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Cellular level/distribution of γ -secretase subunit nicastrin and its
modulator p23 in the brain

by

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ABSTRACT

The processing of amyloid precursor protein (APP) by β - and γ -secretases produces amyloid β (A β) peptide, the principal component of the neuritic plaques found in Alzheimer's disease (AD) pathology. The enzyme γ -secretase is a multimeric protein consisting of presenilins-1/2 (PS1/PS2), nicastrin, anterior pharynx defective 1 (APH-1) and presenilin enhancer-2 (PEN-2). Recently it was discovered that p23, a transmembrane protein involved in intracellular protein trafficking, negatively regulates γ -secretase activity. In the present study, I evaluated the levels/expression of the nicastrin and p23 and their possible colocalization with PS1 in normal adult and developing brains. Additionally, I have studied the alterations of p23 levels in both animal model of neurodegeneration and in postmortem AD brains. Nicastrin and p23 were widely distributed throughout the brain and colocalized in all brain regions with PS1. The levels of nicastrin and p23 were relatively high at the early stages of postnatal development and then declined gradually as age increased. Interestingly, p23 level/expression was found to be altered following kainic acid-induced neurodegeneration in the adult rat brain. Additionally, p23 levels were reduced in the brains of individuals with AD. These results, taken together, suggest that both nicastrin and p23 are expressed in neurons throughout the brain and their levels decline gradually during development to reach an adult profile. Additionally, my results indicate that a decreased level of p23 may contribute to AD pathogenesis by increasing the production of A β -related peptides.

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LIST OF ABBREVIATIONS

a.a	amino acid
A β	Amyloid- β peptide
AD	Alzheimer's disease
NFT	Neurofibrillary tangle
ADAM	A Disintegrin and Metalloprotease
sAPP α	Soluble amyloid precursor protein alpha
AICD	Amyloid precursor protein intracellular domain
APH-1	Anterior pharynx-defective phenotype-1
APOE	Apolipoprotein E
APP	Amyloid precursor protein
ATP	Adenosine triphosphate
BSA	Bovine serum albumen
BACE	β -site APP cleaving enzyme
C83	83 a.a residues C-terminal fragment
C99	99 a.a residues C-terminal fragment
COP	Coat protein
CTF	C-terminal fragment
CuBD	Copper binding domain
DAP	DYIGS and peptidase
DBB	Diagonal band of Broca
DG	Dentate gyrus
DMEM	Dulbecco's modified Eagles medium
ECL	Enhanced chemiluminescence
ER	Endoplasmic reticulum
FAD	Familial Alzheimer's disease
FC	Frontal cortex
FITC	Fluorescein isothiocyanate
GAPDH	Glyceraldehyde-3-phosphate dehydrogenase
GFAP	Glial fibrillary acidic protein

GFP	Green fluorescence protein
GFLD	Growth factor like-domain
HMW	High-Molecular Weight
KPI	Kunitz-type protease inhibitor
NICD	Notch intracellular domain
NP	Neuritic plaques
NSAIDs	Non-steroidal anti-inflammatory drugs
NTF	N-terminal fragment
P23/TMP21	P24 family of transmembrane protein
P75NTR	p75 neurotrophin receptor
PAL	Proline-Alanine-Leucine
PBS	Phosphate-buffered saline
PEN-2	Presenilin enhancer-2
PFA	Paraformaldehyde
PLL	Poly-L-lysine
PS1/PS2	Presenilin1/Presenilin 2
PVDF	Polyvinylidene fluoride
SAD	Sporadic Alzheimer's disease
sAPP β	Soluble amyloid precursor protein beta
SDS	Sodium dodecyl sulfate
TNF	Tumour-necrosis factor
Y2H	yeast two-hybrid assays

CHAPTER – 1

General Introduction and Literature Review

PREFACE: The main objective of this general introduction is to summarize the following issues: i) pathways of APP processing; ii) updated information on structure and assembly of individual γ -secretase components (nicastrin, presenilin, APH-1 and PEN-2); iii) trafficking of the active γ -secretase complex into various subcellular compartments; and iv) the functions of γ -secretase. The remainder of the Introduction deals with the structure and the distribution profile of p23 in peripheral tissues.

Alzheimer's disease (AD) is an adult onset neurodegenerative disorder characterized by a progressive loss of memory followed by deterioration of higher cognitive functions such as praxis, judgment, language and calculation. The average course of AD is approximately a decade, but the rate of progression is variable. Epidemiological data have shown that AD afflicts about 8-10% of the population over 65 years of age and its prevalence doubles every 5 years thereafter. Etiologically, AD is quite heterogeneous and can be caused by mutations in the β -amyloid precursor protein (APP) gene on chromosome 21, the presenilin 1 (PS1) gene on chromosome 14 and the presenilin 2 (PS2) gene on chromosome 1. However, these mutations account for only a small percentage of AD patients, namely those with early age of onset and a family history of the disease. The vast majority of AD patients does not have a familial history of the disease and do not show mutations in these genes (Mullan and Crawford, 1993; Levy-Lahad et al., 1995; Sherrington et al., 1995; Hardy, 1997; St George-Hyslop, 1999). The factors, secondary to causative genes, which play an important role in the pathogenesis of AD, include age, genetic predisposition (e.g. apolipoprotein E, ApoE genotypes) and possibly certain environmental determinants such as head injury and stress (see Poirier et al., 1993; Strittmatter et al., 1993; Muller-Spahn and Hock, 1999; Heininger, 2000).

Neuropathological hallmarks of both familial and sporadic AD include intracellular neurofibrillary tangles (NFTs), extracellular neuritic plaques (NPs), cerebrovascular amyloid deposits, and loss of neurons and synaptic integrity in

specific brain areas. These features are also seen in Down syndrome (DS) brains and, to a limited extent, in the normal aging brain. Structurally, NFTs are mainly composed of paired helical filaments that are derived from hyperphosphorylation of tau, a microtubule-associated protein involved in the stabilization of neuronal cytoskeleton in the normal brain (Iqbal et al., 1989; Selkoe and Schenk, 2003). The NPs, on the other hand, are composed of proteinaceous β -amyloid ($A\beta$) fibrils surrounded by dystrophic neurites and reactive astrocytes as well as microglia. The $A\beta$ fibrils are formed by gradual aggregation of 4 kDa $A\beta$ peptides generated from the precursor protein APP (Hardy et al., 1998; Selkoe and Schenk, 2003). It is suggested that accumulation of $A\beta$ peptide initiates a cascade of events such as gliosis, inflammation and synaptic alterations that eventually lead to loss of neurons in selected brain regions (Hardy et al., 1998). With the help of postmortem histological studies of AD brains it has been demonstrated that NPs are evident predominantly in affected areas such as entorhinal cortex, hippocampus, amygdala, neocortex and certain subcortical nuclei (i.e., nuclei basalis of Meynert, locus ceruleus and dorsal raphe) (Flood et al, 2002; Lazarov et al., 2002; Sheng et al., 2002). The areas which are relatively spared in AD such as striatum, thalamus and cerebellum exhibit diffuse, but not neuritic, plaques (Cole et al., 1993). Although $A\beta$ accumulation *in vivo* has been suggested to play a critical role in the degeneration of neurons leading to the development of AD pathology, at present very little is known about the underlying cellular mechanisms associated with the development of AD pathology. It is believed that a detailed study about the localization of APP and its processing enzymes

involved in the generation of A β peptides may provide a better understanding of AD pathogenesis.

1.1. Amyloid Precursor Protein (APP): APP is a type 1 transmembrane glycoprotein encoded by a gene located on human chromosome 21q21.1. There are at least 10 isoforms of APP which are generated by alternative splicing, of which APP695 is found to be expressed predominantly in neurons, whereas other isoforms such as APP751 and APP770 are expressed mostly in glial cells as well as in certain peripheral cells (Golde et al., 1990; Arai et al., 1991). Structurally, APP contains a large extracellular domain, a single transmembrane domain (TMD) and a small intracellular region. The extracellular region comprises E1 and E2 domains and the E1 domain is further divided into N-terminal growth factor-like domain (GFLD) and a copper binding domain (CuBD). The extracellular E1 domain is connected to E2 domain *via* an acidic, Kunitz-type protease inhibitor (*KPI*) and OX2 domains. The terminal region of the extracellular domain is connected to the cytoplasmic domain of APP *via* TMD (Fig.1.1; Reinhard et al., 2005). Unlike other APP isoforms, APP695 lacks 56 amino acid residues of the KPI motif which basically acts as an inhibitor of all serine proteases (Liu et al., 2000). The A β sequence lies within the membrane spanning domain of APP (Barnham et al., 2003). Accumulated evidence suggests that after synthesis in the endoplasmic reticulum (ER) APP undergoes a wide variety of posttranslational modifications including glycosylation, tyrosine sulfation and phosphorylation. The first two posttranslational modifications (glycosylation and sulfation) help in protein-protein interactions and increasing

stability of the protein itself, whereas phosphorylation may be involved in modulating neuronal excitability (Furukawa et al., 1996). Several experimental approaches have suggested a possible physiological role of APP in synapse formation, cell signaling, long-term potentiation and cell adhesion. However, the exact function of APP has yet not been clearly established (Esteban, 2004; Pearson and Peers, 2006). Mature APP exhibits a rather short half-life and is known to be processed proteolytically either by the non-amyloidogenic α -secretase or amyloidogenic β -secretase pathways.

1.2. The α -secretase pathway: The non-amyloidogenic α -secretase pathway is mediated by a putative α -secretase enzyme. The candidates that can potentially act as α -secretases are members of the ‘A Disintegrin and Metalloprotease’ (ADAMs) family of proteases (Schlondorff and Blobel, 1999), which are expressed ubiquitously in the brain. There are at least 17 ADAM secretases that are known to be expressed in brain, and ADAM8, ADAM9, ADAM10 and ADAM17 are able to cleave APP almost in the middle within the A β domain, thus precluding the formation of full-length A β peptide (Koike et al., 1999; Asai et al., 2003; Lichtenthaler and Haass, 2004). This cleavage releases a soluble ectodomain sAPP α) and a membrane associated 83 a.a.-containing C-terminal fragment that can be further processed by another aspartyl protease called γ -secretase to release a small P3 fragment and an APP intracellular domain (AICD) (see Fig.1.2; Small et al., 1994; Furukawa et al, 1996). In addition to APP, ADAM secretases can cleave other substrates including cytokines and growth

factor (i.e., tumor-necrosis factor, transforming growth factor, heparin-binding epidermal growth factor), certain receptors (i.e., tumor-necrosis factor receptor-I and -II and ErbB4) and other molecules such as fractalkine and angiotensin-converting enzyme (Schlondorff and Blobel, 1999; Hooper et al., 2000)

1.3. The β -secretase pathway: The amyloidogenic β -secretase pathway is mediated by an aspartyl protease called β -secretase which is also referred to as β -site APP cleaving enzyme (BACE1), aspartic protease2, or memapsin 2 (Vattemi et al., 2003). BACE1 is widely expressed in both neuronal and non-neuronal cells but its level is somewhat higher in the brain compared to other tissues (Bennett et al., 2000). There is a homologue of BACE1 called BACE2 which acts as a α -secretase and cleaves the A β sequence between Phe¹⁹ and Phe²⁰, thus preventing the formation of full-length A β peptide (Farzan et al., 2000). The BACE2 gene is located on chromosome 21, whereas the BACE1 gene maps to chromosome 11. In contrast to α -secretase, BACE1 cleaves APP at the N-terminus of the A β sequence, leading to the formation of a soluble N-terminus APP (sAPP β) and a membrane-associated 99 a.a.-containing C-terminal fragment that can subsequently be cleaved by γ -secretase to generate intact A β peptide and the AICD (see Fig.1.2; Vassar et al., 1999). The full-length A β sequence varies in length from 39 to 43 a.a. Under normal conditions ~90% of secreted A β peptides are A β ₁₋₄₀, a soluble form of the peptide that only slowly converts to an insoluble beta-sheet configuration and thus can be eliminated from brain. In contrast, ~10% of secreted A β peptides are A β _{1-42/43}, species that are highly fibrillogenic and

deposited early in individuals with AD and DS (Iwatsubo et al., 1994; Selkoe et al., 1996). The AICD produced in both α - and β -secretase pathways are reported to bind different target proteins and may be involved in various cellular events such as neuronal growth, regulation of gene expression and apoptosis (Hamid et al., 2007).

1.4. Components of the γ -secretase enzyme: The enzyme γ -secretase is a unique class of protease which cleaves its substrate within the hydrophobic transmembrane domain that has already undergone prior ectodomain shedding by other proteases such as α - or β -secretases. This entire process is known as regulated intramembrane proteolysis. The main purpose of intramembrane proteolysis is to clear excess proteins from membranes, and in some cases it is also involved in regulating cellular events by generating active compounds (Wolfe, 2002). The γ -secretase enzyme comprises four different integral proteins, namely PS1 or PS2, nicastrin, anterior pharynx-defective phenotype1 (APH-1) and presenilin enhancer-2 (PEN-2) (see Fig.1.3). All these components are coordinately sequestered into a complex to avoid unwanted proteolysis. The following paragraphs will describe the current state of knowledge of all the components of the γ -secretase complex including PS1/2 that serves as the catalytic component of the enzyme complex.

1.4.1. Presenilins (PS1/PS2): PS1 and PS2 are multiple transmembrane proteins. The PS1 gene is located on chromosome 14 and was discovered in 1995 by genetic linkage studies to be associated with an early-onset form of AD (Rogaev

et al., 1995; Sherrington et al., 1995). The PS2 gene is located on chromosome 1 and was discovered subsequently in another set of early-onset AD families (Levy–Lahad et al., 1995). To date more than 150 mutations have been identified in the PS1 gene, whereas only a few mutations are localized to the PS2 gene. The majority of PS1 mutations are missense mutations that result in single a.a substitutions (Zhang et al., 2009). Interestingly, PS1 and PS2 proteins derived from the two genes share 67% homology and are composed of 467 and 448 a.a, respectively (Fraser et al., 2001). While PS1 knockout mice exhibit an embryonic lethal phenotype with skeletal and brain deformities, PS2 knockout mice are found to be viable and display only mild pulmonary fibrosis and hemorrhages at later stages of development (Shen et al., 1997; Herreman et al., 1999; Steiner et al., 1999). These experimental data suggest that PS1 and PS2, *albeit* sharing some structural homology, may function differently under certain conditions. Accumulated evidence suggests that PS1 and PS2 are relatively conserved across species and share some homology with *C. elegans* proteins such as Sel-12, hop-1 and to some extent SPE-4 (Smialowska and Baumeister, 2006). Knowledge of the detailed structure of PS1 and PS2 can help to understand their interactions with other proteins and their roles in various cellular functions.

Several topological models have been suggested for PS structure, of which the 8-TMD model is most popular (Fraering, 2007), but the 9-TMD model is also gaining some acceptance over the last few years (Li and Greenwald, 1998; Golde et al., 2009). Structurally, PS1 and PS2 have two hydrophilic regions [i.e., the N-terminal fragment (NTF) and the large intracellular loop between 6- and 7-

transmembrane regions] and one hydrophobic C-terminal fragment (CTF) (Fig.1.3). Presenilin is usually synthesized as a ~50 kDa holoprotein in the ER and then undergoes endoproteolysis in the presence of an unknown "presenilinase" enzyme (Thinakaran et al., 1997). The cleavage occurs within the large intracellular loop between TM6 and TM7 domains (i.e., between Thr²⁹¹ and Met²⁹² in PS1 and Ala²⁹⁷ and Met²⁹⁸ in PS2) and it generates a ~ 30 kDa NTF and a ~ 20 kDa CTF that are bound non-covalently in a 1:1 ratio in a high molecular weight complex (Steiner et al., 2000; Haass and Steiner, 2002). In addition to the endoproteolysis, PS1 and PS2 are known to undergo alternative cleavage by the caspase family of proteases, but the significance of this cleavage is currently unknown (van de Craen et al., 1999). The uncleaved PSs are usually degraded by the proteasomes (Kim et al., 1997; Marambaud et al., 1997; Annaert et al., 1999).

Presenilins are not considered to be typical aspartyl proteases as they do not contain sequence homology with the classical aspartyl protease family members, which are characterized by DT/sGT active site motifs. These PS proteins, on the other hand, contain the highly conserved GxGD (residues 382-385 in human PS1) sequence motif in their CTF. The catalytic function of PS1/PS2 is mainly served by the GxGD motif as the aspartyl residues involved in the proteolytic cleavage are embedded within this motif (Steiner et al., 2000). In addition to its catalytic activity, this motif is also involved in substrate identification (Yamasaki et al., 2006). The PSs have another important motif called the proline-alanine-leucine (PAL) motif, which is located within 9-TMD (according to a recently proposed topological 9-TMD model) and is required for ER retention as well as

stabilization of the high-molecular weight complex (Tomita et al., 2002; Kaether et al., 2004; Wang et al., 2004; Wang et al., 2006).

Several research groups focused on dissecting out the role of PS1/PS2 in AD pathogenesis revealed that PSs are the active core of the γ -secretase enzyme complex. This is supported by the evidence that: i) cultures derived from PS1 knockout embryos demonstrated a drastic reduction of both $A\beta_{1-40}$ and $A\beta_{1-42}$ species (De Strooper et al., 1998); ii) PS1 and PS2 double knockout mice showed a complete loss of γ -secretase activity (Herreman et al., 2003; Zhang et al., 2000); iii) mutations on the aspartyl residues of PSs, which are involved in cleavage activity, are found to interfere with the APP as well as Notch processing (Steiner et al., 1999; Wolfe et al., 1999); iv) aspartyl protease inhibitors as well as γ -secretase inhibitors can interfere with γ -secretase activity by binding directly to the aspartyl residues located on PS1/2 proteins (Esler et al., 2000; Li et al., 2000; Seiffert et al., 2000). However, these data did not conclusively indicate that PS1 or PS2 alone can act as γ -secretases as the molecular weight of the active complex (i.e., ~ 200 -250 kDa) is found to be much higher than the molecular weight of either PS1 or PS2 protein, thus raising the possibility that PSs might interact with other proteins to form a high-molecular weight complex that possesses γ -secretase enzyme activity (Yu et al., 1998; Li et al., 2000, Nyabi et al., 2003). Supporting this notion, co-immunoprecipitation as well as a variety of other experimental approaches revealed that PS1 and PS2 can act as a catalytic component of γ -secretase enzyme by interacting directly with three other proteins, namely

nicastatin, APH1 and PEN2 (Capell et al., 2005; Kimberly et al., 2003; Takasugi et al., 2003). Structural analysis further demonstrated that NTF and CTF of PSs are involved in interaction with other components of the γ -secretase enzyme complex, i.e., PS-NTF interacts with the PEN2 and PS-CTF interacts with the nicastrin (Kaether et al., 2004; Kim and Sisodia, 2005; Watanabe et al., 2005). With the help of co-immunoprecipitation, yeast two-hybrid assays and cross-linking studies, it has been shown that the secretory signal sequence residing in the NTF of presenilins may be involved in interactions with APP (Kim et al., 1997; Pradier et al., 1999).

Apart from regulated intramembrane proteolysis, PSs are also involved in various other non-proteolytic functions within cells, including neuronal plasticity, cell adhesion, β -catenin stability, signal transduction mechanisms (Zhou et al., 1997; Georgakopoulos et al., 2000), intracellular calcium signaling, calcium-mediated apoptosis (Shinozaki et al., 1998; Leissring et al. 2000; Choi et al. 2001; Zaidi et al. 2002; Jo et al. 2003) and trafficking of membrane proteins (Katayama et al., 1999; Niwa et al., 1999). This vast array of cellular functions indicates that PSs, in addition to being components of the γ -secretase complex, may interact with a variety of other proteins (Chen and Schubert, 2002). At the cellular level, PS1 and PS2 are ubiquitously expressed in both neuronal and nonneuronal tissues. Using Northern blot analysis and *in situ* hybridization, it has been shown that PS1 mRNA is expressed in higher levels in testis and kidney and relatively lower levels in heart and skeletal muscle (Suzuki et al., 1996; Lah et al. 1997; Nilsberth et al., 1999). In the normal brain, both PS1 and PS2 are found to be widely

distributed in various neuroanatomic regions. The expression levels of the mRNA as well as protein are rather high in the neocortex, hippocampus and cerebellum, but low in olfactory bulb, striatum, thalamus and brain stem nuclei (Lee et al., 1996; Blanchard et al., 1997; Nilsberth et al., 1999). Immunoreactive-PS in adult neurons is localized both in cell bodies and axons (Blanchard et al., 1997), whereas during development its expression is mostly restricted to cell soma (Levesque et al., 1999). At the sub-cellular level, PS1 and PS2 can be detected in ER, Golgi network, nuclear envelope and also on plasma membranes (Anaert et al., 1997; Ray et al., 1999).

1.4.2. *Nicastrin*: Using co-immunoprecipitation as well as other experimental approaches, it was demonstrated that nicastrin can directly interact with PSs and act as a component of the γ -secretase enzyme complex (Yu et al., 2000). The name nicastrin is derived from an Italian village called “Nicastro”, where descendants of an extended family with familial AD were identified (Feldman et al., 1963; Confaloni et al., 2003). Genetic screening studies showed that the nicastrin gene is located on the chromosome 1q23 region which is closely linked to a subset of a late-onset form of AD (Kehoe et al., 1999; Hiltunen et al., 2001). This raises the possibility that nicastrin might play an important role in AD pathogenesis, but so far no AD-related mutations have been identified in the nicastrin coding region (Dermaut et al., 2002).

Nicastrin is a type-I transmembrane glycoprotein comprising 709 a.a. residues with a large N-terminal extracellular domain, a single TMD and a short C-

terminal intracellular region (Fraering et al., 2007). The extracellular domain of nicastrin consists of a signal peptide and a DYIGS (336-340 a.a) motif which is conserved across species (Fig. 1.4; Shah et al., 2005; De Strooper, 2005). The DYIGS motif along with the surrounding a.a. residues is referred to as the “DYIGS and peptidase” (DAP) domain which plays a critical role in protein-protein interactions. The glutamate residue located within the DAP domain (i.e., E³³³) has been shown to be involved in recognizing various substrates cleaved by the γ -secretase enzyme complex (Shah et al., 2005; Beel and Sanders, 2008; Dries et al., 2009). Accumulated evidence suggests that the TMD of nicastrin is involved in the assembly of the γ -secretase complex as well as binding the C-terminal fragment of PSs (Capell et al., 2003), whereas the juxtamembrane region of the extracellular domain is essential for interacting with A β 1 (Walker et al., 2006). To date there is no evidence that nicastrin can directly interact with PEN2.

After synthesis within the ER, nicastrin undergoes partial glycosylation at its N-terminal region which leads to the formation of a highly unstable 110 kDa immature nicastrin (Kimberly et al., 2002; Herreman et al., 2003). The protein is subsequently transported to the post-Golgi compartment where additional glycosylation of the molecule leads to the formation of a more stable 130 kDa mature nicastrin (Edbauer et al., 2002; Leem et al., 2002; Shirotani et al., 2003; Capell et al., 2005). Accumulated evidence suggests that about 16 N-linked glycosylation consensus sequences are evident on the N-terminal domain of nicastrin (Yang et al., 2002). It is of interest to note that only the mature form of nicastrin can preferentially interact with PSs, but activity of the γ -secretase

enzyme complex may not completely depend on the maturity of nicastrin (Herreman et al., 2003). It is suggested that nicastrin helps to stabilize the N- and C-terminal fragments of PSs (Edbauer et al., 2002), whereas PSs are required for the maturation and subsequent trafficking of nicastrin to the membrane surface (Leem et al., 2002). In addition, there is evidence that nicastrin exhibits ~41% of homology with APH-1, a component of γ -secretase which plays a critical role in the assembly of the active enzyme complex (Chen et al., 2001). Recent data indicate that nicastrin is not only involved in regulating the function and assembly of the γ -secretase complex by interacting with PS1 and APH-1 but also in the recognition of various substrates undergoing proteolysis by the γ -secretase enzyme complex (Dries and Yu, 2008). There is evidence that immunoreactive nicastrin is present in selected brain regions including cerebellum and that its level is relatively high during development and then declines gradually to reach the adult profile (Uchihara et al., 2006). However, very little detailed information is currently available about the distribution of nicastrin or its possible colocalization with other components of the γ -secretase complex in the adult mammalian brain.

1.4.3. *Anterior pharynx defective 1 (APH-1)*: APH-1, another component of the γ -secretase complex, is a ~30 kDa polytopic transmembrane protein which spans the membrane 7 times (Lee et al., 2002). The topology of APH-1 revealed that its N-terminal fragment and even-numbered loops face the lumen, whereas the C-terminal fragment and odd-numbered loops reside within the cytosol (Fig.1.3).

Similar to PS1 and nicastrin, APH-1 also undergoes a maturation process by glycosylation as well as endoproteolytic cleavage (Goutte et al., 2002; Fortna et al., 2004). APH-1 contains an important GxxxG motif at its C-terminal fragment which interacts preferentially with immature nicastrin to form a form a ~140-kDa (nicastrin-APH-1) sub-complex (Gu et al., 2003; Hu and Fortini, 2003; LaVoie et al., 2003; Fortna et al., 2004; Niimura et al., 2005). There is also evidence that APH-1 interacts with PSs *via* its GxxxG motif (Tomita et al., 2002; Edbauer et al., 2002). Recent mutagenesis studies indicated that histidine residues (His¹⁷¹ and His¹⁹⁷) located in the 5- and 6-transmembrane domains of APH1 are critical for its interactions with nicastrin as well as PSs (Pardossi-Piquard et al., 2009).

