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OLD AND NEW EVIDENCE FOR NEUROPEPTIDE INVOLVEMENT IN ALZHEIMER'S DISEASE

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Alzheimer's disease (AD) is an irreversible degenerative disorder characterized by degeneration of neurons in different brain areas and by progressive cognitive and functional decline. Various deranged mechanisms play a role in the disease process all inducing neuronal death, the inevitable event occurring in AD. Novel therapeutic approaches using disease-modifying treatment are being investigated with the intention of influencing multiple pathways involved in AD. Because of their putative roles as neurotransmitters, neuromodulators, and neuroregulators in the central nervous system, neuropeptides have been the object of considerable research. Postmortem studies have provided evidence that several neuropeptide-containing neurons are pathologically altered in brain areas of AD patients, as well as in the brain of animal models of AD. In addition, altered levels of neuropeptides have been found in cerebrospinal fluid (CSF) of AD patients, getting insights into the potential role of neuropeptides in the pathophysiology of AD and offering the possibility to identify novel biomarkers of this pathology. The role exerted by neuropeptides seems particularly interesting since they are generally neuroprotective and widely distributed in brain areas responsible for learning and memory processes. The present review summarizes the recent findings on neuropeptide involvement in AD, with a focus on the contribution of thyrotrophin-releasing hormone, cholecystokinin, bradykinin and chromogranin/secretogranin family, describing brain distribution and the role played in AD and in cognitive functions, as well as their neuroprotective properties. Convincing evidence has been provided for the protective role of these neuropeptides against neurodegeneration observed in AD, both *in vitro* and *in vivo*, identifying neuropeptide receptors as potential therapeutic targets.

Alzheimer's disease (AD) is the most common neurodegenerative disease, affecting more than 20 million individuals worldwide. It is clinically characterized by memory defeat and cognitive impairment, accompanied by neuronal loss in the cerebral cortex, hippocampus, basal forebrain, locus coeruleus and dorsal raphe and by a significant damage of basal forebrain cholinergic neurons in brain (Pákáski M, Kálmán J, 2008).

Histopathologically, the hallmarks of AD are the ubiquitous presence of intra-neuronal fibrillary tangles and extracellular deposits of beta amyloid (Aβ) fibrils in senile plaques. While in physiological conditions the phosphorylation-modified tau protein stabilizes the axonal microtubules in the central nervous system (CNS), in AD tau protein may undergo abnormal phoshorylation, hyperphosphorylation and some other modifications (nitration, ubiquitination,

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truncation, shift, prolyl isomerization), leading to intraneuronal amassing of tau protein bringing about a disruption of neuronal cell communication (Zhao et al, 2014).

The amyloid (or Aβ) hypothesis (Hardy and Higgins, 1992) has become the dominant model of AD pathogenesis, indicating a crucial role for the production of Aβ peptides which aggregate into oligomers and further deposit as plaques, and guiding the development of potential treatments. The increased Aβ production leads to an amyloid core formation around which neurites, astrocytes, and glial cells accumulate, thus forming senile plaques and activating an immune system response (Mrak and Griffin, 2001; Tuppo and Arias, 2005).

According to this hypothesis, soluble Aβ oligomers are identified as the principal neurotoxic agent in AD pathology (Haass & Selkoe, 2007). This so-called "amyloid hypothesis" was partially modified by Selkoe and Hardy (2016), indicating the crucial role exerted by neuritic alteration in the immediate vicinity of AD plaques, raising the possibility of decreased efficiency of neurotransmission along them.

The pathophysiology of AD involves disturbances and imbalances occurring in a variety of mechanisms. Besides Aβ production, neurofibrillary tangle accumulation and inflammation, various deranged mechanisms, such as chronic oxidative stress, mitochondrial dysfunction, hormone imbalance, mitotic dysfunction, calcium mishandling, and genetic components play a role in the disease process, all inducing neuronal death, the inevitable event occurring in AD (Anand et al, 2014). The disease has been extensively studied looking for a therapy, however acetylcholinesterase inhibitors and memantine are the only drugs currently approved for treatment, nevertheless providing symptomatic treatment without altering the course of the disease. As a consequence, it is of great importance to develop disease modifying substances that might counteract or slow down neurodegeneration.

The role exerted by neuropeptides seems particularly interesting since they are generally neuroprotective and are involved in learning and memory processes.

Neuropeptides are small proteic molecules (from 3 to 100 amino acids) which mediate or modulate the neuronal communication by binding to specific cell surface receptors: they can act as true neurotransmitters or as neuromodulators (Hallberg, 2015).

Radioimmunoassay and immunohistochemistry have allowed to draw exact distribution maps of individual neuropeptides and their receptors in the central (CNS) and peripheral (PNS) nervous systems. Generally, neuropeptides originate in the body of the nerve cell from precursors with high molecular weight (pre-pro-peptides), usually biologically inactive, whose processing leads to the formation of one or more neuropeptides endowed with biological effects (Hallberg and Nyberg, 2003). Once released, neuropeptides can function as neuromodulators reaching their receptors at a considerable distance from the site of release. For this special form of endocrine transmission the concept has been proposed of volume transmission (VT) and extra-synaptic neurotransmission (Fuxe et al, 2012), further confirmed by the frequent discrepancy observed between neuropeptides and their cognate receptor distribution in many brain areas.

