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Amyloid Beta Peptides, Locus Coeruleus-Norepinephrine System and Dense Core Vesicles

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Abstract

The evolution of peptidergic signaling systems in the central nervous system serves a distinct and crucial role in brain processes and function. The diversity of physiological peptides and the complexity of their regulation and secretion from the dense core vesicles (DCV) throughout the brain is a topic greatly in need of investigation, though recent years have shed light on cellular and molecular mechanisms that are summarized in this review. Here, we focus on the convergence of peptidergic systems onto the Locus Coeruleus (LC), the sole provider of norepinephrine (NE) to the cortex and hippocampus, via large DCV. As the LC-NE system is one of the first regions of the brain to undergo degeneration in Alzheimer's Disease (AD), and markers of DCV have consistently been demonstrated to have biomarker potential for AD progression, here we summarize the current literature linking the LC-NE system with DCV dysregulation and A β peptides. We also include neuroanatomical data suggesting that the building blocks of senile plaques, A β monomers, may be localized to DCV of the LC and noradrenergic axon terminals of the prefrontal cortex. Finally, we explore the putative consequences of chronic stress on A β production and the role that DCV may play in LC degeneration. Clinical data of immunological markers of DCV in AD patients are discussed.

Keywords

amyloid; dense core vesicles; norepinephrine; dopamine- β -hydroxylase; locus coeruleus

Introduction:

The brain has evolved to support two modes of communication: fast acting neurotransmitters and slow acting neuropeptides. The packaging of these diverse signaling molecules into separate secretory compartments of the cell enables an effective means of regulating their exocytosis independently (Zhang, Wu et al. 2011). Thus, small clear synaptic vesicles (SV) generally contain low molecular weight neurotransmitters, while neuropeptides are packaged

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in large dense core vesicles (DCV). Neurotransmitters stored in SVs are primed for fast, phasic release at active zones of the nerve terminal upon depolarization (Wang 2008). Once released into the synaptic cleft, a signal is rapidly transduced via binding of post synaptic receptors and terminated via the degradation or re-uptake of transmitter, clearing the synaptic cleft. In contrast, the second mode of communication, via neuropeptide transmitters, is a slower and long lasting mode of communication that is not spatially or temporally confined, based on their release from separate asynaptic sites and their long half lives (Ludwig and Leng 2006). Thus, while fast acting neurotransmitters provide the brain with massive computational power, spatial and temporal precision, slow acting neuropeptides have broad reaching, long lasting affects that ultimately pass signals between subpopulations of neurons. Thus, the brain is equip with two parallel modes of communication, that are qualitatively different in their outcome. Persistent neuropeptide activity is thought to modulate behavioral states, as the system is designed to initiate global changes in the brain state by diffusing from one population of cells to another (Ludwig and Leng 2006).

The Locus Coeruleus (LC)-Norepinephrine (NE) system is poised to participate in this broad reaching modulatory signaling, as norepinephrine is amongst the prominent catecholamine neurotransmitters known to be stored and undergo co-transmission with neuropeptides from large DCV. The LC is a cluster of noradrenergic neurons located at the base of the fourth ventricle that are recognized as the sole provider of NE to the frontal cortex and hippocampus, and whose broad reaching afferents provide NE to the entire neuraxis. The LC-NE system is critically involved in promoting attention, wakefulness and cognition upon receiving input from hypocretin (orexin) neurons of the hypothalamus (Berridge and Waterhouse 2003). It is also responsive to the neurohormone Corticotropin Releasing Factor (CRF) during both acute and chronic stressors, both cognitive and physical (Van Bockstaele, Colago et al. 1996, Van Bockstaele, Colago et al. 1998, Valentino and Van Bockstaele 2008). The Central Nucleus of the Amygdala (CeA) is a critical source of CRF that can impact on LC activity via effects on dendrites in the rostralateral peri-coerulear region (Van Bockstaele, Colago et al. 1998), thus may serve as a cellular substrate for modulation of brain noradrenergic activity and may serve as a mechanism for the integration of emotional and cognitive responses to stress (Van Bockstaele, Colago et al. 1998, Valentino and Van Bockstaele 2008). Additionally, the LC receives input from a variety of neuropeptides residing in DCVs, and is a point of convergence for multiple peptidergic systems in modulating responsivity to stress.