Accumulated evidence suggests that mammalian APH-1 is present in three different isoforms: APH-1a, APH-1b and APH-1c. Furthermore, there are two C-terminal spliced variants of APH-1a, i.e., the long form APH-1aL comprising 265 a.a residues and the short form APH-1aS comprising 247 a.a residues. The APH-1a shares ~56% homology with APH-1b and APH-1c, whereas APH-1b and APH-1c share ~95% homology. Of all these isoforms, APH-1aL is the predominant form detected in the endogenous γ -secretase complex (Shiratori et al., 2004). More recent studies have shown that various isoforms of APH1 can form different sets of γ -secretase complexes which can substitute each other to mediate proteolytic APP processing (Shiratori et al., 2004; Ma et al., 2005).

The function of APH-1 includes the formation of an APH-1/nicastrin pre-complex and stabilization/trafficking of the γ -secretase complex from the ER to the Golgi-

complex. This is supported by evidence that deletion of APH-1a can significantly reduce the level of mature nicastrin and the formation of the APH-1/nicastrin pre-complex (Ma et al., 2005). Additionally, mutation of APH-1 has been shown to disrupt the export of nicastrin from the ER to the Golgi-complex (Niimura et al., 2005), whereas fibroblasts lacking nicastrin display a reduction in APH1 levels (Vetrivel et al., 2004). Apart from regulating γ -secretase assembly/function, APH-1 has been shown to play a role in the notch signaling pathway during development (Levitan et al., 2001; Francis et al., 2002; Ma et al., 2005).

1.4.4. Presenilin enhancer-2 (PEN-2): PEN-2 and APH-1 were the last components of the complete γ -secretase complex to be identified during genetic studies of *C. elegans* and *D. melanogaster*. Structurally, PEN-2 is a 101 a.a-containing two domain transmembrane protein which is encoded by a gene located on chromosome19 (Fraering et al., 2007). Based on different biochemical approaches, including fluorescent microscopy, epitope tagging and protease protection assays, it has been shown that the two transmembrane domains of PEN-2 are linked by a cytoplasmic loop and their N- and C-terminals fragments usually face the lumen (Francis et al., 2002; Crystal et al., 2003) (Fig.1.3). Accumulated evidence suggests that PEN-2 plays an important role in endoproteolysis as well as stabilization of PS fragments. It is proposed that PEN-2 can bind to PSs even in the absence of the immature nicastrin-APH1 subcomplex (Capell et al., 2005). However, it remains unclear whether the C- and/or N-terminal fragments of PEN-2 are critical for physical interaction with PSs (see Fig. 1.3; Kim and Sisodia, 2005). Recent studies have shown that a functional

DYLSF (90-94 a.a residues) motif located in the C-terminal fragment of PEN-2 may play a role in the physical interaction between PEN-2 and PSs (Hasegawa et al., 2004; Prokop et al., 2004). Recent studies further revealed that the complete length of PEN-2 C-terminal fragment is critical in regulating γ -secretase activity (Hasegawa et al., 2004; Prokop et al., 2004).

1.5. Assembly and trafficking of the γ -secretase complex: All four components of γ -secretase including PS1/PS2 holoproteins (the catalytic core of the protease), nicastrin (involved in substrate selection), APH-1 (promoting the assembly, stabilization and traffic of the complex) and PEN-2 (involved in the maturation of nicastrin and endoproteolysis of PSs), are first synthesized in the ER. After synthesis, nicastrin undergoes rapid maturation *via* N-glycosylation and then forms a stable subcomplex with APH-1. The nicastrin-APH-1 subcomplex subsequently binds to PS1/PS2 holoprotein in the ER to form the ternary Nicastrin/APH-1/PS1 subcomplex. Finally PEN-2 binds to PS1/PS2, which results in rapid endoproteolysis and formation of active N- and C-terminal fragments of PS1/PS2 within the γ -secretase complex (Fig. 1.5). After assembly the majority of the mature γ -secretase complexes (~95%) are transported to the Golgi-complex, whereas only a minority (~5%) is trafficked to other subcellular compartments such as endosomes and plasma membrane. The active γ -secretase complex is transported to the plasma membrane possibly with the help of ER retention signals that may exist within the four components. Once in the plasma membrane, the active complex may be retained at the membrane, or may undergo endocytosis to endosomes/lysosomes and continue to be active in these

compartments. The active γ -secretase complex cleaves APP in the membrane or at the subcellular compartments either *via* amyloidogenic or non-amyloidogenic pathways and then releases the products into the extracellular space and/or cytosol.

1.6. Functions of γ -secretase beyond APP: The active γ -secretase complex, apart from APP, can process more than 80 other substrates, and the majority of them are type 1 transmembrane proteins (Beel and Snader, 2008; Wakabayasi and De strooper, 2008). The most extensively studied γ -secretase substrates so far are: notch, Erb-B4, E-cadherin, N-cadherin, CD44, deleted in colorectal cancer, p75 neurotrophin receptor, low density lipoprotein receptor, Nectin-1, Delta and Jagged. The wide varieties of γ -secretase substrates indicate the versatility of the functions of the enzyme complex (see Table.1).

Table.1 Substrates and functions of γ -secretase

Substrates	Functions	References
Notch	Cell signaling, cell-fate decision during embryogenesis	Shi et al., 2006
Erb-B4, E-and N-cadherins	Cell proliferation and differentiation, cell adhesion and cell migration	Koike et al.1999
Deleted in colorectal cancer	Axonal development, synaptic transmission and memory processing	Parent et al., 2005
P75-NTR, low density lipoprotein receptor as well as CD44	Transcriptional regulation	Jung et al., 2003; Cui et al 2006
Nectin-1	Cell adhesion and regulation of synaptic function	Kim et al., 2002
Delta and Jagged	Down regulation of notch signaling	Ikeuchi and Sisodia, 2003

1.7. Modulation of γ -secretase activity: Since γ -secretase is involved in the processing of APP leading to the generation of A β peptide, which plays a critical role in the development of AD pathogenesis, one of the major goals for AD therapy is to identify γ -secretase inhibitors/modulators which can attenuate A β production without affecting other processes. So far only non-steroidal anti-inflammatory drugs (NSAIDs) and adenosine triphosphate (ATP) have been shown to decrease A β peptide levels without altering notch processing (Fraering et al., 2005; Wolfe, 2008). There is evidence that activity of γ -secretase can also be modulated by other proteins such as p23, CD147, phospholipase D, calsenilin and the members of the X11/Mint family (Verdile et al., 2007). However, only p23 has so far been shown to directly modulate γ -secretase activity both *in vitro* and *in vivo*.

1.8. Structure and function of p23: Accumulated evidence indicates that p23 is a 24 kDa type I transmembrane protein belonging to the p24 family of proteins. These proteins are basically considered as putative cargo receptors which are abundant in coat protein I and II (COPI and COPII) vesicles. The COPI vesicles transport proteins within the Golgi-network as well as from the Golgi to the ER, whereas COPII vesicles transport newly synthesized proteins from the ER to the Golgi complex. Based on conserved C-terminal sequence, the family of p24 proteins is categorized into four subfamilies: p24- α , p24- β , p24- γ and p24- δ . The protein p23 belongs to the p24- δ subfamily (Dominguez et al., 1998). All the p24 proteins have certain structural similarities including a large luminal domain with

two conserved cysteine residues linked by a disulfide bridge, a C-terminally located transmembrane stretch and a short 12-20 a.a-containing cytoplasm tail with a sequence motif (Fig.1.6). The C-terminal motif of p24 proteins binds to COPI and COPII vesicles (Sohn et al., 1996; Harter and Wieland, 1998; Belden and Barlowe, 2001). However, with the help of biochemical and morphological analysis, it has been demonstrated that p23 is not enriched in purified COP-coated vesicles and its cytoplasmic tail is not required for association with COPI under *in vitro* conditions (Blum et al., 1999). Experimental evidence further indicates that members of the various p24 subfamilies can interact and form tetrameric complexes containing one representative of each subfamily (Belden and Barlowe, 1996; Fullekrug et al., 1999; Marzioch et al., 1999). Most of the p24 proteins depend on each other in terms of stability, localization and/or transport. Hence, the formation of hetero-oligomers seems to be coupled intimately to their functions. The family of p24 proteins, apart from acting as COP vesicle cargo receptors, is known to be involved in a variety of other functions such as transport of proteins from the ER and Golgi, regulation of COP vesicle budding, ER quality control and organization of the Golgi apparatus (Blum and Lepier, 2008).

The cellular distribution of p24 family of proteins has not been studied extensively in mammalian tissues including the brain. Recently, with the help of RT-PCR, the p23 transcripts have been detected in various mouse tissues including liver, kidney, spleen, heart muscle, small intestine and brain (Sohn et al., 1996; Strating et al., 2009). At the subcellular level, the majority of p23 has been shown to be localized in the cis-Golgi network and intermediate

compartments (Sohn et al., 1996; Nickel et al., 1997; Rojo et al., 1997). There is evidence that p23 is expressed predominantly in the nephrogenic zone of the kidney during development and then declines markedly in the adult stage, suggesting that p23 is a developmentally regulated gene and may direct the intracellular trafficking or secretion of proteins responsible for nephrogenesis (Baker and Gomez, 2000).

In 2006 Chen and colleagues showed for the first time that p23 is a member of the high molecular weight PS complex which can negatively modulate APP cleavage by the active γ -secretase complex (Chen et al., 2006). Down regulation of p23 by siRNA in cell cultures demonstrated a drastic reduction of A β production. Vetrivel et al. (2007) subsequently demonstrated that p23 can mediate two distinct but interrelated functions: i) it negatively modulates γ -secretase activity and reduces A β production and ii) it promotes maturation and trafficking of APP to the cell surface. Apart from these functions, very little is currently known about the cellular distribution of p23 in the brain or its potential role in regulating AD pathogenesis.

1.9. Hypothesis and Objectives: In general, it is evident from the aforementioned studies that much is known about the structure, assembly and intracellular trafficking of γ -secretase components. At the cellular level, the distribution profile of PSs in the central nervous system has been studied quite extensively (Siman et al., 2003). In contrast, very little information is currently available on the levels and/or cellular distribution of nicastrin, APH-1, PEN-2 or

p23. Given the critical role of the γ -secretase complex in AD pathogenesis, I hypothesized that all four components of γ -secretase and its modulators are widely distributed in the adult brain and are selectively altered in AD pathology. To address this issue, I have undertaken two following objectives as a part of my MSc project:

1.9.1 Objective-1: to determine the level and cellular distribution of nicastrin and its possible colocalization with PS1 during development and in adult rat brains.

1.9.2 Objective-2: to establish the levels and cellular distribution of p23 in rodent brains and their possible alterations in an animal model of excitotoxicity as well as in AD pathology.

Amyloid Precursor Protein structure (APP)

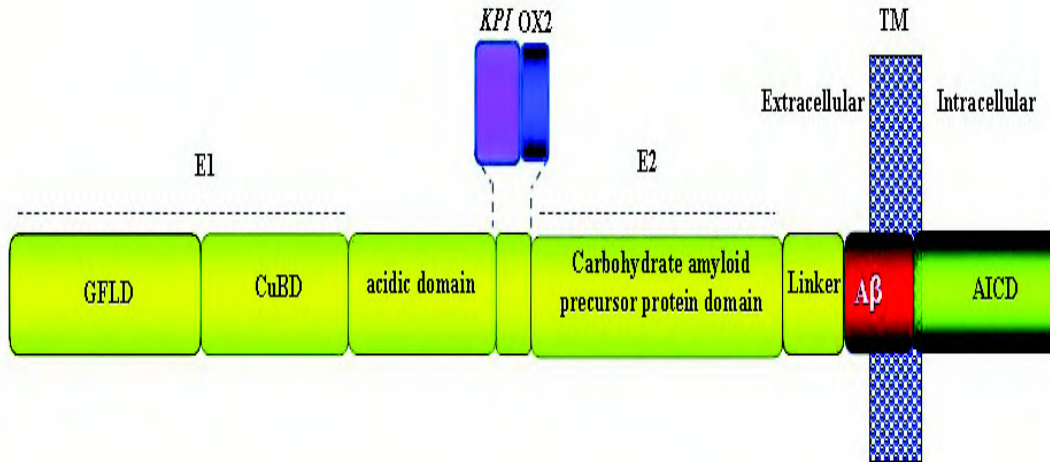


Fig. 1.1: APP is type 1 transmembrane protein that comprises a large extracellular domain, a transmembrane domain and a short APP intracellular domain (AICD). The extracellular domain comprises the E1 and E2 regions and the E1 region is linked to E2 *via* an acidic domain, KPI and OX2 domains. The E1 region is again divided in to two domains, an N-terminal growth factor-like domain (GFLD) and a copper-binding domain (CuBD). The last part of the extracellular domain is a linker. Aβ indicates that the amyloid β-peptide sequence lies within transmembrane domain. The Kunitz-type protease inhibitor domain (KPI) is present in APP isoforms 751 and 770, whereas the OX2 sequence is present only in the APP770 isoform (Adopted and modified from Reinhard et al., 2005).

Processing of APP by α - and β -secretase pathways

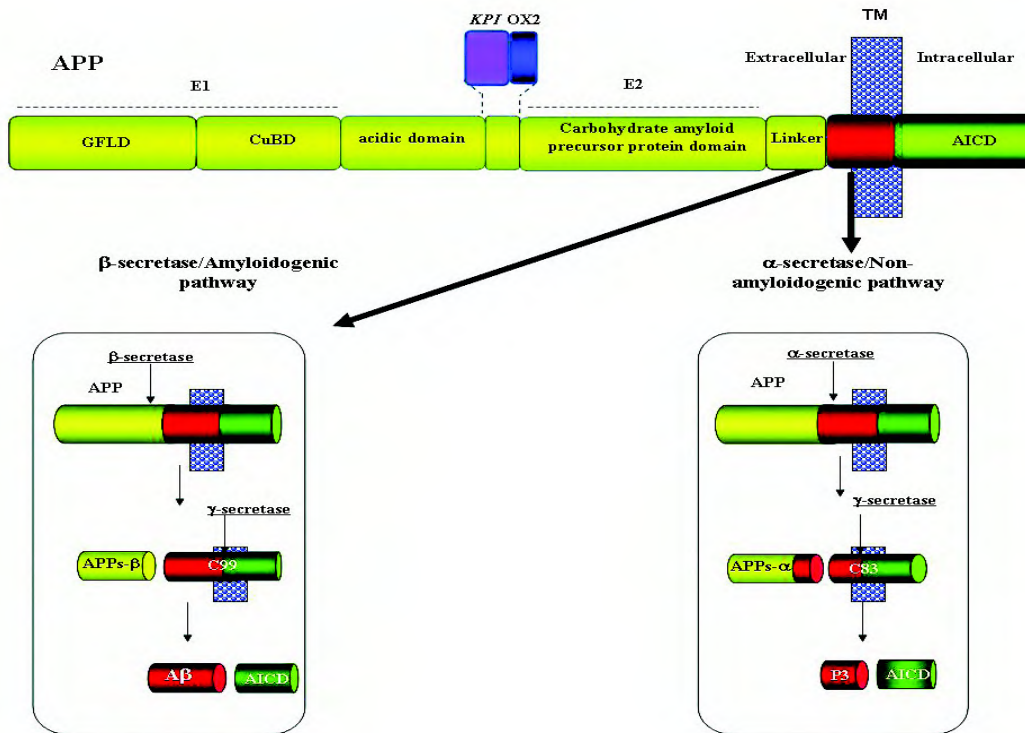


Fig. 1.2: APP undergoes cleavage *via* either α - or β -secretase pathways. The enzyme α -secretase cleaves APP in the middle of the A β sequence, thus precluding the formation of intact A β peptide. This cleavage results in formation of a soluble APP α (sAPP α) and a membrane bound 83 amino acid (a.a)-containing C-terminal fragment (C83). Subsequent cleavage of C83 by γ -secretase results in release of 3kDa P3 peptide and the APP intracellular domain (AICD). The enzyme β -secretase cleaves APP at the N-terminus of the A β sequence, leading to the formation of a soluble N-terminus APP (sAPP β) and a membrane-associated 99 a.a.-containing C-terminal fragment that can subsequently be cleaved by γ -secretase to generate intact A β peptide and the AICD. The complete sequence of A β peptide varies in length from 39-43 a.a residues. Among the various A β peptides, A β 42 is the major protein component of the amyloid plaque and it aggregates more rapidly than other forms (Adapted and modified from Fraering, 2007).

Components of the γ -secretase enzyme complex

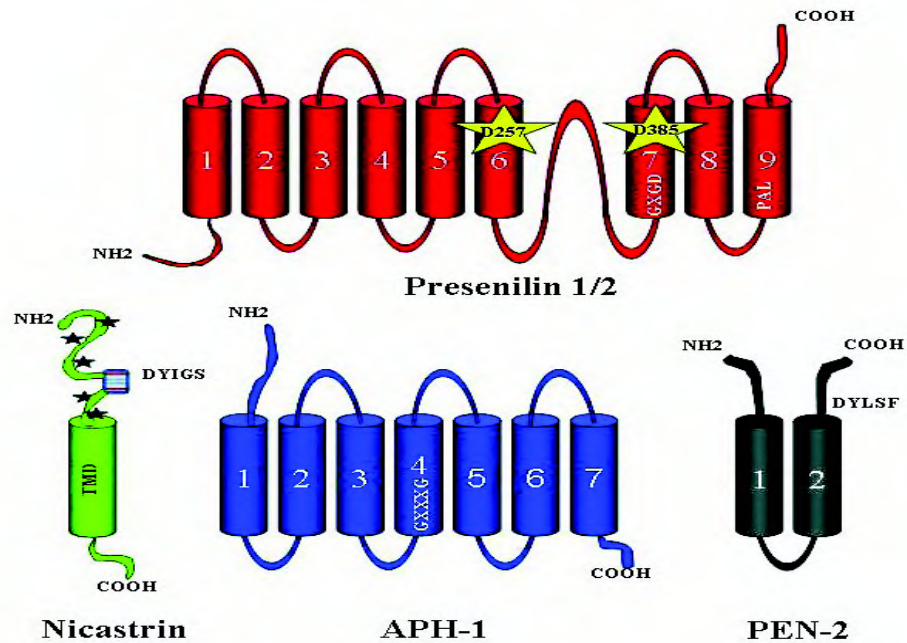


Fig. 1.3: The γ -secretase complex consists of four core components: presenilin1/2 (PS1/2), nicastrin, APh-1 and PEN-2. PS1/2 comprises an N-terminal fragment (NTF), a large intracellular loop between 6- and 7-transmembrane regions (TMDs) and one hydrophobic C-terminal fragment (CTF). The 6- and 7-TMDs contain catalytic aspartic acid residues that participate in the proteolytic cleavage of the peptide bond of substrates (D257 and D385 marked as yellow stars). The GxGD motif located in 7-TMD is also important for catalytic activity of PS as well as substrate recognition. The (PAL) motif located within 9-TMD is required for ER retention as well as stabilization of the high-molecular weight complex. Nicastrin is a type-I transmembrane glycoprotein comprising a large N-terminal extracellular domain, a single TMD and a short C-terminal intracellular region. APh-1 contains 7 TMDs in its structure. The GxxxG motif which lies within the 4-TMD plays a critical role in interacting with nicastrin as well as PS1/2. PEN-2 comprises 2 TMDs that are linked by a cytoplasmic loop. Their N- and C-terminal fragments usually face the lumen. The DYLSF (90-94 a.a residues) motif located in the C-terminal fragment of PEN-2 may play a role in the physical interaction with the PS1/2 (Adapted from Fraering, 2007).

Predicted topology of Nicastrin

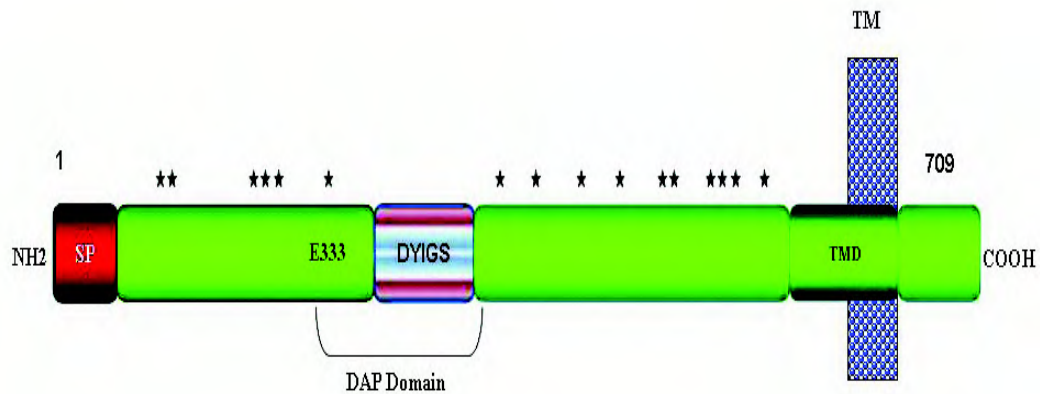


Fig. 1.4: Nicastrin is type 1 transmembrane glycoprotein that comprises an N-terminal signal peptide (SP), a large N-terminal hydrophilic extracellular domain with 16 N-linked glycosylation sites (marked as stars), one hydrophobic transmembrane domain (TMD) and a short hydrophilic C-terminus of 20 a.a residues (COOH). The conserved DYIGS motif (336-340 a.a residues) lies within extracellular domain. The DYIGS motif along with the surrounding a.a residues, including the glutamate residue at position 333(E333), is referred to as the DAP domain. DYIGS and E333 play an important role in substrate selection (Adapted from Fraering, 2007 and An Herreman, 2003)

Fig. 1.5: Assembly and trafficking of γ -secretase components

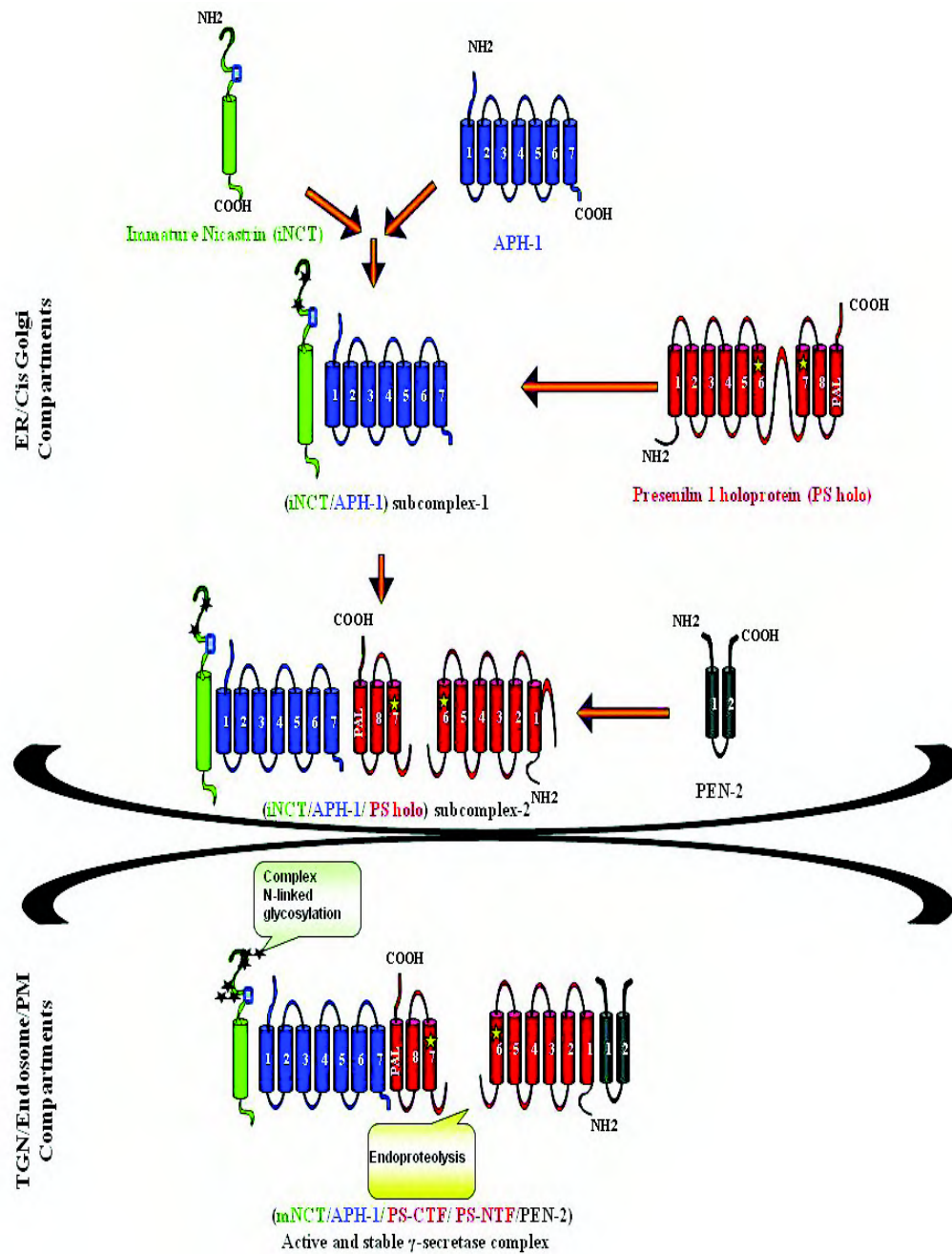


Fig. 1.5: Assembly and trafficking of γ -secretase components

Shortly after its synthesis, nicastrin (Nct) is partially *N*-glycosylated in the ER to form immature nicastrin (imNct). APH-1 interacts with imNct in the ER and forms a stable (imNct/APH-1) subcomplex-1. This subcomplex-1 then binds to PS1/2 holoprotein to form the subcomplex-2 i.e., imNct/APH-1/PS. The final step in assembly occurs in the ER, where PEN-2 binds to PS holoprotein and forms a subcomplex-3 i.e., imNct/APH-1/PS/PEN-2. The incorporation of PEN-2 into the subcomplex-2 leads to the rapid endoproteolysis of PS holoprotein in its large intracellular loop between TMD6 and TMD7 (indicated by arrow). The resulting PS fragments i.e., PS-NTF and PS-CTF represent the active forms of PS within the γ -secretase complex in the Golgi and/or ER. Finally, while most (~95%) of the γ -secretase complex cycles between the ER and the Golgi, a minority (~5%) of the γ -secretase complex is trafficked to plasma membrane (PM) and endosomes *via* a trans-Golgi-network (TGN). The imNct further undergoes complex *N*-glycosylation and converts to a mature form (mNct), which leads to the conformational change and subsequent formation of an active γ -secretase complex (Adapted from Fraering, 2007).

