Alzheimer's disease genes in zebrafish (Danio rerio)

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DECLARATION

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Portrait (1918/1919)

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ABBREVIATIONS

aa	amino acid
AD	Alzheimer's disease
AICD	APP intracellular domain
APP	amyloid precursor protein
CAM	cell adhesion molecule
CHD	cell adhesion molecule homology domain
CNS	central nervous system
CSF	cerebrospinal fluid
ECM	extracellular matrix
FAD	early-onset familial Alzheimer' disease
FGF	fibroblast growth factor
FGFR	fibroblast growth factor receptor
HSPG	heparan sulphate proteoglycan
Ig domain	immunoglobulin-like domain
LOAD	late-onset sporadic Alzheimer's disease
MHB	midbrain-hindbrain boundary
NICD	Notch intracellular domain
PNS	peripheral nervous system
PSEN	presenilin

Progress is impossible without change
and those who cannot change their minds
cannot change anything

George Bernard Shaw

ABSTRACT

Accumulation of amyloid β peptide (Aβ) is an early event in the cascade of neurogenerative processes leading to Alzheimer's disease (AD). Aβ is generated by enzymatic cleavages by two sequentially acting proteases, β-secretase and γ-secretase, which liberate the Aβ from its precursor, the amyloid precursor protein (APP). Aberrant processing of the APP by γ-secretase has been suggested to lead to increased formation of a neurotoxic Aβ variant and deposition of amyloid plaques, consequently leading to Alzheimer's disease. Presenilin is essential for cleavage of APP and may be the catalytic subunit of γ-secretase, an intramembrane-cleaving protease. γ-secretase is a multi-protein complex, which in addition to Presenilin consists of three other core components, Nicastrin, APH-1 and PEN-2, that constitute its biological activity. AD pathology is likely to involve the perturbation of numerous molecular mechanisms and signalling pathways, including FGF signalling.

Whereas considerable effort has been undertaken to understand the molecular pathogenesis of Alzheimer's disease, and some genes likely to play pivotal roles in the progression of the disease have been identified, fundamental aspects of its aetiology remain unresolved. Importantly, the normal functions of the major AD-related genes identified so far, and the identity of the genetic networks they interact with, are unknown. Recently it has been realised that genes associated with Alzheimer's disease, and other degenerative diseases, also play important functions during embryogenesis.

The interactions between many important signalling pathways are highly conserved during embryo development and may control cellular responses in widely different embryonic structures, such as the brain, heart and somites (Pires-daSilva and Sommer, 2003). Consequently, it is likely that some of the highly conserved AD-related genes, for example the *presenilins*, may be components in evolutionarily preserved signalling networks operating to control the development of many different embryonic tissues.

This suggests that investigations of the genetic mechanisms controlling developmental processes in different embryonic tissues may be a relevant approach in order to gain insight into the normal biological functions of AD-related genes and, ultimately, the cause of neurodegeneration, as well as other pathological conditions, in the adult. This in mind, I decided to dissect the functions of Alzheimer's disease genes during embryo development using the zebrafish (*Danio rerio*) as a model organism.

In chapter 1 our current understanding of the key mechanisms and pathways involved in AD pathology is reviewed. In chapter 2 and 4 the embryonic expression patterns of the zebrafish *presenilin* and *APP* genes are investigated. In chapter 3 the role of γ-secretase activity during embryogenesis is analysed using a potent γ-secretase inhibitor. In chapter 5 the expression pattern of zebrafish *fibroblast growth factor receptor 1*, a central component in FGF signalling, is described in order to establish the foundation for further investigations of possible interactions between established Alzheimer's disease genes and other pathways in embryonic zebrafish.

Chapter 1

Alzheimer's disease: neurodegeneration and beyond

1.1. Epidemiology, aetiology and pathology of Alzheimer's disease

Alzheimer's disease (AD) accounts for more than 50% of all cases of dementia (Dugue et al., 2003). Estimates of prevalence vary, but 1-5% of the population over the age of 65, and 20-40% of the population over 85 may be affected by AD (Small et al., 1997), with approximately 15 million individuals affected worldwide (Puglielli et al., 2003). Because of the ongoing increase in life expectancy, by 2050 it is expected that approximately 25% of people living in Western societies will be over 65 years of age, one third of whom are likely to develop AD (Sloane et al., 2002). The economic costs associated with treatment and care of AD patients is substantial at present (Small et al., 2002). However, the negative societal and economic impact of this disease is expected to worsen substantially with the demographic transitions worldwide. Therefore, it is imperative to understand the molecular mechanisms underlying AD in order to develop effective measures for population screening, and drug treatment to cure afflicted individuals and/or prevent onset of the disease.

