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Localization of endogenous amyloid- β to the coeruleo-cortical pathway: consequences of noradrenergic depletion

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Abstract

The locus coeruleus (LC)-norepinephrine (NE) system is an understudied circuit in the context of Alzheimer's disease (AD), and is thought to play an important role in neurodegenerative and neuropsychiatric diseases involving catecholamine neurotransmitters. Understanding the expression and distribution of the amyloid beta ($A\beta$) peptide, a primary component of AD, under basal conditions and under conditions of NE perturbation within the coeruleo-cortical pathway may be important for understanding its putative role in pathological states. Thus, the goal of this study is to define expression levels and the subcellular distribution of endogenous $A\beta$ with respect to noradrenergic profiles in the rodent LC and medial prefrontal cortex (mPFC) and, further, to determine the functional relevance of NE in modulating endogenous $A\beta_{42}$ levels. We report that endogenous $A\beta_{42}$ is localized to tyrosine hydroxylase (TH) immunoreactive somatodendritic profiles of the LC and dopamine- β -hydroxylase (D β H) immunoreactive axon terminals of the infralimbic mPFC (ILmPFC). Male and female naïve rats have similar levels of amyloid precursor protein (APP) cleavage products demonstrated by western blot, as well as similar levels of endogenous $A\beta_{42}$ as determined by enzyme linked immunosorbent assay. Two models of NE depletion, DSP-4 lesion and D β H knockout (KO) mice, were used to assess the functional relevance of NE on endogenous $A\beta_{42}$ levels. DSP-4 lesioned rats and D β H KO mice show significantly lower levels of endogenous $A\beta_{42}$. Noradrenergic depletion did not change APP cleavage products resulting from β -secretase processing. Thus, resultant decreases in endogenous $A\beta_{42}$ may be due to decreased neuronal activity of noradrenergic neurons, or, by decreased stimulation of adrenergic receptors which are known to contribute to $A\beta_{42}$ production by enhancing γ -secretase processing under normal physiological conditions.

Keywords

Amyloid; Norepinephrine; Dopamine- β -hydroxylase; Adrenergic receptors; Stress

INTRODUCTION

Neuropeptides are unique and diverse-acting transmitters that may exert prolonged effects in modulating neuronal activity and behavior. A relatively new addition to the repertoire of over 100 known physiological neuropeptides is amyloid beta (A β), the protein originally identified as the monomeric subunit of the extracellular plaques that are a primary component of Alzheimer's disease (AD) neuropathology. Over a decade has passed since the recognition of A β as an endogenous neuropeptide that undergoes physiological metabolism in the central nervous system (Haass, Schlossmacher et al. 1992, Seubert, Vigo-Pelfrey et al. 1992, Shoji, Golde et al. 1992); and yet its physiological function is not well understood. The A β peptide is derived from the type I transmembrane protein amyloid precursor protein (APP) composed of 695–770 amino acids, and may undergo proteolytic cleavage via two divergent pathways (Haass, Schlossmacher et al. 1992). Under physiological conditions, a majority of APP is processed via the non-amyloidogenic pathway, in which APP is cleaved by α -secretases present on the plasma membrane, resulting in the formation of sAPP α and the carboxyl terminal C83 fragment. Once APP undergoes proteolysis by α -secretases, it can no longer form the A β fragment because cleavage occurs within the A β domain (LaFerla, Green et al. 2007). In the amyloidogenic pathway, APP is a substrate for the aspartic protease β -secretase (BACE-1), which cleaves APP on the luminal side of the membrane, releasing a soluble APP β fragment (sAPP β), and carboxyl terminal C99 fragment (Vassar, Bennett et al. 1999). BACE-1 cleavage generates the new N-terminus corresponding to the first amino acid of A β (LaFerla, Green et al. 2007), and subsequent cleavage of this fragment, between 38–41 amino acids by the γ -secretase, releases A β . BACE-1 and γ -secretase have been localized to intracellular compartments that contain a highly acidic pH at physiological conditions, optimal for BACE-1 and γ secretase activity. These compartments include the trans-Golgi network, the endoplasmic reticulum, and endosomal, lysosomal, and mitochondrial organelles (Mizuguchi, Ikeda et al. 1992, Xu, Greengard et al. 1995, Kinoshita, Shah et al. 2003), all of which may be sites for constitutive intracellular A β production (Wertkin, Turner et al. 1993, LaFerla, Green et al. 2007). A series of elegant in vivo microdialysis studies suggest that there are several cellular mechanisms that contribute to A β peptide production in the central nervous system (Cirrito, Yamada et al. 2005, Cirrito, Kang et al. 2008), which may have important implications for its subcellular localization. The majority of A β is produced at the synapse, in a synaptic activity- and endocytosis-dependent manner, accounting for approximately 60% of total A β , while approximately 30% is thought to arise from the secretory pathway of the soma (Cirrito, Yamada et al. 2005, Cirrito, Kang et al. 2008).

Recently, the locus-coeruleus (LC)-norepinephrine(NE) system has been identified as an underappreciated and understudied circuit in the context of AD (Ross, McGonigle et al. 2015). The LC is a cluster of noradrenergic neurons located at the base of the fourth ventricle and is recognized as the sole provider of NE to the frontal cortex and hippocampus, and whose broad-reaching afferents provide NE to the entire neuraxis (Foote et al., 1983). The LC-NE system is critically involved in promoting attention, wakefulness and cognition (Berridge and Waterhouse 2003). It is also responsive to the neurohormone corticotropin releasing factor (CRF) during exposure to both acute and chronic cognitive and physical

stressors (Van Bockstaele, Colago et al. 1996, Van Bockstaele, Colago et al. 1998, Valentino and Van Bockstaele 2008). NE is produced by dopamine-beta-hydroxylase (D β H), an enzyme that resides in large dense-core vesicles (LDCV) and small synaptic vesicles within the axon terminals of LC neurons. NE is released from small synaptic vesicles under conditions of depolarization, and may be concurrently released with neuropeptides from LDCV, which are the site of neuropeptide synthesis and storage (Wang 2008). NE is also thought to undergo volume transmission, in which the release of NE is not confined to the synapse, but can diffuse to interact with adrenergic receptors (AR) on surrounding neurons and glial cells (Ridet, Rajaofetra et al. 1993, Benarroch 2009). This feature of NE transmission becomes particularly important when considering the role of NE in modulating central inflammatory responses to a variety of cytotoxic insults (Feinstein, Heneka et al. 2002). Thus, the LC-NE system is poised to initiate global changes in the brain microenvironment at both synaptic and circuit levels, regulating multiple aspects of neuronal functioning.

