SYMPATHETIC INNERVATION, NOREPINEPHRINE CONTENT, AND NOREPINEPHRINE
TURNOVER IN ORTHOTOPIC AND SPONTANEOUS MODELS OF BREAST CANCER

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ABSTRACT

Activation of the sympathetic nervous system (SNS) drives breast cancer progression in preclinical breast cancer models, but it has yet to be established if neoplastic and stromal cells residing in the tumor are directly targeted by locally released norepinephrine (NE). In murine orthotopic and spontaneous mammary tumors, tyrosine hydroxylase (TH)+ sympathetic nerves were limited to the periphery of the tumor. No TH+ staining was detected deeper within these tumors, even in regions with a high density of blood vessels. NE concentration was much lower in tumors compared to the more densely innervated spleen, reflecting the relative paucity of tumor TH+ innervation. Tumor and spleen NE concentration decreased with increased tissue mass. In mice treated with the neurotoxin 6-hydroxydopamine (6-OHDA) to selectively destroy sympathetic nerves, tumor NE concentration was reduced approximately 50%, suggesting that the majority of tumor NE is derived from local sympathetic nerves. To evaluate NE utilization, NE turnover in orthotopic 4T1 mammary tumors was compared to spleen under baseline and stress conditions. In non-stressed mice, NE turnover was equivalent between tumor and spleen. In mice exposed to a stressor, tumor NE turnover was increased compared to spleen NE turnover, and compared to non-stressed tumor NE turnover. Together, these results demonstrate that NE in mammary tumors is derived from local sympathetic nerves that synthesize and metabolize NE. However, differences between spleen and tumor NE turnover with stressor exposure suggest that sympathetic NE release is regulated differently within the tumor microenvironment compared to the spleen. Local mammary tumor sympathetic innervation, despite its limited distribution, is responsive to stressor exposure and therefore can contribute to stress-induced tumor progression.

1. INTRODUCTION

Activation of the sympathetic nervous system (SNS), release of norepinephrine (NE), and adrenergic receptor (AR) signaling regulates solid tumor growth and metastasis (reviewed in (Cole et al., 2015)). AR-expressing cells reside within tumors and within extra-tumoral organs that receive abundant sympathetic innervation, such as spleen and bone marrow (Campbell et al., 2012; Katayama et al., 2006; Mendez-Ferrer et al., 2008). In ovarian and pancreatic tumors, psychosocial stressors and environmental conditions that activate the SNS elevate tumor NE concentration (Eng et al., 2015; Lutgendorf et al., 2011; Thaker et al., 2006). In prostate cancer, the density of tumor sympathetic nerves correlates with poor clinical outcomes (Magnon et al., 2013). These reports provide evidence for local release of NE within the microenvironment of solid tumors, but in breast cancer, local sympathetic innervation and release of NE have yet to be evaluated.

Several experimental approaches have been used to characterize sympathetic innervation and NE release in peripheral tissues. Immunohistochemical detection of tyrosine hydroxylase (TH), the rate-limiting enzyme in catecholamine synthesis, provides insight into the abundance and anatomical location of sympathetic nerves (Felten and Olschowka, 1987; Lorton et al., 2009). The neurotoxin 6-hydroxydopamine (6-OHDA) selectively ablates sympathetic nerves and markedly depletes NE in innervated organs (Lorton et al., 1990; Madden et al., 1994). 6-OHDA treatment has been used in experimental cancer models to investigate the impact of the SNS on solid tumor progression (Magnon et al., 2013; Raju et al., 2007). Tissue NE concentration is an indicator of the density of sympathetic innervation (e.g. see (Bellinger et al., 2008)), but the majority of tissue NE is located within the neuron and cannot directly be distinguished from released NE

(Eisenhofer et al., 2004). With SNS activation, tissue NE concentration can increase, decrease, or not change due to the dynamic balance between processes that regulate tissue NE concentration: NE synthesis, release, reuptake, and metabolism (Eisenhofer et al., 2004). Therefore, NE turnover has been used as an index of sympathetic activation under a variety of conditions (Bellinger et al., 2008; Jones and Musacchia, 1976; Migliorini et al., 1997).

NE turnover is measured by treating mice with α -methyl-*p*-tyrosine (AMPT), an inhibitor of TH. Inhibition of NE synthesis produces a decline in tissue NE. The rate of NE decline is a function of the rate of released NE and its subsequent metabolism. Quantitative measures of NE turnover include 1) turnover rate, the amount of NE synthesized and degraded per gram of tissue per hour, and 2) turnover time, the time required to synthesize the steady-state tissue pool of NE (Bellinger et al., 2008; Jones and Musacchia, 1976). Under conditions that activate the SNS, a higher rate of NE turnover indicates greater NE utilization as defined by the processes of synthesis, release, reuptake, and metabolism. Thus, NE turnover measured in mammary tumors is an indicator of NE synthesis and release from sympathetic nerve fibers within the tumor microenvironment.

Here we have characterized tumor sympathetic innervation, NE content, and NE turnover at baseline and with stress exposure in preclinical models of breast cancer. We have focused on an orthotopic mammary model commonly used to study highly metastatic breast cancer preclinically, the mammary adenocarcinoma 4T1. We have also investigated innervation and NE content in spontaneous mammary tumors from the MMTV-PyMT transgenic mouse. In this mouse line, over-expression of the polyoma middle T antigen in the mammary epithelium produces mammary tumors that progress from premalignant hyperplastic lesions to late stage metastatic disease, histopathologically mimicking human

breast cancer (Lin et al., 2003). The results demonstrate limited distribution of TH+ sympathetic nerves in orthotopic and spontaneous mammary tumors, but the capacity for NE turnover under baseline and stress conditions indicates that AR-expressing tumor cells and/or host stromal cells within mammary tumors can be targeted by local NE release.