This transmission mode expands the classical concept of synaptic transmission based on a communication point by point and emphasizes the capacity of the transmitter molecules, especially peptides, to influence large target areas.

Unlike classical neurotransmitters, inactivated by specific synaptic reuptake mechanisms, neuropeptide activity is disabled by enzymatic proteolysis mediated by various extracellular peptidases, or is induced by the decrease of their concentration due to dilution. This neuropeptide degradation can lead to the formation of fragments that have similar or very different biological activities in respect to parent peptides (Nyberg and Hallberg, 2007).

Considering the large distribution of neuropeptides in the CNS and their ability to modulate cognitive functions, it is not surprising that numerous neuropeptides are differentially affected in AD (Table I).

The role exerted by neuropeptides in AD has been recently reviewed. Van Dam et al, (2013) summarized pathophysiological mechanisms and

Table I. *Changes in neuropeptide levels in AD patients and AD animal models, compared to the corresponding controls (healthy patients or wild type animals).*

	AD human brain		AD human CSF		AD animal models	
Pep tide	**Levels**	**References**	**Levels**	**References**		**References**
TRH	↓	Biggins *at al.*, 1983 (amigdala) Luo *at al.*, 2002 (hippocampus) Yong-Hong *at al.*, 2013 (blood)	↑	Pekary *at al.*, 1991 (TRHGly)		
CCK	↓	Perry *at al.*, 1981 (cortex) Sagar *at al.*, 1984 (hippocampus) Mazurek and Beal, 1991 (cortex) Lofberg *at al.*, 1996 (cortex)			↓	Diez *at al.*, 2003 (hippocampus of APP23 mice)
	↑	Struble *at al.*, 1987 (amyloid plaques) Perry *at al.*, 1981 (amyloid plaques)			↑	Diez *at al.*, 2003 (hippocampus, cortex and amyloid plaques of V717F mice)
BK					↑	Iores-Marcal *at al.*, 2006 (BK fragment in CSF of Aβ infused rats)
CgA	↑	(amyloid plaques): Marksteiner *at al.*, 2002, Rangon *at al.*, 2003, Lechner *at al.*, 2004, Willis et al 2011	↓	Blennow et al 1995; Eder et al 1998; Simonsen et al 2007; Perrin et al 2011; Jahn et al 2011; Mattson et al 2012; Paterson et al 2014; Wildsmith *at al.* 2014		
CgB	↓	Marksteiner et al 2002 (hippocampus) Lechner *at al.* 2004 (cortex)	↓	Eder et al 1998; Mattson et al 2010, 2012, 2013		
	↑	(amyloid plaques): Marksteiner et al 2002; Lechner *at al.* 2004; Willis *at al.* 2008, 2011				
	AD human brain		**AD human CSF**		**AD animal models**	
Peptide	**Levels**		**References**		**Levels**	
SgII	↓	Marksteiner et al 2002 (hippocampus) Lechner *at al.* 2004 (cortex)	↓	Eder et al 1998; Matsson et al 2010; Spellman *at al.* 2015	↑	Willis *at al.*, 2008 (amyloid plaques of V717I and K670M/ N671L mice)
	↑	Kaufmann 1998 (amyloid plaques, hippocampus, cortex) Marksteiner et al 2002 (amyloid plaques) Lechner *at al.* 2004 (amyloid plaques)				

SgIII	↑	Plà *at al.* 2013 (cortex)				
7B2	↑	Winsky-Sommerer *at al.*, 2003 (cortex) Helwig et al 2013 (amyloid plaques)			↑	Helwig et al 2013 (amyloid plaques of APP/PSEN1 mice)
ProSAAS	↑	Wada et al 2004 Hoshino *at al.* 2014 (amyloid plaques)	↓	Jahn *at al.* 2011	↑	Hoshino *at al.* 2014 (brain of APdE9 mice)
VGF		Cocco *at al.* 2010 (cortex)	↓	Carrette el al 2003; Jahn *at al.* 2011; Wujte *at al.* 2012; Spellman *at al.* 2015; Hottla *at al.* 2015; Hendricson *at al.* 2015		

therapeutic opportunities of vasopressin and oxytocin, somatostatin, neuropeptide Y (NPY), corticotropin releasing hormone (CRH), urocortin, galanin, vasoactive intestinal peptide (VIP), neurotensin, opioid peptides, angiotensin and substance P (SP). Willis et al, (2011) and Severini et al, (2016) reviewed the involvement of chromogranins and SP in AD, respectively.

The aim of the present review is to update information on the activity of old and new neuropeptides involved in AD, i.e. thyrotrophin-releasing hormone, cholecystokinin, bradykinin and chromogranin/secretogranin family.

THYROTROPIN-RELEASING HORMONE

Thyrotrophin-Releasing Hormone (TRH) was the first hypothalamic releasing hormone to be isolated and characterized. The name TRH derives from its action on the anterior pituitary, where it stimulates, *in vivo* and *in vitro*, not only the synthesis and release of thyrotrophin (TSH), but also of prolactin (PRL), and in some species also of growth hormone (GH) (Joseph-Bravo et al, 2015). TRH is a tripeptide, identified in 1969, derived from a 242-amino precursor acid protein (ProTRH). In addition to its neuroendocrine function, stimulating the thyroid gland, TRH has functions of neurotransmitter and neuromodulator in both the CNS and PNS. It was demonstrated to co-localize and to be co-secreted with other neurotransmitters in different nerve cell types in both CNS and peripheral tissues (Hrabovszky and Lipositis, 2008). ProTRH mRNA and TRH itself are widely distributed throughout the brain in extra-hypothalamic regions, including the olfactory system, reticular thalamic nucleus, amigdala, the hippocampus, piriform cortex, and striatum, where it plays a neuromodulatory role (Jackson and Reichlin, 1974; Pekary, 1998) (Fig. 1).