Previous studies have used N-(2-chloroethyl)-N-ethyl-2-bromobenzylamine (DSP-4), an LC selective neurotoxin, to induce LC degeneration to understand its putative role in A β ₄₂ neuropathology and AD. Rats treated with exogenous human A β ₄₂ seven days after DSP-4 injection show significant LC degeneration and have five-fold increases in A β ₄₂ deposition (Heneka, Galea et al. 2002). DSP-4 injection in mice coexpressing the swedish mutant of the amyloid precursor protein and the presenilin 1 DeltaExon 9 mutant (APP/PS1) results in LC degeneration, increased inflammation, accelerated cerebral amyloidosis and exacerbated spatial memory deficits (Jardanhazi-Kurutz, Kummer et al. 2010). These findings are particularly intriguing considering the known modulatory role of NE at the tripartite synapse (Feinstein, Heneka et al. 2002, Heneka, Galea et al. 2002), the putative function of A β neurotransmission on neurons and glia, and the finding that AR are among the G protein-coupled receptors that have been shown to contribute to A β production (Thathiah and De Strooper 2011). Specifically, β ₂AR and α ₂AR are localized to terminal regions of the LC such as the medial prefrontal cortex (mPFC) and have been shown to have a direct influence on APP processing machinery residing at the synapse (Thathiah and De Strooper 2011). The synthetic NE precursor, L-threo-3,4-dihydroxyphenylserine (L-DOPS), when administered to 5xFAD transgenic mice, increases NE levels, decreased astrocyte activation and Thioflavin-S staining with concurrent increases in mRNA levels of neprilysin and insulin degrading enzyme (IDE) (Kalinin, Polak et al. 2012). Additionally, the finding that NE or isoproterenol, via activation of β ₂AR on microglia cells, upregulates IDE, a degrading enzyme of A β , further supports a role for NE as an important regulator of A β (Kong, Ruan et al. 2010). While there is clear evidence to support a role for NE in the modulation of A β production and clearance at the synapse, the neuroanatomical substrates of this interaction have not been defined. This study aims to characterize the basal frequency and distribution of A β in noradrenergic terminals of the infralimbic mPFC (ILmPFC) of naïve rodents, as well as the functional role of NE in modulating endogenous A β ₄₂.

MATERIALS AND METHODS

Animals

Adult male and female Sprague Dawley rats (250–300 g, n=36) were acquired from Jackson Laboratories (Bar Harbor, ME, USA). Male and female heterozygous and knockout (KO) D β H mice (15–35 g, n=16) were bred at the University of Pennsylvania as described previously (Murchison, Zhang et al. 2004). Because genetic deletion of D β H is lethal during development, first adrenergic agonists and then a synthetic precursor of NE, L-threo-3,4-dihydroxyphenylserine (Lundbeck Pharmaceuticals, Deerfield, IL), was administered to the fetuses via the maternal drinking water (Thomas, Matsumoto et al. 1995). Postnatally, D β H KOs are viable without further intervention. Because D β H is not rate-limiting for the synthesis of NE, heterozygous mice have normal levels of NE and were used as controls (Thomas, Marck et al. 1998). Additionally, male wild-type (WT) and KO mice for APP (15–35g, n=6) were acquired from Jackson Laboratories and used for antibody optimization and as negative controls for the A β ₄₂ enzyme-linked immunosorbent assay (ELISA). Animals were housed 2 per cage at 25°C and kept on a 12-hour light-dark cycle, with free access to food and water. All procedures were approved by the Drexel University College of Medicine Institutional Animal Care and Use Committee in accordance with the revised Guide for the care and use of Laboratory Animals (1996). Efforts were taken to minimize pain and discomfort, and to limit the number of animals used.

DSP-4 Injection

DSP-4 is a blood-brain barrier-permeable neurotoxin that can be administered into the intraperitoneal (i.p.) cavity to achieve selective loss of LC noradrenergic neurons (Ross, Johansson et al. 1973). DSP-4 administration results in a retrograde degeneration that is dose-dependent, requiring at least 40–50 mg/kg to deplete approximately 80% of NE in the cortex, and primarily affects neurons originating from the LC but not the small percentage of fibers ascending from A1 and A2 cell groups (Marien, Colpaert et al. 2004). Adult male and female rats (n=16) were given an i.p. injection of either DSP-4 (n=8; 4 female, 4 male) or saline (n=8; 4 female, 4 male) (Sigma Aldrich, St Louis, MO). DSP-4 was injected at a dose of 50 mg/kg, which produces a rapid and long-lasting decrease in NE and D β H (Ross and Renyl 1976). Rats were sacrificed 7 days following injection.

Tissue Preparation

Male and female rats used for immunohistochemistry (IHC) were deeply anesthetized using isoflurane (Vedco, St. Joseph, MO) and subsequently transcardially perfused through the ascending aorta with the following fixatives: 10 ml of 1000 units/ml heparinized saline, 50 ml of 3.75% acrolein in 2% formaldehyde and 200 ml of 2% formaldehyde in 0.1 M phosphate buffer (PB) pH 7.4. Sections from the acrolein-fixed cases (n=6; 3 male, 3 female) were used for immunoelectron microscopy, as acrolein fixation yields optimal tissue preservation for ultrastructural analysis. For light and immunofluorescence microscopy, the same procedure was conducted with 4% formaldehyde in 0.1 M PB without acrolein (n=6; 3 male, 3 female). The brains were removed, cut into 4–5 mm coronal blocks, stored in 2% formaldehyde fixative for an additional 30 minutes and then sectioned (30–40 μ m) on a vibrating microtome (Vibratome; Pelco EasiSlicer, Ted Pella, Redding, CA) for electron

microscopy, or a cryostat (Microm HM 50, Microm International, Waldorf, Germany) for light and fluorescence microscopy. Brains were cut in 40 μ m coronal sections, and appropriate ILmPFC or LC sections were selected for IHC processing using the rat brain atlas of Paxinos and Watson (Paxinos, 1986). The ILmPFC was the focal point of quantitative analysis using electron microscopy and micrographs used in figures 3 and 4 were taken specifically from ILmPFC.

Immunohistochemical Labeling

Primary Antisera—Acrolein-perfused tissues were incubated in 1% sodium borohydride in 0.1 M PB to remove reactive aldehydes, and then blocked in 0.5% BSA in 0.1 M Tris-buffered saline (TBS). Tissue sections were incubated for 48 hours at 4°C in primary antibody in 0.1% BSA and 0.25% Triton X-100 in 0.1 M TBS. Single-labeled tissues were incubated at a concentration of 1:1,000 in mouse anti-MOAB2 (Kerafast, Boston, MA), a pan-specific antibody directed to amino acids 1–4 of monomeric A β ₄₂ that can detect intraneuronal A β ₄₂ and does not recognize APP; for characterization see (Youmans, Tai et al. 2012). Alternatively, single-labeled tissues were incubated in rabbit anti-D54D2 at a concentration of 1:100 (Cell Signaling, Beverly, MA), an antibody that is highly sensitive and detects endogenous A β ₄₂, A β ₄₀, A β ₃₉, A β ₃₈, and A β ₃₇. Dual-labeled tissues were incubated in a cocktail of mouse anti-MOAB2 (Kerafast, 1:1,000) plus rabbit anti-D β H (Immunostar, Hudson, WI; 1:1000) (for specificity and characterization, see (Van Bockstaele, Biswas et al. 1993) or a cocktail of rabbit anti-D54D2 (Cell Signaling, 1:100) plus mouse anti-D β H (EMD Millipore, Billerica, MA, 1:600).

Light Microscopy—Tissue sections were incubated for 48 hours with mouse anti-MOAB2 or rabbit anti-D54D2. They were then rinsed and incubated in biotinylated donkey anti-mouse, or donkey anti-rabbit secondary antibodies (1:400, Jackson ImmunoResearch), respectively, for 30-minutes followed by rinses in 0.1M TBS. Subsequently, sections were incubated for 30 minutes in avidin-biotin complex (ABC kit, Vector Laboratories). MOAB2 or D54D2 was visualized by the peroxidase reaction with 0.02% diaminobenzidine and 10 μ l of 30% H₂O₂ in 0.1 M TBS.