Topology of p23

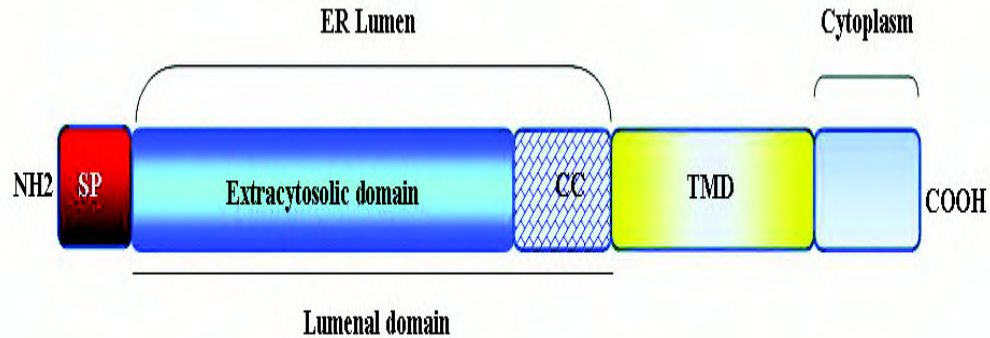


Fig.1. 6: p23 is a type I transmembrane protein which comprises a signal peptide (SP) at the amino terminus (NH₂) region, an extracytosolic domain, a coiled-coil domain (CC), a single transmembrane domain (TMD) and a short cytoplasmic tail (COOH). The extracytosolic domain and the CC domain together are called the luminal domain since both lie within the luminal region of the ER. The cytoplasmic tail of p23 is responsible for its localization in the ER/Golgi compartment, whereas the luminal domain is responsible for its localization in the plasma membrane. The TMD of p23 is involved in interactions with the γ -secretase components (Adapted from Blum et al., 1996).

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CHAPTER - 2

Cellular distribution of γ -secretase subunit nicastrin in the developing and adult rat brains

PREFACE: Although much is known about nicastrin structure, intracellular trafficking and its assembly into the γ -secretase complex, information on its distribution in the brain is very limited. This study was carried out to examine the cellular expression and distribution profile of nicastrin in adult and developing rat brains. We also investigated the possible colocalization of nicastrin with PS1 at the cellular level.

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2.1. Introduction

An invariant feature associated with Alzheimer's disease (AD) pathology is the presence of neuritic plaques containing a compact deposit of amyloid fibrils surrounded by dystrophic neurites, activated microglia and reactive astrocytes (Cordell et al., 1994; Price et al., 1998; Sekoe et al., 2003). The amyloid filaments are composed of 39-43 a.a amyloid β ($A\beta$) peptides, which are derived from sequential proteolysis of amyloid precursor protein (APP) by β -secretase and γ -secretase (Glenner et al., 1984; Kang et al., 1987; Sekoe et al., 2003). While the β -secretase has been identified as an aspartyl protease called β -APP cleaving enzyme (BACE), recent data suggest that γ -secretase activity resides in a high molecular weight multimeric protein complex composed of at least four core components, i.e., presenilin 1 or 2 (PS1 or PS2), nicastrin, anterior pharynx defective-1 (APH-1) and presenilin enhancer-2 (PEN-2) (Strooper et al., 2003; Sisodia et al., 2003; Vetrivel et al., 2006). More recently, cell surface type I transmembrane glycoprotein CD147, and a member of the p24 family of transmembrane proteins, p23/TMP21, involved in vesicle trafficking between the ER and Golgi apparatus, have been identified as regulatory subunits of the γ -secretase (Zhou et al., 2005; Chen et al., 2006). In addition to processing of APP, γ -secretase cleaves C-terminal stubs derived from at least 30 other type I membrane proteins including Notch, releasing cytosolic domains that in some cases migrate to the nucleus and regulate transcription of select target genes (Strooper et al., 2005). At present, however, the significance of various components of the γ -secretase and their role in regulating the biological activity of

the secretase remain unclear.

Nicastrin, an integral component of the γ -secretase complex, is a transmembrane glycoprotein with a long hydrophilic N-terminal domain containing multiple glycosylation sites, a hydrophobic transmembrane domain and a short hydrophilic C-terminal tail (Yu et al., 2000; Chen et al., 2001). Nicastrin was first identified in a *C. elegans* genetic screen as *aph-2*, an essential component of GLP-1/Notch signaling pathway in early embryos (Goutte et al., 2000). In the same year, the mammalian homolog of *aph-2* was independently identified by biochemical methods as a protein bound to PS1, and was named nicastrin (Yu et al., 2000). Nicastrin deficiency leads to lethal Notch or PS1/PS2 like phenotype with impaired γ -secretase cleavage of APP and Notch (Hu et al., 2002; Li et al., 2003). Additionally, fibroblasts derived from nicastrin knock-out mice failed to generate A β peptide as a consequence of destabilized γ -secretase complex, thus indicating the significance of nicastrin in regulating the γ -secretase activity (Li et al., 2003). Studies from biochemical approaches such as RNAi and protein overexpression have indicated that nicastrin may first bind to APh-1 to form a nicastrin-APH-1 subcomplex prior to its association with PS1 and PEN-2 (Edbauer et al., 2002; Kaether et al., 2002; Zhang et al., 2005). Nevertheless, biogenesis, maturation, stability and the steady-state levels of γ -secretase components are co-dependent. For example, the heavily glycosylated type I membrane protein nicastrin does not mature or exit the ER in cells lacking PS1 expression (Leem et al., 2002). The function of nicastrin within the γ -secretase complex is beginning to emerge.

Ectodomain shedding is a prerequisite for γ -secretase cleavage of substrates (Struhl et al., 2000). The extracellular domain of nicastrin binds to C-terminal stubs generated by ectodomain shedding of type I membrane protein substrates, thus recruiting substrates for cleavage by the γ -secretase (Shah et al., 2005).

Although much is known about the processing, assembly and intracellular trafficking of nicastrin, very little information is currently available on the anatomical localization of this protein either in the adult or in the developing mammalian brain. Additionally, it remains to be defined whether the cellular distribution profile of nicastrin overlaps or differs from that of other core components of the γ -secretase complex (PS1, APH-1 and PEN-2). While co-localization of nicastrin with other γ -secretase components will provide an anatomical basis for the site of γ -secretase activity, variations in their distribution may raise the possibility for a γ -secretase-independent function of nicastrin. Earlier studies have shown that nicastrin protein and/or its mRNA are constitutively expressed in a variety of cell lines and tissues including the brain (Sato et al., 2001; Hebert et al., 2004; Confaloni et al., 2005). Using mouse lines bearing targeted mutations in their PS1 and/or APP genes, nicastrin immunoreactivity has been demonstrated to be distributed predominantly in neurons throughout the neuroaxis (Siman et al., 2004). More recently, the relative amount of cerebellar nicastrin located in active site of synaptogenesis was found to be altered during postnatal development, suggesting a possible role for the γ -secretase complex in synapse formation and maintenance (Uchihara et al., 2006).

However, cellular distribution and/or levels of nicastrin in postnatal or adult rat brains remain to be established. Here, using Western blotting and immunohistochemistry, we report that nicastrin immunoreactivity is widely distributed in all major regions of developing and adult rat brains. Additionally, we have demonstrated that nicastrin immunoreactivity is co-localized with PS1 in all regions of the adult rat brain, thus providing an anatomical basis for nicastrin's function as an integral component of the γ -secretase complex.

2.2. Materials and Methods

2.2.1. *Materials*: Sprague-Dawley rats obtained from Charles River (St Constant, Quebec) were used in the study. Adult male rats (225-275 g) and postnatal rats from different developmental stages i.e., postnatal day 1 (P1), P7 and P21 were housed and maintained in accordance with the University of Alberta Policy on the handling and treatment of laboratory animals. The characterization of a polyclonal nicastrin antiserum raised against synthetic peptide corresponding to residues 689-709 (SP718) and a polyclonal PS1 antisera raised against N-terminal (PS1_{NT}) fragment has been described previously (Lee et al., 1996; Leem et al., 2002; Thinakaran et al., 1998). Commercially available goat polyclonal nicastrin antiserum (N-19) was purchased from Santa Cruz Biotechnology Inc. (California, USA). Polyacrylamide electrophoresis gels (4-20%) were from Invitrogen (Burlington, Canada), PVDF membranes were from BioRad (California, USA), anti-glyceraldehyde-3-phosphate dehydrogenase (GADPH) antiserum was from Abcam Inc. (Cambridge, USA) and the enhanced chemiluminescence (ECL) kit was from New England Nuclear (Mississauga, Canada). Donkey anti-goat Texas Red and donkey anti-rabbit fluorescein isothiocyanate (FITC) conjugated secondary antibodies were from Jackson ImmunoResearch (West Grove, PA, USA). Horseradish peroxidase-conjugated protein A and anti-actin antiserum were purchased from Sigma (St. Louis, USA), while elite Vectastain ABC kit was from Vector Laboratories (Burlingame, USA). All other chemicals of analytical grade were purchased from either Fisher Scientific or Sigma Chemical.

2.2.2. Immunoblotting: Six adult rats were decapitated, their brains removed immediately and areas of interest (i.e., frontal cortex, parietal cortex, septum, striatum, hippocampus, thalamus, hypothalamus, cerebellum and brainstem) dissected out and homogenized in Tris lysis buffer [50 mM Tris-HCl (pH 7.4), 10 mM MgCl₂, 0.1% bovine serum albumin (BSA), 5 mM phenylmethylsulfonyl fluoride, 10 µg/ml leupeptin and 10 µg/ml aprotinin]. In parallel, four rats from each of the following postnatal developmental stages i.e., P1, P7 and P21 were decapitated and brain regions of interest (i.e., cortex, hippocampus, cerebellum and brainstem) were dissected out and homogenized in Tris-lysis buffer. Tissues were then processed for immunoprecipitation by incubating with SP718 nicastrin antiserum as described earlier (Leem et al., 2002; Vetrivel et al., 2004). The immune complexes were precipitated by protein A/G PLUS-agarose, separated by 4-20% polyacrylamide gel electrophoresis for 90 min before being transferred to PVDF membranes. Membranes were then blocked for 1 hr in phosphate-buffered saline (PBS) supplemented with 10% non-fat milk and 0.2% Tween-20 (PBST) and incubated overnight at 4°C with N-19 nicastrin antibody (1:1000). Membranes were washed three times with PBST, incubated with horseradish peroxidase-conjugated protein A (1:2500) for 1 hr at room temperature and then exposed to X-ray films using an ECL detection kit. Blots depicting the presence of nicastrin in the adult rat brain were then stripped and sequentially reprobed with anti-PS1_{NT} and anti-GADPH (1:1000) antibodies. Blots depicting the presence of nicastrin at different stages of developing brains were reprobed with anti-actin (1:500) antibodies and quantified using an MCID image analysis system

as described previously (Hawkes et al., 2003). The data from the developing rat brains (which are presented as mean \pm S.E.M) were analyzed using one way ANOVA followed by Tukey's post-hoc analysis, with significance set at $p < 0.05$.

2.2.3. Immunohistochemistry: Eight adult male rats were deeply anesthetized with 8% chloral hydrate (VWR Canlab, Montreal, Canada), prior to perfusion with 0.01M PBS, pH 7.2, followed by 4% paraformaldehyde (PFA) or Bouin's solution. Six postnatal rats from each stage of development (i.e., P1, P7 and P21) were anesthetized by cooling on ice (P1) or with isoflurine gas (i.e., P7 and P21) and then perfused with 0.01M PBS followed by 4% PFA. Brains were removed, post fixed overnight in the same fixative and stored at 4°C in 30% PBS/sucrose. Coronal brain sections (20 and 40 μ m) were cut on a cryostat and then processed following free-floating procedure as described earlier (Hawkes et al., 2003). For enzyme-linked procedure, sections were first washed with PBS, boiled for 15 min in 10 mM sodium citrate buffer (pH 6) and then treated with 1% hydrogen peroxide for 30 minutes prior to overnight incubation with either rabbit/goat antinicastrin antibody (1:1000) or rabbit anti-PS1_{NT} antibody (1:000) at room temperature. Subsequently, sections were exposed to appropriate secondary antiserum (1:200) for 1 hr at room temperature, rinsed with PBS and incubated with avidin-biotin reagents for an additional hr at room temperature. Finally, the sections were developed using the glucose-oxidase-diaminobenzidine tetrahydrochloride-nickel enhancement method, as described earlier (Hawkes et al., 2003; Jafferli et al., 2000). The specificity of the nicastrin antibody was determined by omission of the primary antibody and by pre-adsorption of the

diluted antiserum with 10 μ M peptide antigen. Nicastrin staining was not observed in sections in which the primary antibody was either omitted or preadsorbed with excess peptide antigen. Immunostained sections were examined under a Zeiss Axioskop-2 microscope and the photomicrographs were taken with a Nikon 200 digital camera and exported to the Adobe Photoshop 6.0 program. For double immunofluorescence staining, adult rat brain sections (20 μ m) were incubated overnight at room temperature with goat anti-nicastrin (1:50) and rabbit anti-PS1_{NT} (1:200) antisera. After incubation with primary antisera, sections were rinsed with PBS, exposed to Texas Red- or FITC-conjugated secondary antibodies (1:200) for 2 hr at room temperature, washed thoroughly with PBS and then cover-slipped with Vectashield mounting medium. Immunostained sections were examined under a Zeiss Axioskop-2 fluorescence microscope and the photomicrographs were taken with a Nikon 200 digital camera and exported to the Adobe Photoshop CS2 program for further processing (Hawkes et al., 2006). The rat brain atlas of Paxinos and Watson (1986) was used to define and name anatomical structures.

2.3. Results

2.3.1. Immunoblotting: We first performed immunoblot analysis to examine the distribution of nicastrin in the adult rat brain. As shown in Fig 2.1, the nicastrin antiserum recognized one band with apparent molecular weight of 120 kDa corresponding to the mature glycosylated nicastrin. The nicastrin, as evident from a representative immunoblot, was present in all major regions of the brain including frontal cortex, parietal cortex, hippocampus, thalamus, hypothalamus, striatum, septum, cerebellum and brainstem. The overall expression of nicastrin was found to be relatively lower in the septum and cerebellum compared to other regions of the brain (Fig. 2.1). Reprobing of the blots with polyclonal PS1 antibodies PS1_{NT} revealed the presence of a 30 kD PS1 N-terminal fragment in all major regions of the brain such as frontal cortex, parietal cortex, hippocampus, thalamus, hypothalamus, striatum, septum, cerebellum and brainstem (Fig. 2.1).

2.3.2. Distribution of nicastrin immunoreactivity in the adult rat brain:

Nicastrin immunoreactivity, as observed with two different polyclonal nicastrin antisera (i.e., SP718 and N-19), is widely distributed throughout the adult rat brain in select neuronal populations within the basal forebrain, cerebral cortex, hippocampus, amygdala, thalamus, hypothalamus, cerebellum and brainstem (Figs. 2.2-2.4). However, the intensity of staining differed in a neuron- and region-specific manner. The specificity of the immunostaining was established by preadsorption of the antibody with excess antigen, which abolished the immunolabeling (Fig. 2.4). In the following sections, we describe the distribution profile of nicastrin immunoreactivity observed in specific brain regions.

Basal forebrain and basal ganglia: Nicastrin immunoreactivity was observed in all subfields of the basal forebrain including the septum, diagonal band complex (Fig. 2.2A) and nucleus basalis of Meynert. In septal nuclei, a group of multipolar cells were moderately labeled, whereas in the diagonal band complex, some weakly labeled neurons were found intermingled with moderately labeled neurons (Fig. 2.2A). A few nicastrin-immunoreactive neurons were also seen in the bed nucleus of the stria terminalis and nucleus basalis of Meynert. A population of small neurons was also encountered in the globus pallidus (Fig. 2.2B) and ventral pallidum, whereas the entopeduncular nucleus displayed few nicastrin immunoreactive cells. A number of nicastrin positive neurons were found scattered throughout the caudate putamen (Fig. 2.2C). These neurons, which were usually located in between unstained myelinated fascicles, were multipolar with short processes.

Cerebral cortex: Nicastrin immunoreactive neurons were detected in most layers of the neocortex with varying degrees of staining intensity. Characteristically, the labeling was high in layers IV-VI, moderate in layers II-III and relatively low in layer I (Figs. 2.2D, 2.2E). The laminar distribution of nicastrin labeled neurons was particularly striking in cingulate cortex and in the frontoparietal cortex. In general, a number of moderately stained smaller multipolar neurons were visible in layers II-III, whereas intensely labeled pyramidal neurons with vertically oriented apical dendrites were seen in layers IV and V of the cortex (Fig. 2.2E). Layer VI, on the other hand, was characterized by some scattered multipolar neurons with moderate somatodendritic labeling. In the piriform cortex, intensely

labeled nicastrin immunoreactive neurons were common in both the pyramidal and polymorphic layers, intermingled with a smaller population of weakly labeled neurons.

Amygdala: Several groups of moderately labeled nicastrin-immunoreactive cell bodies were evident in the cortical, medial and basolateral amygdaloid nuclei (Fig. 2.2G). Additionally, some multipolar cells exhibiting rather weak immunoreactivity were also apparent in the anterior amygdaloid area and central amygdaloid nucleus. Most of the immunostaining in the amygdaloid nuclei was found to be associated predominantly with cell bodies (see Fig. 2.2G inset).

Hippocampus: The hippocampal formation showed some of the most intense nicastrin immunoreactivity in the brain (Fig. 2.3A-2.3C). Within the Ammon's horn, strong labeling was apparent in the CA1-CA3 pyramidal cells and their apical dendrites (Fig. 2.3A, 2.3B), but occasional multipolar neurons were also found scattered in the strata oriens and stratum radiatum. Within the dentate gyrus, granule cell somata were moderately labeled (Fig. 2.3C), whereas little nicastrin immunoreactivity was evident in the adjacent molecular layer. A number of nicastrin-positive polymorphic neurons were also observed in the hilus region of the hippocampus (Fig. 2.3C).

Hypothalamus and Thalamus: In the hypothalamus rather strong neuronal labeling was observed in the supraoptic (Fig. 2.2F) and paraventricular (Fig. 2.3D) nuclei, whereas neurons located in the ventromedial nucleus, dorsolateral

hypothalamic areas and arcuate nucleus (Fig. 2.3E) showed rather weak to moderate labeling. The immunoreactivity in the median eminence was confined exclusively to neuropil (Fig. 2.3E). A number of medium-sized nicastrin immunostained neurons were observed throughout the thalamus (Fig. 2.3F). These neurons which were moderately labeled were evident in ventral and lateral portions of the thalamus (Fig. 2.3F) and in the habenular nucleus.

Midbrain: Moderate somatodendritic labeling was observed in the superficial gray layers of the superior colliculus and in the central gray matter. The substantia nigra pars reticulata was characterized by multipolar neurons with moderate nicastrin immunoreactivity (Fig. 2.3G), whereas the pars compacta exhibited rather weak labeling. Neurons of the red and oculomotor nuclei displayed rather intense immunoreactivity (Fig. 2.3H). Moderately labeled neurons were also apparent in the intermediate gray layer of superior colliculus and mesencephalic trigeminal nucleus.

Brainstem: Nicastrin immunoreactivity was visible at all brainstem levels. A population of large multipolar neurons was encountered in the pontine reticular nucleus, whereas numerous moderately labeled neurons were seen in the inferior colliculus (Fig. 2.4A), abducens nucleus and reticulotegmental nucleus of pons. However, strong labeling was evident particularly in the motor trigeminal nucleus, pontine nucleus (Fig. 2.4B) and in the facial (Fig. 2.4C) as well as vestibular nuclei (Fig. 2.4D).

Cerebellum: A common pattern of intense nicastrin immunoreactivity prevailed throughout the cerebellum (Fig. 2.4E, 2.4F). In the cortex, Purkinje cells were heavily stained and often seen in continuity with their stained dendritic shafts extending into the molecular layer (Fig. 2.4E). The granule cells exhibited rather weak staining, whereas a number of deep cerebellar nuclei showed numerous moderately labeled immunoreactive cell bodies and dendrites.

2.3.3. Co-localization of nicastrin and PS1 in the adult rat brain: To determine the possible co-localization of nicastrin and PS1 in the adult rat brain, we first established the normal distribution profile of PS1 immunoreactivity using a single labeling procedure and then performed double labeling experiments in sections from different brain regions. In agreement with earlier reports (Elder et al., 1996; Lee et al., 1996; Blanchard et al., 1997; Kim et al., 1997; Lah et al., 1997; Yan et al., 2004), PS1-immunoreactive neurons and fibers were distributed throughout the brain including the septal/diagonal band complex, nucleus basalis of Meynert, striatum, cerebral cortex, hippocampus, amygdala, thalamus, hypothalamus, median eminence, brainstem motor nuclei and cerebellar Purkinje cells (Figs. 2.5 and 2.6). Dual-labeling experiments revealed that nicastrin is co-localized with PS1-positive neurons in virtually all regions of the brain including vertical and horizontal limbs of the diagonal band complex (Fig. 2.5B, 2.5C), striatum (Fig. 2.5E, 2.5F), cerebral cortex (Fig. 2.5I, 2.5J), hippocampal formation (Fig. 2.5L, 2.5M), median eminence (Fig. 2.6B, 2.6C), thalamus (Fig. 2.6E, 2.6F), brainstem motor nuclei (Fig. 2.6H, 2.6I) and cerebellar Purkinje cells (Fig. 2.6K, 2.6L). It is apparent from our double labeling experiments that nicastrin and PS1 are co-

localized not only in cell bodies but also in dendrites/neuropil in discrete regions of the brain.

2.3.4. Nicastrin immunoreactivity in the postnatal developing rat brain: Earlier studies have indicated that PS1 expression is relatively high at early stages of development and then declines gradually to reach an adult profile of distribution (Moreno-Flores et al., 1999; Fakla et al., 2000; Keino et al., 2003; Wines-Smuelson and Shen, 2005). To establish whether nicastrin expression levels and distribution are also developmentally regulated, we performed immunoblotting and immunocytochemical analyses of the cortex, hippocampus, cerebellum and brainstem obtained from P1, P7 and P21 brains (Fig. 2.7A-2.7L). The relative levels of nicastrin in all these brain regions were found to be highest at P1 followed by a gradual decline to attain the levels found in adults. However, the chronological alterations were more evident in hippocampal and brainstem regions than in the cortex or cerebellum (see Fig. 2.7A, 2.7D, 2.7G, 2.7J). At the cellular level, nicastrin immunoreactivity is widely distributed at all stages of the postnatal developing brains (Fig. 2.7B, 2.7C, 2.7E, 2.7F, 2.7H, 2.7I, 2.7K, 2.7L). At P1 and P7, nicastrin immunoreactivity was localized predominantly in neuronal cell bodies, whereas at P21 immunoreactivity was apparent not only in the cell bodies but also in dendrites/neuropil of all brain regions. The cortical regions of P1 and P7 brains exhibited rather homogenous nicastrin immunoreactivity in all layers without any laminar distinction, whereas cortex from P21 brain showed subtle variation in labeling intensity in different layers (Fig. 2.7B, 2.7C). In the hippocampus, intense nicastrin immunoreactivity at P1

and P7 was evident primarily in the CA1-CA2 pyramidal cell layers and granular cell layers of the dentate gyrus. The CA3 pyramidal cell layer showed relatively lower intensity of immunoreactivity. The distribution profile of nicastrin did not exhibit significant alteration during the course of development but its intensity, as evident from P21 hippocampal formation, was found to be drastically decreased (Fig. 2.7E, 2.7F). As for the brainstem motor nuclei, nicastrin immunoreactivity was apparent mostly in cell bodies at P1 and P7, whereas a number of dendrites/neuropil, in addition to motoneuron cell bodies, was also labeled at P21 stage (Fig. 2.7H, 2.7I). Interestingly, the cerebellum of P1 and P7 brains showed robust nicastrin immunoreactivity in cell bodies of Purkinje cells and some punctate staining in the external granular layer. At P21, labeling intensity was relatively lower but often seen in dendritic shafts of the Purkinje cells (Fig. 2.7K, 2.7L).