TRH has been shown to produce a variety of behavioral changes and neuropharmacological effects independent of its thyrotrophin-releasing properties, by acting via interaction with two different membrane receptors. TRH receptor 1 (TRH-R1) and receptor 2 (TRH-R2) are typical G-protein-coupled receptors, coupling to Gq ang G11, pertussin-toxin-insensitive G proteins that activate PLC-β (Gershengorn and Osman, 1996). Consistent with the endocrine functions, THR-R1 predominates in hypothalamic nuclei, however, is present in brainstem regions and spinal cord motoneurons where it is involved in autonomic and somatomotor control. THR-R2 mRNA is widely distributed throughout the brain with highest levels in the thalamus, cerebral and cerebellar cortex, medial habenulae, medial geniculate nucleus, pontine nuclei, and the entire reticular formation (Heuer et al, 2000). TRH has been proposed to play a role in AD. Significant differences in hypothalamic or pituitary functions were observed by Albert et al,

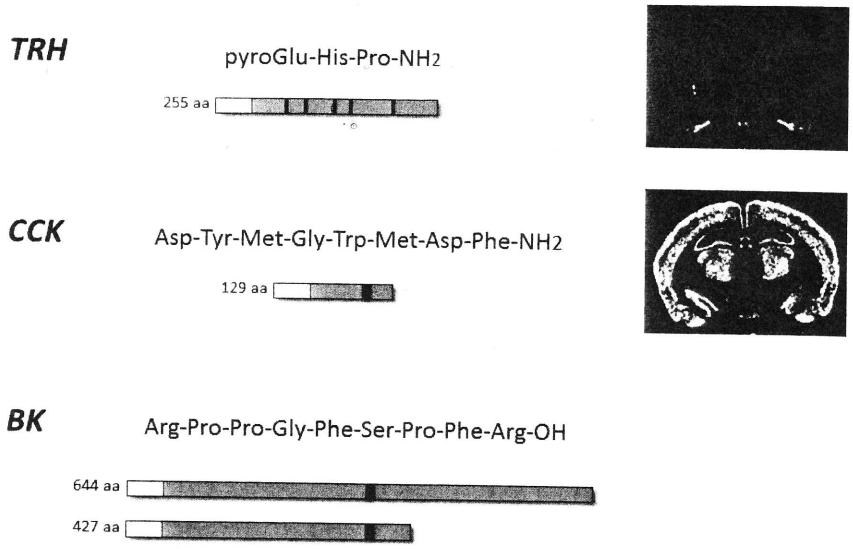

Fig. 1. *TRH, CCK and BK precursor sequence and brain distribution. The scheme represents the approximate length and the number of amino acids of the precursor of TRH, CCK and BK and their amino acid sequence. The leader signal for secretory protein is indicated as light shadowed block. The black blocks indicate the biological active fragments identified. In the right panels are shown images of in situ hybridization for the corresponding gene expression in mouse CNS (Source from Allen Brain Atlas, mouse.brain-map.org).*

(1993) between patients with senile dementia of AD type and control subjects undergoing the TRH test, while other studies failed to demonstrate a significant impairment under TRH stimulation (Gomez et al, 2000). Other works, focusing on the TRH-TSH-thyroid axis, confirmed an abnormal function of hypothalamic or pituitary functions in AD patients showing that, compared to healthy controls, the AD patients had significantly lower levels of TRH in the blood (Yong-Hong et al, 2013).

During the last decades, the relationship between TRH and AD in human subjects has been extensively studied. Increased levels of TRH were found in CSF (Pekary et al, 1991), and decreased levels in hippocampal regions (Luo et al, 2002) and amigdala (Biggins et al, 1983), despite other studies not describing significant differences (Yates et al, 1983; Bouras et al, 1986; Nemeroff et al, 1989; Banky et al, 1992). TRH has been shown to have neuroprotective functions in primary neuronal cultures (Koenig et al, 1996) due, at least in part, to its potent inhibition of GSK-3 via PKC-mediated phosphorylation (Luo and Stopa, 2004). The neuroprotective activity of TRH, together with the known facilitation of cholinergic functions, suggested a therapeutic potential of TRH in AD. It was, in fact, demonstrated that TRH administration can improve memory function in AD patients (Yarbrough and Pomara, 1985; Mellow et al, 1989) and also in the fimbria-fornix lesioned rat model (Bennet et al, 1997).

Possible mechanisms for TRH neuroprotection have been proposed. Among others, it has been suggested that a signal transduction pathway linking TRH with GSK3β activity and tau phosphorylation could be responsible for the formation of neurofibrillary tangles associated with dementia of AD. By binding to its receptor, mostly the TRH-R2 subunit receptor, TRH should be able to activate the G-protein coupled receptor (GPCR), finally triggering the MAPK signal pathway to inhibit GSK3β activity and prevent tau phosphorylation (Luo and Stopa, 2004).