Electron Microscopy—The secondary antibodies used for electron microscopy were biotinylated anti-rabbit for D β H and TH labeling at 1:400, and 1 nm gold particle conjugated goat anti-mouse IgG (Electron Microscopy Science, Hatfield, PA) at 1:50 for MOAB2. Immunoperoxidase labeling of D β H and TH was performed as described above. Electron-dense labeling of MOAB2 was detected via silver intensification of immunogold MOAB2 particles using a silver enhancement kit (Aurion R-GENT SE-EM kit, Electron Microscopy Science). Tissues were prepared for visualization under the electron microscope as previously described, with osmification, serial dehydration, flat-embedding, and tissue sectioning at 74 nm on an ultramicrotome (Commons, Beck et al. 2001). Sections were collected on copper mesh grids and examined using an electron microscope (Morgani, Fei Company, Hillsboro, OR). Digital images were viewed and captured using the AMT advantage HR HR-B CCD camera system (Advance Microscopy Techniques, Danvers, MA). Immunofluorescence and electron micrograph images were prepared using Adobe Photoshop to adjust the brightness and contrast.

Immunofluorescence—For confocal microscopy, detection of immunofluorescence labeled antigens utilized two combinations of secondary antibodies. The first combination included secondary antibodies, tetramethylrhodamine-5-isothiocyanate (TRITC)-conjugated donkey anti-mouse IgG for D β H detection and fluorescein isothiocyanate (FITC)-conjugated donkey anti-rabbit IgG for D54D2 A β detection. The second combination included TRITC-conjugated donkey anti-rabbit IgG for D β H detection and FITC-conjugated donkey anti-mouse IgG for MOAB2 A β detection at 1:200 dilutions (Jackson Immunoresearch). Tissue sections underwent serial dehydration, were mounted on slides, and coverslipped using DPX (Aldrich). Slides were then viewed using an Olympus IX81 inverted confocal microscope (Hatagaya, Shibuya-Ku, Tokyo, Japan), with helium and argon laser excitation wavelengths of 488, 543 and 635. The microscope is also equipped with filters (DM 405-44, BA505-605, and BA 560-660) and the Olympus Fluoview ASW FV1000 program. Fluorescence confocal images were assembled and adjusted for brightness and contrast in Adobe Photoshop.

Controls

Prior to dual and triple IHC experiments, antibodies were optimized using immunoperoxidase staining and light microscopy under a variety of experimental conditions including perfusion reagents (paraformaldehyde or acrolein), a series of antibody dilutions, and incubation period (overnight at room temperature or 48 hours at 4°C). In addition to negative control groups that do not contain primary antibody, control groups containing primary but no secondary were used to reveal any endogenous fluorescence or peroxidase activity. Experiments were also run with liver tissue sections, known to be devoid of APP, as an additional control for antibody specificity. Common pitfalls of IHC have been well described (Fritschy 2008), and have been addressed in these optimization studies. The utilization of KO animals is regarded as the gold standard for antibody specificity, thus APP KO mice were utilized during optimization procedures of each A β antibody, as well as in western blot and sandwich ELISA experiments to estimate background signal. Following fixation and primary antibody incubation under the optimal conditions determined for each antibody, the tissues were subject to immunoperoxidase labeling techniques as previously described (Van Bockstaele, Colago et al. 1996). Tissues were dehydrated in a series of escalating alcohol concentrations and coverslipped with Permount (Fischer, Hampton, NH) for viewing on a light microscope (Olympus BX51, Japan, D10BXF Camera, Diagnostic Instruments, USA), or coverslipped with DPX for viewing on a confocal microscope (Olympus).

To ensure specificity of the biotinylated and gold-conjugated secondary antibodies for quantification at the ultrastructural level, control tissue taken directly from the plastic-tissue interface was processed in parallel, with omission of the primary antisera for A β and D β H, and was devoid of immunolabeling. As an additional control, tissue sections taken from APP KO animals were also run in parallel with naïve rat sections. No peroxidase or immunogold non-specific labeling was detected in APP KO sections (Figure 4A). Single spurious immunogold-silver labeling may contribute to false positive labeling, indicated by the presence of single immunogold-silver particles on blood vessels, myelin or nuclei (Reyes 2006b, 2007, Van Bockstaele et al 1996a,b). To estimate the extent of spurious labeling, a

randomized sampling of 100 myelin processes was evaluated for immunogold labeling; while spurious single gold particles were present (Figure 4B), there was minimal labeling for 2 or more gold particles (1/100), thus a criterion of 2 or more gold particles was upheld for quantification purposes.

Ultrastructural Analysis

Quantification and analysis of A β localization at the EM level was conducted as previously reported (Commons, Beck et al. 2001, Oropeza, Mackie et al. 2007). The regions selected for analysis included the ILmPFC and the LC. Adequate preservation of ultrastructural morphology was one of the criteria imposed when selecting tissue sections to be used for ultrastructural analysis. A minimum of 3 sections per region of each animal from 6 animals were used for the analysis. At least 10 grids containing 4–7 thin sections each were collected from plastic-embedded sections of the mPFC and LC from each animal. Quantitative evaluation of immunoreactive elements was applied only to the outer 1–3 μ m of the epon–tissue interface where penetration of antisera is optimal. To prevent the inclusion of spurious labeling in quantification, only profiles with a minimum of 2 gold particles were considered immunoreactive and used for quantification. For dual labeling, only micrographs containing both peroxidase and gold–silver markers were used for the tissue analysis to ensure that the absence of one marker did not result from uneven penetration of markers (Leranth and Pickel, 1989). Examination of serial sections was used to determine synaptic associations of axon terminals not always apparent in single sections.

Cellular elements were isolated and classified based on Fine Structure of the Nervous System (Peters 1991). Somata were identified by the presence of a nucleus, Golgi apparatus, and smooth endoplasmic reticulum. Proximal dendrites contained endoplasmic reticulum, were apposed to axon terminals, and were larger than 0.7 μ m in diameter. Synapses were verified by the presence of a junctional complex, a restricted zone of parallel membranes with slight enlargement of the intercellular space, and/or associated postsynaptic thickening. A synaptic specialization was only designated to the profiles that form clear morphological characteristics of either Type I or Type II (Gray 1959). Asymmetric synapses were identified by thick postsynaptic densities (Gray's Type I; Gray 1959), while symmetric synapses had thin densities both pre- and postsynaptically (Gray's Type II; Gray 1959). An undefined synapse was defined as an axon terminal plasma membrane juxtaposed to a dendrite or soma devoid of recognizable membrane specializations and no intervening glial processes. Axon terminals were distinguished from unmyelinated axons based on synaptic vesicle presence and a diameter of greater than 0.1 μ m.

Data from 6 (n=3 male, n=3 female) animals were used in the characterization of the anatomical substrate for A β -NE interactions. For each animal, comparable levels of the mPFC and LC were selected for ultra-thin sectioning. For each animal, dendritic and axon-terminal profiles were sampled from at least 5 copper grids of ultrathin tissue sections near the tissue-plastic interface. Approximately 500 profiles from each animal were scanned, and approximately 300 of these fit all criteria below and were included in the analysis. Dendrites with a maximal cross-sectional diameter between 0.7 μ m and 5 μ m met the criteria for analysis, and a mix of dendrites of sizes from across this range was included in the analysis.

Large profiles were excluded to avoid the bias towards positive labeling of larger structures. Extremely small, large, longitudinal and irregularly shaped profiles were excluded from the analysis due to possibly higher perimeter/surface ratios and risk of biasing the silver grain counts towards the membrane. Any profiles containing large, irregularly shaped silver grains of more than 0.25 μm were excluded from the analysis. Cellular profiles that failed to meet any of the described criteria were excluded from analysis.