AD is a complex and genetically heterogenous disease, characterised by progressive memory loss and other cognitive and behavioural deficits (Cummings, 2000). The main neuropathological characteristics include the presence of intracellular neurofibrillary tangles (NFT) consisting of hyperphosphorylated microtubule-associated tau protein, and extracellular amyloid β-peptide (Aβ) deposits in plaques and around cerebral blood vessels (Selkoe, 2001). The aetiology of AD is still unclear. However, epidemiological studies have identified two distinct forms of AD. An early-onset form which is characterised by autosomal dominant mutations in a small number of genes, and a late-

onset form involving genetic and environmental risk factors that increase the risk of developing the disease (Selkoe, 2001). Early-onset familial AD (FAD) accounts for 5-10% of AD cases (Selkoe, 2001). The onset of the disease has been reported at approximately 30 years of age (Houlden et al., 2001), but most commonly, mental impairment is evident in afflicted individuals of 40-50 years of age (Selkoe, 2001). Presently, FAD has been linked to mutations in the genes for the *amyloid precursor protein* (APP), *presenilin 1* (*PSEN1*), and *presenilin 2* (*PSEN2*). Together, they account for approximately 40% of FAD cases, with a predominance of causative mutations in *PSEN1* (Czech et al., 2000). Late-onset sporadic AD (LOAD) accounts for 90-95% of AD cases, and affected individuals show AD pathology above 60 years of age (Selkoe, 2001). Only the *apolipoprotein E* gene (*APOE*) has been consistently associated with sporadic AD (Strittmatter et al., 1993). APOE is one of the major apolipoproteins in the plasma and the principal cholesterol carrier protein in the brain (Puglielli et al., 2003). This suggests that cholesterol may play a role in the pathogenesis of the disease. In support of this proposition, epidemiological studies have implicated high levels of plasma cholesterol as an environmental risk factor in AD (Jarvik et al., 1995; Kuo et al., 1998). *APOE* is a polymorphic gene with three common alleles present in humans, termed *APOE2*, *3* and *4* (Strittmatter and Bova Hill, 2002). The *APOE4* allele has been shown to increase the risk of developing AD in a dose-dependent manner. Individuals that are homozygous for *APOE4* alleles (approximately 2% of the population) are five times more likely to develop AD than are homozygotes for *APOE3* (Selkoe, 2001). However, it should be emphasised that *APOE4* is a risk factor for AD, and is neither necessary nor sufficient to cause AD.

Regardless of the differences in age of onset of the disease and the kind of causative lesions involved in the two forms of AD, they both exhibit very similar histopathogenetic events. Most prominent is the abnormal accumulation of Aβ in amyloid deposits and cerebral blood vessels (Selkoe, 2001). In most forms of FAD this involves the increased production of the 42-amino acid variant of Aβ (Aβ42), which promotes the aggregation of total Aβ into amyloid fibrils (Selkoe, 2002). In contrast to FAD, in LOAD the accumulation of Aβ is the result of a complex interplay between genetic and environmental factors, affecting the balance of generation, deposition, degradation and clearance (Selkoe, 2001). Nevertheless, the strong resemblance of the phenotypic characteristics suggests that information about the mechanism of the inherited form of AD, caused by mutations in the *APP* and *presenilin* genes, is likely to be directly relevant to our understanding of the pathogenesis of the common, sporadic form of AD.

1.2. APP and the biogenesis of Aβ

Aβ is a normal product of APP metabolism (Haass et al., 1992; Seubert et al., 1992). Aβ exists as two small peptides of either 40 or 42 amino acids (aa). The 40 aa peptide (Aβ40) is the predominant species produced under normal physiological conditions, while Aβ42 accounts for only 5-10% of total Aβ peptides (Suzuki et al., 1994). In contrast, in familial AD abnormal APP processing leads to an increase in the amount of Aβ42 to 15-40% of total Aβ (Scheuner et al., 1996). The Aβ species are derived from