CHOLECYSTOKININ

Cholecystokinin (CCK), a peptide originally discovered as a gastrin-like molecule of the gastrointestinal tract, plays an important role in

the release of pancreatic enzymes, gall bladder contraction, and gastric motility. As recently reviewed (Beinfeld handbook 2013 ,738-743), CCK is one of the most abundant and widely expressed neuropeptides in the brain, essentially as CCK8 amide, in the sulphated form. CCK shares the same five carboxyl-terminal amino acids with gastrin, which is believed to have evolved from CCK. CCK has been shown to be present in microgram quantities in the brain, distributed in high levels in the hippocampus, amygdala, septum, olfactory tubercles, caudate nucleus and the hypothalamus, ventral tegmental area and substantia nigra (Schiffmann et al, 1991) (Fig. 1).

CCK co-localizes with several key neurotransmitters and neuromodulators, prevalently γ-aminobutyric acid (GABA), as well as endocannabinoids, dopamine, serotonin and vasoactive intestinal peptide (Hokfelt et al, 1980; Somogyi et al, 1984; Kosaka et al, 1985; Katona et al, 1999), providing additional evidence for its wide-ranging role in physiological functions in neuronal networks.

CCK interacts with two G-protein (G_q and G_{11}) receptors, activating a variety of intracellular signal transduction pathways (Williams et al, 2002). Protein kinase C activation is the main signaling for CCKA receptor (CCKA-R), while adenylyl cyclase is for CCKB receptor (CCKB-R). CCKA-R is relatively specific for sulphated CCK8, while CCKB-R (identical to the gastrin receptor) interacts also with un-sulphated CCK8, CCK4 and gastrin and represents the main CCK receptor in the brain. CCK acts as an excitatory neurotransmitter modulating the release and function of other neurotransmitters and is involved in diverse normal behaviors, such as learning and memory, feeding, nociception and satiety. The central importance of CCK in neuronal networks is also reflected by its involvement in a variety of neurological and neuropsychiatric disorders including anxiety, panic attacks, schizophrenia and epilepsy (Lee and Soltesz, 2011). Several reports suggest a modulatory role of CCK in memory processing (Flood et al, 1987, Kovács and De Wied, 1994), an aspect of crucial relevance in AD, in which memory and other cognitive functions are impaired. It was demonstrated that CCK enhances memory retention and protects cholinergic neurons against basal forebrain lesions (Sugaya et al, 1992).

Alterations in the CCK system have also been correlated with AD. Indeed, despite the content of CCK in the brain of AD patients being generally relatively unchanged, in the most severe cases it was found to be reduced (Perry et al, 1981; Sagar et al, 1984; Mazurek and Beal, 1991; Löfberg et al, 1996). In addition to reduction in CCK content in brain tissues, neuritic plaques in the AD brain have been demonstrated to contain CCK (Struble et al, 1987; Perry et al, 1981).

Moreover, the CCK receptors are also affected, as a significant down-regulation was demonstrated of both CCKA and CCKB receptors in the brain of patients with AD and with mild cognitive impairment, suggesting that these receptors could play a role in AD development (Hokama et al, 2014; Lin et al, 2014).

Alterations in CCK content have been found also in AD animal models. Unlike the decrease in peptide levels reported in studies on AD human brain, increased levels of CCK were demonstrated in hippocampus and cortex, as well as in neuritic plaques of 18- and 26-month-old transgenic mice overexpressing V717F human beta-amyloid precursor protein (Diez et al, 2000). By contrast, in APP23 mice a decrease in CCK immunoreactivity was shown in hippocampal mossy fibers (Diez et al, 2003).

BRADYKININ

The kinins bradykinin (BK) and kallidin, also called Lys^{10}-BK, are oligopeptides released in the plasma or interstitial fluid after the cleavage of kininogens by kallikreins, a family of serine proteases. Plasma kallikrein and tissue kallikrein 1 (KK1) are the main enzymes involved in kinin source in blood and tissue, respectively. Kininogen-1 gene (KNG1) is a glycoprotein that contains the BK sequence in its mid portion (Fig. 1).

Kinins are potent vasodilators, promote natriuresis and diuresis, and have beneficial cardiovascular effects, however, they also promote pain and inflammation (Bhoola et al, 1992). Components of the kallikrein-kinin system are present in blood,

heart, aorta, brown adipose tissue, adrenal and lung. In the brain, they are localized in the cerebral cortex, brain stem, cerebellum, hypothalamus, hippocampus, and pineal gland, among others. They are found surrounding blood vessels, in neurons and glial cells (Raidoo and Bhoola, 1998).

In human plasma, BK is rapidly metabolized by kininases, among which aminopeptidase P and carboxypeptidase N. However, the major degradation pathway of BK involves also kininase II and angiotensin-converting enzyme (ACE), which is also responsible for the conversion of inactive angiotensin I into the vasopressor angiotensin II (Boola et al, 1992).