2. MATERIALS AND METHODS

2.1 Mice. Female BALB/cByJ mice (6-8 weeks of age), NOD.SCID (6-8 weeks of age), and MMTV-PyMT mice (5-6 weeks of age) were purchased from The Jackson Laboratories (Bar Harbor, ME) and housed 3-4 per cage with food and water *ad libitum* on a 12:12 h light:dark cycle. Mice were adapted to the vivarium for 2 weeks prior to use. Mice were euthanized by pentobarbital overdose (200 mg/kg, intraperitoneally (IP)) followed by cervical dislocation. All procedures were approved by the University of Rochester Committee on Animal Resources.

2.2 Cell Lines. 4T1 tumor cells (ATCC CRL-2539) and MDA-MB-231 cells (ATCC CRM-HTB-26; referred to as MB-231) were purchased from American Tissue Type Collection (Manassas, VA). MDA-MB-231BR, a brain-seeking variant of MB-231 was obtained from Dr. T. Yoneda (University of Texas Health Science Center, San Antonio, TX). 4T1 was grown in RPMI containing L-glutamine and supplemented with 10% fetal calf serum (FCS) and penicillin and streptomycin (P/S). MB-231 and MB-231BR were maintained in DMEM containing L-glutamine and supplemented with 10% FCS and P/S. All cell lines were tested monthly for mycoplasma contamination and new cells were obtained from frozen stock after 12 weeks in culture.

2.3 Tumor Implantation. BALB/c mice were injected with 2×10^5 4T1 tumor cells in sterile saline into the right inguinal mammary fat pad (orthotopic injection) under isoflurane gas anesthesia. NOD.SCID mice were injected orthotopically with 1×10^6 MB-231 or MB-231BR cells under ketamine/xylazine (90/9 mg/kg IP) anesthesia.

2.4 Chemical Sympathetic Ablation. 6-hydroxydopamine (6-OHDA; Sigma-Aldrich, St. Louis, MO) was dissolved in sterile saline containing 0.01% ascorbate (Sigma-Aldrich) immediately prior to injection. In BALB/c mice, 6-OHDA was administered IP 4 and 2 days prior to orthotopic 4T1 tumor implantation and thereafter every 5 days to prevent reinnervation and maintain a long-term sympathectomy (Lorton et al., 1990). Vehicle controls were injected IP with 0.01% ascorbate in sterile saline. MMTV-PyMT mice were injected 2 times with vehicle or 100 mg/kg 6-OHDA (2 days apart) at 9 weeks of age and sacrificed 1 week after the second injection.

2.5 Tissue Harvest. Spleen and tumors were dissected free of fat and weighed. Larger spleens and tumors were divided into 100-200 mg pieces. In the NE turnover experiments, the entire spleen or tumor was homogenized for catecholamine determination. Tissue was frozen on dry ice and stored at -80°C until further processing.

2.6 NE Determination. Tissue was homogenized 10% w/v in ice-cold 0.01 N hydrochloric acid. Homogenates were kept on ice at all times to minimize catecholamine degradation. NE concentration was determined by ELISA (Rocky Mountain Diagnostics; Colorado Springs, CO) following the manufacturer's instructions. Appropriate homogenate dilutions were pre-determined. Absorption was measured at 450 nm using a multiwell plate reader

(Synergy HT, Biotek Instruments Inc, Winooski, VT). Curve fitting and sample concentration calculations were conducted with Gen5 software (Biotek).

In experiments evaluating the relationship between tissue NE concentration and tissue weights from MMTV-PyMT mice (Fig. 3G,H), NE concentration data was pooled from 3 different experiments using 3 different groups of mice ranging in age from 9 to 12 weeks old and 3 ELISAs. For the same comparison in 4T1 tumor and spleen (Fig. 3E,F), NE concentrations were determined in the same group of mice using a single ELISA.

2.7 Dual Stressor Exposure. See Fig. 6a for diagram. BALB/c mice were housed 3 per cage upon arrival. Two weeks later, mice in the stressed group were transferred to single housing (social isolation). Group-housed controls remained in their original housing. Six days after initiating social isolation, all mice were injected with 1×10^5 4T1 tumor cells orthotopically. Eight days later, singly-housed mice were subjected to restraint stress in a non-compressing 110-ml tube for 3 consecutive days for 2 h per day between 9 and 11 A.M. Mice were returned to their original home cage after each session. During this time period, group-housed controls remained in their home cages. Twenty-four h after the final restraint stress session, NE turnover was measured in all mice. This time point was chosen based on pilot studies demonstrating differences between stress-induced spleen and tumor NE concentration.

2.8 NE Turnover. NE turnover was determined based on previous publications (Bellinger et al., 2008; Jones and Musacchia, 1976; Vaughan et al., 2014). On the day of NE turnover determination, 4T1 tumor-bearing mice were divided into 3 groups: 1) No injection (No Inj); 2) injected IP with α -methyl-DL-p-tyrosine methyl ester HCl (300 mg/kg AMPT;

Sigma-Aldrich, St. Louis, MO dissolved in sterile saline); or 3) injected IP with sterile saline (VEH). Mice in the No Inj group were sacrificed at time 0 h. Mice in the VEH and AMPT groups were injected at time 0 and sacrificed 4 h later (Bellinger et al., 2008; Vaughan et al., 2014). Tumors and spleen were harvested from all mice for NE determination.