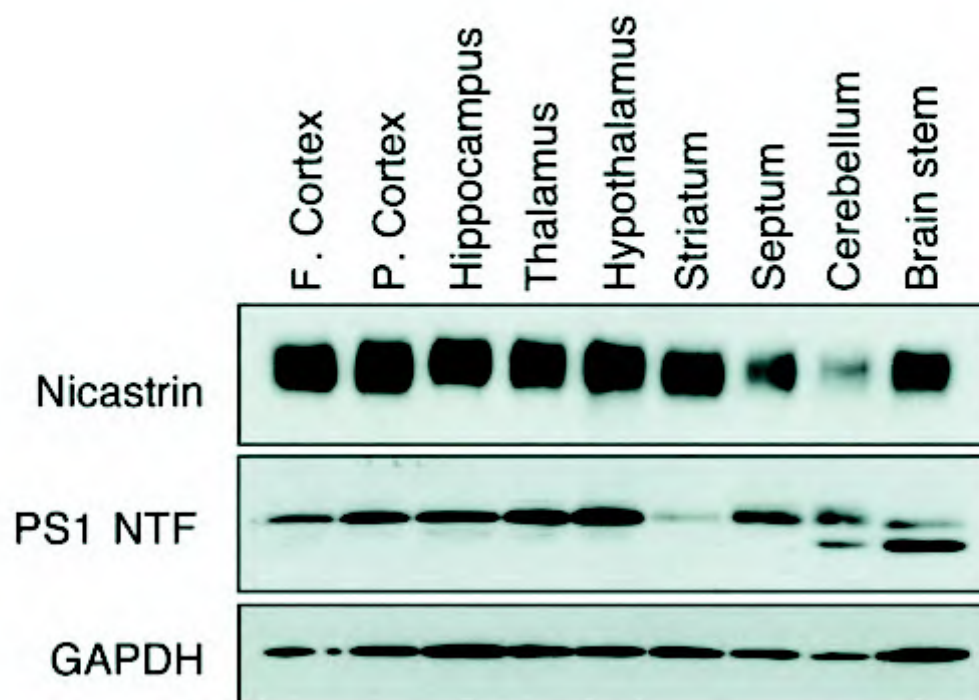


Fig. 2.1: Immunoblot analysis of nicastrin in different brain regions of the adult rat. Nicastrin (NCT) antiserum reacts with one major band of approximately 120 kDa, corresponding to the mature complex glycosylated form of nicastrin. The same membrane was sequentially probed with PS1_{NT} and glyceraldehyde-3-phosphate dehydrogenase (GAPDH) antibodies. *F. cortex*, frontal cortex; *P. cortex*, parietal cortex.

Figure. 2.2

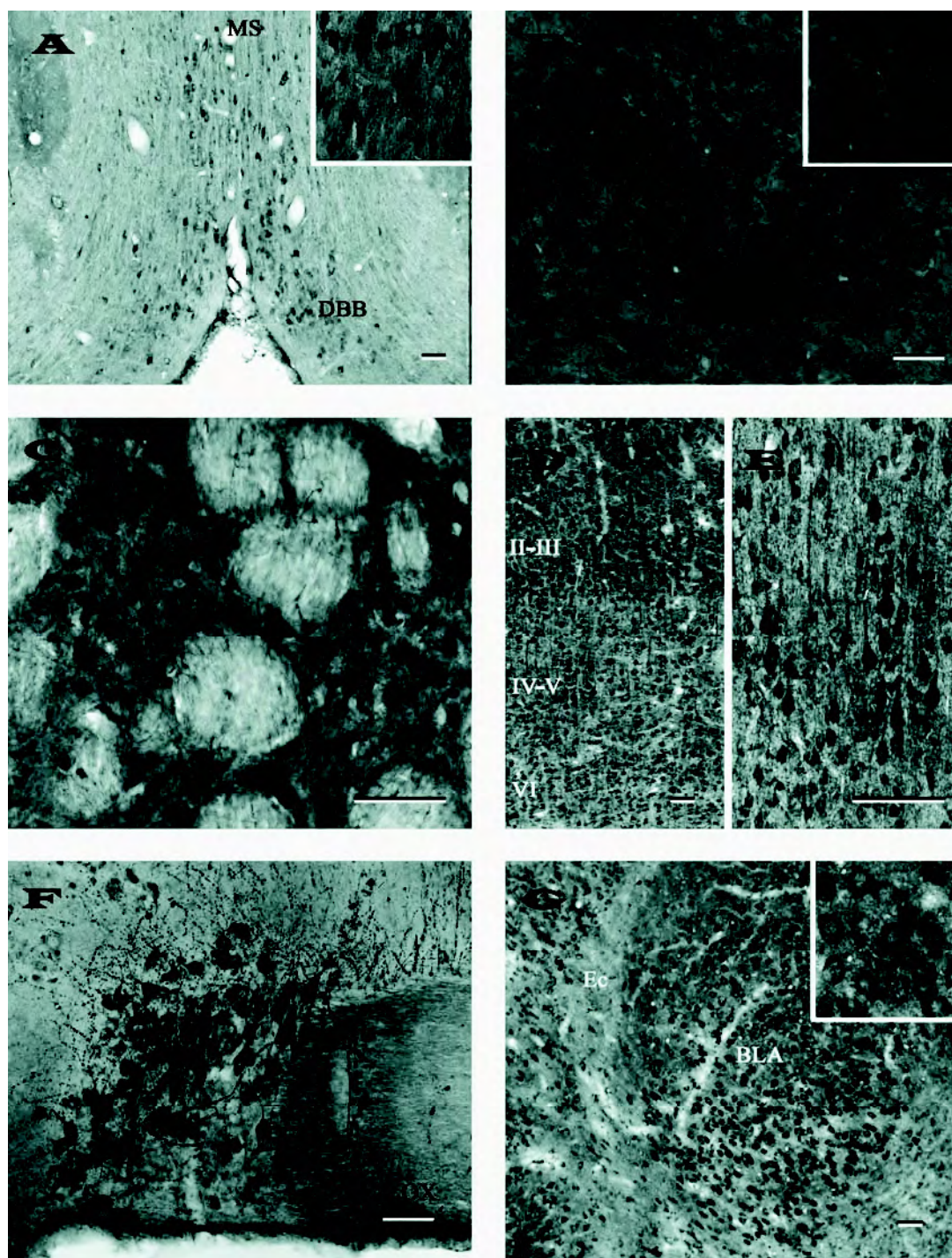


Fig. 2.2: Photomicrographs of transverse sections of adult rat brain showing the distribution of nicastrin immunoreactive neurons and fibers in the diagonal band of Broca (A), globus pallidus (B), striatum (C), cortex (D, E), supraoptic nucleus (F) and amygdaloid nucleus (G). Note that neurons of the diagonal band of Broca, globus pallidus and striatum are moderately labeled, whereas supraoptic and amygdaloid nuclei exhibited rather intense immunoreactivity. The staining intensity in the neocortex is variable: weak labeling in layer I, moderate staining in layers II-III (D) and strong immunoreactivity in deep layers IV-VI (D and E). Insets in (A), (B) and (G) show neuronal labeling at higher magnification. Representative photomicrographs of striatum (C) and cortex (D, E) were obtained with N-19 antiserum, whereas others (A, B, F, G) were acquired following labeling with SP718 antiserum. Scale bar = 50 μ m. *MS*, medial septum; *DBB*, diagonal band complex; *OX*, optic chiasm; *Ec*, external capsule; *BLA*, basolateral amygdala.

Figure. 2.3

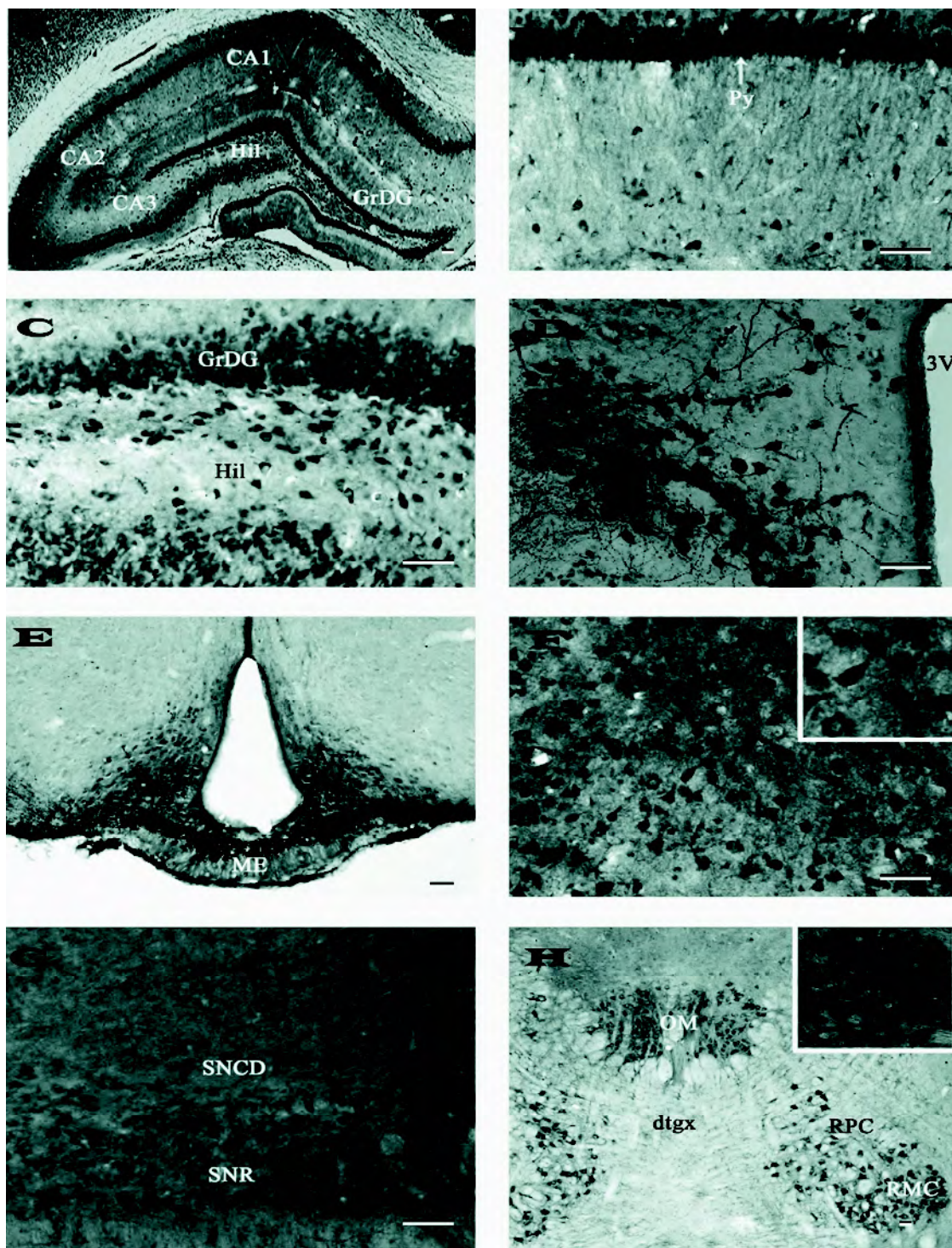


Fig. 2.3: Photomicrographs of transverse sections of adult rat brain showing the distribution of nicastrin immunoreactive neurons and fibers in the hippocampus (A), CA1 pyramidal cell layer of the Ammon's horn (B), granule cell layer and hilus of the dentate gyrus (C), paraventricular nucleus of the hypothalamus (D), arcuate nucleus/median eminence (E), medial thalamic nucleus (F), substantia nigra (G) and oculomotor and red nuclei (H). Note intense labeling in neurons of the pyramidal cell layer, hilus and paraventricular nucleus and fibers in the median eminence. Insets in (F) and (G) show neuronal labeling at higher magnification. Representative photomicrographs of medial thalamic nucleus (F) and substantia nigra (G) were obtained with N-19 antiserum, whereas others (A, B, C, D, E, H) were acquired following labeling with SP718 antiserum. Scale bar = 50 μ m. *Hil*, hilus; *GrDG*, granular cell layer of the dentate gyrus; *Py*, pyramidal cell layer; *3V*, third ventricle; *Arc*, arcuate nucleus; *ME*, median eminence; *SNCD*, substantia nigra compact part; *SNR*, substantia nigra reticular part; *OM*, oculomotor nucleus; *dtgx*, dorsal tegmental decussation; *RPC*, red nucleus periventricular part; *RMC*, red nucleus magnocellular part.

Figure.2.4

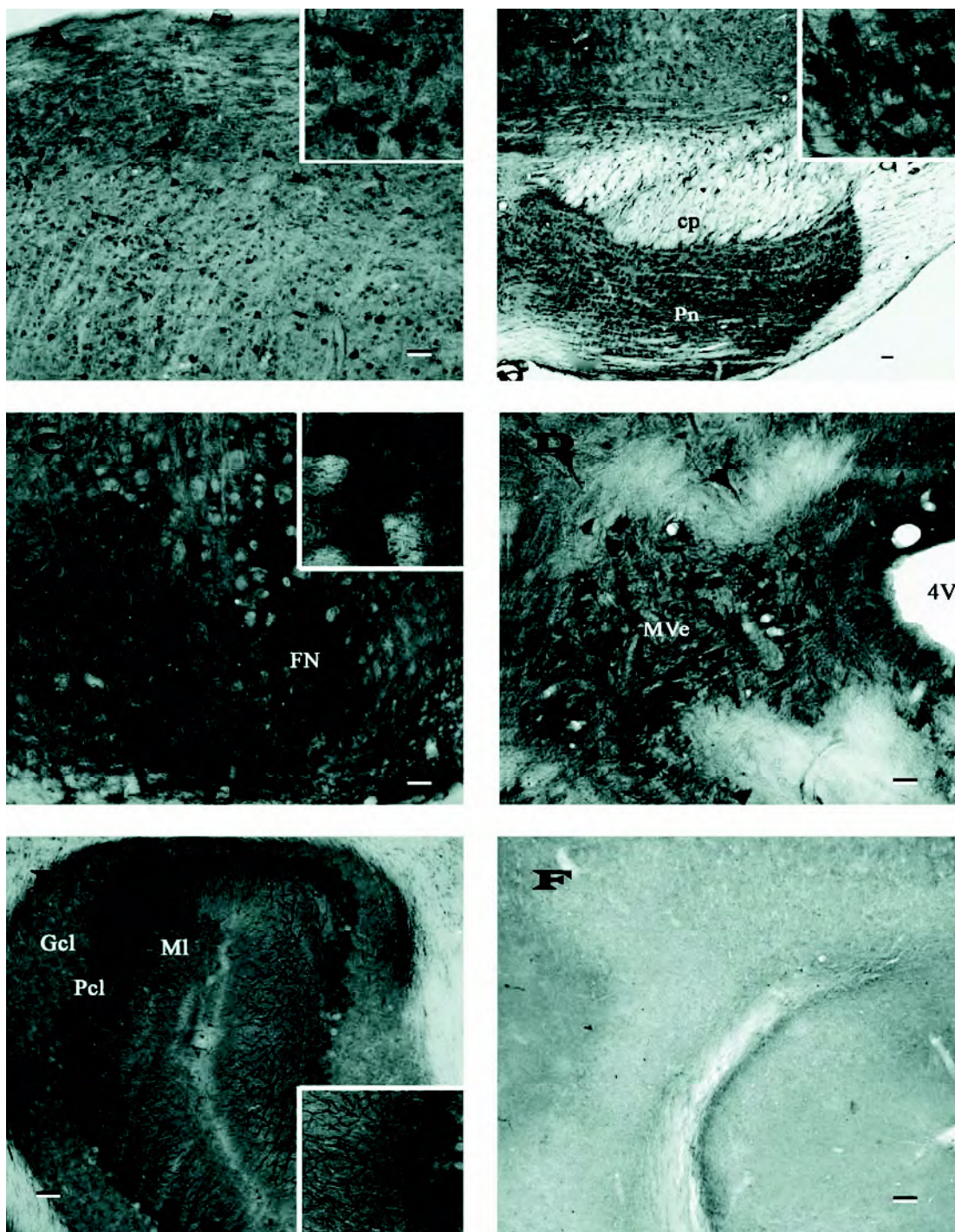


Fig. 2.4: Photomicrographs of transverse sections of adult rat brain showing the distribution of nicastrin immunoreactivity in the inferior colliculi (A), pontine nucleus (B), facial nucleus (C), vestibular nucleus (D) of the brainstem and in the cerebellum (E). Note intense labeling of the brainstem neurons and the Purkinje cells of the cerebellum. F, represents a cerebellar section processed following preabsorption of the antibody with 10 μ M purified rat nicastrin. Inset in (A), (B), (C) and (E) show neuronal labeling at higher magnification. All photomicrographs were acquired following labeling with SP718 antiserum. Scale bar = 50 μ m. *cp*, cerebral peduncle; *Pn*, pontine nuclei; *FN*, facial nucleus; *Mve*, medial vestibular nucleus; *Gcl*, granular cell layer; *Pcl*, Purkinje cell layer; *ML*, molecular layer.

Figure. 2.5

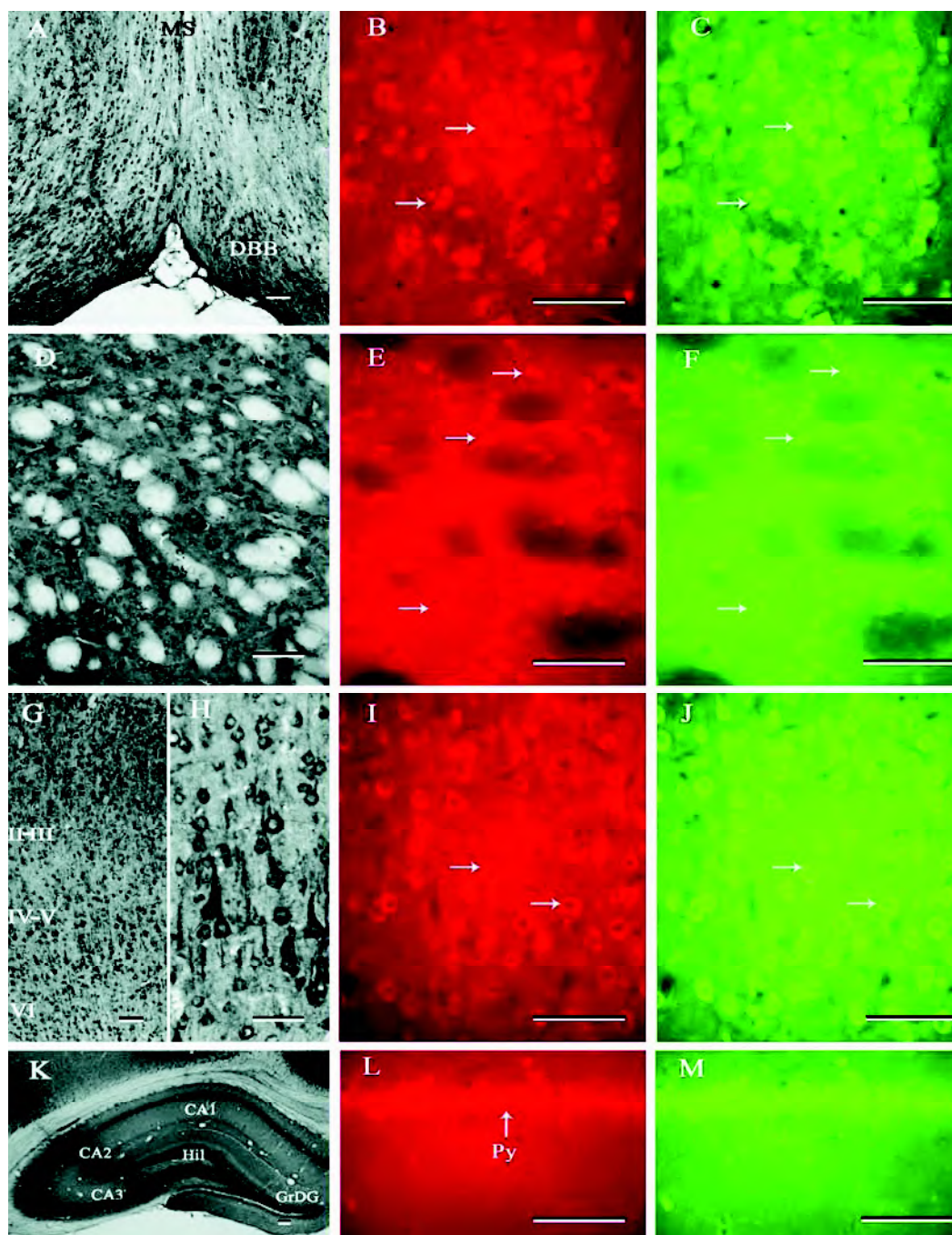


Fig. 2.5: Photomicrographs of transverse sections of adult rat brain showing the distribution of PS1 immunoreactivity and its co-localization with nicastrin in the diagonal band of Broca (A-C), striatum (D-F), cortex (G-J) and hippocampal formation (K-M). Note the widespread distribution of immunoreactive PS1 in various regions of the adult rat brain (A, D, G, H, K). Double labeling experiment showed that PS1 is co-localized with nicastrin in all neurons located in the diagonal band of Broca (B, C), striatum (E, F), cortex (I, J) and hippocampus (L, M) of the adult rat brain. Scale bar = 50 μ m. *MS*, medial septum; *DBB*, diagonal band complex; *Hil*, hilus; *GrDG*, granular cell layer of the dentate gyrus; *Py*, pyramidal cell layer.

Figure.2.6

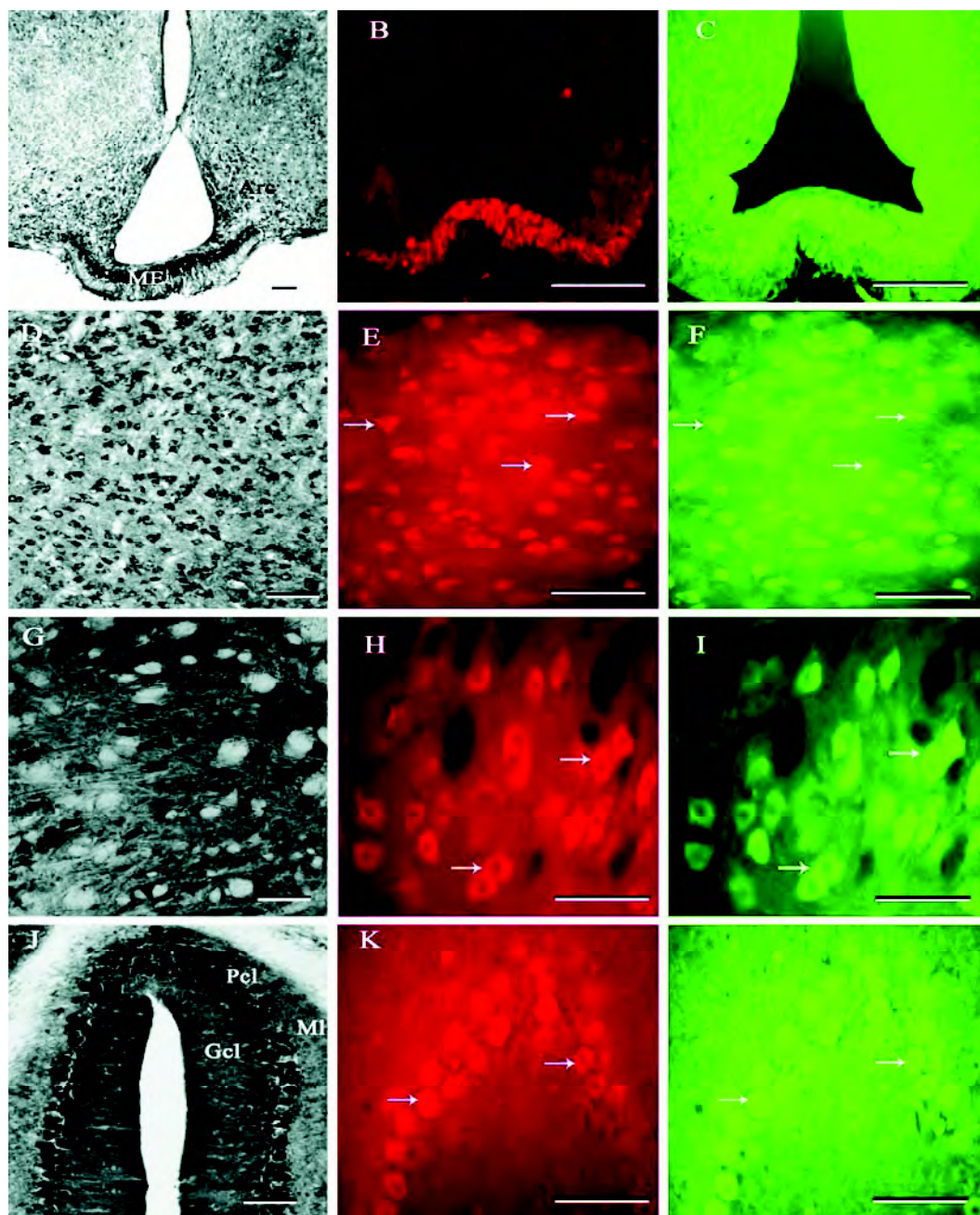


Fig. 2.6: Photomicrographs of transverse sections of adult rat brain showing the distribution of PS1 immunoreactivity and its co-localization with nicastrin in the median eminence (A-C), thalamus (D-F), brainstem (G-I) and cerebellum (J-L). Note the widespread distribution of immunoreactive PS1 in various regions of the adult rat brain (A, D, G, J). Double labeling experiment showed that PS1 is co-localized with nicastrin in all neurons and/or nerve terminals located in the median eminence (B, C), thalamus (E, F), brainstem (I, J) and cerebellum (L, M) of the adult rat brain. Scale bar = 50 μ m. *ARC*, arcuate nucleus; *ME*, median eminence; *Gcl*, granular cell layer; *Pcl*, Purkinje cell layer; *MI*, molecular layer.