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 finding that dense core vesicle specific markers are dysregulated in AD, and that the LC is amongst the earliest regions to undergo degeneration, brings to light an important question regarding the role of DCV dysregulation in LC-NE system dysfunction, and the potential role of DCVs in degenerative conditions of catecholaminergic neurons. In this review, we will start by synthesizing recent and previous research regarding DCV and neuropeptide storage and release, then move into current literature on the impact of neuropeptide transmission on the integrity and responsivity of the LC-NE system, and

finally, review clinical evidence that dense core vesicle markers may play a role in neurodegenerative disease states, particularly in catecholamine neuronal populations.

i. Neuropeptide Storage and Release

Neuropeptides are synthesized in the cell body and condensed into DCV (Wang 2008). DCV can be released in the soma as well as in the nerve terminal far away from active zones. Formation of DCVs is a multi-step process that involves the sorting of DCV cargo into immature secretory granules in the trans golgi network (TGN) (Aixa Alfonso 2010). These immature granules do not yet contain a dense core and are not stimulus-responsive. Subsequently, immature vesicles may be remodeled to form mature DCV, a process that involves cargo condensation and changes in protein composition (Wang 2008, Aixa Alfonso 2010). The granin family of acidic glycoproteins are quantitatively the major constituents of DCV in endocrine and neuroendocrine cells and are thought to be the driving force in the physical process of secretory granule formation due to their ability to aggregate under conditions of acidic pH and high calcium concentration. The granin family is comprised of Chromogranin A (CgA), chromogranin B (CgB/secretogranin I (SGI)), and Chromogranin C (CgC/secretogranin II (SGII)) (Fischer-Colbrie, Hagn et al. 1986, Fischer-Colbrie, Hagn et al. 1987, Fischer-Colbrie and Schober 1987, Gerdes, Rosa et al. 1989, Huttner, Gerdes et al. 1991, Winkler and Fischer-Colbrie 1992, Hendy, Bevan et al. 1995, Gerdes and Glombik 2000). Of particular importance, numerous lines of evidence suggest that aggregation of CgA serves as a molecular switch for DCV biogenesis (Kim, Tao-Cheng et al. 2001, Kim, Tao-Cheng et al. 2002, Loh, Kim et al. 2004, Kim, Gondre-Lewis et al. 2006). Thus, biophysical properties of DCV cargo, particularly CgA, allow cooperative, low affinity interactions that effectively sort these proteins by causing aggregation away from other soluble proteins destined for other pathways leading to TGN (Aixa Alfonso 2010). It is thought that interaction of luminal cargo, such as CgA, with the TGN membrane could nucleate aggregation and anchor the aggregate to the membrane (Aixa Alfonso 2010). During the processing and remodeling phase of maturation, neuropeptides can be sorted to different DCVs within the same cell before undergoing anterograde transport to axon terminals via kinesin motor proteins (Fisher, Sossin et al. 1988, Sossin, Sweet-Cordero et al. 1990, Perello, Stuart et al. 2008) (Depicted in Figure 1).

DCV are cell-type specific carriers that store unique combinations of proteins, including multiple neuropeptides (Hokfelt, Broberger et al. 2000, Salio, Lossi et al. 2006), until an extracellular stimulus triggers fusion with the plasma membrane (Aixa Alfonso 2010). Recent proteomics studies utilizing combined nano-high performance liquid chromatography (HPLC) Chip MS/MS tandem mass spectrometry reveal that DCV contain proteins of distinct functional categories including neuropeptides, neurohormonal factors, protease systems, neurotransmitters, enzymes, reduction/oxidation regulatory proteins, transporters, receptors, ATPases, protein folding, lipid biochemistry, signal transduction, exocytosis, calcium regulation as well as structural and cell adhesive proteins (Wegrzyn, Bark et al. 2010). Neuron-like chromaffin cells have been utilized extensively for studies of regulated secretion of neurotransmitters from DCV, and have advanced knowledge of enzymes that produce such neurotransmitters in the brain (Carmichael and Winkler 1985, Njus, Kelley et al. 1985). Most notably, DCVs of the adrenal chromaffin cells contain

catecholamine neurotransmitters and their synthesizing enzymes alongside neuropeptide transmitters (Winkler, Apps et al. 1986). Within NE neurons, the core of DCV contain soluble proteins, peptides and lipids in addition to NE and adenosine triphosphate (ATP). It is approximated that thirty-eight percent of the total vesicle protein is in the core, and of that, approximately fifty-three percent is the NE synthesizing enzyme Dopamine- β -Hydroxylase (DBH) (Gupta, Bark et al. 2010, Wegrzyn, Bark et al. 2010)