NE turnover rate, the rate constant, k_{NE} , and turnover time were determined by plotting $\log_{10}$ NE concentration versus time following AMPT treatment (Bellinger et al., 2008). Linear regression analysis of $\log[NE]$ versus time relationship was performed using individual data points, representing individual mice, obtained at 0 and 4 h after AMPT injection. The slope and standard error of the regression coefficient were computed by the least-squares method. $\log[NE]$ concentration at a give time, t , was determined using the equation $\log[NE] = \log[NE]_0 - 0.434k_{NE}t$ where $[NE]_0$ is the initial concentration of NE. $[NE]_0$ was determined by averaging NE concentration of the non-injected and VEH-injected groups. Based on this equation, the following variables were calculated:

Turnover rate (NE synthesized and degraded per gram of tissue per hour) = $[NE]_0 \times k_{NE}$

Turnover time (time required to synthesize the steady-state tissue pool of NE) = $1/k_{NE}$

Rate constant k_{NE} (fraction of the NE concentration lost per unit time) = $(-)\text{slope}/0.434$

2.9 Cytokine Quantification. Tumors were homogenized (10% w/v) in ice-cold RIPA buffer containing HALT protease inhibitor cocktail (Pierce, Rockford, IL). Vascular endothelial growth factor (VEGF) and IL-6 were measured using mouse-specific Quantikine ELISA kits (R & D Systems, Minneapolis, MN) according to the manufacturer's instructions. Absorption was measured at 450 nm using a multiwell plate reader (Synergy HT, Biotek Instruments Inc, Winooski, VT). Curve fitting and sample concentration calculations were conducted with Gen5 software (Biotek).

2.10 Imaging and Image Analysis. Tumors were fixed in 4% paraformaldehyde for 72 h, followed by incubation in 10% sucrose and 30% sucrose for 24 h each. Tissue sections (20 μ m thick) were washed 3X for 10 minutes each in phosphate buffered saline (PBS), then incubated in blocking buffer (5% normal goat serum, 0.2% Triton X-100, 0.5% bovine serum albumin in PBS) for 1 h at room temperature. For TH and CD31 blood vessel fluorescent detection in 4T1 and MMTV-PyMT tumors, tissue sections were incubated in blocking buffer containing primary antibodies against TH (1:100, chicken anti-tyrosine hydroxylase, Abcam ab76442) and CD31/PECAM-1 (1: 25, rat anti-CD31/PECAM-1, Abcam ab7388) overnight in a hydrated chamber at 4°C. Negative controls were tissue sections incubated in blocking buffer only with no primary antibody. Following overnight incubation, sections were washed 3X with PBS, then incubated with species-appropriate secondary antibodies (TH, 1:500, goat anti-chicken AlexaFluor 647; CD31/PECAM-1, 1:500, goat anti-rat AlexaFluor 594) in secondary antibody buffer (2% normal goat serum, 0.25% bovine serum albumin in PBS) for 1 h at room temperature. Tissue sections were rinsed 3 times with PBS, then mounted with DAPI-containing ProLong Gold anti-fade reagent (Life Technologies), cover-slipped, and stored in the dark at 4°C until epifluorescent or confocal imaging.

Epifluorescent images were taken on a BX51 Olympus microscope with appropriate emission filters and mounted with a Spot Pursuit RT color digital camera (Diagnostic Instruments, Sterling Heights, MI). Confocal images (Fig.1L-N) were collected using a Zeiss LSM 510 Meta confocal laser scanning microscope. Each fluorescence channel was structured as follows: DAPI: 405 nm diode laser excitation, 475 nm long-pass emission filter; AlexaFluor 488: 488 nm Argon laser excitation, 505 nm long-pass emission filter;

AlexaFluor 594: 543 nm Helium:Neon laser excitation, 505–550 nm band pass emission filter. All images were collected as 10 μm Z-axis stacks with 1 μm intervals at 1024x1024 pixel resolution with a 0.80 μsec pixel dwell time. The confocal pinhole diameter was set to 1 airy unit for all channels. Maximum projected images were subsequently false-colored, background corrected and despeckled using ImageJ software (NIH).

For DAB immunohistochemistry, MB-231 tumor tissue sections were incubated with 0.3% hydrogen peroxide in methanol for 30 minutes at room temperature followed by 3 washes for 5 minutes each with water to remove endogenous peroxidase activity. After blocking with blocking buffer for 1 h at room temperature, sections were incubated with rabbit anti-TH (1:500; Abcam) overnight at 4°C. The substrate reaction was developed using Vectastain ABC Elite kit with biotinylated goat anti-rabbit IgG (PK-4001; Vector Laboratories) following the manufacturer's instructions.

FITC-conjugated anti-F4/80 antibody (Abcam ab60343) was used to detect F4/80+ macrophages by epifluorescent imaging as described above. For image analysis using ImageJ, positive F4/80+ staining was quantified as follows: mean background signal was calculated as the average pixel value of 3 unstained tissue sections. F4/80-stained sections were masked to exclude non-tissue area, then each tissue section was thresholded to the mean background signal. Percent positive F4/80 staining was quantified as $[(\# \text{ pixels above mean background signal} / \text{pixel tissue area}) \times 100]$.