Biological effects of kinins are produced by activation of two transmembrane receptors coupled to G proteins (Gα and Gq), namely B1 and B2 receptor (Regoli and Barabe, 1980). Most actions of kinins are mediated by B2 receptor, which has high affinity for BK and is considered a constitutive receptor (Regoli et al, 1998). On the other hand, B1 receptor possesses higher affinity to des-Arg^9-BK and Lys-des-Arg^9-BK and has limited distribution in tissues under physiological conditions. However, it is mainly expressed in pathological conditions such as chronic inflammation, infection or injury (Regoli and Barabe, 1980). Activation of kinin receptors induces phospholipase C stimulation and promotes intracellular calcium mobilization, as well as release of nitric oxide (NO), especially on neurons and blood vessels (Marceau, Regoli, 2004).

The role exerted by kallikrein-kinin system in AD and other neurological disorders was recently reviewed (Viel and Buck, 2011; Naffah-Mazzacoratti et al, 2014). It was demonstrated that proteolytic enzymes levels are altered in AD (Ladror et al, 1994). Among these, intracerebral kallikrein seems to play an important role in the pathogenesis of AD, as demonstrated by a reduced kallikrein-like enzyme activity, due to a reduction in the gene expression in cerebral tissue of AD patients (Aoyagi et al, 1990). Likewise, increased expression of kallikrein 10 and kallikrein 6 were observed in CSF, plasma and whole blood of patients with AD, showing a strong relationship between the kallikrein-kinin system and brain degeneration (Diamandis et al, 2000; 2004).

Additionally, an activation of the contact/kinin system in CSF of patients with AD has been reported as the result of an anionic interaction of residues within the region 1–11 of $A\beta_{1-42}$ with factor XII, inducing kallikrein generation. This finding seems to be characteristic for brain of AD patients, since in the CSF of patients with neuroimmune inflammatory disease (multiple sclerosis, chronic inflammatory demyelinating polyneuropathy) there was no evidence of increased cleavage of high molecular weight kininogen (Bergamaschini et al, 2001).

According to Farrall et al, (2009), the frontal cortex of patients with AD shows high levels of plasma kallikrein as well as its mRNA. This finding and the high enzyme activity suggest that kinin production could influence cerebral blood flow and vascular permeability altered in AD.

A more direct evidence for BK involvement in AD pathology was demonstrated analyzing BK release and its processing in brain and cerebrospinal fluid (CSF) of rats infused chronically with Aβ (Iores-Marçal et al, 2006). In CSF of animals infused with Aβ, BK concentration was significantly increased, however, in the brain of Aβ group, only a BK fragment was detected. These results suggest that the kallikrein-kinin system is activated in this AD animal model, and that BK is efficiently inactivated by kininases in brain. Since it was reported that in cell cultures BK can increase alpha-secretase processing of APP, inducing decreased $A\beta_{1-40}$, a major constituent of amyloid plaques, BK inactivation could ultimately contribute to the increased senile plaque deposits in the rat brain (Nitsch et al, 1998).

In addition to the variations in kallikrein-kinin enzymatic system, also BK receptors expression appeared to be modified in AD, mainly related to neuroinflammation (Marceau and Bachvarov, 1998, Viel and Buck, 2011). Indeed, in cultured skin fibroblasts from AD patients an increase in the number of BK receptors was reported (Huang et al, 1995), as well as biochemical abnormalities in B2 receptor functions (Jong et al, 2002).

Moreover, increased expression of B1 receptor was found in hippocampal astrocytes of AD mice. In the same work, the ability of B1R antagonists

to abrogate amyloidosis and cerebrovascular and memory deficits was demonstrated, providing evidence for a harmful role for B1R in AD pathogenesis (Lacoste et al, 2013), despite other experimental data indicating that in Tg-SwDI mice B1R activation plays an important role in limiting the accumulation of Aβ in AD-like brain (Passos et al, 2013).

It was also shown that chronic i.c.v. injection of $A\beta_{1-40}$ promotes significant increase in densities of kinin B1 and B2 receptors, mainly in brain regions related to cognitive behavior (Viel et al, 2008). Nevertheless, a single i.c.v. injection of aggregated $A\beta_{1-40}$ induced an increase in B1 receptor expression in hippocampus, but did not modify B2 expression in the same area (Prediger et al, 2008). These variable results suggest that the involvement of the kinin system in Aβ toxicity could be a function of the quantity of Aβ, as well as a function of the exposure time of tissue to the peptide.

On the whole, while B1R is certainly involved in neuroinflammation related to AD, B2R preferentially seems to mediate neuroprotective effects. Activation of B2R by BK was demonstrated to reduce inflammation and neuronal death (Noda et al, 2007) and to promote neurogenesis (Trujillo et al, 2012). Furthermore, it was shown that activation of B2 receptors, but not B1 receptors, up-regulates mRNA for nerve growth factor (NGF) in glial cells, establishing a neuroprotective condition (Noda et al, 2007a).

New advance in the role exerted by BK receptors in AD animal models is represented by the availability of B1 and B2 knockout mice. Following Aβ infusion, B1 knockout mice did not show any difference in memory behavior compared to control animals with the same treatment, while B2 knockout mice resulted in a significant reduction in memory consolidation (Amaral et al, 2010). These data demonstrate that, following chronic infusion with Aβ, B1 receptor could play an important role in the neurodegenerative process, while B2 receptor could have a neuroprotective role. This is further confirmed by the increased number of Aβ plaques found in B2 knockout mice infused with Aβ, pointing to B2 receptor as a potential therapeutic target in AD (Caetano et al, 2015).