Protein Extraction

Naïve rats, DSP-4- and saline-treated rats, WT and D β H KO and APP KO mice used for western blotting and ELISA analyses were briefly exposed to isoflurane and then euthanized by decapitation via guillotine. Brain tissue was rapidly removed on ice from each animal using a trephine and razor blades, and the mPFC or LC was micro-dissected from each. The mPFC and LC regions of each animal were homogenized with a pestle and extracted in radioimmunoprecipitation assay lysis buffer (Santa Cruz Biotechnology, Santa Cruz, CA) on ice for 20 min. Lysates were cleared by centrifugation at 13,000 rpm for 12 min at 4° C. Protein concentrations of the supernatants were quantified using the bicinchoninic acid protein assay reagent (Pierce, Rockford, IL).

Western Blotting

Cell lysates derived from mPFC- or LC-micropunched brain tissue from 3 male and 3 female naïve rats containing equal amounts of protein (20 μg) were diluted with Novex® 2X Tris-Glycine Sodium Dodecyl Sulfate sample buffer (Invitrogen, Carlsbad, CA) containing dithiothreitol (Sigma) and separated on 4–20% Tris-Glycine polyacrylamide gels and then electrophoretically transferred to Immobilon-P PVDF membranes (Millipore, Bedford, MA). Membranes were incubated in antibodies directed against the C-terminus of APP β derived (1:200; ThermoFisher Scientific, Frederick, MD), and N-terminus of APP overnight and then secondary antibodies conjugated to IR dye680 goat anti-mouse IgG (LI-COR, Lincoln, NE; 1:20,000) or IR dye800 donkey anti-rabbit IgG (LI-COR; 1:15,000) for 60 minutes to probe for the presence of proteins. Following incubation in secondary antibody, membranes were washed and exposed for various lengths of time up to 2 minutes in the Odyssey (LI-COR) to optimize exposures. APP β and α protein expression was readily detected by immunoblotting in rat frontal cortex and LC extracts. APP α and APP β immunoreactivity (-ir) was visualized as a band that migrates between approximately 125-90 kDa. Blots were incubated in stripping buffer (Restore Stripping Buffer, Pierce) to disrupt previous antibody-antigen interactions and then re-probed with GAPDH (1: 2,000; ProteinTech Rosemont, IL) to ensure proper protein loading. To ensure the accuracy of the LC microdissection, membranes were probed with antibodies directed against tyrosine hydroxylase (TH) (1:500; Immunostar, Hudson, WI). Prior to quantification blots were pseudocolored to black and white in separate files for analysis. The density of each band was quantified using Image J software. Raw values for each band were normalized to GAPDH loading control bands. Subsequently, normalized values from blots run in triplicate were averaged for final quantification. Values for baseline male and female characterization studies were analyzed by student's t-test, while DSP-4 treated and D β H-KO experiments were analyzed by one-way ANOVA with Tukey's Multiple Comparisons.

ELISA

Sandwich ELISA was conducted in accordance with the instructions provided in the High Sensitivity A β ₄₂ kit (Wako Chemicals, Richmond, VA) and NE kit (Eagle, Nashua, NH). Cell lysates from 5 male and 5 female naïve rats containing equal amounts of protein (20 ug) were used for A β ₄₂ kit. Of the 5 male and 5 female naïve rat cell lysates that were subjected to A β ₄₂ analysis, cell lysates containing 20ug of protein from 3 male and 3 female were also used in the NE kit for correlation analysis. Each sample was run in technical triplicate. A standard curve was run for each replicate and was used to estimate the concentration of A β ₄₂ or NE in each sample. Values for baseline male and female characterization studies were analyzed by student's t-test, while DSP-4 treated and D β H-KO experiments were analyzed by one-way ANOVA with Tukey's Multiple Comparisons.

RESULTS

Immunoreactivity for A β is visualized in the ILmPFC using light microscopy

Immunoperoxidase detection of A β was conducted in the rat frontal cortex using two different antibodies, MOAB2 and D54D2, and light microscopy (Figure 1). There are a number of limitations that should be addressed while using IHC detection (see (Fritschy 2008) for full review). Considered the gold standard for antibody optimization, the specificity of MOAB2 and D54D2 antibodies was validated when tissue sections taken from mice null for APP were run in parallel with sections of WT mice and naïve rats and demonstrated no immunoreactivity (Figure 1 C-C' and E-E'). Additional control sections for each experiment were run in parallel, using the same reagents, in which either the primary or secondary antisera was omitted, or liver sections (Figure 1 C'', E''), known to be devoid of APP, were used with the rest of the protocol identical, and no immunoreactivity was observed (Figure 1 C-C'', E-E''). MOAB2- and D54D2-ir showed similar distribution in somatodendritic and varicose processes (Figure 1B-B' and 1D-D'). Immunoreactivity was most abundant in layer V, moderate in layers II and III, and sparse in layer IV. At higher magnification within layer V, immunoperoxidase labeling indicated that A β was associated with processes resembling pre- and postsynaptic cellular profiles (Figures 1 B'' and 1D''), consistent with its putative pre- and postsynaptic roles (Rodrigues, Gutierrez et al. 2014).

Basal Expression Levels of BACE1 and APP Cleavage Products in the mPFC Are Similar between Male and Female Naïve Rats

Western Blot analysis was conducted to assess basal APP cleavage product expression in the mPFC of naïve rats (Figure 2A). Our results (Figure 2B) reveal no significant difference ($p=0.25$) between sAPP α fragments in naïve male ($0.88 \pm \text{SD } 0.045$) and female rats ($0.74 \pm \text{SD } 0.05$). No significant difference ($p=0.71$) between sAPP β fragments in naïve male ($0.89 \pm \text{SD } 0.06$) and female rats ($0.87 \pm \text{SD } 0.07$) were observed. In line with these results, BACE1 expression was also not significantly different ($p=0.62$) between naïve male ($0.89 \pm \text{SD } 0.18$) and female ($0.86 \pm \text{SD } 0.04$) rats.

Endogenous A β ₄₂ in Naïve Rat mPFC Positively Correlates with NE

In order to determine the endogenous levels of A β ₄₂ in the mPFC, we conducted an ELISA (Wako Chemicals) designed to be sensitive enough to detect endogenous A β with the highest signal to noise ratio of commercially available kits (Teich, Patel et al. 2013). Tissue lysates from APP KO mice were run to estimate background, with an average concentration (0.23 pg/mg $\pm$ SD 0.26) that was significantly decreased ($p=0.0013$) compared to WT mouse lysate (2.1 pg/mg $\pm$ SD 0.48). Consistent with previous reports of endogenous A β ₄₂ in the low picomolar range (Figure 2C), the average concentration of A β ₄₂ in naïve male rats (1.28 pg/mg $\pm$ SD 0.36) was similar to that for naïve female rats (1.56 pg/mg $\pm$ SD 0.43), showing no significant difference ($p=0.63$). We then sought to determine the extent to which endogenous A β ₄₂ levels are related to endogenous NE levels in the mPFC under basal conditions. In order to accomplish this, a NE ELISA was conducted on mPFC homogenate derived from 3 of the same animals used in the A β ₄₂ ELISA. NE levels were then compared to the A β ₄₂ value obtained from each animal using Pearson's correlation; this demonstrated a significant positive correlation ($r=0.8217$; $p=0.045$). This finding establishes a relationship between NE and endogenous A β ₄₂, and it supports other reports indicating that NE may influence levels of A β ₄₂ via interactions with adrenergic receptors on neurons or microglia to promote production or degradation of A β ₄₂, respectively (Ni, Zhao et al. 2006, Kong, Ruan et al. 2010, Yu, Wang et al. 2011, Chen, Peng et al. 2014).