2.11 Statistical Analysis. Statistical analyses were conducted with GraphPad PRISM software. For two-group comparisons, an F-test for variance was conducted to compare variances. If variances were not significantly different, an unpaired two-tailed student's t-

test was employed. If variances differed ($p < 0.05$), group comparisons were conducted using the non-parametric Mann-Whitney U-test. One-way ANOVA was used to compare more than two experimental groups. Significant interactions or main effects were analyzed post-hoc by Holm-Sidak or Fisher's LSD tests. If variance differed significantly between groups, the non-parametric Kruskal-Wallis test was employed with significant effects analyzed by Dunn's multiple comparison test. Correlations between tissue NE concentration and the corresponding tissue mass in individual animals were calculated using the Pearson correlation coefficient. For all tests, $p < 0.05$ was considered statistically significant. The number of mice per group ranged from 6-11 as indicated in the figure legends.

3. RESULTS

3.1 Sympathetic TH+ innervation in Orthotopic Mammary Tumors and in Spontaneous MMTV-PyMT Mammary Tumors

Immunohistochemical staining was used to detect TH+ nerve fibers and CD31+ blood vessels in spleen and in orthotopic and spontaneous mammary tumors. The spleen is included for comparison in these studies because splenic sympathetic innervation, NE content and turnover have been previously described (Felten and Olschowka, 1987; Bellinger et al., 2008; Jones and Musacchia, 1976) and because the spleen is involved in mammary tumor progression (DuPre and Hunter, 2007). In spleens from 4T1-bearing mice, a dense network of TH+ fibers was detected surrounding blood vessel (Fig. 1A,B). By comparison, orthotopic mammary tumors displayed sparse TH+ innervation. Single TH+ nerve fibers were detected in orthotopic MB-231 (Fig. 1C), MB-231BR (Fig. 1D) and 4T1

mammary tumors (Fig. 1E-H). These fibers were limited to the outer regions of the tumor (Fig. 1E,F). As in the spleen, tumor TH+ fibers were often found in close proximity to CD31+ blood vessels (Fig. 1D,F,G) but were also found in the parenchyma distant from blood vessels (Fig. 1F,G). No TH+ innervation was detected towards the center of the tumor, even in non-necrotic areas with a high density of CD31+ blood vessels (Fig. 1H). In spontaneous MMTV-PyMT tumors, TH+ innervation was detected surrounding premalignant masses at 7 weeks of age coursing between adipocytes, detected as red autofluorescent spheres (Fig. 2A,B) and in regions devoid of CD31+ blood vessels (Fig. 2C). At the advanced carcinoma stage, TH+ innervation was sparse, but occasional single TH+ nerve fibers could be detected distant from tumor blood vessels (Fig. 2D-F) and associated with tumor blood vessels (Fig. 2G).

3.2 Reduced NE concentration with increased tissue mass

To determine if tumor NE concentration varies with size of the tumor, NE was measured in 4T1 and MMTV-PyMT mammary tumors over time. For comparison, splenic NE concentration was determined in the same mice. In 4T1 orthotopic tumors, increased tumor weight (Fig. 3A) was associated with reduced NE concentration (Fig. 3B). In 4T1-bearing mice, spleen mass increased with tumor growth (Fig. 3C). As spleen weight increased, splenic NE concentration decreased (Fig. 3C,D). Note that splenic NE concentration was ~10X greater than tumor NE, reflecting the higher density of sympathetic innervation. A significant inverse relationship between tissue weight and NE concentration was observed for 4T1 tumors (Fig. 3E) and spleen (Fig. 3F). In MMTV-PyMT mice, the relationship between individual MMTV-PyMT tumor or spleen weight and the corresponding NE concentration was plotted using MMTV-PyMT mice ranging in age from

9-12 weeks, representing early to late stage adenocarcinoma. A trend toward an inverse relationship in tumor and spleen weight versus NE concentration was detected in MMTV-PyMT mice (Fig. 3G,H; Pearson correlation, $p=0.07$; $p=0.052$, respectively).

3.3 Chemical ablation of sympathetic nerves depletes NE in mammary tumors

To determine if NE content in breast tumors is sensitive to ablation of sympathetic nerves, mice were treated with the selective neurotoxin 6-OHDA (Kostrzewa and Jacobwitz, 1974; Lorton et al., 1990). In 4T1-bearing mice treated with 6-OHDA throughout the 3-weeks of tumor growth, 4T1 tumor NE concentration was reduced $\sim 45\%$ (Fig. 4A) and $\sim 75\%$ in the spleen (Fig. 4B) compared to vehicle controls. In 9-10 week old MMTV-PyMT mice treated for one week with 6-OHDA, sympathectomy reduced tumor and spleen NE $\sim 60\%$ and $\sim 80\%$, respectively (Fig. 4C,D). Furthermore, in sympathectomized BALB/c mice, 4T1 tumor and spleen weight were significantly reduced at sacrifice (Fig. 4E,F). In conjunction with the 6-OHDA-induced reduction in 4T1 tumor weight, sympathectomy decreased the density of F4/80+ tumor associated macrophages (Fig. 4G) and reduced tumor IL-6 (Fig. 4H) along with a non-significant decrease in tumor VEGF (Fig. 4I). No alterations in 4T1 tumor metastases were detected in 6-OHDA-treated mice compared to vehicle controls (data not shown).