Another point to be considered is the potential activity of ACE inhibitors in AD (Zou and Michikawa, 2008). ACE inhibitors (such as enalapril, ramipril and many others) are well-established as important antihypertensive drugs, liable to block conversion of inactive angiotensin I into the vasopressor angiotensin II. However, they also increase half-life of BK, enhancing levels of circulating BK and potentiating BK receptors. At least *in vivo*, ACE inhibitor activity is mainly mediated by B2 receptors, as demonstrated by the inhibitory effect exerted by B2 receptor antagonists (Marceau and Regoli, 2004). The ability of ACE inhibitors to modulate the kinin system could be responsible, at least in part, for the observed neuroprotective activity.

CHROMOGRANIN/SECRETOGRANIN FAMILY

The chromogranin/secretogranin family represents an extended but functionally conserved family of proteins, including chromogranins (chromogranin A and chromogranin B), secretogranins (secretogranin II and secretogranin III), and related proteins (7B2, NESP55, proSAAS, and VGF). They are localized in secretory vesicles and are variously distributed in endocrine, neuronal and neuroendrocrine cells, as well as in the immune system and occasionally in other tissues, subserving essential roles in the regulated secretory pathway that is responsible for controlled delivery of peptides, hormones, neurotransmitters, and growth factors. In the brain, they are widely localized in different areas (Fig. 2).

The first granins identified were chromogranin A (CgA) and chromogranin B (CgB), purified from adrenal medulla, but other proteins were successively added to this family, all sharing some commune features. They are large acidic proteins, sometimes glycosylated or sulphated, having the ability to bind calcium and, although very soluble, to aggregate in the acidic compartment, inducing the formation of dense core granules in the presynaptic structures. Granins regulate different functions, acting as chaperons for protein sorting, modulating prohormone convertase activity and regulating secretory vesicle content release. Moreover, through the secretory pathway, most of them are proteolitically

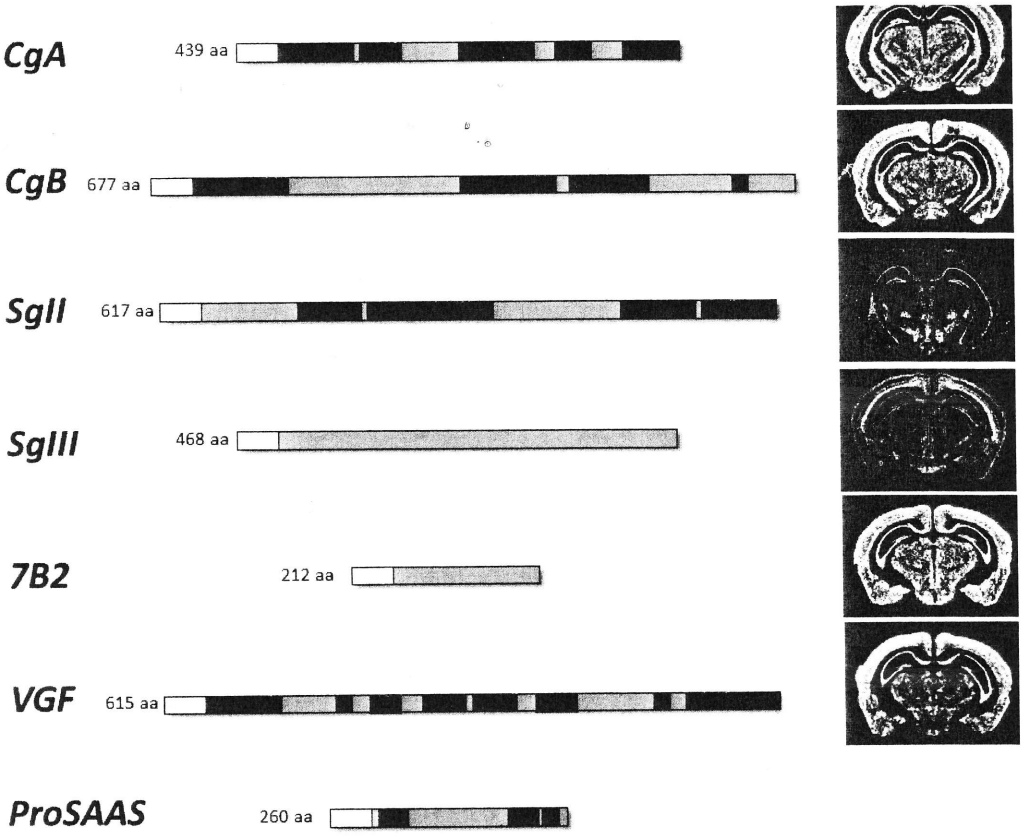

Fig. 2. *Granin precursors sequence and brain distribution. The scheme represents the approximate length and the number of amino acids of the precursor of CgA, CgB, SgII, SgIII, 7B2, VGF and ProSAAS. The leader signal for secretory protein is indicated as light shadowed block. The black blocks indicate the biological active fragments identified. In the right panels are shown images of in situ hybridization for the corresponding gene expression in mouse CNS (Source from Allen Brain Atlas, mouse.brain-map.org).*

processed in different biological active peptides that are stored in large dense core granules and released upon secretory stimulation (for extensive review see Bartolomucci et al, 2011). Despite repeated attempts to demonstrate the existence of cognate receptors, no evidence for specific granin receptors, or the precise mechanisms of action has been produced to date. Since granins are largely distributed in the CNS and are involved in synaptic functions, several studies investigated the potential utility of granins as diagnostic biomarkers for AD and other neurodegenerative diseases (Bartolomucci et al, 2011, Willis et al, 2011). However, due to the processing of these large precursor molecules in many different neuropeptides, it is difficult to recognize the precise identity of these fragments because often Authors refer to the whole precursor.