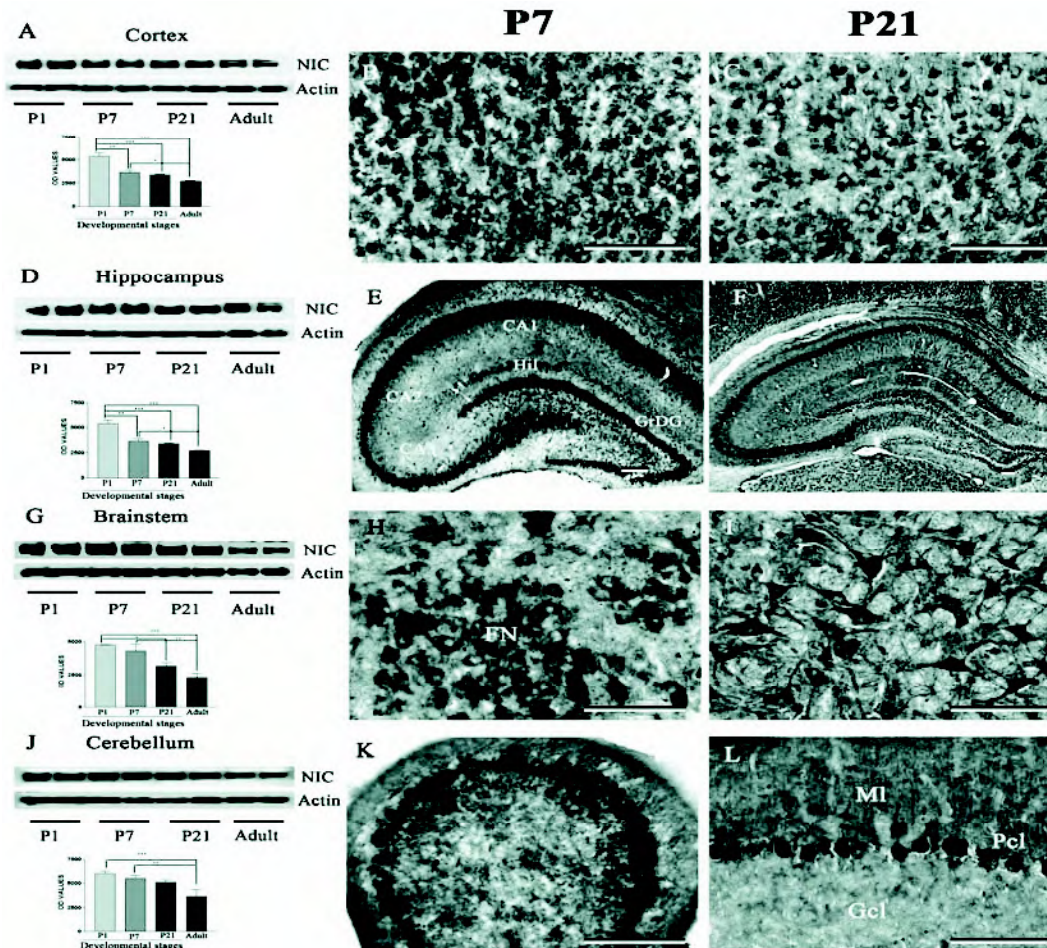


Fig. 2.7: Immunoblotting and immunohistochemical staining showing the levels and expression of nicastrin in the cortex (A-C), hippocampus (D-F), brainstem (G-I) and cerebellum (J-L) of postnatal rat brains at different stages of development. Note the gradual decrease in nicastrin levels at all four regions of the brain with the progress of the development (A, D, G, J). Immunohistochemical staining revealed that nicastrin expression is relatively high in the cortex (B, C), hippocampus (E, F), brainstem (H, I) and cerebellum (K, L) of P7 (B, E, H, K) compared to P21 (C, F, I, L) postnatal brains. All immunocytochemical photomicrographs were acquired following labeling with SP718 antiserum. Scale bar = 50 μ m. * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$. Hil, hilus; GrDG, granular cell layer of the dentate gyrus; FN, facial nucleus; Gcl, granular cell layer; Pcl, Purkinje cell layer; MI, molecular layer.

2.4. Discussion

The present study provides the first comprehensive cellular distribution of nicastrin, an integral subunit of γ -secretase complex, in postnatal developing and adult rat brains. As evident from our immunoblotting and immunohistochemical analyses, nicastrin is widely expressed throughout the brain and the level of expression is developmentally regulated during postnatal stages. Additionally, nicastrin immunoreactivity extensively overlaps with PS1 distribution in various regions of the adult rat brain, thus providing an anatomical basis for a physiological role for γ -secretase complex in a wide spectrum of neurons located throughout the brain.

A potential concern regarding any immunological study is the specificity of the antiserum employed for the immunolabeling. The nicastrin and PS1 antisera used in the present study have been previously characterized (Lee et al., 1996; Leem et al., 2002; Vetrivel et al., 2004). The specificity of antisera is further confirmed by our immunoblotting experiment, which showed that nicastrin antiserum essentially reacted with one major band of approximately 120 kDa corresponding to the mature complex glycosylated forms of the protein in all brain regions examined. The anti-PS1_{NT} antibody visualized a single band with a molecular weight of approximately 30 kDa, equivalent to human PS1-NTF. For immunohistochemistry, standard immunological controls, including omission of the primary antiserum and preincubation of the antisera with excess antigen, eliminated staining, thus indicating that the nicastrin and PS1 antisera specifically recognize the endogenous nicastrin and PS1, respectively. As for nicastrin, this is

further substantiated by evidence that two nicastrin antisera reproducibly stained similar groups of neurons and fibers in the rat brain.

Our results clearly indicate that nicastrin is expressed in all major areas of the adult rat brain. At the cellular level, nicastrin immunoreactivity predominantly appears to be associated with neurons and their processes. Although no positive staining was readily apparent in morphologically identifiable glial cells, the expression of nicastrin in astrocytes and/or microglia in normal adult rat brain cannot be formally ruled out based on the present findings. Interestingly, in addition to the neuronal soma, neuropil labeling was apparent in many brain regions, suggesting that nicastrin is localized in dendrites and/or axon terminals. However, the intensity of immunoreactivity in select neuronal populations varies distinctly among different regions of the brain. Areas that express relatively high levels of nicastrin include the cortex (layers IV and V), pyramidal and granule cell layers of the hippocampus, selected hypothalamic nuclei, Purkinje cells of the cerebellum, pontine nucleus and motoneurons of the brainstem. Moderate neuronal labeling was apparent in the basal forebrain areas, amygdala, thalamus, superior colliculus, midbrain areas and granule cells of the cerebellum.

In keeping with the adult distributional profile, analysis of postnatal rats showed widespread distribution of nicastrin in various brain regions including cerebral cortex, hippocampus, brainstem and cerebellum. However, nicastrin expression decreased with the progress of postnatal development. It is also of interest to note that nicastrin expression was apparent predominantly in cell bodies at the early

stages of development, whereas during later stages immunoreactivity was evident both in the cell bodies as well as dendrites/neuropil. Furthermore, nicastrin expression markedly decreased in the hippocampus and brainstem as compared with the level of expression in cortex and cerebellum, thus suggesting that developmental profile of nicastrin is possibly regulated differentially in various brain regions.

To the best of our knowledge, the previous immunocytochemical report regarding nicastrin distribution in the developing and adult rat brain is mostly restricted to the analysis of cerebellum (Uchihara et al., 2006). Consistent with this study, our results show nicastrin expression is developmentally regulated and it is localized in both the Purkinje cells as well as granule cell layer of the cerebellum. Furthermore, the present study extends previous findings on two accounts by revealing that i) the levels and distribution of nicastrin are developmentally regulated not only in the cerebellum but also in the cerebral cortex, hippocampus and brainstem regions, and ii) nicastrin immunoreactivity in the adult rat brain is distributed throughout the neuroaxis including the basal forebrain, cortex, amygdala, thalamus, hypothalamus, substantia nigra and brainstem. These results are very much compatible with the findings reported in the adult mouse brain (Siman et al., 2004). However, variation in the intensity of labeling is discernible in certain brain areas such as the cortex where a gradient of immunoreactivity was evident in the adult rat brain in contrast to the uniform labeling across the cortical layers in the mouse brain. Additionally, neuropil of stratum radiatum and stratum lacunosum moleculare of the adult rat hippocampal formation, unlike the mouse

hippocampus, did not exhibit significant nicastrin immunoreactivity (Siman et al., 2004). These apparent discrepancies could relate either to the animal species or to the epitope specificity of nicastrin antisera used in both the studies. At present, the distribution profile of nicastrin mRNA has not been thoroughly investigated at the cellular levels in the adult rat brain, but high levels of nicastrin transcripts have been detected in the cortex, hippocampus and cerebellum of the adult rat brain by RT-PCR assay. Interestingly, in addition to the full length nicastrin mRNA, the expression of an alternative spliced variant lacking exon 3 has also been found to be expressed preferentially in embryonic and adult nervous system (Confaloni et al., 2005).

Earlier studies have shown that nicastrin can be co-immunoprecipitated and co-localized with PS1 in a variety of cells/neurons (Yu et al., 2000; Baulac et al., 2003; Leem et al., 2002; Pasternak et al., 2003; Siman et al., 2003; Uchihara et al., 2006). Consistent with previous reports (Elder et al., 1996; Lee et al., 1996; Blanchard et al., 1997; Kim et al., 1997; Lah et al., 1997; Yan et al., 2004), we find that PS1 is distributed widely in the adult rat brain. Additionally, our double labeling experiments showed that PS1 is co expressed with nicastrin in almost all brain regions investigated in the present study, leading to the suggestion that nicastrin most likely acts as an integral component of the γ -secretase complex in the brain rather than mediating any effects of its own. However, it is of interest to determine whether nicastrin and PS1 are localized at the same subcellular site within neurons along with other components of the γ -secretase complex. At present, no report is available on the cellular distribution of APH-1 or PEN-2 in

the adult rat brain, but a recent immunocytochemical study showed that four components of the γ -secretase complex exhibit a coincident anatomical localization in the adult transgenic mouse brain (Siman et al., 2003). Some subtle variations have been reported in the cellular distribution profile among the four γ -secretase subunits in selected brain regions as well as in certain peripheral tissues. For example, PEN-2 immunoreactivity, compared to other γ -secretase components, was found to be rather intense in the mossy fiber terminal zone of the hippocampus (Siman et al., 2003), whereas nicastrin, but not PS1, was expressed at relatively high levels in muscle membranes (Ilaya et al., 2004). Whether these differences reflect unique functions of PEN-2 or nicastrin independent of the γ -secretase remains to be established.

Nicastrin was initially identified as a cofactor of PS1 within the γ -secretase complex that cleaves several membrane proteins including APP and Notch (Strooper et al., 2005). It has recently been shown that the extracellular domain of nicastrin is involved in the substrate recognition, whereas the transmembrane domain of the protein is critical in the assembly and trafficking of the γ -secretase complex to the cell surface (Capell et al., 2003; Shah et al., 2005). The significance of this protein is further highlighted by RNAi and gene knockout studies, which clearly demonstrated that nicastrin is essential for the assembly of PS1/ γ -secretase complex and secretion of A β peptide (Yang et al., 2002; Hu et al., 2002; Leem et al., 2002; Li et al., 2003; Zhang et al., 2005). Since A β -related peptides are produced constitutively (Selkoe et al., 2003), it is likely that

distribution of nicastrin observed in a variety of neuronal populations represents cellular sites of active γ -secretase complex, which mediates APP processing in the developing as well as adult rat brain. Interestingly, we find that nicastrin is expressed both in the brain regions that are vulnerable in AD pathology such as cortex and hippocampus, and also in relatively unaffected regions such as striatum and cerebellum. This is somewhat compatible with a recent study which showed that γ -secretase subunits are expressed at similar levels in amyloid-rich (e.g., deep entorhinal cortex) and amyloid-poor (superficial entorhinal cortex) brain regions of mice bearing targeted mutations in the PS1 and APP genes (Siman et al., 2004). Thus, it is likely that neuronal vulnerability or amyloid deposition in AD may depend on factors other than the differential distribution of γ -secretase complex in the brain.

As a component of the γ -secretase complex, nicastrin is also involved in the recognition and cleavage of a variety of other integral membrane proteins such as Notch, Nectin-1a, E-cadherin and ErbB-4 receptor (Ni et al., 2001; Kim et al., 2002; Marambaud et al., 2002; Vetrivel et al., 2006). There is evidence that peptides generated from these cleavages are capable of modulating functions in the developing as well as the adult brain (Strooper et al., 2005). Earlier studies have shown that neurogenesis in the rat brain is mostly completed by birth, with neuronal differentiation, migration and synaptogenesis continuing for several weeks (Bayer et al., 1986; Marty et al., 2002). The pronounced expression of nicastrin in the early postnatal brains, together with the evidence that γ -secretase

plays a critical role in development (Figuerola et al., 2002; Sarkar et al., 2003) raise the possibility that nicastrin as an integral component of γ -secretase complex may be involved in regulating neuronal differentiation, maturation and/or synaptogenesis during development. Additionally, it is likely that widespread nicastrin expression in the adult brain may also contribute, at least in part, in the γ -secretase processing of substrates to regulate normal synaptic function in the adult brain (Parent et al., 2005). In conclusion, the present study demonstrates clearly that nicastrin is widely distributed in the adult rat brain, which may provide an underlying basis to define precisely the site of γ -secretase activity and other functions of the protein, if any, in normal and AD brains.

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CHAPTER - 3

Localization and regional distribution of p23/TMP21 in the brain

PREFACE: Recently it has been suggested that p23, which is involved in protein trafficking can also play a critical role in AD pathogenesis. Much information is available on its distribution in peripheral tissues but not in the brain. This study was carried out to determine the level and cellular distribution of p23 in rodent brains and its possible alterations in an animal model of excitotoxicity as well as in AD pathology.

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3.1. Introduction

Alzheimer's disease (AD) is pathologically characterized by the cerebral deposition of 39- to 43-a.a β -amyloid peptides ($A\beta$) in the brain of afflicted individuals. Sequential proteolysis of amyloid precursor protein (APP) by β - and γ -secretases generate $A\beta$ (Iwatsubo, 2004; Vassar, 2004). γ -Secretase is a multimeric transmembrane protein complex containing presenilins (PS1 or PS2), nicastrin, APH-1 and PEN-2 as core subunits (Iwatsubo, 2004). Recently, p23 (also called TMP21) was identified as regulatory components of γ -secretase complex (Chen et al., 2006). p23 is a member of the p24 family of proteins, which are highly conserved type I transmembrane proteins involved in the coat protein (COP) I and II vesicle-mediated cargo transport between the ER and the Golgi (Barlowe, 2000; Blum et al., 1999). The p24 family of proteins has been proposed to regulate COP vesicle budding, ER quality control, organization of the Golgi apparatus, and the formation of tubular transport intermediates (Bethune et al., 2006; Simpson et al., 2006).

In mammals, members of the p24 family proteins function as homo- and heterodimers (Jenne et al., 2002). A yeast *S. cerevisiae* mutant strain with deletion of all members of the p24 family was viable and showed delay in the secretory pathway trafficking of select proteins (Belden and Barlowe, 2001; Springer et al., 2000). In the nematode *C. elegans*, reducing the activity of the p24 family member SEL-9 increased the cell surface accumulation of a transport-defective mutants of the Notch homologues, GLP-1 and LIN-12 (Kehoe et al., 1999). In the fly *Drosophila*, loss of function mutations in p24 homologues reduced signaling

associated with the TGF- β homologue, Dpp (Bartoszewski et al., 2004). Mice with homozygous disruption of p23 alleles exhibited early embryonic lethality, underscoring the physiological significance of p24 family proteins (Denzel et al., 2000).

An important link between p23 function and A β production was uncovered when it was found that p23 associates with γ -secretase complexes (Chen et al., 2006). Interestingly, reducing p23 expression results in increased γ -secretase cleavage of APP in intact cells and cell-free A β assays (Vetrivel et al., 2007). Moreover, knockdown of p23 expression conferred biosynthetic stability to nascent APP, allowing its efficient maturation, surface accumulation, and cleavage by α - and β -secretases (Vetrivel et al., 2007). These studies clearly establish that p23 is a negative modulator of A β production.

At present, there is no published report on the distribution and relative abundance of p23 in the central nervous system. Here, we performed detailed characterization on the localization of p23 in the adult rodent and human brain, and during the postnatal developmental stages in rodents. Our studies show that p23 is widely expressed in major brain areas, and co-localizes with γ -secretase core subunits PS1 and nicastrin in neurons. Interestingly, we show that steady-state p23 levels decline during postnatal development in rat and mouse brain. This age-related decline in p23 expression may be an intrinsic factor that significantly impacts on APP processing and A β burden in the aging nervous system.

3.2. Materials and methods

3.2.1. *Animals and Autopsy Material:* Sprague-Dawley rats obtained from Charles River (St Constant, Quebec) and B6C3F1/J mice obtained from The Jackson Laboratory (Bar Harbor, Maine) were used in the study. Animals were housed and maintained in accordance with the Institutional Policy on the handling and treatment of laboratory animals. Rat and mouse brain harvested during embryonic and postnatal developmental stages were prepared essentially as described previously (Lee et al., 1996). For p23 immunostaining, well-characterized post-mortem human brain tissue samples from frontal cortex, hippocampus and cerebellum of AD (n = 8; age 78.3 ± 1.1 yrs; postmortem delay, 29 ± 6.4 hrs) and age-matched control (n = 6; age 72.8 ± 2.3 yrs; postmortem delay, 26.2 ± 4.2 hrs) were obtained from the Brain Bank of the Douglas Hospital Research Center, Montreal, Canada. Human brain tissues used for the Western blot analysis were obtained at autopsy, 1-10 h post-mortem, in the Brain Resource Center, The Johns Hopkins University School of Medicine. Brain tissue from seven controls free of any neurological diseases (age 30, 40, 59 [two individuals], 67, 75 and 86 years), and eight cases of sporadic AD (age 55, 56, 59, 61 [two individuals], 65, 76 and 80) were used. All cases of sporadic AD were pathologically confirmed by consortium to establish a registry for Alzheimer's disease (CERAD) criteria and brains were stored at -70°C . Brain tissue from three patients with familial early-onset AD (FAD) carrying the PS1 I143T mutation (age 36, 38 and 40 years) and from one patient with the PS1 G384A mutation (age 40 years) were obtained at autopsy in the Born-Bunge Foundation (Dermaut et al., 2002) and stored at -70°C .

3.2.2. Kainic acid administration: Thirty two adult male rats were injected intraperitoneally with kainic acid dissolved in normal saline (12 mg/kg) or an equal volume of saline (0.2-0.25 ml) as described previously (Kar et al., 1997). Brains were harvested from control (n = 8) and kainic acid-injected rats at 12 h, 2 days or 12 days following the injection (n = 8 for each time point) and processed for Western blotting or immunohistochemistry as described below.

3.2.3. Cell culture: Primary mixed brain cultures were cultured from cortical tissue harvested from embryonic day 15 mouse embryos and maintained as described previously (Parent et al., 2005). For immunofluorescence studies, neurons were cultured on glass coverslips coated with 0.1% polyethyleneimine in 15 mM borate buffer [pH 9]. HeLa cells stably expressing GFP-tagged N-acetylgalactosaminyltransferase-2 were cultured in Dulbecco's modified Eagle's medium supplemented with 10% fetal bovine serum. Mouse N2a neuroblastoma cells were maintained in 45% Dulbecco's modified Eagle's medium and 50% Opti-MEM (Invitrogen, Carlsbad, CA) supplemented with 5% fetal bovine serum.

3.2.4. Antibodies: The polyclonal p23 antiserum was raised against a synthetic peptide corresponding to N-terminal residues 32-57 of mouse p23 (Vetrivel et al., 2007) and affinity purified using an Ultralink Immobilization kit (Pierce, Rockford, IL). Rabbit polyclonal antisera PS1_{NT} and α -PS1Loop, and polyclonal antiserum raised against PEN-2 have been described previously (Thinakaran et al., 1998; Vetrivel et al., 2004). Monoclonal A β antibody was a generous gift from Dr. S. Newman, Smith Kline Beecham Pharm, UK. The following

antibodies were purchased from commercial sources: PS1 mAb (Affinity Bioreagents, Hornby, ON, Canada), polyclonal goat nicastrin antiserum (Santa Cruz Biotechnology Inc., Santa Cruz, CA); rabbit polyclonal A β -1 antibody (Zymed laboratories, San Francisco, CA); glial fibrillary acidic protein (GFAP) mAb, ED1 mAb (Serotec, Oxford, UK); GM130 mAb and N-Cadherin mAb (BD Transduction Laboratories, San Jose, CA); PSD-95 mAb (Upstate Biotech Inc., Charlottesville, VA), GAPDH mAb (Abcam Inc., Cambridge, MA), β -actin mAb and synaptophysin mAb (Sigma-Aldrich, Mississauga, ON, Canada). Donkey anti-goat Texas red, donkey anti-rabbit FITC and CY3-conjugated secondary antibodies were from Jackson ImmunoResearch (West Grove, PA), and Alexa 488- and 555-conjugated secondary antibodies were from Invitrogen (Carlsbad, CA).

3.2.5. Protein Analyses: Total protein lysates from post-mortem human brain tissue and mouse brain harvested during embryonic and postnatal developmental stages were prepared essentially as described previously (Lee et al., 1996). Brain regions of interest were dissected from six normal adult rats and four rats from each of the postnatal developmental stages P1, P7 and P21. Tissues were homogenized in ice-cold RIPA-lysis buffer [20 mM Tris HCl (pH 8), 150 mM NaCl, 0.1% SDS, 1 mM EDTA, 1% Igepal CA-630, 50 mM NaF, 1 mM NaVO₃, 10 μ g/ml leupeptin and 10 μ g/ml aprotinin]. Similarly, hippocampal regions from control (n = 4) and kainic acid-treated rats (4 animals from each time point) were dissected out and homogenized in RIPA-lysis buffer. Proteins were separated in

4-12% or 4-20% NuPAGE gels (Invitrogen, Burlington, Canada) and immunoblotted with 1:1000 dilution of p23 antibody or appropriate dilutions of the loading control and marker antibodies. For peptide competition, antibodies were preincubated with 40 $\mu\text{g/ml}$ of an unrelated peptide (control) or the p23 peptide immunogen at 4°C overnight prior to incubation with a blot containing N2a total protein lysate. N2a cells grown to subconfluency were lysed in 1% CHAPSO and used for co-immunoprecipitation as described previously (Chen et al., 2006). Polyclonal antibody *STC261* raised against stanniocalcin 2 (Ito et al., 2004) was used as negative control to establish specificity for the co-immunoprecipitation. For quantification, the immunoblots were developed by a chemiluminescence method and exposed to X-ray films for various lengths of time to ensure that the signals were not saturated. Optimal exposures were quantified using standard densitometry, and a calibration step tablet was used to convert raw optical densities to relative fold difference in signal intensity essentially as described (<http://rsb.info.nih.gov/ij/docs/examples/calibration/>) using Metamorph software (Molecular Devices Corporation, Downingtown, PA). Normalized signal intensities were compared between sporadic AD (mean age 64.13 ± 3.2) and FAD cases (mean age 38.5 ± 1) and their respective age-matched controls (older controls mean age 69.2 ± 5.1 and young controls mean age 35 ± 5 , respectively). The data are presented as mean \pm S.E.M, and statistical significance was analyzed by *t*-tests.

3.2.6. Immunohistochemistry: Eight normal adult rats and 16 rats from a kainic acid-treated experimental group (i.e., 4 saline-treated control and 4 rats from each

time-point) were deeply anesthetized prior to perfusion with PBS, pH 7.2 followed by 4% paraformaldehyde. Similarly, four postnatal rats from each stage of development (i.e., P1, P7 and P21) were anesthetized and perfused with PBS followed by 4% paraformaldehyde or Bouin's solution. After perfusion, brains were post-fixed overnight in the same fixative and then stored at 4°C in 30% PBS/sucrose. Human brain tissues were immersion fixed in formalin and then stored in 30% PBS/sucrose. Brain tissues were coronally sectioned (20 and 40 μ m) on a cryostat and then processed for either enzyme-linked immunoperoxidase or by a double immunofluorescence method as described earlier (Kodam et al., 2008). For the immunoperoxidase procedure, sections were washed with PBS, treated with 1% hydrogen peroxide for 30 minutes, treated with 10 mM sodium citrate buffer and autoclaved for 20 min. Sections were then incubated overnight with p23 antisera (1:1000), exposed to avidin-biotin reagents for 1 hr at room temperature and then developed using a glucose-oxidase-diaminobenzidine tetrahydrochloride-nickel enhancement method (Kodam et al., 2008). The specificity of the p23 antibody was determined by omission of the primary antibody, peptide competition (see above), or by incubating the sections with preimmune serum. Immunostained sections were examined under a Zeiss Axioskop-2 microscope and the photomicrographs were taken with a Nikon 200 digital camera and exported to the Adobe Photoshop 6.0 program for further processing. The rat brain atlas was used to define and name anatomical structures (Paxinos and Wats 1986).