3.4 NE Turnover in 4T1 Mammary Tumors

NE turnover was measured to determine if mammary tumor sympathetic innervation has the capacity to regulate steady state NE under baseline conditions and with stress exposure. In these experiments, NE was measured in 3 groups of mice (1) not injected (No Inj) and sacrificed at time 0, (2) VEH or (3) AMPT injected at time 0 and sacrificed 4 later. No Inj and VEH groups were used to test the assumption that tissue NE

was at steady state over the course of the 4 h turnover experiment (Bellinger et al., 2008; Jones and Musacchia, 1976). In the first experiment, NE turnover was determined in non-stressed group-housed BALB/c mice 8 days after tumor cell injection. Tumor and spleen weights did not differ between groups (data not shown). AMPT treatment to inhibit NE synthesis significantly reduced tumor (Fig. 5A; ANOVA, $p < 0.0001$) and spleen NE (Fig. 5B; Kruskal-Wallis, $p = 0.002$) compared to the No Inj and VEH groups. Tumor and spleen NE concentration did not differ significantly between No Inj and VEH groups indicating that steady state assumptions were met; therefore these groups were combined to calculate NE concentration at time 0. The slope of the line generated by plotting individual log NE concentrations at time 0 (No Inj + VEH groups) and 4 h (AMPT group) was determined by linear regression analysis (Fig. 5C,D). In both spleen and tumor, the slope of the lines differed significantly from no decay, i.e. slope = 0 ($p < 0.05$; Fig. 5C,D, Table 1). NE turnover time and the turnover constant k_{NE} were equivalent between spleen and tumors (Table 1). Note that the NE turnover rate, the product of $[NE]_0$ (average of No Inj and VEH groups) and k_{NE} , was greater in the spleen reflecting higher splenic NE concentration compared to tumor NE concentration (Table 1).

Next, NE turnover was measured in 4T1 tumor-bearing mice exposed to a dual stressor consisting of socially isolated mice exposed to acute restraint stress (2 h restraint stress per day, 3 consecutive days; see timeline in Fig. 6A). Tumor cells were injected orthotopically 6 days after initiating social isolation, and NE turnover was measured 8 d later, 24 h after the final restraint stress session. Stressed mice were divided into the 3 groups described above: No Inj, VEH, and AMPT. An additional non-stressed, group-housed (GH) cohort was sacrificed at time 0. Tumor and spleen weights did not differ between

groups (data not shown). In the tumors, NE concentration differed significantly across groups (Fig. 6B; ANOVA, $p=0.002$). In the stressed mice, AMPT reduced tumor NE compared to the VEH group and the No Inj group (Fig. 6B). Because the No Inj and VEH groups did not differ, these groups were combined at time 0, and the slope of NE decay was determined. In the tumors, the slope of the line was significantly different from zero ($p=0.0008$; Fig. 6C; Table 2). By contrast, splenic NE concentration did not differ significantly across groups (Fig. 6D; ANOVA $p=0.13$). To determine the slope of the NE decay line, the stressed No Inj and VEH groups were combined for time 0. For the spleen, the slope of the line did not differ significantly from zero ($p=0.09$; Fig. 6E; Table 2). The splenic NE turnover time was much longer (20.8 h) than tumor NE turnover time (5.8 h) (Table 2). Differences in the response between spleen and tumor were also noted in the measures of tissue NE. Tumor NE concentration in the stressed No Inj group was elevated compared to the non-injected, group-housed mice (Fig. 6B). This elevation was not apparent in the spleen (Fig. 6D).

To compare NE turnover rates in the non-stressed mice to the stressed mice, NE turnover rates in the stressed mice were divided by the corresponding NE turnover rates in the non-stressed mice. With stressor exposure, tumor NE turnover rate increased 2.7-fold compared to that of non-stressed mice (stressed, 5.6 vs. non-stressed, 2.1; Tables 1,2). In striking contrast, stressor exposure reduced NE turnover rate in the spleen to 20% that of non-stressed mice (stressed, 23.7 vs. non-stressed, 118.6; Tables 1,2). Thus, 24 h after the final restraint stress exposure, mammary tumors display greater NE utilization compared to baseline conditions, but in the same mice, NE turnover is greatly reduced in the spleen.

This result suggests different mechanisms regulate tumor and spleen NE release with stressor exposure.

4. DISCUSSION

Preclinical models have linked SNS activation to breast cancer progression and metastasis (Shakhar and Ben-Eliyahu, 1998; Sloan et al., 2010; Szpunar et al., 2013; Thaker et al., 2006), but the presence of mammary tumor sympathetic innervation and its capacity to respond to stress exposure have not been described. We detected sparse sympathetic nerve fibers in orthotopic and spontaneous murine mammary tumors that correlated with low tumor NE concentration. Ablation of sympathetic nerves reduced tumor NE by approximately 50% indicating that sympathetic innervation is the source of the majority of NE in mammary tumors. NE utilization, as measured by NE turnover, was readily detected in mammary tumors under baseline conditions and with stressor exposure. Moreover, stress-induced tumor NE turnover was greater in tumors compared to spleen, suggesting that the tumor microenvironment regulates stress-induced NE release and availability. Thus, SNS activation and NE release within mammary tumors must be considered as a local mechanism by which stressor exposure regulates mammary tumor progression.

4.1 Limited distribution of sympathetic nerve fibers in mammary tumors and functional impact of peripheral SNS ablation

The limited distribution of sympathetic TH+ nerve fibers to the outer regions of orthotopic mammary tumors suggests that NE release may regulate entrance of stromal cells into the tumor and tumor/stromal cell activity in a highly localized area. No sympathetic TH+ innervation was detectable towards the center of the tumor, even in

regions with dense vascularity. This parallels our unpublished observations in core biopsies from human breast tumors where we have been unable to detect TH+ innervation.