A β Immunoreactive Puncta are Co-localized with D β H in the ILmPFC and BNST

In order to investigate the anatomical substrates of the relationship between NE and endogenous A β ₄₂, dual immunofluorescence experiments were designed to test the hypothesis that noradrenergic axon terminals contain A β peptides. Dual labeling of A β and the NE-synthesizing enzyme D β H was performed using immunofluorescence detection and visualized using confocal microscopy. Localization of MOAB2 or D54D2 and D β H indicated abundant labeling of D β H and moderate intensity labeling of MOAB2 and D54D2 in the ILmPFC (Figure 3). Additionally, numerous fibers exhibited both fluorophore labels indicating co-existence between A β and D β H-ir (Figure 3). Sections were also taken from the Bed Nucleus of the Stria Terminalis (BNST), a group of interconnected nuclei that receives abundant noradrenergic input, to test for MOAB2-ir, and they showed similar labeling of A β but sparse co-localization with D β H, in contrast to the ILmPFC.

Ultrastructural Analysis of A β with D β H in the ILmPFC

Dual-label EM was used to characterize the ultrastructural localization of A β in relation to D β H-positive profiles, using a combination of immunogold and peroxidase labeling. When examining dual-labeled tissue at the ultrastructural level for the co-localization of A β and D β H, MOAB2-ir was found predominantly in axon terminals, many of which were also D β H positive. A β distribution and immunogold localization percentages were drawn from quantification of 956 profiles (approximately 300 per animal, $n=3$). Of the 259 A β (MOAB2)-positive profiles examined, 18.14% (47/259) were distributed in unlabeled dendritic processes, while 81.8% (212/259) were localized to presynaptic axon terminals. Approximately 23% (212/909) of the total axonal profiles examined exhibited A β (MOAB2)-immunogold labeling in the ILmPFC. The frequency of A β (MOAB2)-labeling

in D β H-ir axonal profiles was quantified to further investigate the hypothesis that A β is present in noradrenergic axon terminals. Of the 259 A β (MOAB2)-positive profiles examined, 185 profiles also contained D β H immunoperoxidase, constituting 71.4% of the total A β (MOAB2)-positive population analyzed. Of 882 D β H labeled profiles, 20.9% (185) contained 2 or more A β (MOAB2)-immunogold particles. Of the dually labeled D β H- and A β -ir axon terminals that formed synapses with unlabeled dendrites, approximately 25.9% (48/185) exhibited symmetric synapses (Figure 4C,D), while 5.94% (11/185) exhibited asymmetric synapses (Figure 4E,F), and 68% (126/185) were considered undefined (Figure 4G,H).

Basal Expression of BACE1, APP Cleavage Products, and Endogenous A β_{42} in the LC Are Similar Between Male and Female Naïve Rats

Western Blot analysis was conducted to assess basal APP cleavage product expression in the LC of naïve male and female rats (Figure 5A). Our analysis (Figure 5B) revealed no significant difference ($p=0.74$) between sAPP α fragments in naïve male ($0.79\pm\text{SD } 0.16$) and female rats ($0.74\pm\text{SD } 0.19$). No significant difference ($p=0.34$) between sAPP β fragments in naïve male ($0.77\pm\text{SD } 0.02$) and female rats ($0.83\pm\text{SD } 0.1$) were observed either. In line with these results, BACE1 expression was not significantly different ($p=.41$) between naïve male ($0.84\pm\text{SD } 0.13$) and female ($0.74\pm\text{SD } 0.13$) rats. Analysis of TH expression revealed no significant differences ($p=.64$) between male ($0.89\pm\text{SD } 0.07$) and female ($0.85\pm\text{SD } 0.14$) rats, thus ensuring the accuracy of the LC microdissection and approximately equivalent loading of LC tissue. The average concentration of A β_{42} in the LC of naïve male rats ($0.84\text{ pg/mg}\pm\text{SD } 0.13$) was similar to naïve female rats ($0.74\text{ pg/mg}\pm\text{SD } 0.13$), showing no statistically significant difference ($p=0.41$).

Ultrastructural Analysis of A β_{42} Immunoreactivity in the Somatodendritic Processes of the LC

In order to investigate the hypothesis that at least a portion of A β_{42} co-localized with D β H in the ILmPFC may be derived from the LC, we conducted immunoelectron microscopy and ultrastructural analysis on naïve male and female rat LC tissue sections. We examined the frequency and distribution of A β_{42} and its co-localization with TH. TH is the first (Nagatsu, Levitt et al. 1964) and rate limiting step (Levitt, Spector et al. 1965) of catecholamine biosynthesis, and is abundantly expressed in the LC (Pickel, Joh et al. 1975) in contrast to D β H, which is found in noradrenergic axon terminals (Hartman, Zide et al. 1972). When examining dual-labeled tissue at the ultrastructural level for the co-localization of A β_{42} and TH, MOAB2-ir was found predominantly in somatodendritic processes, many of which were also TH positive. A β_{42} distribution and immunogold localization were drawn from quantification of a total of 1552 profiles (approximately 250 per animal, $n=3$ male, $n=3$ female). Approximately 38.6% (473/1552) of the total TH-ir somatodendritic profiles examined exhibited A β (MOAB2)-immunogold labeling in the LC. Of the 611 total A β_{42} (MOAB2)-positive profiles examined, 77.4% (473/611) were distributed in TH-ir dendritic processes, while 20.6% (126/611) were localized to TH-ir soma. Of the dually labeled TH- and A β -ir dendrites that formed synapses with unlabeled axon terminals, approximately 6.76% (32/473) exhibited symmetric synapses (Figure 6 A), while 21.1% (100/473) exhibited asymmetric synapses (Figure 6), and 68.3% (323/473) were considered undefined

(Figure 6 B). Interestingly, of the 126 A β ₄₂ (MOAB2)-positive and TH-ir soma, there were 11 occurrences of immunogold particles labeling for A β ₄₂ (MOAB2) associated with endoplasmic reticulum structures (11/126; 8.73%), and 17 occurrences of immunogold particles associated with mitochondria (17/126; 13.5%).

NE Depletion Results in Lower Levels of Endogenous A β ₄₂

To determine if NE has a functional role in modulating levels of endogenous A β ₄₂, two models of NE depletion were utilized. Levels of A β ₄₂ were assessed by ELISA, and APP cleavage products by western blot. In the first model, DSP-4 was utilized to induce selective LC neuronal death. Western blot analysis indicated that D β H expression in DSP-4-treated animals was significantly reduced ($p=0.009$) compared to saline (Tukey's adj. $p=0.01$) and naïve rats (Tukey's adj. $p=0.02$). Additionally, ANOVA revealed that DSP-4 treated rats ($3.125 \pm \text{SD } 1.5$) had significantly reduced levels of NE compared to saline treated ($9.4 \pm \text{SD } 1.12$) and naïve rats ($8.3 \pm \text{SD } 1.3$) ($p=0.02$) as quantified by ELISA. DSP-4 treated male and female rats (0.54 pg/mg ; $\text{SD } 0.4$) showed significantly lower levels of A β ₄₂ as quantified by ELISA (ANOVA $p=0.005$), compared to naïve ($1.15 \text{ pg/mg} \pm \text{SD } 0.43$; Tukey's adj. $p=0.01$) and saline-treated ($1.186 \text{ pg/mg} \pm \text{SD } 0.18$; Tukey's adj. $p=0.009$) controls. We probed this further by correlating the A β ₄₂ values of DSP-4 or saline-treated animals to NE values obtained from ELISA conducted on mPFC homogenate derived from the same animal. While DSP-4-treated animals showed a positive correlation (Pearson's $r=0.747$) between A β ₄₂ and NE levels, it did not reach statistical significance ($p=0.08$). In contrast, a significant positive correlation between A β ₄₂ and NE was observed with saline treatment (Pearson's $r=0.913$; $p=.03$), as would be expected based on our findings in the naïve rats. Western blot analysis of APP- α ($p=0.18$), APP β ($p=0.19$), and BACE 1 ($p=0.53$) expression showed no significant changes between naïve, DSP-4- and saline-treated rats.