Interestingly, the recent literature supports a model in which neuropeptides are nearly always co-packaged with smaller neurotransmitters (Bean, Zhang et al. 1994, Salio, Lossi et al. 2006). This presents a complex question as to how the exocytosis of small neurotransmitters and neuropeptides may remain independently controlled when released from a common compartment. To this end, investigators have extensively studied two vastly different modes of exocytosis—kiss-and-run, and full fusion. Chromaffin cells can release catecholamine and neuropeptide transmitters simultaneously from the same vesicle under conditions of full fusion (Whim 2006), however, depending on the size and frequency of stimulation, norepinephrine alone may be released during kiss-and-run fusion (Fulop, Radabaugh et al. 2005). These studies critically advanced our understanding of quantal release from DCV by utilizing amperometric analysis of quantal size combined with electrophysiological stimulation techniques. In using these combined techniques investigators were able to determine that at basal firing rates (under 15 Hz) quantal size and rate of catecholamine release was drastically reduced compared to stimulation conditions that mimicked stress (greater than 15 Hz). With specialized structure, kinetics, and sensitivity, the LDCV serve a specialized function in reorganizing neuronal networks, providing a substrate for prolonged behavior (Ludwig and Leng 2006).

ii. Regulation of Locus Coeruleus-NE System: Peptidergic Convergence and DCV

Physiologically, the LC is poised to switch between two modes of discharge activity that dictate behavioral outcomes in response to task- and stress- related stimuli [for extensive review see (Aston-Jones and Cohen 2005, Valentino and Van Bockstaele 2008)]. The LC tonic discharge rate is related to task-focused states and states of arousal. During passive, or unstressed conditions, the LC exhibits low levels of tonic discharge that are associated with decreased attention to task related stimuli and disengagement from the environment. During task-focused states, LC tonic firing is optimized for high responsivity to sensory stimuli. This state is characterized by electronic coupling of LC neurons and phasic bursts of firing. In contrast, during the stress response, LC tonic activity exceeds this optimal firing threshold, promoting hyperarousal and scanning of the environment. This state is characterized by high tonic, low phasic, uncoupled firing, and may be elicited by CRF during the stress response.

A number of peptidergic systems converge onto the LC to counteract responsivity to stress, thus highlighting the importance of DCVs that store and release such peptides in maintaining the integrity of the LC-NE system. One such system is the endogenous opioid system, particularly enkephalin, which densely innervates the nuclear core of LC neurons, peri-coerulear dendritic zones, and overlaps with CRF in the rostral LC. Enkephalin is able to decrease LC firing and responsivity by binding to and activating μ -opioid receptors

(MOR) densely present on LC somatodendritic processes and coupled to inhibitory G proteins resulting in neuronal hyperpolarization. While endogenous opioids such as enkephalin are not released tonically to control LC activity, they are released following the stress response to selectively decrease tonic activity without affecting phasic activity. Thus, the result is an enhanced sensory evoked response (Valentino and Wehby 1988). Another neurochemical system that promotes stress resilience is Neuropeptide Y (NPY). NPY is a highly conserved 36 amino acid peptide derived from preproNPY that is abundantly expressed in the central nervous system. Similarly, to MOR, NPY receptors are inhibitory G-protein coupled, resulting in decreased excitability of LC neurons. It has been hypothesized that NPY serves as an opposing system to the excitatory effects of pro-stress neurotransmitters such as CRF and NE (Heilig, Koob et al. 1994, Eaton, Sallee et al. 2007, Sah and Geraciotti 2013, Enman, Sabban et al. 2015). This hypothesis is supported by studies demonstrating that NPY is poised to modulate CRF and NE as it is frequently contained within the same neuroanatomical brain structures, and results in physiological and behavioral outputs opposite to these pro-stress neurotransmitters (reviewed in (Kask, Harro et al. 2002, Sajdyk, Shekhar et al. 2004, Shekhar, Truitt et al. 2005, Sah and Geraciotti 2013, Enman, Sabban et al. 2015).