In MMTV-PyMT mice, mammary tumor development closely mimics clinical breast cancer stages of development histologically and phenotypically (Lin et al., 2003). Sympathetic innervation was detected during premalignancy, coursing amongst adipocytes. This finding raises the possibility that SNS hardwiring of mammary adipose tissue is a mechanism by which catecholamines can regulate adipocytes and, mammary adipose tissue metabolism to modulate early mammary tumor progression (Volden et al., 2013; Zhang et al., 2009). The sparse TH+ sympathetic innervation detected in late-stage adenocarcinoma reflects the restricted distribution of sympathetic innervation to blood vessels and smooth muscle in the normal mammary gland (Eriksson et al Neuroscience, 1996). This limited innervation contrasts to the dense sympathetic innervation found in other types of solid tumors, such as pancreatic and prostate cancer, where the healthy tissues receive rich sympathetic innervation. Like breast cancer, SNS activation facilitates tumor progression in pancreatic and prostate cancers (Kim-Fuchs et al., 2014; Magnon et al., 2013). Pancreatic and prostate tumors also exhibit a pathologic feature called perineural invasion that is not frequently present in breast cancer. In perineural invasion, tumor cells penetrate the neural sheath, and use the nerves to spread to distant sites. In pancreatic and prostate cancer, perineural invasion is detected at high frequency and is associated with poor prognosis (Ayala et al., 2008; Liebig et al., 2009). By contrast, perineural invasion has been described in a low frequency of breast tumors, and is not prognostic in breast cancer (Duraker et al., 2006; Liebig et al., 2009). These distinctions, together with the scarcity of sympathetic TH+ nerves in the preclinical mouse models

described here, provide evidence that nerves are not well integrated into mammary tumors in clinical breast cancer.

Consistent with the low density of nerve fibers, NE concentration in orthotopic and spontaneous mammary tumors was low compared to the more densely innervated spleen. In 4T1 tumor-bearing mice, tumor and spleen NE concentration decreased as tissue volume increased over time. A similar relationship has been demonstrated in enlarged draining lymph nodes in response to immunization (Lorton et al., 1997). In MMTV-PyMT mice, a trend toward an inverse association was detected between tumor weight and tumor NE. The smaller range of MMTV-PyMT tumor weights, the range of tumor stages (from early to late carcinoma), and pooling 3 separate experiments may have contributed to variability that reduced our ability to detect a significant association.

The declining NE concentration with tumor expansion combined with the paucity of tumor TH+ nerves in locations other than the tumor periphery demonstrate that sympathetic nerves are not incorporated into the expanding tissue volume. Breast tumors may not release sufficient guidance/supporting factors to direct sympathetic nerves into the tumor, despite evidence for nerve growth factor expression by breast cancer cells (Dolle et al., 2003). Alternatively, the inflammatory microenvironment of the tumor may actively repel sympathetic nerves as described in rheumatoid arthritis (Klatt et al., 2012). These results raise the possibility that the impact of local sympathetic innervation of the tumor may be greatest early in tumor growth, as has been demonstrated in preclinical prostate cancer (Magnon et al., 2013). Based on restricted mammary tumor sympathetic innervation, we speculate that as the tumor grows, a greater proportion of AR-expressing target cells become more distant from the site of released NE. In response to diminished NE

availability, AR sensitivity would increase, but AR-expressing cells distant from nerve fibers would be unlikely to encounter released NE. In the context of tumor cell AR signaling, however, the impact of circulating catecholamines is currently unknown.

Despite the low tumor NE concentration, ablation of sympathetic nerve fibers further reduced NE concentration in 4T1 and MMTV-PyMT mammary tumors. This result indicates that the majority of NE present within orthotopic mammary tumors is derived from sympathetic nerve fibers. The relatively inefficient 6-OHDA-induced NE depletion in tumors compared to spleen may be due to several reasons. 1) A proportion of tumor NE may be derived from circulating NE derived from the adrenal medulla, which is not susceptible to sympathetic nerve ablation (Kostrzewa and Jacobwitz, 1974). We do not believe circulating NE is a significant contributor to tumor NE in sympathectomized mice because plasma NE did not differ between vehicle and 6-OHDA treated mice (data not shown). 2) The detection limit of the ELISA (20 pg/ml NE) would preclude measurement of very low levels of NE. 3) NE may be derived from potential non-neuronal cell sources within the tumor (Flierl et al., 2007; Jenei-Lanzl et al., 2015), although non-neuronal cells expressing TH were not detected immunohistochemically. 4) 6-OHDA may not sufficiently penetrate the tumor parenchyma to access all sympathetic nerves. Poor tissue perfusion leads to organs described as '6-OHDA-resistant' (Kostrzewa and Jacobwitz, 1974). In these organs, NE depletion is not as great as '6-OHDA-sensitive' organs such as the spleen. Leaky tumor vessels and poor delivery of chemotherapeutic drugs described in solid tumors (Jain, 2001) may similarly preclude effective delivery of 6-OHDA.