We then evaluated a genetic model of NE depletion for alterations in endogenous A β ₄₂. We conducted ELISA analyses on male and female mice null for the D β H gene or on heterozygous control mice that have normal levels of NE (Thomas, Marck et al. 1998). Significantly lower A β ₄₂ levels was observed in male and female D β H KO mice compared to heterozygous controls (ANOVA $p=0.002$). Western blots were used to determine if lower A β ₄₂ levels result from altered BACE1 expression; however, no significant changes were observed ($p=0.8$), consistent with unaltered sAPP β ($p=0.3$) and β C-terminal fragments ($p=0.15$). Correspondingly, there were no significant changes in APP α ($p=0.08$) or ADAM10 expression in precursor ($p=0.26$), mature ($p=0.67$), or active forms ($p=0.3$). Thus, the significant reduction in A β ₄₂ monomers is likely the result of an interaction at the level of the γ -secretase. Of particular relevance may be the β_2 AR, which is known to alter APP processing by promoting the activity of the γ -secretase by physically interacting with the $\alpha 1A$ subunit.

DISCUSSION

Methodological Considerations

To our knowledge, this is the first report of endogenous A β in noradrenergic terminals of the ILmPFC. This study utilized two different antibodies to localize endogenous A β ; MOAB2

specifically recognizes the intraneuronal 42 amino acid long A β , while D54D2 recognizes endogenous A β ₄₂, A β ₄₀, A β ₃₉, A β ₃₈, and A β ₃₇ that may result from variable γ -secretase cleavage. Each antibody was determined to be specific using APP KO mice and rat liver slices. The finding that A β is predominantly localized to layer V, with moderate intensity immunoreactivity in layer II/III, is interesting based on our current understanding of cortical layer connectivity, in which layer V receives input from adjacent cortical columns and predominantly sends projections to extracortical nuclei, while layer II/III receives input from other cortical columns and projects to other parts of the cortex. Further, these findings are consistent with the distribution of β ARs within the cortex, which are present in layers II/III and V (Zhou, Sun et al. 2013, Liu, Liang et al. 2014). β ARs of layers II/III and V in the mPFC modulate glutamatergic synaptic transmission via both pre- and postsynaptic mechanisms (Ji, Cao et al. 2008, Liu, Liang et al. 2014), as well as influence APP processing to promote A β production (Ni, Zhao et al. 2006). While the functional consequences of endogenous A β in this region have not been elucidated, our results are consistent with putative mechanisms of A β production from β ARs within layers II/III and V of the mPFC. We found that A β was co-localized with D β H in the BNST and ILmPFC. Our results from immunoelectron microscopy indicated that A β was primarily presynaptic (approximately 82%) in naïve rats, and co-localized with approximately 20% of total D β H-ir axon terminals. To determine the concentration of A β ₄₂ within the mPFC, ELISA was conducted, and it confirmed previous reports indicating that A β ₄₂ is present in the low picomolar range (Puzzo, Privitera et al. 2008, Abramov, Dolev et al. 2009) in naïve male and female rats.

Co-existence of NE and A β ₄₂

The BNST is a group of interconnected subnuclei considered an extension of the amygdala that receives an abundance of noradrenergic input, though not exclusively from the LC, arising from the nucleus of the solitary tract (NTS) (Crestani, Alves et al. 2013). Noradrenergic receptors modulate excitatory and inhibitory transmission of the BNST, and this region is thought to play an important role in the integration of the stress response (Flavin and Winder 2013). A β recognized by the MOAB2 antibody was present in the BNST, although there was not extensive co-localization with D β H under the basal conditions assessed in the present study. It is possible that under conditions of stress, the frequency and distribution of A β in the BNST would be more prominent in D β H-containing axon terminals; however, further studies would be required to investigate this. In contrast, A β was present and co-localized with D β H in the ILmPFC, whose NE input is derived solely from LC neurons. There appeared to be a high frequency of co-localization between A β recognized by the D54D2 antibody and D β H, which may reflect the detection of multiple A β lengths ranging from 37–42 amino acids. In contrast, MOAB2 showed moderate co-localization with D β H in the ILmPFC. The MOAB2 antibody specifically recognizes A β ₄₂ (Youmans, Tai et al. 2012), which is known to be synthesized within intracellular compartments under basal conditions, and to accumulate both intra- and extracellularly under pathological conditions (LaFerla, Green et al. 2007).

To investigate A β ₄₂ further, we conducted immunoelectron microscopy studies using the MOAB2 antibody, which revealed a predominantly presynaptic distribution of A β with less

frequent occurrences of postsynaptic A β under basal conditions. This is consistent with previous reports indicating the localization of APP and β -secretase to the presynaptic compartments, within the synaptosomal fraction of tissue lysates (Caporaso, Takei et al. 1994, Tomimoto, Akiguchi et al. 1995, Ikin, Annaert et al. 1996, Abramov, Dolev et al. 2009, Del Prete, Lombino et al. 2014). A β_{42} was also present in somatodendritic profiles of the LC, wherein approximately 8% of A β immunogold-labeled particles were associated with endoplasmic reticulum, along with BACE-1 and APP-cleavage fragments, indicating that endogenous A β synthesis may occur within LC cell bodies. Other mechanisms of A β_{42} production in the ILmPFC may involve adrenergic receptors. For example, it is possible that the presynaptic α_2 ARs, which act as autoreceptors that regulate NE synthesis and release (Strosberg 1993, Berridge and Waterhouse 2003) and have been implicated in the regulation of APP processing, could contribute to the generation of this presynaptic pool of A β . It has been demonstrated that the α_{2a} AR may facilitate amyloidogenic processing of APP (Figure 9) via the endocytic and secretory pathways by interrupting an interaction between APP and SorLa, a retromer protein that retains APP in the Golgi compartment under normal physiological conditions. It has been proposed that by disrupting this interaction, α_{2a} AR facilitates APP transport to endosomal compartments where it may be proteolytically cleaved (Chen, Peng et al. 2014). Interestingly, a genetic mutation in the SORLA/SORL1 gene confers increased risk for Alzheimer's disease (Rogaeva, Meng et al. 2007). While this interaction may provide some insight, the exact mechanism by which A β is produced under basal physiological conditions within the presynaptic compartment, and the contribution of NE, remains unclear.