In addition to inhibitory peptidergic interactions that modulate LC responsivity to stressors, accumulating evidence suggests that the LC may also autoregulate by releasing NE from somatodendritic processes. The distinct kinetics of stimulus-secretion coupling in somata is regulated by action potential firing patterns. Quantal release events from somatodendritic sites of LC neurons are detected only when firing rate is high (15-20 Hz) (Berridge and Waterhouse 2003, Huang, Wang et al. 2007, Huang, Zhu et al. 2012). The high frequency action potential most likely elevates the level of residual calcium in LC somata which leads to NE release, as shown in chromaffin cells (Duan, Yu et al. 2003). NA in central neurons is stored both in small synaptic (granular) vesicles and LDCV (Hokfelt 1968), and such vesicles are also present in both the cell soma and dendrites of LC neurons (Hokfelt 1967, Shimizu, Katoh et al. 1979, Groves and Wilson 1980). Thus, one question that may arise in considering LC autoregulation via NE release, is the vesicle pool responsible for LC regulation and the timing of release. To this extent, combined amperometry and patch clamp studies of somatic transmitter release from LC neurons in brain slices have allowed for accurate measurement of the timing of quantal NE release following LC neurons firing or depolarization. Compared with the fast synaptic transmission in the range of milliseconds, the latency of LC somatic release is in the range of seconds, which is 100-1000 times slower (Huang, Wang et al. 2007). These studies were able to provide some evidence for exocytotic release from LDCV after K⁺ stimulation and tannic acid treatment. While these investigators acknowledge that we do not know the extent to which the NE-storing SV (Shimizu, Katoh et al. 1979) participate in the somatic release process, the fact that NA secretion preferentially occurs at high action potential frequency may suggest that large DCV are mainly involved (Huang, Wang et al. 2007). The physiological significance of somatic release is to produce negative feedback and to down regulate neuronal hyperactivity, which consequently inhibits NE release from axon terminals of LC to many brain areas.

Based on the high-stimulation dependent release of somatic NE, stress and stimulus-induced LC hyperactivity in firing may trigger this auto-inhibitory mechanism of alleviating

hyperactivity [Reviewed in (Huang, Zhu et al. 2012, Trueta and De-Miguel 2012). NE released from the soma then binds to and activates alpha-2a adrenergic receptors present on LC neurons, which are coupled to inhibitory g-proteins and result in neuronal hyperpolarization and decreased sensitivity of LC neurons to stimulation. Systemic application of alpha-2a antagonists increase NE release in LC and projection areas (Callado and Stamford 1999, Callado and Stamford 2000). In addition to autoinhibition of release, activation of alpha 2a receptors by NE hyperpolarizes LC neurons and reduces firing rate (Williams, Henderson et al. 1985). This negative feedback via alpha 2a receptor enables an autoregulatory mechanism and may play an important role in LC physiology. If these negative feedback mechanisms are damaged, LC neuron hyperactivity induced by wide range of stressors would not be inhibited and therefore contribute to many disorders such as epilepsy, ADHD, sleep and arousal disorders, PTSD, depression, schizophrenia, AD and PD (Valentino and Van Bockstaele 2008). Such effect may be protective for LC neurons which may be particularly vulnerable to damage induced by high energy demands (Feinstein, Kalinin et al. 2016).

Recently, endogenous Amyloid Beta (A β) peptides, a primary component of AD neuropathology, have been localized within the coeruleo-cortical circuit, and have been shown to positively correlate with levels of NE. In the coming sections, we review the well characterized metabolic pathway of the A β peptide, the putative role of NE in regulating endogenous A β levels, and the emerging evidence that DCV may be a cellular substrate of A β and NE co-secretion.

iii. Endogenous Amyloid Beta Production and Putative Physiological Function

A β has been recognized as an endogenous neuropeptide that undergoes physiological metabolism in the central nervous system for over a decade and we still lack a basic understanding of its physiological function. Early studies demonstrating the existence of endogenous 4 kilodalton A β peptides, identical to those deposited as extracellular plaques in Alzheimer's disease (AD), were conducted in human mononuclear leukemic and neuroblastoma (M17) cell lines (Shoji, Golde et al. 1992). In the same year, additional studies of endogenous A β in non-transfected primary neuronal cultures demonstrated for the first time that A β peptides are produced and secreted under normal conditions, and could be readily immunoprecipitated from the culture media (Haass, Schlossmacher et al. 1992). Furthermore, a study utilizing ELISA and affinity chromatography demonstrated the existence of A β in the cerebrospinal fluid, plasma, and conditioned medium of human mixed brain cells grown *in vitro*, demonstrating for the first time that A β is produced and released both *in vitro* and *in vivo* in humans (Seubert, Vigo-Pelfrey et al. 1992).