a) Chromogranin A

CgA, a 439-amino-acid protein, was the first identified granin and the most extensively studied (Winkler and Fischer-Colbrie, 1992). CgA was postulated to be an immunostimulator in AD, contributing to neuroinflammation started by Aβ peptides (Heneka et al, 2010). Indeed, intense staining for CgA was demonstrated in about 30%

of Aβ plaques in AD cortical samples, frequently surrounded by hyperactivated microglia (Marksteiner et al, 2002; Rangon et al, 2003; Lechner et al, 2004; Willis et al, 2011). CgA increase in neuritic plaques correlates with an increase in Catestatin (CST, an internal fragment of CgA), able to activate pro–caspase-1 (Wu et al, 2013).

There is evidence that CgA activates microglia to a reactive phenotype and stimulates the release of microglial cytotoxins, suggesting that this peptide may contribute to the continued and neurotoxic activation of microglia in AD (Lechner et al, 2004).

CgA staining increase in the amyloid plaques inversely correlates with CgA levels found in the CSF, significantly reduced in patients with AD or taupathies (Blennow et al, 1995; Eder et al, 1998; Simonsen et al, 2007; Perrin et al, 2011; Jahn et al, 2011; Mattson et al, 2012; Paterson et al, 2014; Wildsmith et al, 2014). Among CgA derived peptides, only Serpin (26 aa at C-terminal) showed a neuroprotective activity, as demonstrated *in vitro*, in AtT20 neuronal cell line (Koshimizu et al, 2011). To date, no neuroprotective functions have been identified for Vasostatin, Pancrestatin and Chromacin, the other major forms of the CgA-derived neuropeptides, endowed with neuroendocrine activities.

b) Chromogranin B

CgB, a pre-pro-protein of 677 amino acids, shares several features with CgA, including wide expression throughout the endocrine and nervous systems, acidic protein backbone, random-coil structure, and heat stability. CgB is abundantly expressed in many neurons and peptidergic endocrine cells (Bartolomucci et al, 2011).

Immunostaining of post-mortem brains from AD patients showed positive CgB reactivity in amyloid plaques, more prominent in hippocampal regions (Marksteiner et al, 2002; Lechner et al, 2004; Willis et al, 2008, 2011), while levels of CgB peptides in CSF of AD patients were significantly decreased, compared to control subjects (Eder et al, 1998; Mattson et al, 2010; 2012; 2013).

c) Secretogranin II

SgII is a 617 amino acid pre-pro-protein highly conserved across evolution and, together with CgB is the major soluble constituent of the large dense core vesicles of presynaptic structures (Fischer-Colbrie et al, 1995). Several studies showed an increased SgII immunostaining in amyloid plaques, while a significant reduction in immunoreactivity was described in different brain areas of AD patients (Kaufmann, 1998; Marksteiner et al, 2002; Lechner et al, 2004). Alterations in SgII content have been reported also in animal models of AD. Indeed, in brain of transgenic mice overexpressing human APP751 with the London (V717I) and Swedish (K670M/N671L) mutations, about 40% of amyloid-beta plaques were associated with SgII, however no immunostaining reduction was observed in specific brain areas, compared to controls, as otherwise reported in AD patients (Willis et al, 2008).

From SgII processing three major products are obtained: Secretoneurin (SN, 33 aa sequence, near the N-terminal portion), EM-66 (central portion) and Manserin (40 aa sequence, near the C-terminal region). Consistent with the decreased level of CgA and CgB found in in CSF of AD patients, also SN or other SgII fragments were found to be reduced (Eder et al, 1998; Matsson et al, 2010; Spellman et al, 2015). In addition, SN neuropeptide was demonstrated to promote *in vitro* and *in vivo* neuroprotection after oxygen/glucose deprivation, suggesting an anti-apoptotic activity through Jak2/Stat3 signaling pathway (Shyu et al, 2008).

d) Secretogranin III

SgIII is an acidic 468 amino acid secretory protein, well conserved during evolution, from mammals to fish. It has been identified as a specific binding protein for CgA, suggesting that it can play a central role in secretory granule biogenesis (Hosaka and Watanabe, 2010).

Although different fragments of SgIII have been detected in several neuroendocrine cell types from various species (Holtious et al, 1996), no biologically active peptides derived from SgIII have been described. In the cerebral cortex of AD patients increased levels of SgIII were observed in dystrophic neurites surrounding amyloid plaques (Plà et al, 2013). Additionally, SgIII was detected in CSF but

no significant differences between AD patients and control subjects were reported (Perrin et al, 2011).

e) 7B2

The smaller granin 7B2 (212 amino acids) is perhaps the most evolutionarily conserved member of the granin family, containing a C-terminal peptide acting as inhibitor of the prohormone convertase 2 (PC2), responsible for the proteolytic processing of many precursor proteins, among which neuropeptides precursors (Mbikay et al, 2001). Alterations, often conflicting, in PC2 and 7B2 levels have been shown in AD brain, related to a dysregulation in the level of different neuropeptides. A marked decrease in the ratio of the PC2 precursor to the total enzymatic pool was observed in the frontal cortex of AD patients, corresponding to an increase in the binding protein 7B2 (Winsky-Sommerer et al, 2003). On the contrary, other studies reported increased levels of PC2 in AD brain, while no differences were detected in the levels of 7B2 (Yakovlera et al, 2007). Likewise, no significant differences were found in the 7B2 immunoreactivity in various brain regions obtained from patients with AD and from control subjects (Iguchi et al, 1987).