The finding that approximately 25.9% (48/185) of D β H and A β dual-labeled axon terminals exhibited symmetric synapses (Type II; inhibitory)(Figures 3C,D) is consistent with the evidence that NE, via the stimulation of ARs, modulates GABAergic inhibitory synapses. Previous studies demonstrate the ability of NE to promote the induction of synaptic plasticity by modulation of GABAergic inhibition, as well as the ability of NE to enhance the frequency and amplitude of GABAergic inhibitory postsynaptic currents and potentiate GABAergic processes (Waterhouse, Moises et al. 1980), effects that may be exerted differentially depending on the region of the brain and the receptor subtype activated (Tully and Bolshakov 2010). The finding that A β is localized to a subset of noradrenergic terminals that likely modulate GABAergic activity is of interest, as it would support the notion that under physiological conditions A β_{42} may facilitate the actions of NE, but also serve as an anatomical substrate by which accumulation of A β_{42} could perturb NE modulation of GABAergic transmission. This is relevant considering that the activity of GABA $_A$ receptors is thought to underlie gamma oscillations (Traub, Cunningham et al. 2003), which are frequently found to be abnormal in EEG recordings of AD patients (Koenig, Prichep et al. 2005). As noted above, NE also may modulate glutamatergic transmission via the activation of β ARs (Ji, Cao et al. 2008, Zhou, Sun et al. 2013), although it appears that A β was not frequently localized to D β H-containing axon terminals forming asymmetric synapses (approximately 5.94%; 11/185) (Figure 3E,F). The observation that 68% (126/185) of dual-labeled profiles were considered undefined (Figure 3 G,H) may reflect the propensity of monoaminergic neurotransmitters such as NE to undergo volume transmission. The presence of A β in D β H-containing axon terminals without clearly delineated synapses is consistent

with the notion that peptide neurotransmitters, such as A β , exert their actions in a prolonged and spatially non-restricted fashion. The present study has localized A β to D β H-ir terminals of the ILmPFC, thus providing evidence of an anatomical substrate for the potential regulated co-secretion of NE and A β from noradrenergic terminals.

Noradrenergic Depletion Reduces Endogenous A β_{42}

DSP-4 administration resulted in lower levels of endogenous A β_{42} . Previous studies have used the DSP-4 model of NE depletion to establish the role of the LC-NE system in A β_{42} deposition by administering exogenous A β_{42} seven days after DSP-4 injection and LC degeneration, and have shown five-fold increases of A β_{42} deposition (Heneka, Galea et al. 2002). These studies described an A β_{42} -dependent inflammatory response exacerbated by noradrenergic loss that is characterized by an increase in neuronal iNOS and glial IL-1 β mRNA levels (Feinstein, Heneka et al. 2002). Importantly, DSP-4 administration alone did not induce a significant inflammatory response (Heneka, Galea et al. 2002), supporting the notion that NE acts as modulator of inflammation that may act to suppress or engage the inflammatory cascade depending on the microenvironment and the inflammatory stimulus present (Feinstein, Heneka et al. 2002, Hinojosa, Caso et al. 2013). Thus, in the present study, it is unlikely that the observed reductions in endogenous A β_{42} are the result of an increased inflammatory response induced by DSP-4 or by depletion of NE. Instead, the observed reductions may be due to the loss of LC terminals that harbor A β_{42} .

In another model of NE deficiency, D β H KO mice, reductions in endogenous A β_{42} were also observed. D β H KO mice have increases in dopamine (DA) content that vary with brain region (Heneka, Galea et al. 2002). However, it seems less likely that alterations in DA contributed to reductions in A β_{42} because DA receptors are not among the G protein-coupled receptors known to play a role in APP processing (Thathiah and De Strooper 2011). On the other hand, β_2 AR and α_2 AR are known to promote A β_{42} production (Figure 9) by altering APP processing when stimulated by NE (Ni, Zhao et al. 2006, Thathiah, Horre et al. 2013, Chen, Peng et al. 2014). It is thought that the joint endocytosis of β_2 AR and APP from the plasma membrane following receptor activation will promote amyloidogenic processing by increasing the availability of APP to act as a substrate for γ -secretase in the endosomal compartment (Thathiah and De Strooper 2011). It has also been proposed that β_2 AR is able to modulate A β production via its association with β -arrestin2, which increases the catalytic activity of the γ -secretase complex by physically interacting with the $\alpha 1A$ subunit (Thathiah, Horre et al. 2013). In line with this, studies in a transgenic animal model of AD have shown that β_2 AR antagonists reduce levels of A β_{40} and A β_{42} (Ni, Zhao et al. 2006), and the therapeutic potential of targeting β ARs in AD has been reviewed (Yu, Wang et al. 2011). Thus, reduced A β_{42} levels observed in D β H KO mice may be the result of decreased interactions between NE and β_2 AR and α_2 AR (Figure 9).

Functional Implications

In conclusion, the findings of the present study reaffirm the importance of investigating NE in modulating endogenous A β_{42} levels in the rodent and in the human. We've demonstrated here that NE levels correlate with endogenous A β_{42} levels, and that reductions in NE correspond to reductions in endogenous A β_{42} . It is imperative to consider that these results

occur in the absence of neurodegenerative events, and that once the neurodegenerative cascade has been initiated, alterations in NE may have drastically different effects on A β ₄₂, based on the neuroprotective action of NE and the deleterious effects of its depletion in the inflammatory state (Feinstein, Heneka et al. 2002, Heneka, Galea et al. 2002). The current study also highlights the importance of monitoring NE and perturbations in NE levels in early or prodromal phases of AD, which resonates with previous studies identifying LC cell death as an early marker of disease (Chalermpananupap, Kinkead et al. 2013). Another facet of the relationship between NE and A β ₄₂ that warrants investigation is the influence of stress on A β ₄₂ levels under physiological conditions, as NE is elevated during the stress response (Curtis, Lechner et al. 1997, McCall, Al-Hasani et al. 2015), and chronically elevated in those with stress-related psychiatric disorders such as anxiety. This may be an important area of future investigation in light of recent findings that elevated A β ₄₂ in cognitively normal adults is associated with anxiety (Lavretsky, Siddarth et al. 2009), and that anxiety related to elevations in A β ₄₂ are also associated with more rapid cognitive decline in patients with mild cognitive impairment (Ramakers, Verhey et al. 2013, Pietrzak, Lim et al. 2015). While mechanisms of interaction between A β ₄₂ and neurodegenerative and psychiatric disease are still elusive, they are profoundly important for the diagnosis and management of neurodegenerative disease (McKeith and Cummings 2005). Thus targeting and monitoring NE in early stages of disease may prove to be important for the stratification of patient populations in clinical trials and potentially for individualized treatment strategies.

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Figure 1. A β peptides are Localized to Mouse ILmPFC

A. Schematic diagram adapted from the rat brain atlas (Paxinos 1998) depicting the mPFC and delineates infralimbic (IL) and prelimbic (PL) regions, as well as cortical layers III and V. **B.** and **B'** are high-magnification micrographs and enlarged regions, respectively, where punctate MOAB2 staining resembles presynaptic boutons. **C.** and **C'** high-magnification micrographs of tissue sections taken from the same level of the ILmPFC showing immunoperoxidase labeling of A β_{37-42} recognized by the D54D2 antibody in the ILmPFC. A β_{37-42} are most abundant in cortical layer V, with moderate-intensity staining in layer III. Scale bar B–C = 50 μ m

Figure 2. Basal APP Cleavage Product Expression and A β ₄₂ Levels in the Rat mPFC

A. Similar intensity bands migrated between 125-90 kDa for APP β and APP α , and 68 kDa for BACE1 under basal conditions for naïve male and female rats. **B.** Quantification of western blot APP α cleavage products shows no significant differences between male and female naïve rats. Similarly, no significant differences between sAPP β fragments in naïve male and female rats were observed. BACE1 expression was also not significantly different between naïve male and female rats. **C.** Using tissue lysate derived from WT and APP KO mice, ELISA for A β ₄₂ detected significant differences between WT and APP KO mice with minimal background signal. When tissue homogenate derived from male and female naïve rats was evaluated by ELISA, A β ₄₂ was in the low picomolar range with no significant difference between sexes. **D.** Tissue homogenate from each naïve male and female rat evaluated by NE ELISA revealed a significant positive correlation between NE and A β ₄₂ levels in the mPFC. *Values with asterisk are significantly different ($p < 0.05$) from each other.