Subsequent studies investigating the synthesis of A β revealed that it is derived from the type I transmembrane protein Amyloid Precursor Protein (APP) composed of 695-770 amino acids (Haass, Schlossmacher et al. 1992). Under normal physiological conditions, a majority of APP is cleaved by α -secretases present on the plasma membrane, resulting in the formation of sAPP α and the carboxyl terminal C83 fragment, precluding the formation of A β (LaFerla, Green et al. 2007). Alternatively, APP may undergo proteolytic cleavage by the aspartic protease β -secretase (BACE-1), which cuts 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 results in the formation of a new N-terminus with the first amino acid of A β (LaFerla, Green et al. 2007). Subsequent cleavage of this fragment, between 38-41 amino acids by the γ -secretase results in the release of the A β peptide. BACE-1 and γ -secretase, have been localized to a number of intracellular compartments including the trans Golgi network (TGN), the endoplasmic reticulum (ER), endosomal, lysosomal, and mitochondrial membranes (Mizuguchi, Ikeda et al. 1992, Xu, Greengard et al. 1995, Kinoshita, Shah et al. 2003), all of which may be sites for intracellular A β production (LaFerla, Green et al. 2007). The intracellular production of A β under constitutive conditions was demonstrated for the first time in the NT2N human cell line, derived from teratocarcinoma cells that were treated with retinoic acid (Wertkin, Turner et al. 1993).

Another wave of progress came from electrophysiology studies in brain slices of APP-overexpressing transgenic mice, which demonstrated that neuronal activity modulates the production and secretion of A β peptides. Further, these studies demonstrated that subsequently, A β may act post synaptically to selectively depress excitatory synaptic transmission on neurons overexpressing APP and surrounding neurons that do not overexpress APP, thus forming the basis of the first hypothesis that A β may participate in a negative feedback loop controlling neuronal excitability (Kamenetz, Tomita et al. 2003). The role of synaptic activity in modulating A β production was confirmed via in vivo microdialysis studies in wildtype and APP transgenic mice (Cirrito, Yamada et al. 2005), and later found to be endocytosis dependent (Cirrito, Kang et al. 2008).

Elegant in vivo microdialysis studies suggest that there are at least three mechanisms that contribute to A β production. Approximately sixty percent of A β peptides are produced at the synapse, in a synaptic activity- and endocytosis-dependent manner (Cirrito, Yamada et al. 2005, Cirrito, Kang et al. 2008). About ten percent is thought to arise from a pathway that requires endocytosis but is independent of synaptic activity, and remains sensitive to dynamin-DN. Finally, the remaining pool of A β , comprising 30% of total ISF A β levels, may be the product of several mechanisms, including A β produced within the secretory pathway (Busciglio, Gabuzda et al. 1993) or A β diffusing from brain areas that are not affected by dynamin-DN or TTX. Alternatively, this last pool may be a factor of incomplete inhibition of endocytosis or some small contribution of altered A β elimination.

The production of A β is also largely dictated by the subcellular localization of APP. APP is commonly found embedded in the plasma membrane; however, it may also be internalized via endocytosis where it encounters the β - and γ -secretases that reside in acidic intracellular compartments (Haass, Kaether et al. 2012). Typically, the internalization of APP favors amyloidogenic processing and the formation of A β , whereas its presence on the surface confers increased likelihood that it will encounter the α -secretase known to produce nontoxic, soluble APP α and C83 fragments (Hong, Huang et al. 2014). Based on the cumulative evidence that synaptic A β production is neuronal activity- and endocytosis-dependent, it has been proposed that under conditions of neuronal activation, depolarization will result in vesicle fusion and release, and ultimately will require the recycling of synaptic vesicles (Cirrito, Kang et al. 2008). It is during the recycling of the synaptic vesicles that

greater amounts of APP may be internalized into the intraneuronal compartments where β - and γ - secretases reside, producing A β fragments that will then be released into the extracellular space (Cirrito, Kang et al. 2008). Since Kamenetz et al. first suggested the participation of A β in a negative feedback loop, it has been shown that A β has the ability to enhance calcium permeability pre-synaptically resulting in increased probability of vesicle release (Abramov, Dolev et al. 2009), or may act post-synaptically to suppress neuronal activity by promoting receptor internalization (Snyder, Nong et al. 2005, Wang, Yuen et al. 2011, Ulrich 2015), and long term depression (LTD) (Li, Hong et al. 2009, Palop and Mucke 2010), supporting the notion that A β has a physiological role in the central nervous system as a modulator of neuronal activity (Kamenetz, Tomita et al. 2003).

iv. Amyloid Beta Peptides, Norepinephrine and Dense Core Vesicles: Significance for LC Autoregulation