In 4T1-bearing mice, sympathetic nerve ablation reduced tumor weight in association with decreased F4/80+ tumor associated macrophages and tumor IL-6. These

results are consistent with other reports of 6-OHDA-induced reductions in tumor progression (Magnon et al., 2013; Raju et al., 2007) and links between β -AR activation and tumor associated macrophages (Sloan et al., 2010). However, because sympathetic ablation is not restricted to the tumor, it is not possible to attribute the 6-OHDA-induced effects solely to loss of tumor sympathetic innervation. For example, SNS activation may also facilitate 4T1 tumor progression by regulating hematopoietic cell differentiation and trafficking patterns between bone marrow, spleen, and tumor (Katayama et al., 2006; Mendez-Ferrer et al., 2008). Ablation of sympathetic innervation of the bone marrow and spleen may reduce the tumor-promoting hematopoietic response and thereby reduce tumor mass. Furthermore, our finding that 4T1 metastasis to the lung was not altered in sympathectomized mice was unexpected. Given the evidence that β -AR or α 2-AR activation increased tumor metastasis to the lung in orthotopic breast tumor models (Sloan et al., 2010; Szpunar et al., 2013), we predicted that loss of sympathetic input would reduce lung metastases. One possible explanation is that 4T1 cells express no functional AR (Szpunar et al., 2013), unlike the mammary tumor line used by Sloan and colleagues, and the inability of sympathetic ablation to modulate metastasis may reflect the inability of 4T1 tumor cells to respond directly to SNS activation. Another explanation for the inability of sympathectomy to alter lung metastasis is compensatory adrenal corticosterone or epinephrine production in sympathectomized mice that may minimize the impact of loss of sympathetic input on metastatic processes. These possibilities remain to be investigated.

4.2 NE turnover in mammary tumors

NE turnover in mammary tumors provides further evidence that tumor NE is derived from sympathetic nerves. The results provide an indication of the dynamic range of

tumor sympathetic nerve activity under baseline and stress conditions. Under baseline conditions, splenic NE turnover rate and turnover time determined in the present report were equivalent to previous reports (Bellinger et al., 2008; Jones and Musacchia, 1976). NE turnover in mammary tumors was equivalent to the spleen. With stressor exposure, splenic NE decay was much slower in stressed mice compared to non-stressed mice. This reduced NE turnover in the spleen occurred 24 h after the last restraint stress exposure, and may reflect a recovery mechanism to restore splenic NE to steady state. On the other hand, stress-induced NE turnover at this time point was significantly greater in tumors compared to spleen, in conjunction with elevated tumor NE concentration in stressed mice compared to non-stressed controls. The significance of the differences in NE turnover between tumor and spleen with regard to regulation of the host response to tumor progression remains to be determined.

The differences in tumor versus spleen NE turnover with stress exposure may reflect unique features of the tumor microenvironment. First, low tumor sympathetic nerve density and NE concentration may lead to differential regulation of NE release in the tumor compared to the spleen. In the spleen, presynaptic α_2 -AR autoinhibition of NE release may be active, limiting stress-induced NE release (Trendelenburg et al., 1999). In contrast, tumor NE may not be sufficient to activate α_2 -AR autoinhibition, producing a more sustained NE release and a greater dependence on NE synthesis to maintain NE pools with stressor exposure. A second possibility is the proinflammatory tumor microenvironment that includes the cytokines G-CSF, TNF- α and IL-1 β , all of which have been demonstrated to modify NE, NE release or reuptake (Katayama et al., 2006; Lucas et al., 2012; Reynolds et al., 2005; Tsumori et al., 1998). Third, hypoxia and acidosis can regulate TH expression and

catecholamine release. The low oxygen/low pH microenvironment within the expanding tumor may differentially regulate NE synthesis and release in tumors compared to that of the normoxic spleen (Fernandez-Morales et al., 2014; Gozal et al., 2005; Hui et al., 2003; Rico et al., 2005). Alternatively, the differences in NE turnover between tumor and spleen may reflect regulation at the level of the central nervous system leading to differential sympathetic output (Kvetnansky et al., 2009).

In summary, we have demonstrated that sympathetic innervation is the primary source of NE in orthotopic and spontaneous mammary tumors. Importantly, mammary tumor NE content and turnover was modulated by stressor exposure. Stress-induced SNS activation differed between tumors and spleen, suggesting differences in NE availability with stress exposure. Limited sympathetic innervation and decreasing NE concentration with tumor growth implies that the impact of released NE is diminished as the tumor volume expands and the distance between sympathetic nerve fibers and AR-expressing target cells becomes greater. Nonetheless, the demonstration of tumor NE turnover suggests locally released NE can directly target AR-expressing cells. Our results demonstrate that assessment of NE release and availability is critical for establishing local release of NE within the primary tumor and within extra-tumoral organs that participate in tumor progression in order to propose safe and effective therapies targeting NE release and AR activation in cancer treatment.

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FIGURE LEGENDS

Fig. 1. TH+ sympathetic innervation in spleen and orthotopic mammary tumors.

(A, B) High density TH+ sympathetic innervation (green) associated with CD31+ blood vessels (red) in the spleen of a 4T1-bearing mouse. (C) TH+ innervation (brown) in an MB-231 orthotopic tumor with DAB detection. (D) TH+ innervation surrounding a large blood vessel in an MB-231BR orthotopic tumor. (E,F,G) Orthotopic 4T1 tumor TH+ nerve fibers at 10X (E), 20X (F), and 40X (G). Region indicated in (E) shown at 20X in (F). White line in (E) indicates border of tumor. In (F, G), white arrows depict TH+ nerve fibers not closely associated with blood vessel. Yellow arrows show CD31+ blood vessels not closely associated with TH+ nerve fibers. White arrowheads indicate TH+ nerves located near blood vessels. (H) Region of CD31+ blood vessels in 4T1 tumor with no TH+ innervation. Magnification and scale indicated in each image

Fig. 2. TH+ sympathetic innervation in spontaneous mammary tumors from

MMTV-PyMT mice. TH+ innervation in MMTV-PyMT mice at 7 weeks (A-C) and 15 weeks (D-G) of age. In (D-G), DAPI nuclear stain was used to detect tumor cells. (E,F) White arrows indicate TH+ innervation not associated with CD31+ blood vessels. (G) White arrowheads indicate TH+ innervation associated with CD31+ blood vessels. All fluorescent images except D-F were imaged using an epifluorescent microscope. Confocal images (D-F) were processed as described in methods. Magnification and scale indicated in each image.