Figure 3. A β Co-localizes with D β H in the Rat ILmPFC

Confocal fluorescence micrographs showing D β H (red) co-localized with A β ₄₂ (green, MOAB2 antibody) in the ILmPFC and Bed Nucleus of the Stria Terminalis (BNST). A β ₃₇₋₄₂ recognized by the D54D2 antibody also co-localize with D β H in the ILmPFC. MOAB2- and D54D2-ir appear punctate, consistent with presynaptic structures such as axon terminals containing D β H. D β H was visualized using tetramethylrhodamine-5-isothiocyanate (TRITC)-conjugated donkey anti-mouse or -rabbit IgG, while MOAB2 or D54D2 was visualized using fluorescein isothiocyanate (FITC).

Figure 4. $A\beta_{42}$ is Localized to $D\beta H$ Immunoreactive Rat Axon Terminals

A. Representative electron micrographs of ILM-PFC sections taken from APP KO mice showing no MOAB2-ir. **B.** As an additional control, 100 randomly selected myelin profiles were assessed for spurious immunogold-silver particles. Single immunogold-silver particles were present in the myelin, as depicted; however, there were rare occurrences of two or more immunogold-silver particle labeling in these myelinated structures. Thus, a criterion of two or more immunogold-silver particle labeling was required during quantification of $A\beta$ -ir profiles. **C, D.** $D\beta H$ immunoperoxidase labeling of axon terminals ($D\beta H$ -t) that also contain two or more $A\beta_{42}$ immunogold-silver particles (arrowheads) that form symmetric synapses (arrows) with unlabeled dendrites. **E.** $D\beta H$ -t that also contains two or more $A\beta_{42}$ immunogold-silver particles (arrowheads) that forms asymmetric synapses (arrows) with an unlabeled dendrite, and is also directly adjacent to a soma. **F.** $D\beta H$ -t that also contains two or more $A\beta_{42}$ immunogold-silver particles (arrowheads) that forms asymmetric synapses (arrows) with a dendritic spine, and is also in the vicinity of an unlabeled dendrite (ud) and

glial process. **G,H.** D β H-t that also contains several A β ₄₂ immunogold-silver particles (arrowheads), whose synapses are undefined (curved arrows).

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Figure 5. Basal APP Cleavage Product Expression and Endogenous Aβ₄₂ Levels in the Rat LC
A. Similar intensity bands migrated between 125-90 kDa for APPβ and APPα, 60 kDa for TH and 68kDa for BACE1 under basal conditions for naïve male and female rats. **B.** Quantification of western blot APP cleavage products shows no significant difference between sAPPα fragments in naïve male and female rats. No significant differences between sAPPβ fragments in naïve male and female rats, or BACE1 expression between naïve male and female rats were observed. When tissue homogenate derived from the microdissected LC of male and female naïve rats was evaluated by ELISA, expression was determined to be in the low picomolar range with no significant difference between sexes.

Figure 6. $A\beta_{42}$ is Localized to TH-immunoreactive Somatodendritic Processes in the Rat
A. TH immunoperoxidase labeling of a dendrite (TH-d) that also contains two or more $A\beta_{42}$ immunogold-silver particles (arrowheads) that forms a symmetric synapse (arrows) with an unlabeled axon terminal (ut). **B.** TH-d and $A\beta_{42}$ immunogold-silver particles (arrowheads) that does not make any clearly defined synaptic contacts (curved arrows). **C.** TH-d exhibiting greater than two $A\beta_{42}$ immunogold-silver particles (arrowheads) along points of synaptic connections. Interestingly, this TH-d contacts two unlabeled axon terminals (ut), one of which forms a symmetric synapse (arrows), and the other an asymmetric synapse (arrows), demonstrating convergent signaling of inhibitory and excitatory input onto the same TH-d.

Figure 7. DSP-4-treated Rats Show Decreased Levels of Endogenous Aβ₄₂.

A. Similar intensity bands migrated between 125-90 kDa for APPβ and APPα, 15 kDa for APPβ C-terminal fragments, and 68 kDa for BACE1. **B.** Western blot analysis of APPα, APPβ, and BACE 1 expression showed no significant changes between naïve, DSP-4- and saline-treated rats. DβH expression in DSP-4-treated animals was significantly reduced compared to saline and naïve rats. DSP-4-treated male and female rats showed significantly decreased levels of Aβ₄₂ expression as quantified by ELISA compared to naïve and saline-treated controls using one-way ANOVA. DSP-4-treated animals showed a positive correlation between Aβ₄₂ and NE levels that were not statistically significant. Saline-treated controls showed a significant positive correlation between Aβ₄₂ and NE using Pearson's Correlation. *Values with asterisk are significantly different (p<0.05) from each other.

Figure 8. DβH KO Mice Show Decreased Levels of Endogenous Aβ₄₂

A. Similar intensity bands migrated between 125-90 kDa for APPβ and APPα, 15 kDa for APPβ C-terminal fragments, 68 kDa for BACE1, and 100, 80, and 60 kDa for precursor, mature, and active ADAM10, respectively, between control and DβH KO mice. **B.** Western blots show no significant changes in BACE-1 expression, sAPPβ or β C-terminal fragments in DβH KO mice. Additionally, there were no significant changes in APPα or ADAM10 expression in precursor, mature, or active forms. A significant decrease in Aβ₄₂ levels was observed in male and female DβH KO mice compared to heterozygous controls using one-way ANOVA. *Values with asterisk are significantly different (p<0.05) from each other.

Figure 9. Influence of LC Neurons on Endogenous A β ₄₂ and Potential Mechanisms by which NE Depletion Results in Reduced A β ₄₂ Levels

Under conditions of LC neuronal activation, synaptic vesicle recycling may increase A β ₄₂ production at NE synapses in the prefrontal cortex and other projection regions. Postsynaptic β_2 adrenergic receptors (AR) are thought to increase A β ₄₂ production when activated by NE in two ways. **1.** The joint endocytosis of β_2 AR and APP from the plasma membrane following receptor activation will increase the availability of APP to act as a substrate for γ -secretase in the endosomal compartment (Thathiah and De Strooper 2011). **2.** It has also been proposed that β_2 AR is able to modulate A β production via its association with β -arrestin2, which increases the catalytic activity of the γ -secretase complex by physically interacting with the $\alpha 1A$ subunit (Thathiah, Horre et al. 2013). Thus, in the absence of NE neurotransmission, there may be decreased activity of the γ -secretase, along with decreased receptor internalization, resulting in reduced levels of A β ₄₂. Presynaptic α_{2a} AR are also known to increase A β ₄₂ production when activated by NE by interrupting an interaction between APP and SorLa, a retromer protein that retains APP in the Golgi compartment. It has been proposed that by disrupting this interaction, α_{2a} AR facilitates APP transport to endosomal compartments where it may be proteolytically cleaved (Chen, Peng et al. 2014). Thus in the absence of NE, SorLa may effectively retain APP in the Golgi compartment, preventing its proteolytic cleavage, thereby reducing A β ₄₂ production.