While it has yet to be investigated *in vivo*, APP, β - and γ - secretases have been localized to neuron-like chromaffin cells in vitro (Toneff, Funkelstein et al. 2013). Further, this important study demonstrated that under conditions of KCl induced depolarization or forskolin treatment, A β peptides underwent regulated co-secretion with other peptides and catecholamine neurotransmitters. Amongst co-secreted neuropeptides were galanin, enkephalin, NPY and catecholamine neurotransmitters including dopamine, norepinephrine, and epinephrine (Toneff, Funkelstein et al. 2013). From DCV, NE is amongst the monoaminergic transmitters known to undergo volume transmission, characterized by the ability to travel a short distance to interact with adrenergic receptors on synaptic sites on surrounding neurons and glial cells after being released from the presynaptic terminal (Ridet, Rajaofetra et al. 1993, Benarroch 2009). This is consistent with observations at the ultrastructural level, that nearly all noradrenergic varicosities in the cerebral cortex and hippocampus are non-synaptic (Hokfelt 1968, Descarries, Watkins et al. 1977, Umbriaco, Garcia et al. 1995, Aoki, Venkatesan et al. 1998, Aoki, Venkatesan et al. 1998).

Adrenergic receptors (ARs) are metabotropic G-protein coupled (GPCRs) whose distribution have been well characterized at the ultrastructural level. In the prefrontal cortex, α 2A receptors are mostly localized to axon terminals, dendritic shafts, and astrocytic processes, all lacking morphologically identifiable synaptic junctions (Aoki, Venkatesan et al. 1998). Similar distributions at post synaptic sites of the prefrontal cortex have been described for β 2-AR. This is especially important given that ARs have been shown to have a direct influence on β - and γ -secretase activity at the synapse (Thathiah and De Strooper 2011), and that NE or isoproterenol, via activation of the β 2 adrenergic receptor (β 2AR) on microglia cells, up-regulates insulin degrading enzyme (IDE) a degrading enzyme of A β . These findings support a role for NE as an important regulator of A β (Kong, Ruan et al. 2010), and are consistent with the role of NE as an modulator of the central immune response at the tripartite synapse (Feinstein, Heneka et al. 2002, Heneka, Galea et al. 2002).

Preliminary data from our lab have localized SGII and endogenous A β peptides to TH immunoreactive dendrites in the LC of naïve rats (n=6; 3 male, 3 female). Immunoperoxidase labeling for TH was combined with sequential dual immunogold-silver labeling where the immunogold-silver particles were differentiated based on their size.

Obtaining different sized immunogold-silver particles was achieved by incubating with one ultra-small gold conjugate, followed by silver enhancement, and then incubating with the second ultra-small gold conjugate, followed by additional silver enhancement (Yi, Leunissen et al. 2001). This resulted in two groups of silver-enhanced particles: smaller particles that were enhanced once and larger particles that were enhanced twice (Yi, Leunissen et al. 2001, Jin, Kittanakom et al. 2010). Immunoperoxidase- and dual immunogold-silver labeling (large and small gold-silver particles, for A β and SGII, respectively) were localized in the same tissue section (Figure 1). Gold-silver labeling for A β and SGII were readily distinguishable from each other. A β was identified by large-sized gold-silver particles (>50 nm cross-sectional diameter; average size 33.3nm) while SGII was labeled using small-sized gold-silver particles (<40 nm cross-sectional diameter; average size 67.9nm). A β -immunogold-silver particles appeared as black punctate particles within axon terminals while SGII-immunogold-silver particles appeared as smaller black punctate particles within the dendrites and in axon terminals. Immunogold-silver labeling for A β and SGII was also clearly distinguishable from the immunoperoxidase reaction product. Of 819 TH-labeled somatodendritic profiles, 17.7% (145/819) contained A β immunoreactivity while 82% (674/819) did not contain A β immunoreactivity. Of the 145 TH-labeled somatodendritic profiles that contained A β immunoreactivity, 69% (101/145) also contained SGII immunoreactivity. Of the 819 TH-labeled somatodendritic profiles, approximately 15% (121/819) also contained SGII-immunoreactivity. The majority (91% 92/101) of TH-immunoreactive dendrites that contained both A β and SGII immunoreactivity did not show recognizable synaptic specialization.