Fig. 3. NE concentration declines in mammary tumors with increasing tumor mass.

Orthotopic 4T1 tumor (A) weight and (B) NE concentration, and (E) inverse

relationship between tumor weight and NE concentration. In the same animals, splenic (C) weight and (D) NE concentration, and (F) the inverse relationship between spleen weight and NE concentration. In MMTV-PyMT mice, (G) tumor and (H) spleen NE concentration is weakly inversely related to tissue NE concentration. In A-D, results expressed as mean $\pm$ SEM, n=5-6 per group at each time point. The data from each individual animal in A-D was used to create the scatter plots in E, F. For G, H, tissue weights and the corresponding NE concentration from individual MMTV-PyMT mice aged 9-12 weeks. Statistical analysis: (A) ANOVA, $p < 0.0001$; Holm-Sidak's test, *** $p < 0.001$; ** $p < 0.01$; * $p < 0.05$; (B) Kruskal-Wallis, $p = 0.0008$; Dunn's multiple comparison test * $p < 0.05$; (C) Kruskal-Wallis, $p < 0.0001$; Dunn's * $p < 0.05$; (D) ANOVA, $p = 0.0006$; Holm-Sidak's test, * $p < 0.05$; (E-H) Linear regression analysis with Pearson correlation.

Fig. 4. Sympathetic Ablation Depletes NE in 4T1 and MMTV-PyMT Mammary

Tumors. NE concentration in tumor and spleen from (A, B) 4T1-bearing mice and (C, D) MMTV-PyMT mice. (E) 4T1 tumor and (F) spleen weight. 4T1 tumor (G) F4/80+ staining and quantification, (H) IL-6, and (I) VEGF. Results expressed as mean $\pm$ SEM, n=9-10 for 4T1-BALB/c mice; n=3-4 for MMTV-PyMT mice. Asterisks indicate significance based on t-test or non-parametric Mann-Whitney (M-W) U test ($p < 0.05$).

Fig. 5. AMPT treatment reduced NE in 4T1 Tumors and Spleen of Non-stressed

BALB/c mice. NE turnover was determined 8 d after orthotopic injection of 4T1 cells. NE concentration was determined in (A) tumor and (B) spleen in mice that were not injected (No Inj) and sacrificed at time 0 or injected with VEH or AMPT at time 0 and

sacrificed 4 h later. NE decay over time in (C) tumor and (D) spleen was determined by combining mice from No Inj and VEH groups for time 0; mice from the AMPT-injected group make up the 4 h time point (individual mice are represented by circles).

Statistical analyses: A) ANOVA, main effect of treatment, $p < 0.0001$; $**p < 0.01$, $****p < 0.0001$ by Sidak's multiple comparisons test; B) non-parametric Kruskal-Wallis (K-W), main effect of treatment, $p = 0.002$; $*p < 0.05$, $**p < 0.01$ by Dunn's multiple comparison. For (C, D), the p-values shown represent comparison of the slope to horizontal (slope = 0). Results expressed as mean $\pm$ SEM. No Inj, $n = 6$, vehicle, $n = 9$; AMPT, $n = 9$.

Fig. 6. NE turnover in Stress-Exposed BALB/c Mice. (A) Timeline for dual stress exposure; SI = social isolation; RS = restraint stress; NE turnover was measured 24 h after the final restraint stress exposure in orthotopic 4T1 tumors and spleen. (B) Tumor NE concentration; (C) Tumor NE decay; (D) Spleen NE concentration; (E) Spleen NE decay. In (C, E) individual mice (circles) from No Inj and VEH groups were combined for time 0; mice from AMPT group make up the 4 h time point. Statistical analyses: (B) ANOVA, main effect of treatment, $p = 0.002$; $*p < 0.05$, $**p < 0.01$, $***p < 0.001$ by Fisher's LSD test; (D) ANOVA, $p = 0.13$; (C, E) Slope of the line was determined by regression analysis. The p-values shown represent comparison of the slope to horizontal (slope = 0). Results expressed as mean $\pm$ SEM, $n = 9-11$ per group.

Table 1

NE Turnover in Spleen and 4T1 Orthotopic Tumors from Non-Stressed BALB/c Mice

	Negative Slope	SEM	kNE $=slope/0.434$	NE turnover time (h) $= 1/kNE$	$[NE]_0$ (ng/g wet wt)	NE turnover rate (ng/g/wet wt/h) $= [NE]_0 * kNE$
TUMOR	0.05829	0.0118	0.1343	7.44	16.0	2.1
SPLEEN	0.05818	0.0121	0.1341	7.46	884.4	118.6

Table 2

NE Turnover in Spleen and 4T1 Orthotopic Tumors with Stressor Exposure

	Negative Slope	SEM	kNE =slope/0.434	NE turnover time = 1/kNE	[NE]₀ (ng/g wet wt)	NE turnover rate (ng/g/wet wt/h) = [NE] ₀ *kNE
TUMOR	0.0750	0.0197	0.1727	5.8	32.3	5.6
SPLEEN	0.0209	0.0120	0.0482	20.8	492.3	23.7

BALB/c-4T1

MMTV-PyMT