA in the right stellate ganglia (32). NET immunoreactivity in stellate ganglia was largely cytoplasmic; a distribution similar to that reported in cultured superior cervical ganglia, brain, and adrenal gland (27, 44). Although cytoplasmic localization predominates in cultured rat superior cervical ganglion cells, they have NE uptake capacity, indicating that NET is trafficked to the plasma membrane (17, 34, 36, 45). It is possible that NET trafficking and function in stellate ganglia occur in a similar manner. In contrast, in transfected HEK cells there is only limited cytoplasmic staining; cell surface NET staining predominates (43).

We observed that all cell bodies in the stellate ganglia were positive for NET; however, not all stellate neurons innervate the heart (37). Some noradrenergic neurons innervate the lung, blood vessels, and piloerector muscles. Also there is a small subset of nonadrenergic (cholinergic) neurons present in these ganglia that are NET-negative in the adult (16). These cholinergic neurons are found in loose clusters at the core of the ganglion and project to the sweat glands and rib peristomeum.

**Relationship of NET to NE Content**

As demonstrated by immunohistochemistry in this study and by others (33, 44), NET protein is found in sympathetic nerve terminals in the heart. The atria are richly innervated by the sympathetic nervous system (25) and contain high amounts of NE per gram (39). We hypothesized that dense sympathetic innervation would correlate with both high NET and NE, such that the atria would have high levels of both and the ventricles would contain less. Our measurements of NE content in the myocardium fit with previous findings that NE concentration is greatest in the atria and least in the ventricles (39, 51). Surprisingly, the total NET protein was highest in the ventricles and lowest in the atria; a negative correlation with NE content. It could be that high tissue levels of NE reduce NET expression; however, in the brain and salivary gland the effects of tissue NE on NET expression are controversial (31, 50); this has not been examined in the heart. Furthermore, since NE and NET differ in their mechanisms of synthesis, there is no reason to believe that synthesis of these two molecules would be directly related. Finally, cardiac NET protein is not exclusively localized to sympathetic fibers that contain NE (20).

Our report of ventricular predominance of NET has functional relevance, suggesting that there is a physiological role for high NET in the ventricles. The ventricles take up more NE per gram of tissue than the atria (19), and this is consistent with our findings of high levels of ventricular NET. Furthermore, the neurotoxin 6-OHDA, an NET substrate used to impair sympathetic terminals and reduce NE content (42, 48), has a greater toxic effect in the guinea pig ventricles compared with the atria (6), indicative of high levels of NET protein in the ventricles. This is similar to our findings in the rat.

---

**Fig. 3.** NET immunoreactivity in cell bodies of sympathetic neurons in the stellate ganglion. Stellate ganglia from normal adult animals were fixed, embedded in paraffin, and sectioned at 5 μM. NET 411 primary antibody was used with Nova Red chromagen such that NET immunoreactivity is shown in red. Images were captured using standard brightfield microscopy. NET immunoreactivity is observed in all neurons of: left stellate (×40 magnification; A); right stellate ganglia (×40 magnification; B); right stellate ganglion neurons at high magnification with cytoplasmic staining of NET observed surrounding large nuclei (×100 oil objective) (n = 4; C); no primary antibody control image counterstained with hematoxylin to show cellular structure and antibody specificity (D); and quantification of NET immunoreactivity indicates that there is no difference between left and right ganglia (P = 0.38; left stellate, n = 518 neurons; right stellate, n = 596 neurons; E). Scale bar = 10 μM.
Consistent with our report of ventricular predominance of NET protein, there are other examples of chamber and transmural differences in NE uptake (10, 22). In addition, there are chamber-specific changes in NET expression in pathologic heart conditions, such as right ventricular failure (35) and pressure overload hypertrophy (7). Since many studies examine whole heart NE uptake, these subtle regional differences may be missed.

**Alternative Sites of NET in the Heart**

It is possible that some of the NET examined in the study was from sources aside from sympathetic fibers. This is supported by findings in stellate ganglionectomized cats showing that uptake of NE still occurs in the sympathetically denervated heart (19), supporting the notion that NET may still exist. Nonneuronal cardiocytes (intrinsic cardiac adrenergic cells) containing NET protein have been described (20, 21). Some intrinsic cardiac neurons, although classically considered parasympathetic, have features of noradrenergic neurons, such as the expression of TH, suggesting that other noradrenergic properties, such as NET expression, would also be present (46). Also, sensory ganglia contain NET protein (30), so it is possible that sensory neurites may contain NET protein as well. These possibilities need to be investigated further.

**Summary**

NET immunoreactivity in the stellate ganglia did not predict a heterogeneous distribution of NET in the heart as both ganglia express the same amount of NET protein. Strikingly, even though NET protein was present in sympathetic nerve fibers colocalized to TH, the abundance of NET protein in the heart chambers was negatively correlated to NE content (i.e., the ventricles contained the most NET and the least NE). A neurotoxic NET substrate (29, 42, 48) reduced NE more in the ventricles than in the atria, supporting the idea that there is more functional NET protein in the ventricles. The higher uptake capacities for NE and 6-OHDA in the ventricles may be due to higher NET protein expression but are not related to stellate ganglion NET expression patterns. Since altered NET function plays a role in the diseased heart, it is important to recognize that the heart is not homogeneous in NE uptake function and that there may be some key regional changes in NET that have not been recognized in whole organ uptake studies.

**Perspectives and Significance**

The innervation of the heart is highly redundant, is not linear, and can display emergent properties (2, 3). Our findings of high NE content and low NET amount in atria vs. ventricles might suggest that with sympathetic stimulation the atrial rate might go up faster and maintain itself for substantially longer periods of time due to a combination of high NE and low NET.
however, this is not the case. Therefore, we must consider further the role of an inverse relationship of NE to NET in the heart chamber. Perhaps, the ventricular predominance of NET protein serves a protective role in preventing excess sympathetic stimulation by NE to the ventricles, which would lead to fatal arrhythmias.

ACKNOWLEDGMENTS

We thank the Michigan State University Histology Laboratory for tissue processing and Dr. Randy Blakely (Vanderbilt University) for the NET primary antibody. Barbara Grant, Dr. Gregory D. Fink, Mohammad Esfahani, and Josh Mastenbrook are recognized for general assistance and support of this project.

GRANTS

This work was supported by a American Heart Association predotoral fellowship (to E. A. Wehrwein) and National Heart, Lung, and Blood Institute Grants P01-HL-70687 (to D. L. Kreulen), HL-084258 (to G. M. Swain).

REFERENCES