Immunofluorescence micrographs demonstrate the co-localization of SGII, A β , and D β H in the prefrontal cortex (Figure 2). To further quantify the frequency of this co-localization, immunoelectron microscopy was also conducted. SGII and A β Peptides were also localized to D β H-immunoreactive axon terminals of the infralimbic medial prefrontal cortex of naïve rats (n=6; 3 male, 3 female). Immunoperoxidase-D β H and dual immunogold-silver labeling (large and small gold-silver particles, for A β and SGII, respectively) were localized in the same tissue section (Figure 3). Gold-silver labeling for A β and SGII were readily distinguishable from each other and were localized to the appropriate cellular structures. A β was identified by large-sized gold-silver particles (>50 nm cross-sectional diameter; average 65nm) while SGII was labeled using small-sized gold-silver particles (<40 nm cross-sectional diameter; average 31 nm). A β -immunogold-silver particles appeared as black punctate particles within axon terminals while SGII-immunogold-silver particles appeared as smaller black punctate particles within axon terminals. Both were present in D β H immunoreactive and unlabeled axon terminals. Immunogold-silver labeling for A β and SGII was also clearly distinguishable from the immunoperoxidase reaction product. Of 631 D β H-labeled axon terminals, 56% (356/631) contained SGII immunoreactivity while 43% (275/631) did not contain SGII immunoreactivity. Of the 356 D β H-labeled axon terminals that contained SGII immunoreactivity, 39% (139/356) also contained A β immunoreactivity. In contrast, only approximately 22% (139/631) of all D β H-immunoreactive terminals were immunoreactive for A β . Of the 139 axon terminals that contained SGII and A β immunoreactivity, approximately 31% (43/139) formed symmetric-type synapses, while

1.4% 2/139) formed asymmetric-type synapses. The remaining 68% (95/139) did not show recognizable synaptic specialization.

The putative physiological role of A β as a modulator of neuronal excitability coupled with the in vivo neuroanatomical studies demonstrating co-localization of TH, A β , and SGII in the LC as well as D β H, A β and SGII in the ILmPFC, suggest that DCV may be a site of regulated co-secretion of A β from NE LC somatodendritic processes and from terminals in projection regions. What is not known, and may be critical to future understanding of the intersection between the LC-NE system and neurodegenerative disease of NE neurons in AD, is whether A β is co-released from DCV under conditions of chronic stress during LC auto regulation. It is tempting to speculate that, under such conditions, a failure to autoregulate the system would lead to increased A β levels in LC soma, predisposing it to plaque formation and subsequent degeneration. Further, one may inquire as to what other catecholamine neuronal populations are vulnerable to degeneration based on the distribution of DCV containing neurons throughout the central nervous system.

v. Implications for Disease: Chromogranin Peptides in AD

Synapse loss is the best correlate for cognitive impairment observed in AD (Terry, Masliah et al. 1991). CgA, SGI, and SGII are well known markers of large DCV, and have recently been identified amongst many synaptic proteins, as biomarkers of AD (Davis, Mohs et al. 1999, Jahn, Wittke et al. 2011, Wildsmith, Schauer et al. 2014). A number of investigators have used alterations in synaptic protein levels and their relationship with A β plaques to identify vulnerable subpopulations of neurons in cognitive deficits observed in AD. Among the first of these studies was conducted in the early 1990's (Brion, Couck et al. 1991), immunolabeling for synaptophysin and CgA was used identify SV and large DCV, respectively. Similarly, to characterize differential synaptic alterations in the brains of post-mortem AD patients, another study utilized CgA, CgB and SGII as markers of large DCV and synaptophysin as a marker of SV. Interestingly, the study found that in AD post-mortem brains, less than 5% of A β plaques contained synaptophysin, while 30% of beta-amyloid plaques co-labelled with CgA, 20% with SGII, and 15% with CgB. This study also brought to light an important interaction between CgA and glial cells, as about 40% of CgA immunopositive plaques and extracellular deposits were surrounded by activated microglia (Lechner, Adlassnig et al. 2004). The authors of the study concluded that CgA is likely to be a mediator between neuronal, glial and inflammatory mechanisms found in AD (Lechner, Adlassnig et al. 2004). Other reports have noted that immunological data suggest a significant increase in levels of Chromogranin A and that these increases were significantly correlated to clinical severity of dementia and neuropathological changes. Further, a high percentage of senile plaques were found to contain CgA and dystrophic neurites, compared to synaptophysin, which was rare (Lassmann, Weiler et al. 1992).

Other studies of a similar nature have demonstrated that there may be regional differences, as the hippocampus was found to have distinctly distributed CgA, CgB, and SGII with an overlap in their distribution patterns (Marksteiner, Kaufmann et al. 2002). The number of CgA immunoreactive plaques was significantly higher than the other chromogranins. In the dorsolateral cortex, the relative number of CgA immunoreactive neuritic plaques was highest