3.2.7. Immunofluorescence labeling: HeLa cells cultured on poly-L-lysine coated glass coverslips were fixed with 4% paraformaldehyde in PBS [pH 7.4] for 20 min and permeabilized using PBS containing 0.5% SDS, 5% β -mercaptoethanol and 10% fetal bovine serum as described (Blum et al., 1999). Neuronal cultures were fixed and permeabilized as above for 15 min. Cells on coverslips were blocked with PBS containing 3% BSA and incubated for overnight at 4°C with affinity-purified p23 antibody (1:500) and anti-GM130 antibody (1:1000) diluted in PBS containing 0.2% Tween-20 and 3% BSA, followed by incubation with Alexa 488- and 555-conjugated secondary antibodies (1:500). Images were acquired as 200 nm z-stacks on a motorized Nikon TE2000 microscope with Cascade II:512 CCD camera (Photometrics, Tucson, AZ) using 100X 1.45 NA Plan-Apochromat oil immersion objective. Image stacks were deconvolved using Huygens software (Scientific Volume Imaging BV, The Netherlands) and processed using Metamorph software (Molecular Devices Corporation, Downingtown, PA). Confocal images of neuronal cultures were acquired with a 100X 1.45 NA Plan-Apochromat oil immersion objective on a Zeiss laser-scanning microscope (Pascal 5) and processed as above.

Adult rat brain sections (20 μ m) were incubated overnight at room temperature with p23 antiserum (1:300) in combination with either goat anti-nicastrin (1:50) or mouse anti-PS1 antibodies. In the case of kainic acid-treated animals, sections were incubated with p23 antiserum along with either anti-GFAP (1:500) or anti-ED1 (1:50) antibodies. Human AD brain sections were treated with 87% formic acid for 7 min prior to incubation with p23 antiserum and A β antibody (1:500).

Sections were incubated with Texas Red-, FITC-, or Cy3-conjugated secondary antibodies (1:200) and mounted using Vectashield mounting medium (Vector Laboratories, Burlingame, CA). Sections were examined under a Zeiss Axioskop-2 fluorescence microscope and the photomicrographs were taken with a Nikon 200 digital camera and exported to the Adobe Photoshop for further processing.

3.3. Results

3.3.1. *Immunoblot analysis of p23 expression in neuroblastoma cells and rat*

brain: Recent studies indicate that p23 functions as a negative modulator of A β production in addition to its documented role in regulating vesicular transport between the ER and the Golgi apparatus (Chen et al., 2006; Vetrivel et al., 2007). Since the localization of p23 in the central nervous system has never been examined, we set out to characterize the expression and distribution of p23 in rat and mouse as well as human brains using immunoblotting and immunohistochemical studies. We first affinity purified a rabbit polyclonal antiserum raised against a synthetic peptide corresponding to the N-terminal luminal residues 32-57 of mouse p23 (Vetrivel et al., 2007). To demonstrate the specificity of the purified antibody, we performed peptide competition studies using a non-specific synthetic peptide (control) or p23 peptide immunogen (described above). As expected, p23 antibody preincubated with the control peptide reacted with a single protein with molecular size of 23 kDa; this reactivity was completely eliminated when the antibody was preincubated with the p23 peptide immunogen (Fig. 3.1A). Previously it was reported that p23 was co-isolated with γ -secretase complex subunits (Chen et al., 2006). In accordance with this study, endogenous γ -secretase complexes co-immunoprecipitated using antibodies against PS1 from 1% CHAPSO lysates of N2a cells which contained small amounts of endogenous p23 in addition to the four core subunits (PS1-derived N- and C-terminal fragments, mature nicastrin, PEN-2 and APH-1) (Fig. 3.1B). Then, we characterized the specificity of the p23 antibody and its ability to recognize endogenous p23 in different regions of the adult rat brain using

immunoblotting. As shown in Fig 3.1C, the antiserum recognized a single major protein with an apparent molecular weight of 23 kDa, corresponding to p23. The p23 protein was expressed in all major regions of the adult rat brain including the septum, striatum, cortex, hippocampus, amygdala, thalamus, hypothalamus, cerebellum and brainstem (Fig. 3.1C).

3.3.2. Subcellular localization of p23: In cultured non-neuronal cells, p23 predominantly resides in cis-Golgi cisternae and adjacent tubulovesicular membranes (Blum et al., 1999; Rojo et al., 1997). In agreement with these previous findings, the p23 antibody employed in our investigation stained the Golgi apparatus in HeLa cells, where it colocalizes with the Golgi-resident enzyme N-acetylgalactosaminyltransferase-2 (Fig. 3.2A). In addition to the predominant Golgi localization, p23 staining of small vesicles is also observed. Next, we examined the localization of p23 in primary mouse cortical neuronal cultures. Consistent with p23 localization in non-neuronal cells, we observed perinuclear staining for p23 mainly in the cell body of neurons. Double immunofluorescence staining with the cis-Golgi marker, GM130 revealed co-localization of p23 with GM130 in cultured mouse cortical neurons (Fig. 3.2B). Similarly, in cultured astrocytes p23 staining was found in the Golgi apparatus where it co-localized with GM130 (Fig. 3.2C). These findings indicate that the main function of p23 in neuronal cells may be similar to that of non-neuronal cells, i.e., the regulation of biosynthetic protein transport.

3.3.3. p23 immunoreactivity in the adult rat brain: Having established the specificity of the p23 antibody, we analyzed the localization of p23 in the brain using frozen sections prepared from the adult rat brain. Results showed that p23 immunoreactivity is widely distributed throughout the adult rat brain including the basal forebrain, basal ganglia, cerebral cortex, hippocampus, thalamus, hypothalamus, cerebellum, and brainstem (Figs. 3.3 and 3.4). We observed region-specific differences in p23 immunoreactivity, which is evident mostly in neurons and fibers but not in glial cells. The specificity of the p23 immunostaining was further established by using preimmune serum, which failed to show specific staining in any given region of the brain (Fig. 3.4F). In the following sections, we describe the overall distribution profile of p23 immunoreactivity observed in specific regions of the brain.

Basal forebrain and basal ganglia: p23 immunoreactivity was evident in all sub-regions of the basal forebrain including the septum, diagonal band complex and nucleus basalis of Meynert. In septal nuclei, several multipolar cells were moderately labeled, whereas in the diagonal band complex, some intensely labeled neurons were found along with some weakly labeled neurons (Fig. 3.3A). A number of rather moderately labeled neurons were also encountered in the bed nucleus of the stria terminalis, nucleus basalis of Meynert, globus pallidus, entopeduncular nucleus and ventral pallidum (Fig. 3.3B). A subset of p23-positive multipolar neurons, located between unstained myelinated fascicles was found scattered throughout the caudate putamen (Fig. 3.3C).

Cerebral cortex: p23 immunoreactive neurons were detected in most layers of the neocortex with varying degrees of staining intensity, i.e., labeling was high in layers II-VI and relatively low in layer I (Figs. 3.3D and 3.E). The laminar distribution of p23 labeled neurons was most striking in the cingulate cortex and the frontoparietal cortex than in other cortical regions. A number of moderately stained smaller multipolar neurons intermingled with strongly labeled neurons were visible in layers II-III, whereas most of the pyramidal neurons located in layers IV and V were intensely labeled with vertically oriented apical dendrites (Fig. 3.3E). Some scattered multipolar neurons with moderate somatodendritic labeling were evident in layer VI of the cortex. In the piriform cortex, intensely labeled neurons were apparent in both the pyramidal and polymorphic layers, intermingled with some weakly labeled neurons.

Hippocampus: The hippocampal formation showed some of the most intense and abundant p23 immunoreactivity in the brain (Fig. 3.3F). Strong labeling was apparent in the CA1-CA3 pyramidal cell layer located within the Ammon's horn. Virtually all pyramidal neurons and their apical dendrites, which were often seen extending into the adjacent *stratum radiatum* layer, displayed intense p23 labeling (Fig. 3.3G). Outside the pyramidal layer, a few medium-sized, multipolar or fusiform p23-immunoreactive neurons were scattered throughout the *strata oriens* and *stratum radiatum* but not in *lacunosum moleculare*. Within the dentate gyrus, granule cell somata were strongly labeled. Large, polymorphic, heavily stained neurons were also present in the hilus (Fig. 3.3H), whereas very little p23 immunoreactivity was observed in the adjacent molecular layer.

Hypothalamus and Thalamus: A rather strong p23 neuronal labeling was observed in the supraoptic and paraventricular hypothalamic nuclei (Fig. 3.3I and 3.3J), whereas neurons located in the ventromedial nucleus, dorsolateral hypothalamic areas and arcuate nucleus exhibited moderate labeling. A number of medium-sized p23-immunostained neurons were observed throughout the thalamus, particularly in ventral and lateral portions (Fig. 3.4A) and in the habenular nucleus.

Amygdala: A number of moderately labeled p23-immunoreactive cell bodies were evident in the cortical, medial and basolateral amygdaloid nuclei (Fig. 3.4B). Some multipolar cells exhibiting rather weak immunoreactivity were apparent in the anterior amygdaloid area and central amygdaloid nucleus. In the amygdaloid nuclei, p23 immunoreactivity was found predominantly associated with cell bodies (see Fig. 3.4B inset).

Midbrain: Moderate p23 labeling was observed in the cell bodies located in the superficial gray layers of the superior colliculus and in the central gray matter. The substantia nigra pars reticulata was characterized by multipolar neurons with moderate to weak p23 immunoreactivity. Neurons of the red and oculomotor nuclei displayed rather intense immunoreactivity. Moderately labeled p23 positive neurons were also apparent in the intermediate gray layer of superior colliculus and mesencephalic trigeminal nucleus.

Brainstem: Strong p23 immunoreactivity was apparent at all levels of the

brainstem. A number of multipolar p23-immunoreactive neurons were encountered in the pontine reticular nucleus, inferior colliculus, abducens nucleus and reticulotegmental nucleus of pons (Fig. 3.4C). Intense labeling was also evident in the motor trigeminal nucleus, pontine nucleus and vestibular as well as facial nuclei (Fig. 3.4D). Additionally, a population of large multipolar process-bearing neurons was encountered in the dorsal raphe nucleus and in dorsal as well as ventral parts of the cochlear nucleus.

Cerebellum: Intense p23 immunoreactivity was evident throughout the cerebellum. In the cortex, Purkinje cells were heavily stained and often seen in continuity with their stained dendritic shafts extending into the molecular layer (Fig. 3.4E). The granule cells exhibited rather weak staining, whereas a number of deep cerebellar nuclei showed numerous moderate to weakly labeled p23-positive cell bodies and dendrites.

3.3.4. Co-expression of p23 with PS1 and nicastrin in the adult rat brain:

Recently we characterized the regional distribution of γ -secretase subunits nicastrin and PS1 in rodent brain and found that both proteins were co-expressed in select neuronal populations throughout the brain (Kodam et al., 2008). To further extend the findings described above, and provide a cellular basis of a functional interrelationship between p23 and the γ -secretase complex, we performed double immunofluorescence labeling of p23 with nicastrin or PS1 in sections from different brain regions. Our results clearly showed that p23 is co-expressed with nicastrin- and PS1-positive neurons in cerebral cortex (Figs. 3.5A-

3.5C, 3.6A-3.6C), hippocampus (Figs. 3.5D-3.5F, 3.6D-3.6F), brainstem motor nuclei (Figs. 3.5G-3.5I, 3.6G-3.6I) and cerebellar Purkinje cells (Figs. 3.5J-3.5L, 3.6J-3.6L). It is apparent from these results that p23 colocalizes with nicastrin and PS1 in neurons located in the aforementioned brain regions.

3.3.5. *p23 immunoreactivity in kainic acid-treated rat brain:* Earlier studies have shown that administration of kainic acid, an agonist for a subtype of ionotropic glutamate receptor, can induce extensive degeneration of hippocampal pyramidal neurons accompanied by hypertrophy of astrocytes and microglial cells (Nadler et al., 2008). To determine whether p23 immunoreactivity is altered following degeneration of neurons in the hippocampus, we evaluated the levels and cellular distribution of p23 following systemic administration of kainic acid into the adult rat. Our Western blotting data showed that steady-state levels of p23 in the hippocampus were not significantly altered at 12 h, 2 d or 12 d following kainic acid administration compared to controls (data not shown). However, at the cellular level we observed that expression of p23 is induced in the reactive glial cells in a time-dependent manner concomitant with a decrease in expression in the pyramidal neurons, which undergo degeneration following the administration of kainic acid (Fig. 3.7A-3.7F). Our double labeling experiments further revealed that expression of p23 was evident in GFAP-labeled reactive astrocytes 12 d after kainic acid treatment, but not detectible in ED1-positive microglia (Fig. 3.7G).

3.3.6. *p23 immunostaining in human AD brains:* We turned our attention to p23 expression in human brain and performed immunostaining of brain tissue from

patients with AD and age-matched controls. We found that p23 immunoreactivity is widely distributed throughout the frontal cortex, hippocampus and cerebellum of control and AD brains (Fig. 3.8A-3.8O). In control brains, p23 immunoreactivity was detected principally in neuronal cell bodies and their processes but not in glial cells. In the frontal cortex, p23-immunoreactive neurons were evident in all layers except layer I, but the staining intensity varied considerably between layers II-VI. A number of moderately stained multipolar neurons were visible in layers II-III, whereas pyramidal neurons with apical dendrites were strongly labeled in layers IV and V of the cortex. Layer VI was characterized by some scattered multipolar neurons with moderate somatodendritic labeling (Fig. 3.8A-3.8B). The hippocampal formation showed rather intense p23 immunoreactivity, primarily in neuronal soma and fibers. Within the Ammon's horn, strong labeling was apparent in the CA1-CA4 pyramidal cell layer, whereas some pyramidal neurons were found to extend their apical dendrites into the adjacent *stratum radiatum* layer. Within the dentate gyrus, granule cell soma exhibited rather weak labeling. Large, intensely labeled polymorphic neurons were present in the hilus, whereas little or no p23 immunoreactivity was evident in the molecular layer (Fig. 3.8E, 3.8F, 3.8H). In the cerebellum, p23 immunoreactivity was evident primarily in Purkinje cells and their dendritic shafts, which often extended into the adjacent molecular layer (Fig. 3.8J). The granule cells and some scattered cell bodies in the molecular layer of the cerebellum exhibited moderate p23 staining.

In AD brains, a number of A β -containing neuritic plaques were evident in both the cortex and hippocampus (Fig. 3.8L and 3.8N). As observed in control brains, p23 immunoreactivity was evident in neurons of the frontal cortex (Fig. 3.8C, 3.8D), hippocampus (Fig. 3.8G, 3.8I) and cerebellum (Fig. 3.8K) of the AD brain. No striking difference in the p23 immunoreactivity was apparent in the cerebellum of the AD brains compared to age-matched controls. However, the labeling of surviving pyramidal neurons in the frontal cortex and hippocampal regions of the AD brains appeared to be less intense than in control brains (Fig. 3.8F, 3.8G). We performed double labeling experiments using p23 and A β antibodies, and found that p23 labeling was mainly associated with neuronal cell bodies, and only occasionally overlapped with A β staining in neuritic plaques in the frontal cortex and hippocampal regions (Fig. 3.8L-3.8O).

To quantitatively assess the levels of p23 in brains of patients with AD and age-matched controls, we performed Western blot analysis of brain homogenates using antibodies against p23 and γ -secretase subunits nicastrin and PS1. Brain cortical tissue from eight sporadic AD cases, four FAD cases from two Belgian AD pedigrees with mutations in the *PSEN1* gene [I143T or G384A substitution; (Dermaut et al., 2002)] and seven age-matched controls were examined. In all brain extracts examined, p23 antiserum reacted with a ~23 kDa polypeptide. The blots were sequentially reprobed with nicastrin, PS1 and GAPDH antibodies. Quantification of immunoblot signals (normalized to GAPDH levels, which serves as the loading control) revealed a significant decrease in the levels of p23

between age-matched controls and AD cases, whereas the levels of γ -secretase subunits nicastrin and PS1 (not shown) remained unchanged between control and AD cases (Table 1). When p23 signals were normalized to that of nicastrin, it was apparent that there was a modest, but significant difference in the levels of p23 between SAD cases relative to age-matched older controls ($35.5 \pm 15.4\%$ decrease in SAD cases; $p < 0.05$). Similarly, when FAD cases were compared to young age-matched controls, there was a significant decrease in the levels of p23 relative to nicastrin ($65.8 \pm 18.9\%$ decrease in FAD cases; $p < 0.03$) (Fig. 3.9C). Thus, p23 is expressed in neurons in human brain, and in the cortex there is a decrease in the steady-state levels of p23 relative to γ -secretase subunits in the brains of individuals afflicted with SAD and FAD.

3.3.7. Postnatal regulation of p23 expression in the brain: Subunits of the γ -secretase are expressed in embryonic brains, and intramembranous γ -secretase cleavage of substrates such as Notch receptors and deleted in colorectal cancer (DCC) is essential for signaling during neuronal development (Vetrivel et al., 2006). To determine the p23 distribution profile during postnatal development of the brain, we performed immunocytochemical analyses of the cortex, hippocampus, cerebellum and brainstem obtained from P1, P7, P21 and adult brains. At the cellular level, we found that p23 immunoreactivity was widely distributed at all stages of the postnatal developing brains. However, at the early stages of development p23 immunoreactivity was restricted primarily to neuronal cell bodies, whereas at later stages (i.e., P21 and adult) immunoreactivity was

apparent not only in the cell bodies but also in dendrites/neuropil of all brain regions (Fig. 3.10A-3.10C). Moreover, we noted that the intensity of p23 labeling decreased gradually during the course of postnatal development.

To determine whether p23 expression is regulated during postnatal development, we performed immunoblotting of mouse brain homogenates prepared at different developmental stages. We found high levels of p23 expression in embryonic mouse brain, but the steady state p23 levels gradually declined after birth to the lower adult level (Fig. 3.10D and 3.10E). In contrast, expression of the presynaptic protein synaptophysin and post-synaptic protein PSD-95 was very low in embryonic brain and increases markedly after birth. N-Cadherin expression was similar during embryonic and postnatal developmental stages and served as a loading control (Fig. 3.10D and 3.10E). The postnatal decrease in the steady-state level of p23 was further confirmed by immunoblot analysis of cortex and hippocampal homogenates of rat brain harvested at P1, P7 and P21 (data not shown). Understanding the functional significance of the decline in p23 expression to amyloid burden in the aging brain remains an active subject of our future investigations.

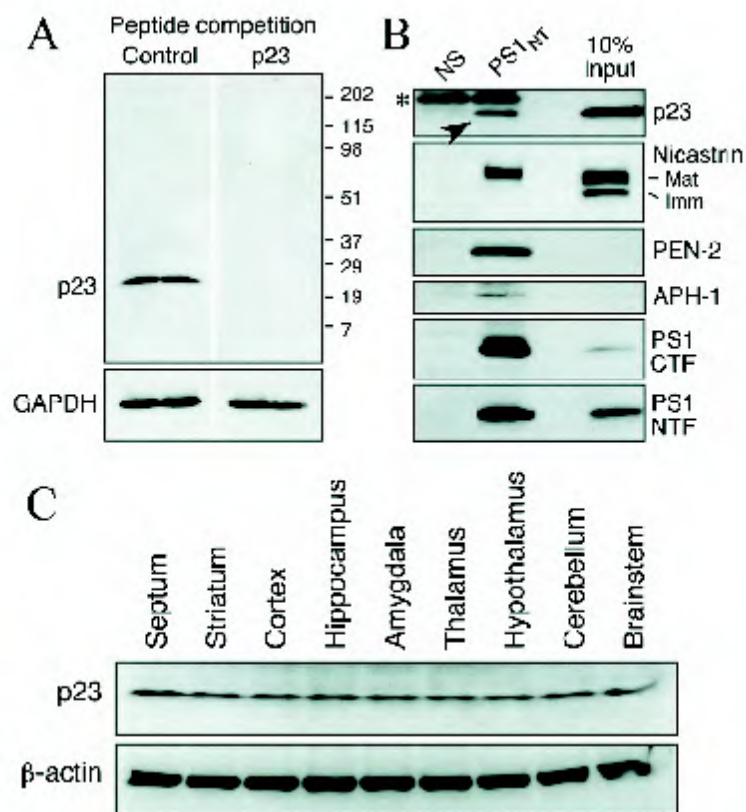


Fig. 3.1: Western blot analysis of p23 expression in adult brain. (A) Characterization of the p23 antibody. Western blot of total lysates from mouse N2a neuroblastoma cells probed with the p23 antibody pre-incubated with saturating concentrations of unrelated peptide (Control) or the p23 peptide immunogen (p23). (B) Co-immunoprecipitation of endogenous p23 with γ -secretase complex. N2a cells were lysed in a buffer containing 1% CHAPSO, and aliquots of lysates (300 μ g protein) were incubated with a non-specific polyclonal (NS) or PS1 antibody (PS1NT). The resulting immunoprecipitates and total lysates corresponding to 10% of the input were probed sequentially with antibodies against p23, nicastrin, PEN-2, APH-1, and PS1 (PS1NT and α -PS1Loop). The arrowhead indicates p23 co-immunoprecipitated by PS1 antibody. (C) Immunoblot analysis of p23 in different regions of the adult rat brain. The blot was reprobed with an antibody against β -actin as the loading control.

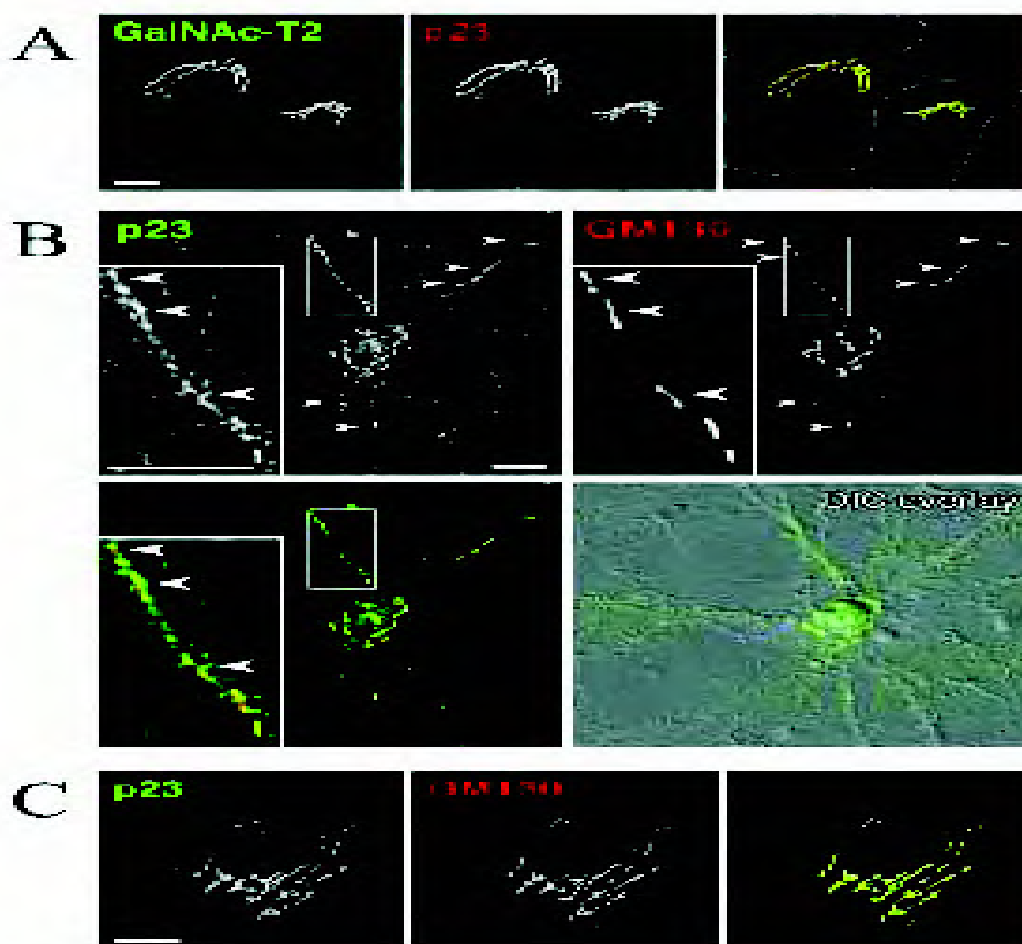


Fig. 3.2: Immunofluorescence localization of endogenous p23 in HeLa cells, cortical neurons and astrocytes. (A) HeLa cells stably expressing GFP-tagged N-acetylgalactosaminyltransferase-2 were analyzed by immunofluorescence staining with p23 antibody. (B) Primary cortical neurons were stained with p23 antibody and a mAb against the cis-Golgi marker GM130. Inserts show higher magnification of the dendritic area indicated by the box. p23 co-localizes with GM130 in the cell body and along the dendrites. Arrowheads point to p23 and GM130 co-localization in dendritic Golgi "outposts" (Horton et al., 2005). (C) Predominant Golgi localization of p23 in cultured astrocytes. Scale bar = 10 μ m