More recently, in the hippocampus and substantia nigra of human AD-affected brains, as well as in the brains of APP/PSEN1 mice, 7B2 was found highly co-localized with Aβ plaques and α-synuclein deposits (Helwig et al, 2013). In the same work it was demonstrated that 7B2 efficiently prevents *in vitro* fibrillation and formation of Aβ aggregates, establishing this neural protein as an anti-aggregation chaperon associated with neurodegenerative diseases. In addition, recombinant 7B2 efficiently blocked the neurocytotoxic effect of $A\beta_{1-42}$, significantly increasing cell viability of Neuro-2A cells (Helwig et al, 2013).

f) ProSAAS

ProSAAS granin is a 260 amino acid precursor protein suggested to function as peptide precursor only in higher vertebrates (Bartolomucci et al, 2011). Like 7B2, it has been suggested to regulate the activity of the pro-hormone convertase PC1 (Lee et al, 2004). The potential role exerted by proSAAS in the pathogenesis of AD was suggested by the evidence that N-proSAAS or proSAAS-like molecules are trapped within the tau fibrils and accumulated in tau inclusions in the AD patients brains (Wada et al, 2004). In addition, in the brain of 12-month-old APdE9 mice, and in the cortex of human AD affected brain, proSAAS immunoreactivity co-localizes with amyloid plaques deposits (Hoshino et al, 2014).

Like other granins, ProSAAS fragments were found decreased in CSF of AD patients in respect to healthy subjects, suggesting that they could represent, together with other neuropeptides, biomarkers for AD (Jahn et al, 2011). As previously reported for 7B2, additional evidence for the role of ProSAAS in AD was demonstrated by the amyloid anti-aggregant activity of an internal ProSAAS fragment (ProSAAS 97-180), shown to efficiently prevent the fibrillation of $A\beta_{1-42,}$ *in vitro* (Hoshino et al, 2014). In the same study, the Authors reported that the recombinant, as well as the endogenously synthesized proSAAS, was able to prevent the neurotoxic effect of $A\beta_{1-42}$ in Neuro2a cells.

g) VGF

The large secretogranin VII, early named VGF, is a 615 amino acid protein precursor of several biological active peptides. A dozen of them have been detected in CSF of patients with neurodegenerative diseases. In CSF of AD patients, both an N-terminal fragment and an internal peptide (VGF 365-375) were found to be decreased (Carrette el al, 2003; Jahn et al, 2011; Wujte et al, 2012; Spellman et al, 2015; Hottla et al, 2015; Hendricson et al, 2015), suggesting that VGF could be a potential biomarker for this disorder. Moreover, in parietal cortex from patients with AD, a reduction in different VGF peptides was shown (Cocco et al, 2010), whereas an increase of VGF expression was reported in peripheral T cells in patients with AD, compared to aged healthy controls (Busse et al, 2015).

Interestingly, it was demonstrated that VGF-derived peptides exert important neuronal stimulatory activity. TLQP-62 (C-terminal VGF peptide) has an antidepressant activity (Lin et al, 2014), increases neuronal electrical excitability in hippocampus neurons (Takker-Varia et al, 2007), while TLQP-21 (a smaller C-terminal VGF peptide)

protects from apoptosis cerebellar granule cells after potassium deprivation (Severini et al, 2008). Moreover, in primary cortical and hippocampal cell cultures, TLQP-21 showed a neuroprotective activity against Aβ toxicity, while other fragments had minor or no neuroprotective effect (Possenti R., unpublished data).

So far, only for TLQP-21 peptide a receptor has been recently discovered, identifying the variously distributed complement C3a receptor-1 (C3AR1) as a target for TLQP-21 (Hannedouche et al, 2013; Cero et al, 2014).

CONCLUSIONS

AD has become a great clinical problem in our society and the prevalence of AD is likely to increase among the aging population worldwide. To date, no treatment has been found that could slow down the progression of the disease or that could prevent cholinergic cell death, as the current therapeutic approach to AD is of a symptomatic type. Since the pathology of AD is very complex and different pathomechanisms are involved, the ultimate goal of a sustainable disease-modifying treatment in AD is to slow down disease progression by addressing the neurodegenerative processes, acting at multiple pathways. The role exerted by neuropeptides seems particularly interesting since they are generally neuroprotective, widely distributed in brain areas responsible for learning and memory processes, and their levels are altered in both human disease and in animal experimental models. Since neuropeptides could represent biomarkers of disease progression, this seems of great potential utility for AD because of inherent difficulties assessing brain function and finding a diagnosis in this pathology. In addition, convincing evidence has been provided for the protective role of several neuropeptides against neurodegeneration both *in vitro* and *in vivo*, identifying neuropeptide receptors as potential therapeutic targets.

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