compared to other cortical areas and was observed in approximately 34% of A β containing plaques. They were distributed throughout all cortical layers with a preferential localization to deeper cortical layers. Another interesting distinction in this region is that the highest density of immunoreactivity was found for CgB, with a layer specific distribution in which SGII immunoreactivity was restricted to the innermost layer of the dentate gyrus whereas CgB was highly and uniformly concentrated throughout the inner molecular layer (Marksteiner, Kaufmann et al. 2002). In the AD brain, the density of SGII- and CgB-like immunoreactivity was significantly reduced in the inner molecular layer of the dentate gyrus, the CA1 area, the subiculum and in layers I, III and V of the entorhinal cortex (Marksteiner, Kaufmann et al. 2002). Another study reported that in general, all 3 chromogranin peptides were detected in hippocampal neuritic plaques, and that the reduction of Chromogranin A-LI in patients with AD was less pronounced with a statistically significant reduction in the entorhinal cortex. Taken together, these studies indicate that chromogranin peptides are markers for human hippocampal pathways and have potential as neuronal markers for synaptic degeneration in Alzheimer's disease (Marksteiner, Kaufmann et al. 2002, Lechner, Adlassnig et al. 2004).

vi. Conclusions

Taken together, the studies described here suggest that DCV may play an important role in preserving the integrity of the LC-NE system under conditions of stress, and that under the degenerative conditions of AD, the dysregulation of DCV and the chromogranin peptides they harbor, may have deleterious effects on the LC. This may result in decreased adaptive responses to stress, thus explaining, in part, increased indices of stress responsivity in AD patient populations. Alternatively, LC degeneration, induced by chronic stress, may have direct implications for chromogranin peptide functioning and regulation. While these possibilities have not been directly explored, evidence in the literature and evidence from our immuno-electron microscopy studies indicate that the LC may be a critical site of interaction of chromogranin peptides, NE and A β . Finally, the synthesis of evidence leads to pertinent questions for further research: What is the role of chronic stress and resultant LC-NE hyperactivity in the aberrant accumulation of CgA peptides? Does CgA peptide dysregulation play a reciprocal role in initiating mechanism of neurodegeneration in the LC? Continued research on the topic is greatly needed to address these questions.

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Highlights

- Several peptidergic systems converge onto the LC to counteract responsivity to stress.
- Evidence in the literature and evidence from our immuno-electron microscopy studies indicate that the LC may be a critical site of interaction of chromogranin peptides, NE and A β .
- The studies described here suggest that DCV may play an important role in preserving the integrity of the LC-NE system under conditions of stress, and that under the degenerative conditions of AD, the dysregulation of DCV and the chromogranin peptides they harbor, may have deleterious effects on the LC.

Figure 1:

Proposed convergence of DCV formation and APP processing in LC cell bodies. In parallel, APP and neuropeptides are synthesized in the endoplasmic reticulum (ER). The modification of neuropeptides and subsequent condensation into DCVs is a multi-step process that occurs in the trans golgi network (TGN) (Aixa Alfonso 2010). APP also undergoes important post-translational modifications in the TGN. Subsequently, immature vesicles may be remodeled to form mature DCV, a process that involves cargo condensation and changes in protein composition (Wang 2008, Aixa Alfonso 2010). During the processing and remodeling phase of maturation, neuropeptides can be sorted to different DCVs within the same cell before undergoing anterograde transport to axon terminals via kinesin motor proteins. We hypothesize that a portion of the DCV resulting from TGN processing and maturation contain A β peptides, or possibly its precursor, APP. The immunoelectron micrograph pictured is a tyrosine hydroxylase immunoreactive cell body in which immunogold labeled monomeric amyloid β (large gold/ arrow heads) are present in the ER structures in parallel with SGII (small gold/ arrows). It is possible that following synthesis, APP and chromogranins are co-packaged during sorting and maturation of DCV in the TGN.

Figure 2:

Immunofluorescence micrograph of SGII, Aβ, and DβH in the prefrontal cortex. SGII (red), Aβ (blue), and DβH (green) are labeled separately and marked by arrow heads. Magenta indicates co-localization between SGII and Aβ, and are marked by a thick arrow. Light blue represents co-localization between DβH and Aβ, marked by a thin arrow. White represents regions in which SGII, Aβ, and DβH were found co-localized and are marked by asterisks. Triple co-localization marked by white, or asterisks demonstrate the presence of SGII-containing vesicles that also contain Aβ, and DβH, and are likely to be DCV.

Figure 3:

Axon terminal of an LC cell body that is triple labeled with peroxidase labeled DβH, small gold labeled SGII indicated by arrows, and large gold labeled Aβ indicated by arrowheads. SGII and Aβ are frequently found in close proximity, away from active zones, suggesting their presence in DCV of the ILMpFC. These DCV may have been transported down the axon terminal from the LC cell body.