Figure. 3.3

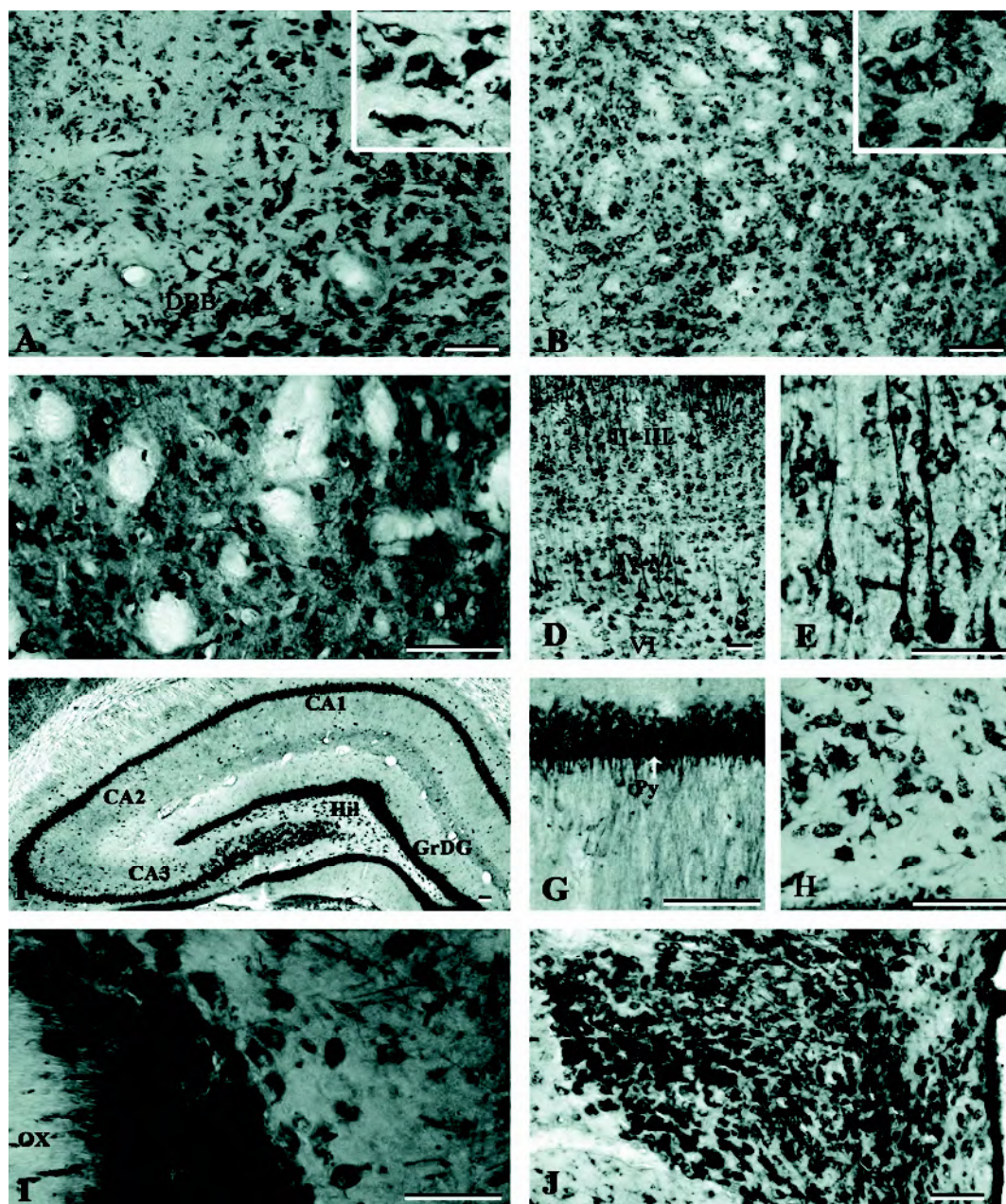


Fig. 3.3: Photomicrographs of coronal sections of adult rat brain showing the distribution of p23-immunoreactive neurons and fibers in the diagonal band of Broca (A), globus pallidus (B), striatum (C), cortex (D, E), hippocampus (F), CA1 pyramidal cell layer of the Ammon's horn (G), polymorphic neurons of the hilus (H) and supraoptic (I) and paraventricular nuclei (J) of the hypothalamus. Note that neurons of the diagonal band of Broca, globus pallidus and striatum are moderately labeled, whereas the hippocampal pyramidal cell layer and supraoptic as well as paraventricular nuclei exhibit rather strong immunoreactivity. The staining intensity in the neocortex is variable: intense in layers IV-V, moderate in layers II-III and VI and relatively low in layer I. Inset in (A) and (B) show neuronal labeling at higher magnification. DBB, diagonal band of Broca; Hil, hilus; GrDG, granular cell layer of the dentate gyrus; Py, pyramidal cell layer; OX, optic chiasma. Scale bar = 50 μ m.

Figure. 3.4

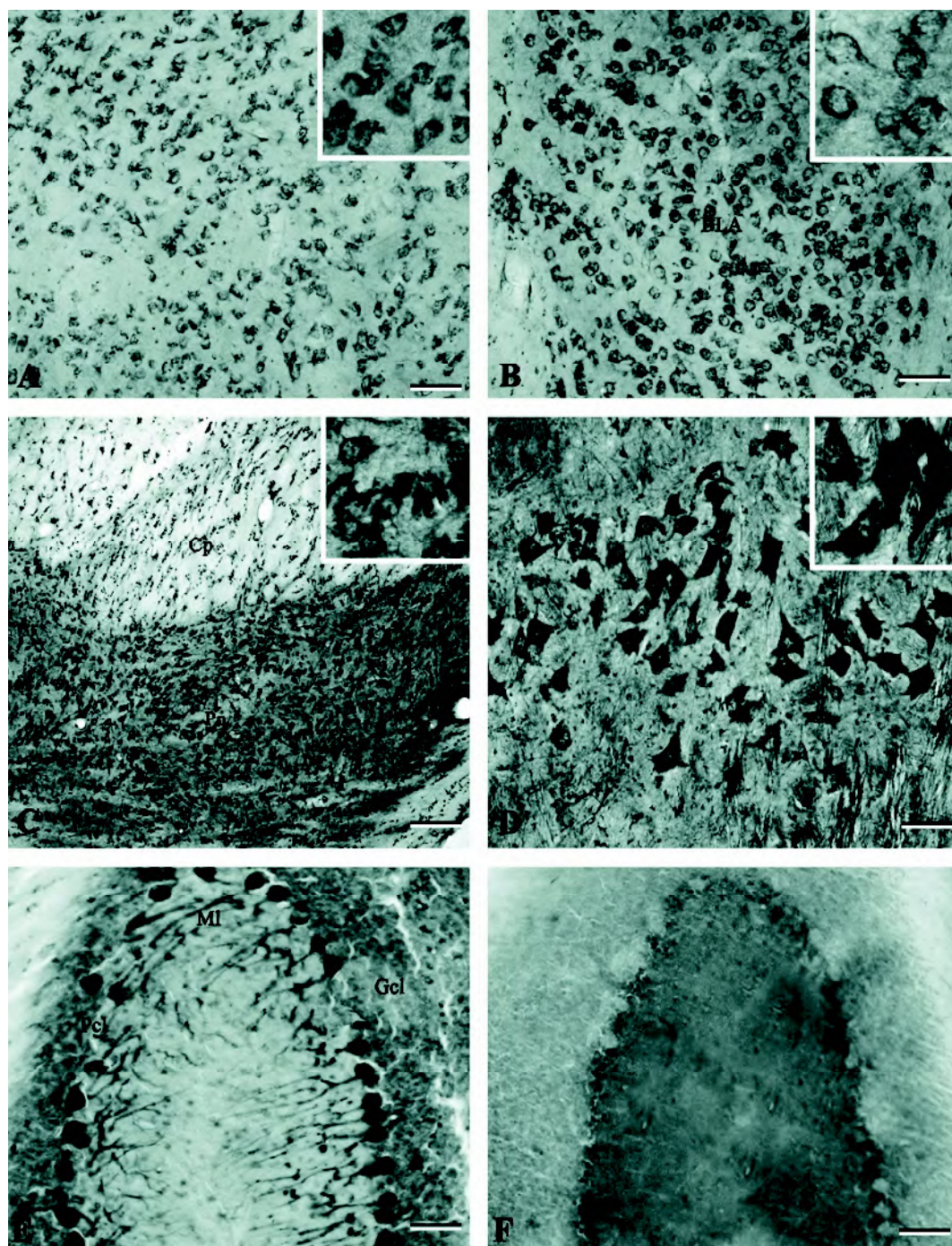


Fig 3.4: Photomicrographs of coronal sections of adult rat brain showing the distribution of p23-immunoreactive neurons and fibers in the medial thalamic nucleus (A), amygdaloid nucleus (B), pontine nucleus (C), facial nucleus of the brainstem (D) and cerebellum (E). Note intense labeling of the brainstem neurons and the Purkinje cells of the cerebellum. F, represents a cerebellar section processed using the preimmune serum. Insets in (A-D) show neuronal labeling at higher magnification. BLA, basolateral amygdale; Cp, cerebral peduncle; Pn, pontine nuclei; Gcl, granular cell layer; Pcl, Purkinje cell layer; Ml, molecular layer. Scale bar = 50 μ m.

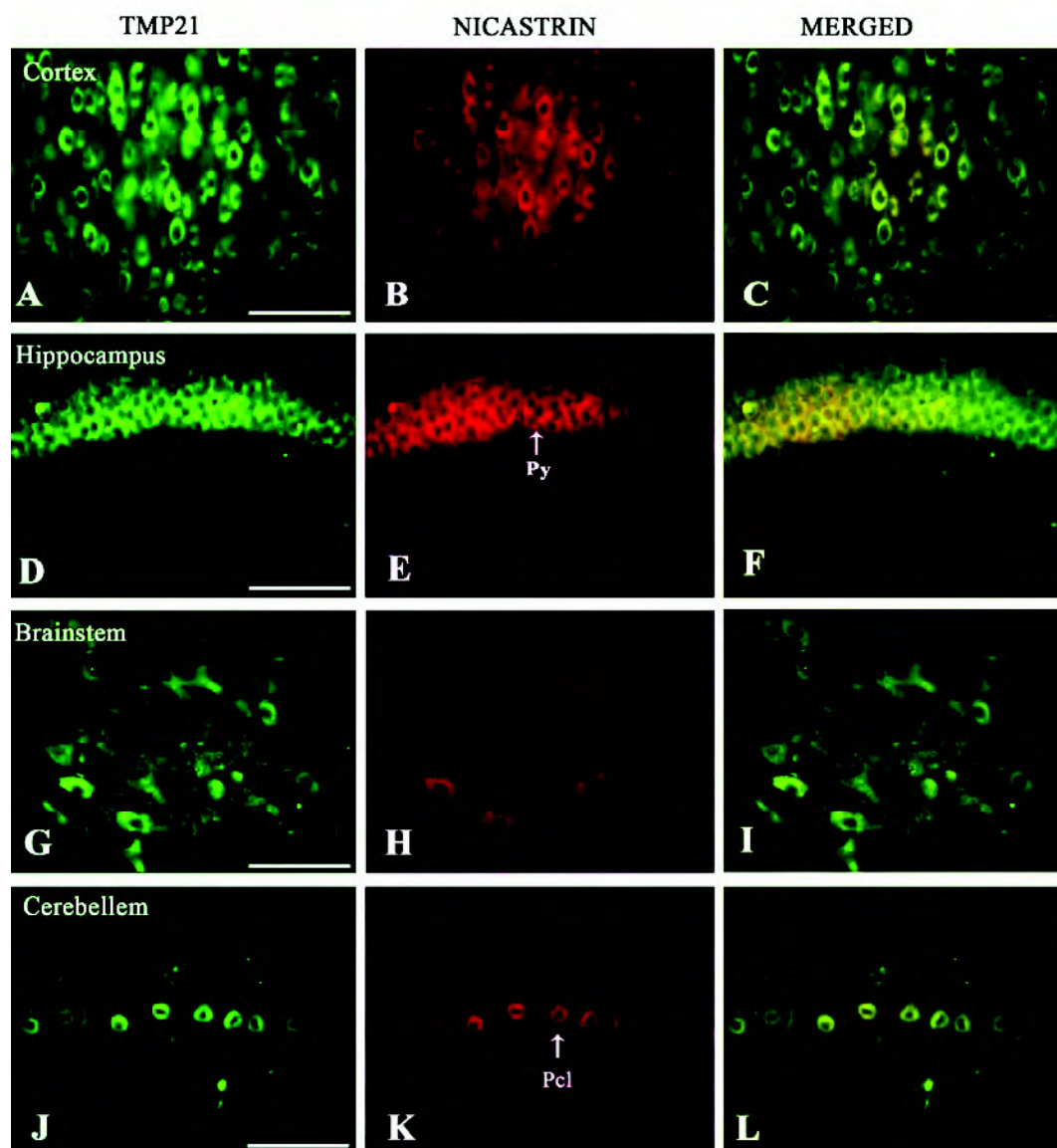


Fig. 3.5: Double immunofluorescence analysis of p23 (A, D, G, J) and nicastrin (B, E, H, K) staining in coronal sections of the adult rat brain cortex (A-C), hippocampus (D-F), brainstem (G-I) and cerebellum (J-L). Note the widespread coexpression of p23 with nicastrin in all neurons located in the cortex, hippocampal pyramidal cell layer (Py), brainstem motoneurons, and cerebellar Purkinje cells (Pcl). Scale bar = 50 μ m.

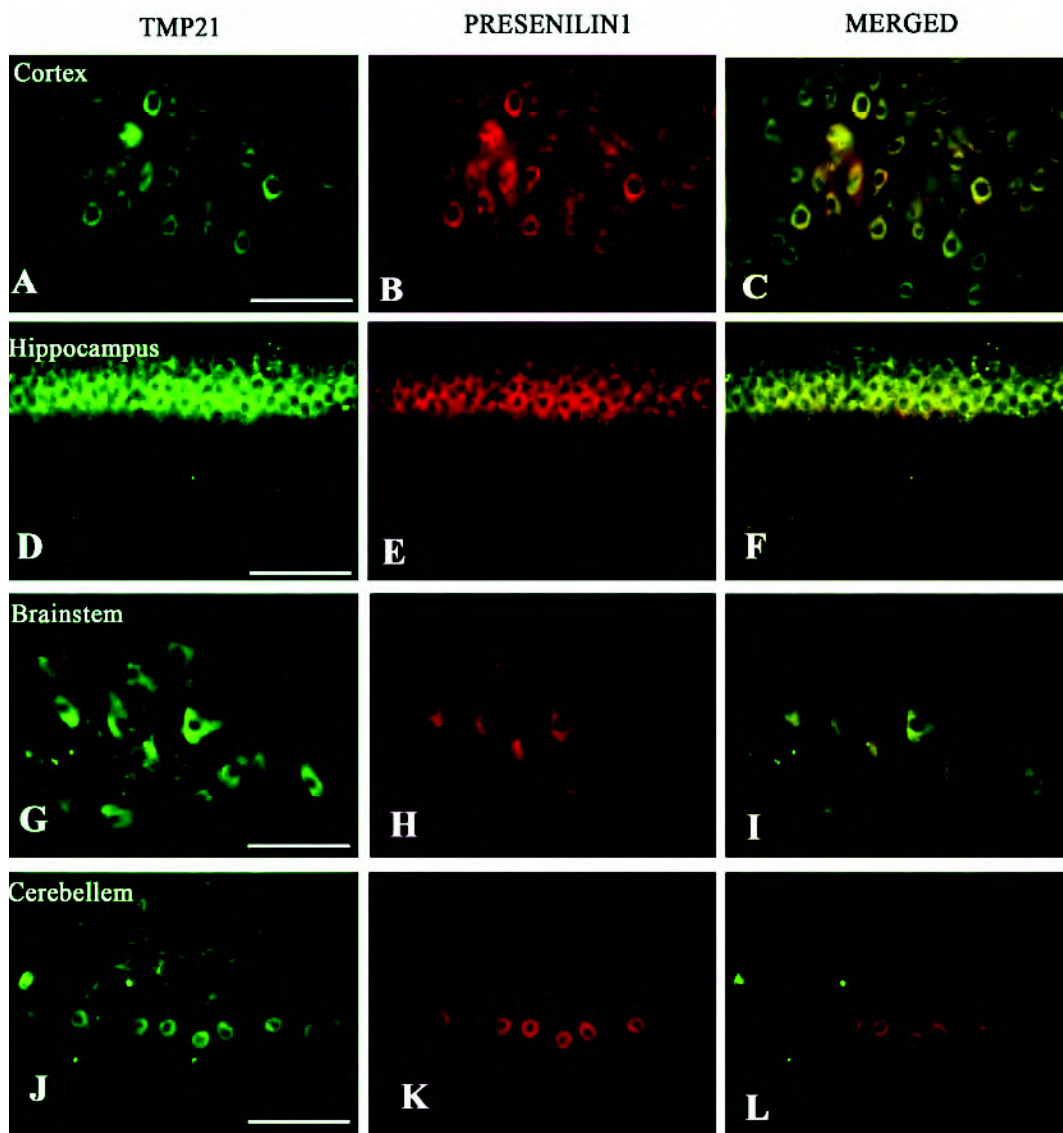


Fig. 3.6: Double immunofluorescence analysis of p23 (A, D, G, J) and PS1 (B, E, H, K) staining in adult rat brain cortex (A-C), hippocampus (D-F), brainstem (G-I) and cerebellum (J-L). Note the widespread coexpression of p23 with PS1 in all neurons located in the cortex, hippocampal pyramidal cell layer (Py), brainstem motoneurons, and cerebellar Purkinje cells (Pcl). Scale bar = 50 μ m.

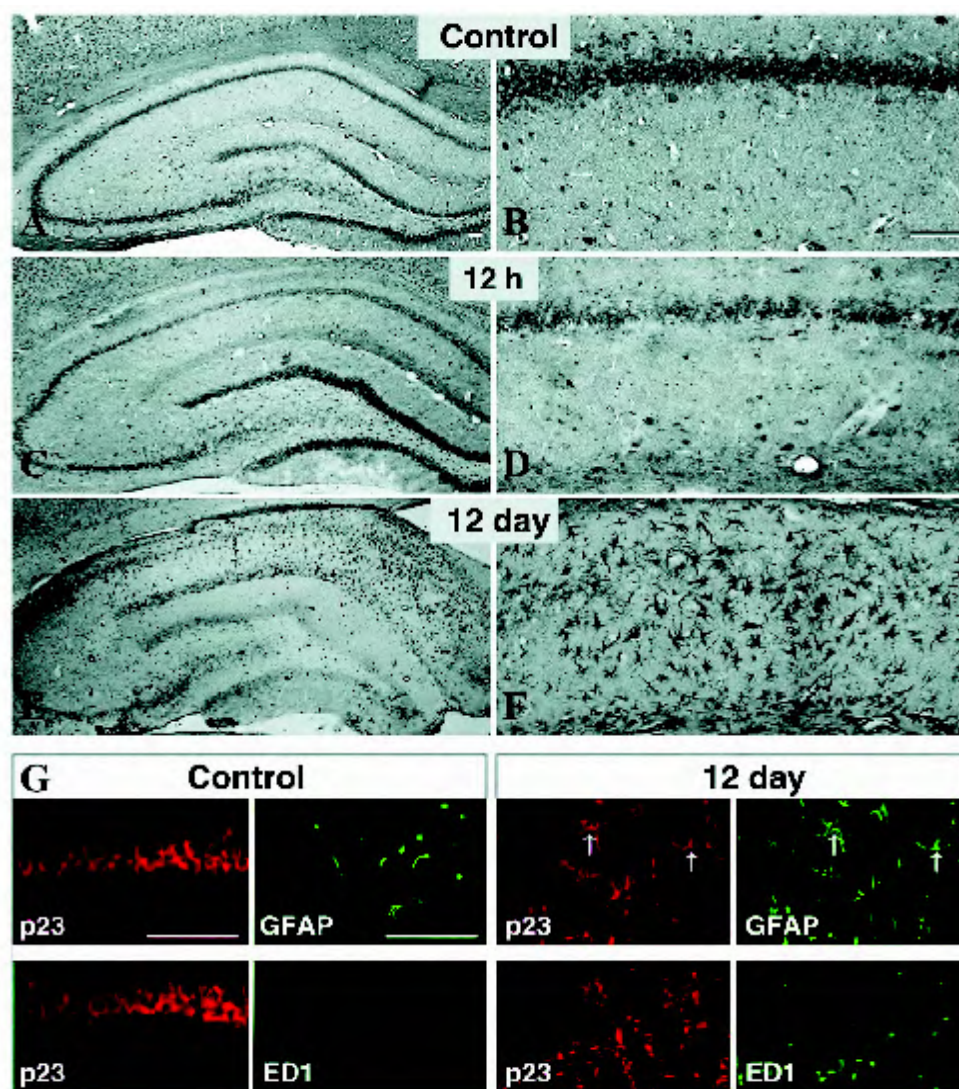


Fig. 3.7: p23 expression in the brain following kainic acid injury. (A-F) The distribution of p23 immunoreactivity in the hippocampal formation (A, C, E) and in the corresponding CA1 sub-field (B, D, F) of control and kainic acid-treated rats analyzed 12 h or 12 day following treatment. Note the time-dependent decrease in neuronal and increase in glial p23 immunoreactivity following the administration of kainic acid. (G) Double immunofluorescence analysis of p23 expression in GFAP-labeled astrocytes and ED1-positive microglia in rat hippocampus. Note that p23 staining is not evident in control brain astrocytes but is highly expressed in reactive astrocytes 12 days following kainic acid administration. p23 staining is undetectable in ED1-positive microglia in control and kainic acid-treated animals. Scale bar = 50 μ m.

Figure. 3.8

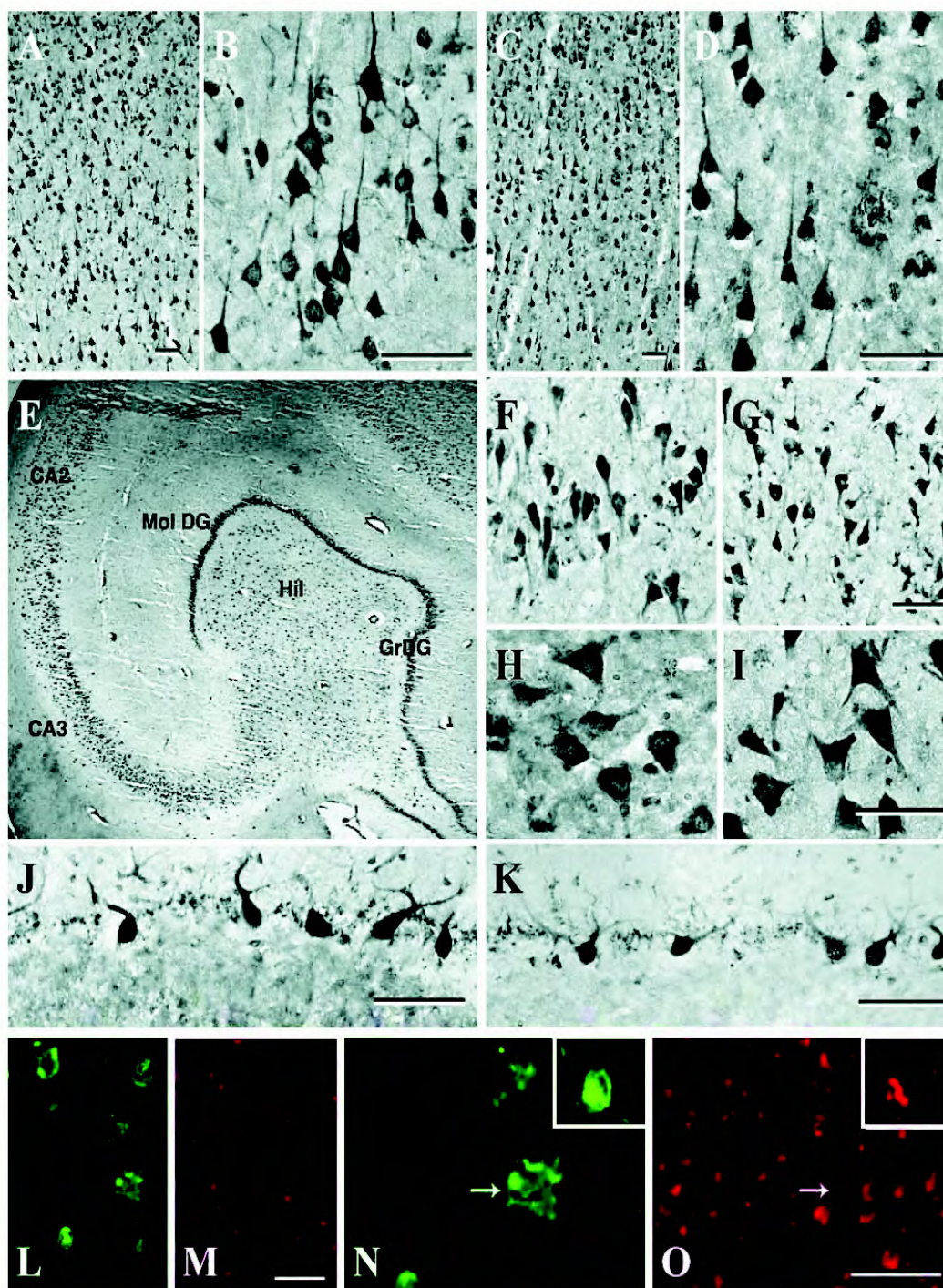


Fig. 3.8: The distribution of p23 immunoreactivity in human brain. Neurons in the frontal cortex (A-D), as well as hippocampal neurons in the pyramidal cell layer (E, F, G), granule cell layer (E) and in the hilus (E, H, I) are strongly labeled, whereas cerebellar Purkinje cells (J, K) are moderately labeled. No marked alteration in the profile of p23 expression was evident in the AD brains (C, D, G, I, K) compared to age-matched controls (A, B, E, F, H, J). (L-O) Double immunofluorescence analysis of A β -positive neuritic plaques (L, N) and p23 expression (M, O) in the frontal cortex of AD brain. Note the lack of overlap between p23 staining and neuritic plaques, except in rare instances (insert). GrDG, granule cell layer of the dentate gyrus; Mol Dg, molecular layer of the dentate gyrus Hil, hilus. Scale bar = 50 μ m.

Figure.3.9

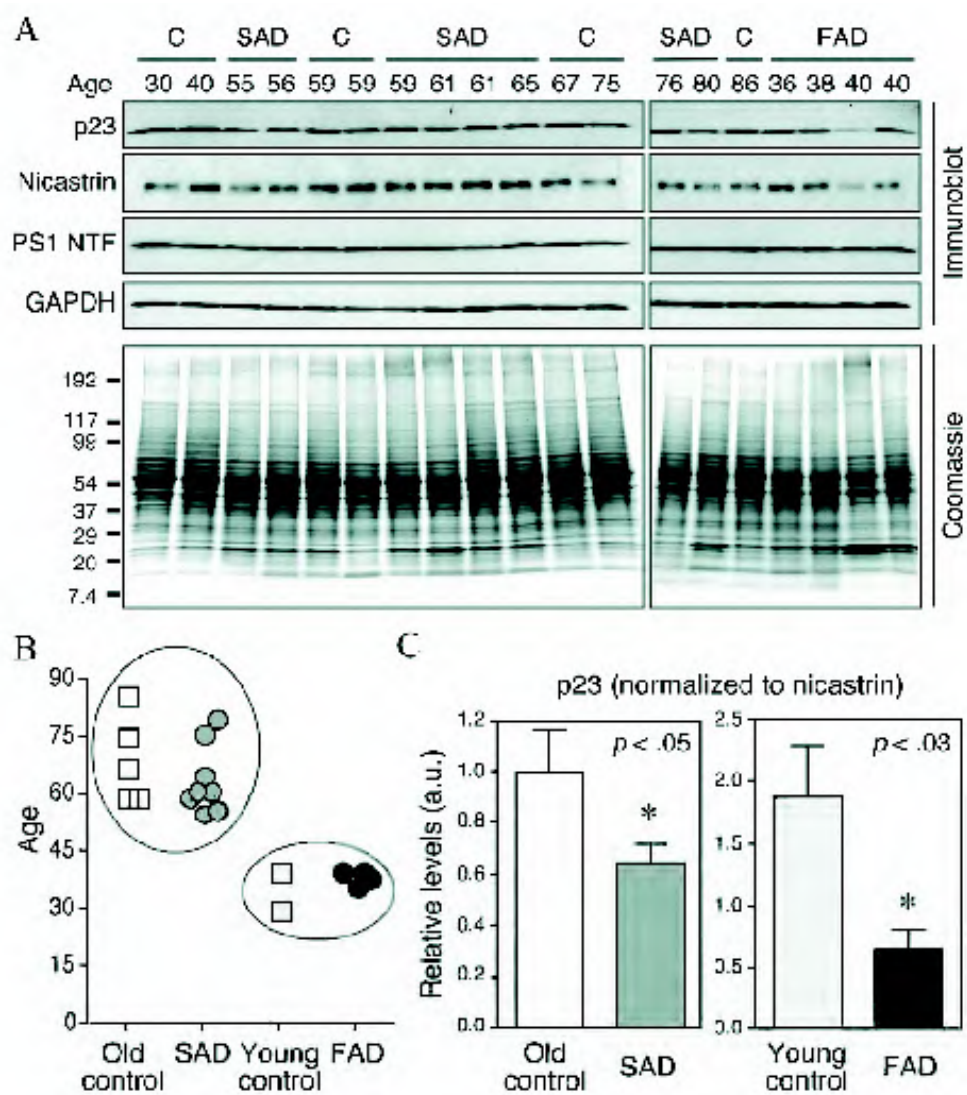


Fig. 3.9: Immunoblot analysis of p23 expression in human cortical brain extracts. (A) Human brain tissue was homogenized in SDS extraction buffer and aliquots of total homogenates were fractionated by SDS-PAGE. The blots were sequentially probed with antibodies against p23, nicastrin, PS1 NTF, and GAPDH. Coomassie stained gels containing aliquots of homogenates is shown in the bottom panel. C, control brains, free of neurological diseases; SAD, sporadic AD brains; FAD, familial early-onset AD patients harboring PSEN1 mutations. (B) The age at death of all individuals was plotted and also indicated on top of each lane in the gel in panel A. The circles enclose SAD patients (mean age 64.13 ± 3.2) and age-matched old controls (mean age 69.2 ± 5.1), as well as FAD patients (mean age 38.5 ± 1) and young controls (mean age 35 ± 5). (C) Signal intensities of p23 and nicastrin were quantified and the ratios of p23 to nicastrin signal intensity in SAD or FAD relative to their respective age-matched controls were plotted. Note that for easier comparison between SAD and FAD in these plots, all values were normalized to old controls.

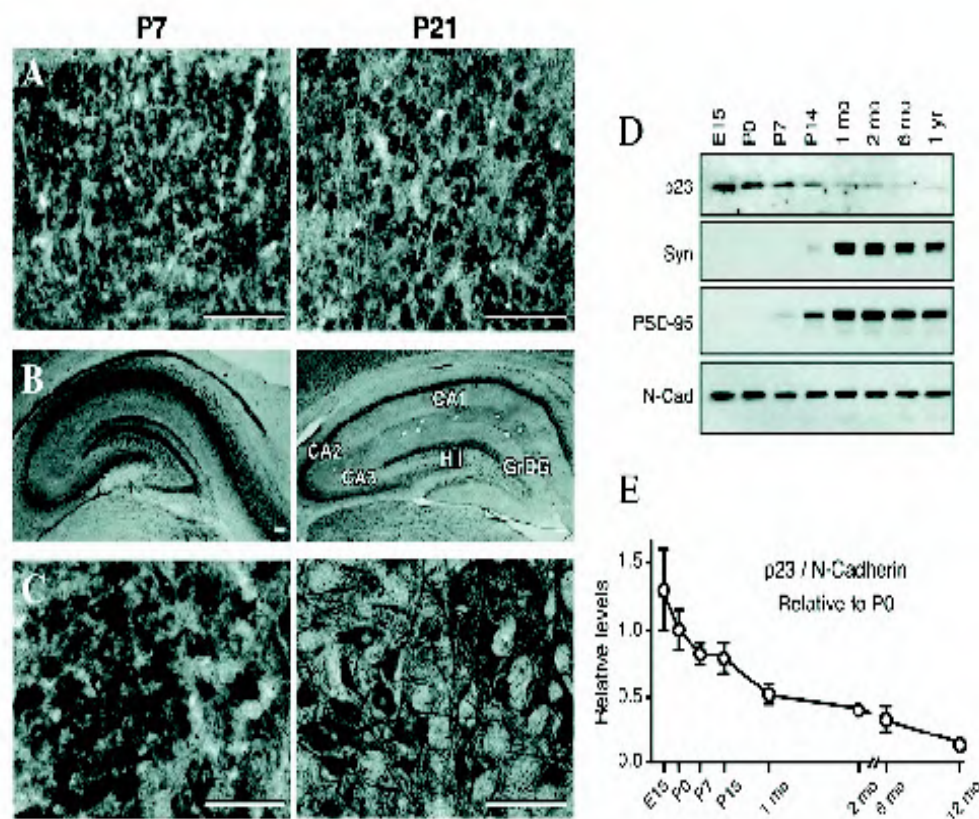


Fig. 3.10: Expression of p23 during postnatal development. Immunohistochemical staining showing the levels and expression of p23 in the cortex (A), hippocampus (B) and brainstem (C) of the postnatal rat brains at postnatal day 7 and 21. Note the relatively stronger p23 staining of neuronal cell bodies at P7 (left panels in A-C), and readily apparent staining of neuronal processes in P21 postnatal brains (right panels in A-C). Hil, hilus; GrDG, granular cell layer of the dentate gyrus. Scale bar = 50 μ m. (D and E) Immunoblot analysis of p23 expression during postnatal brain development. Mouse brains harvested at the indicated stages of embryonic and postnatal development and from adults were separated on SDS-PAGE and analyzed by immunoblotting. Note the high embryonic expression of p23 that gradually declines after birth. Signal intensities of p23 were quantified from analysis of three or more animals and normalized to N-cadherin levels. For comparison, the normalized expression level of each protein at embryonic stage P0 was set to 1 and the level of expression relative to P0 was plotted.

3.4. Discussion

The p24 family of proteins play an important role in vesicular trafficking in the early secretory pathway (Bethune et al., 2006). Homozygous deletion of p23 resulted in early embryonic lethality of mouse embryos, signifying the physiological importance of p23 protein during mammalian embryonic development (Denzel et al., 2000). The recent findings that p23 co-purifies with γ -secretase complex and modulates APP trafficking and A β production illustrate a potentially critical function of p23 in AD pathogenesis (Chen et al., 2006; Vetrivel et al., 2007). Despite the physiological significance of p23 function, knowledge is lacking on p23 expression in the central nervous system. The present study was undertaken to analyze cellular expression and distribution of p23 in rodent and human brain, and in this report we describe the first comprehensive analysis of cellular distribution as well as postnatal expression of p23 in the brain.

Mammalian p24 family proteins exist as monomers and dimers in the ER, Golgi apparatus, and the intermediate compartments (Jenne et al., 2002). Therefore, it was surprising that p23 was identified as a γ -secretase complex component by affinity purification of PS1-associated proteins (Chen et al., 2006). We independently confirmed endogenous p23 association with the PS1 complex by co-immunoprecipitation of endogenous γ -secretase subunits in mouse N2a neuroblastoma cells using an antibody raised against the N-terminal residues 1-65

of PS1 (Thinakaran et al., 1998). Notably, the abundance of p23 in the PS1 complex relative to the input in our experiments raises the possibility that p23 associates with only a subset of γ -secretase complexes (Fig. 1B). This is an important observation because previous siRNA knockdown studies indicate that p23 is a negative modulator of A β production (Chen et al., 2006; Vetrivel et al., 2007). Thus, it appears that A β production by p23 association with γ -secretase may be regulated by the abundance of “free” p23 that is not present as a heterodimer with other members of the p24 family.

Our Western blot analysis revealed that p23 is expressed in all major areas in rat brain. However, immunohistochemical analysis showed clear variation in the intensity of staining in different regions of the brain as well as labeling of individual cells. At the cellular level, there is more intense staining in neuronal populations than in glial cells. Strong staining was evident in hippocampus, neocortex and, more importantly, pyramidal neuronal cell populations. Some of these regions are most vulnerable to pathological changes associated with AD. Co-localization of p23 with PS1 and nicastrin, two core components of γ -secretase, in neuronal populations of the cortex and hippocampus indicate that a subset of γ -secretase complexes in these neurons could possibly have p23 associated with them. Similarly, the p23 immunostaining pattern in human brain matched that of rat brain and showed intense staining in hippocampal neuronal populations. Immunoblot analysis of p23 expression showed reduced signals for p23 in brain tissue from sporadic AD and FAD brains bearing *PSEN1* I143T and

G384A mutations, relative to respective old and young age-matched controls, respectively (Fig. 9 and Table 1). The observation that the levels of p23 are significantly reduced relative to γ -secretase subunits in AD tissue suggests that this decrease in p23 levels may have functional importance in the modulation of APP processing by the γ -secretase similar to what has been shown in cultured cell lines (Chen et al., 2006; Vetrivel et al., 2007). Thus, we suggest that the balance between the levels of p23 and γ -secretase is one of the factors that influences the extent of amyloid burden in the brains of individuals with AD.

Immunofluorescence analysis of primary neuronal cultures revealed predominant localization of p23 with the Golgi marker GM130 in neurons (Fig. 2). Restricted localization of p23 to Golgi membranes is expected based on previous studies on p23 in non-neuronal cells such as HeLa (Blum et al., 1999). Interestingly, p23 immunoreactivity is prominently observed in neuronal soma as well as apical dendrites of pyramidal neurons in the cortex and hippocampus, and the dendrites of cerebellar Purkinje neurons (Figs. 3 and 4). Based on the co-localization of p23 with GM130 in the dendrites of primary cortical neurons, the dendritic staining in brain tissue likely represents localization of p23 in Golgi outposts that are known to partition into larger dendrites in neurons (Horton et al., 2005). As a major function for p23 is to regulate vesicular trafficking in the early secretory pathway, it remains to be determined whether p23-bound γ -secretase complexes are selectively targeted to Golgi outposts in the dendrites. Moreover, the lack of p23 staining in axons is noteworthy because APP C-terminal fragments containing the

entire A β sequence are transported in the fast component of anterograde transport of neurons in the central nervous system (Buxbaum et al., 1998) and are thought to be cleaved by γ -secretase at or *en route* to the neuronal terminals to release A β near synapses. Based on our findings, it is unlikely that the subset of γ -secretase in the axonal compartment can be negatively regulated by p23.

Previous studies suggest a general role for p23 in the recruitment of the small GTPase ADP-ribosylation factor-1 to the donor Golgi membrane during COP I-vesicle formation, which is a highly conserved process in eukaryotic cells (Gommel et al., 2001). Therefore it was surprising that p23 immunohistochemical staining was only weakly detected in astrocytes in rat or human brain sections. Nevertheless, we were able to observe strong p23 labeling in cultured astrocytes, where the staining co-localized with the Golgi marker GM130 (Fig. 2). Furthermore, kainic acid-induced neurodegeneration selectively elevated p23 expression in astrocytes but not in microglial cells in rat brain. It would be interesting to determine whether p23 function in the astrocytes is involved in neurodegeneration or protection against seizure-induced brain damage. It should be noted that this is only a preliminary observation and closer scrutiny on the mechanism of astrocytic upregulation of p23 awaits further detailed investigation in the future. Nevertheless, the results described above show that p23 is expressed in neuronal populations throughout the brain, and under certain conditions can be upregulated in astrocytes.

Finally, our studies also revealed remarkable postnatal developmental changes in p23 expression in the brain. We found that the steady-state level of p23 was high during the early stages of embryonic development, and then declined to the lower adult level a few months after birth. Notably, p23 expression markedly declined in the first few weeks after birth, at a time when presynaptic and post-synaptic proteins begin to express in the brain. Moreover, this age-dependent decrease in p23 expression is particularly interesting and significant with reference to A β burden in the brain because of the negative regulation of A β production by p23 (Chen et al., 2006; Vetrivel et al., 2007). As described above, successful interaction of p23 with γ -secretase depends on the abundance of p23 that is not complexed with other p24 family proteins. We suggest that the high abundance of p23 during the early stages of development likely allows p23 to negatively modulate A β production by association with the γ -secretase, thus keeping A β levels under tight control in young animals. In contrast, as the animals age, the decline in the steady-state level of p23 could markedly influence the availability of free p23 to interact with the γ -secretase complex, thus relieving p23-mediated negative control of A β production. We are in the process of testing the above hypothesis by generating p23 transgenic mice.

3.5. Acknowledgements

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CHAPTER – 4

Genereal Discussion

The present study clearly revealed that nicastrin, a component of the γ -secretase complex, and p23, a negative regulator of γ -secretase activity, are widely distributed throughout the brain, suggesting a critical role for the enzyme in normal functioning of the nervous system as well as in AD pathology. The key findings of my study are:

- i) The proteins nicastrin and p23 are mostly distributed in neurons of the basal forebrain, cortex, hippocampus, thalamus, hypothalamus, cerebellum and brainstem of the normal adult brain. They are colocalized with PS1 in all brain regions studied.
- ii) The levels/expression of nicastrin and p23 are relatively higher during early stages of brain development and then decline gradually to reach the adult profile.
- iii) In *in vitro* culture studies p23 coimmunoprecipitates with other components of the γ -secretase complex and is localized mostly in the Golgi complex both in neurons as well as astrocytes.
- iv) Kainic acid administration induces expression of p23 in reactive astrocytes,

but not in microglia, of the adult rat hippocampal formation.

- v) The steady-state level of p23 is significantly decreased in AD brains compared to age-matched controls. In parallel, p23 immunoreactivity appears to be less intense in the cortex and hippocampus but not in the cerebellum of the AD brains compared to the respective controls. Neuritic plaques located in the hippocampal and cortical regions of the AD brain are occasionally found to express p23 immunoreactivity.

The overall significance of the aforementioned findings was discussed rather extensively in the respective Chapters. The main focus of this section is to integrate these results in the context of normal brain function and in relation to AD pathology. Additionally, I will highlight future directions of work that may reveal further the significance of these proteins in the regulation of normal brain function and AD pathology.

4.1. The distribution of immunoreactive nicastrin and p23 in the brain: My results demonstrated that nicastrin and p23 are widely expressed throughout brain, including in basal forebrain, basal ganglia, cerebral cortex, amygdala, hippocampus, hypothalamus, thalamus, midbrain, cerebellum and brainstem. Interestingly, the profile of nicastrin distribution was found to be very similar to that of p23 in almost all regions of the brain including those which are affected (i.e., cortex and hippocampus) and unaffected (i.e., hypothalamus, thalamus and cerebellum) in AD pathology. For example, in cortex both components showed

moderate labeling in layer II-III, whereas cortical layers IV-V displayed rather intense immunoreactivity. Similarly, strong immunoreactive nicastrin and p23 were apparent in the CA1-CA3 pyramidal cells but not in the adjacent molecular layer of the hippocampus. In basal forebrain both components exhibited a rather moderate labeling. The overlapping pattern of staining was also apparent in the hypothalamus, thalamus and cerebellum. These results raise the possibility that the γ -secretase complex and its regulator are involved in functions other than AD pathogenesis.

It is of interest to note that nicastrin and p23 immunoreactivities in the adult rodent brain, under normal conditions, are confined mostly to neurons and are not apparent either in astrocytes or microglia. However, our *in vitro* studies revealed the presence of p23 in cultured astrocytes. Consistent with our *in vitro* data, some earlier studies have shown that nicastrin and PS1/PS2 transcripts are also expressed in cultured astrocytes (Lee et al., 1996; Confaloni et al., 2005). Although the underlying cause of the differential distribution of p23 in *in vitro* and *in vivo* paradigms remains to be determined, it is possible that cultured astrocytes, in contrast to those in the normal brain, are somewhat reactive. This notion is partly supported by our observation that astrocytes when activated following kainic acid administration are also found to express p23 (Vetrivel et al., 2008) as well nicastrin (Kodam et al., unpublished results). Some recent studies have further shown that reactive astrocytes can express both PS1 and nicastrin following traumatic brain injury (Nadler et al., 2008). However, the functional significance of the redistribution of p23 and components of γ -secretase into

reactive astrocytes in various experimental paradigms remains to be defined.

4.2. Subcellular localization of nicastrin and p23: Previous studies have shown that p23 in peripheral tissues is mostly associated with cis-Golgi cisternae and intermediate compartments (Sohn et al., 1996; Nickel et al., 1997; Rojo et al., 1997). Consistent with these results, we have demonstrated that p23 is localized in the cis-Golgi compartments of cultured neurons and astrocytes. These results suggest that p23 may possibly be involved in regulating protein trafficking within cells both in peripheral tissues as well as brain. Unlike p23, nicastrin is widely distributed in different subcellular compartments, including the peri-nuclear region, Golgi network, lysosomes, mitochondria, plasma membrane and, to a lesser extent, the ER, thus suggesting that it may have different roles in specific cellular sites (Yang et al., 2002; Hansson et al., 2004). This is partly supported by the evidence that nicastrin located in the plasma membrane, along with other components of the γ -secretase complex, may be involved in the generation of secreted $A\beta_{1-40}$ peptide, whereas its localization in the ER, Golgi and lysosomes may involve regulation of the production of intracellular $A\beta_{1-42}$ peptide (Morais et al., 2008). Apart from influencing $A\beta$ production, nicastrin localized in various subcellular compartments may be involved differentially in regulating assembly, trafficking and/or substrate recognition for the γ -secretase enzyme complex (Capell et al., 2003; Morais et al., 2003; Shah et al., 2005). Unlike nicastrin, very limited information is available on the significance of p23 localization in cis-Golgi compartments in relation to AD pathology. Some recent studies have

indicated that p23 may perform dual roles at the subcellular level: i) it is involved in trafficking of APP from the ER/Golgi to the plasma membrane to facilitate the accumulation and maturation of APP and ii) it is involved in the modulating the γ -secretase cleavage that leads to decreased production of A β -related peptides (Vetrivel et al., 2007). It would be of interest to study not only the possible colocalization of p23 and nicastrin in the Golgi compartment but also to explore the functional significance of their coexistence at the subcellular level.

4.3. Co-expression of nicastrin, p23 and PS1 in the adult rat brain: We and others have shown that PS1 and nicastrin display overlapping distributional profiles, but there are some minor discrepancies. For example, PS1 immunoreactivity in the hippocampus moderately labeled the neuropil of stratum lucidum, stratum lacunosum molecular and stratum molecular, whereas a low level of nicastrin immunoreactivity was evident in these layers (Siman et al., 2003; Kodam et al., 2008). Similar discrepancies were also noticed in peripheral tissues, especially in the skeletal muscle, where PS1 mRNA levels were found to be markedly lower than nicastrin mRNA (Nilsberth et al., 1999; Ilaya et al., 2004). These discrepancies could either be due to variation of methods used or to different roles of these proteins apart from acting as components of the γ -secretase complex. Earlier studies have demonstrated that endogenous p23 is coimmunoprecipitated with all components of the γ -secretase complex including nicastrin (Chen et al., 2006). However, evidence of their coexpressions in different brain regions/cell populations is lacking. Our results using single as well as double labeling methods showed that p23 is colocalized with nicastrin and PS1 in various

brain regions, including the pyramidal cells of cortex and hippocampus, Purkinje cells of cerebellum and motor neurons of brainstem. These findings provide an anatomical substrate for the potential role of p23 in regulating the activity of the γ -secretase complex in different brain regions. More recently, it has been shown that p23, by interacting with the γ -secretase complex *via* its transmembrane domain, regulates the activity of the enzyme (Paradossi-Piquard et al., 2009). However, the interaction of p23 with individual components of the γ -secretase complex and its relevance to the formation/activity of the enzyme remains unclear.

4.4. Postnatal regulation of nicastrin and p23 in the brain: Earlier reports suggest that sel-12, a homolog of PSs, mediates developmental cell-fate decisions elicited by the Notch/*lin-12* signaling pathway (Levitan and Greenwald, 1995). Furthermore, human PS1 could substitute for sel-12, thus indicating that PS1/PS2 may be involved in the Notch signaling pathway during embryonic development in mammals (De strooper et al, 1998; Koo and Kopan, 2004). This is further supported by evidence that i) PS1 knockout mice show early embryonic lethality (Donoviel et al., 1999; Herreman et al., 1999) and ii) PS1 mRNA expression levels are high in developing brain and are widely distributed throughout the neuroaxis (Lee et al., 1996). Similarly, recent studies have shown that nicastrin is also involved in Notch signaling as well as APP processing (Yu et al., 2000; Chung and Struhl, 2001; Edbauer et al., 2002; Hu et al., 2002). Mice lacking the nicastrin gene are found to resemble notch and PS1 knockout mice, thus providing support for its role during development (Swiatek et al., 1994; Conlon et al., 1995;

Huppert et al., 2000; Li et al., 2003; Nguyen et al., 2006). Consistent with these results, I showed that levels and expression of nicastrin are markedly high in discrete brain regions during development, thus suggesting its possible involvement in the Notch signaling pathway apart from APP processing. Another important finding of my study is that nicastrin was localized predominantly in neuronal cell bodies at earlier stages of development, whereas in the adult brain it is apparent in both cell bodies and dendrites/neuropils. Given the evidence that nicastrin may participate in neuronal differentiation during neurogenesis (Sarkar and Das, 2003), it is possible that high expression of nicastrin in cell bodies may be involved in the differentiation and/or maturation of neurons in selected brain regions. My result demonstrated that p23, like nicastrin, is also expressed at rather high levels during development and then declines to reach the adult profile. The high abundance of p23 during development may modulate A β production by its association with the γ -secretase complex. Additionally, p23 may have a critical role in regulating embryonic development since knocking down the p23 gene generates embryonic lethality (Denzel et al., 1999).

4.5. Immunoreactive p23 in AD brains: Previous studies have demonstrated that both PS1 and PS2 exhibit similar widespread distributions in AD and age-matched control brains. However, neither PS1 nor PS2 was found to be present in A β -containing neuritic plaques in AD brains (Mathews et al., 2000). Consistent with these studies, our results showed for the first time that the p23 immunoreactivity is widely distributed throughout the frontal cortex, hippocampus and cerebellum of AD and age-matched control brains. However, in

contrast to PS1/2 expression, the intensity of p23 immunoreactivity was reduced in surviving hippocampal and cortical pyramidal neurons in AD brains compared to control samples. Additionally, p23 immunoreactivity was occasionally evident in A β -containing neuritic plaques. Supporting immunohistochemical data, the level of p23 was also significantly reduced in AD brains compared to age-matched control tissues. These results, when analyzed with reference to *in vitro* studies (Vetrivel et al., 2007), clearly suggest that reduced levels of p23 may be involved in the increased production/levels of A β peptides that are inherently associated with AD pathogenesis. However, it needs to be determined from future studies whether p23 levels/expression are selectively altered in the affected *vs* unaffected regions of AD brains and their possible involvement, if any, in the generation of neuritic plaques or neurofibrillary tangles in AD pathology.

In summary, the present study demonstrates that nicastrin and p23 are widely distributed in major brain areas of adult and developing brain and are co-expressed with each other as well as with PS1. These results suggest that they might have a wide spectrum of roles in normal brains as well as in AD pathology. Furthermore, reduced levels/expression of p23 in AD brains raise the possibility that p23 may play a critical role in AD pathogenesis by influencing increased production of A β peptide from its precursor APP.

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