the **amyloid precursor protein (APP)** through the amyloidogenic pathway, involving coordinated enzymatic cleavages performed by two proteases termed β- and γ-secretase, respectively (**FIG. 1.1 AND 1.2**) (Ling et al., 2003). APP is a type 1 integral glycoprotein, which is heavily N- and O-glycosylated during its passage through the secretory pathway (Moir et al., 1992). Only a minor fraction of the total APP pool is found at the cell surface, due to proteolytic processing of APP and endocytosis of APP holoprotein (Kuentzel et al., 1993; Sambamurti et al., 1992). β-secretase cleavage is executed by an aspartyl protease termed BACE1 (Sinha et al., 1999; Vassar et al., 1999; Yan et al., 1999), which leads to the shedding of the APP ectodomain (sAPPβ) and retention of a membrane-bound intermediate APP peptide (C99) (**FIG. 1.1**) (Haass and Steiner, 2002). The soluble sAPPβ is secreted into the extracellular space and has been found in the cerebrospinal fluid (CSF) (Palmert et al., 1989; Weidemann et al., 1989). The C99 fragment is the substrate for γ-secretase cleavage, which results in the generation of Aβ and the C-terminal-derived **APP intracellular domain (AICD)** (Haass and Steiner, 2002). γ-cleavage is unusual as it occurs within the cell membrane. γ-secretase is a multi-protein complex, presumably with aspartyl-protease-activity, with the Presenilins as core components (De Strooper, 2003; Fortini, 2002). Amyloidogenic processing through the combined action of β- and γ-secretases constitute a minor pathway in most cell types, except neurons (Tienari et al., 1989). APP is predominantly processed through the non-amyloidogenic pathway, involving successive proteolysis by α- and γ-secretase (**FIG. 1.1**) (Weidemann et al., 1989). APP at the cell surface can undergo α-secretase-mediated shedding of its ectodomain (sAPPα), which, similar to sAPPβ, is secreted into the extracellular space and found in the CSF (Palmert et al., 1989; Weidemann et al., 1989).

The identity of α-secretase has not been established, but members of the ADAM family sheddases, ADAM10 and ADAM17, have been shown to cleave APP at the α-secretase cleavage site *in vitro* (Buxbaum et al., 1998; Kojro et al., 2001). sAPPα is potentially a biological active molecule, which has been implicated in synapse formation (Morimoto et al., 1998), and may also serve important functions in memory formation and consolidation (Huber et al., 1993; Meziane et al., 1998), as well as in memory retention (Roch et al., 1994). The 83 aa C-terminal fragment (C83) retained in the membrane, following α-secretase cleavage, can undergo γ-secretase-mediated proteolysis to release the p3 peptide plus the AICD (**FIG. 1.1 AND 1.2**).

Different *APP* species have been identified in a wide range of human tissues. They are generated by alternative splicing and include mRNAs encoding APP proteins consisting of 695 and 751 amino acids (APP695 and APP751) (**FIG. 1.3**) (Kang et al., 1987; Ponte et al., 1988). APP751 differs from APP695 by the insertion of a 56 amino acid (aa) long domain with similarity to Kunitz-type protease inhibitor (KPI) domains (Ponte et al., 1988; Tanzi et al., 1988). The APP695 isoform is predominantly expressed in neurons (Kang et al., 1987). The APP751 has been demonstrated to have protease inhibitory activity (Kitagushi et al., 1988), however, the significance of this finding in relation to its physiological roles remain unclear. Two other major isofoms consisting of 714 and 770 amino acids (APP714 and APP770), including the OX-2 domain only or both the OX-2 and KPI domains, have also been described (Golde et al., 1990; Tanzi et al., 1988). These two isoforms have not been associated with AD pathology.

1.3. The amyloid hypothesis

Amyloid plaques consist of numerous proteins and other components. The major protein constituent is Aβ (Atwood et al., 2002). The identification of Aβ as an invariant feature of AD led to the hypothesis that Aβ is not only a hallmark of AD, but is a causative agent in the development of AD pathology. According to **THE AMYLOID CASCADE HYPOTHESIS** neurotoxic accumulation of Aβ in amyloid plaques triggers a pathological cascade leading to neuronal dysfunction and death (**FIG. 1.4**) (Hardy and Selkoe, 2002; Hardy and Higgins, 1992). Mounting evidence supports this idea (Selkoe, 2001). First, FAD mutations in the *APP*, *PSEN1* and *PSEN2* genes increase total Aβ, and/or increase the relative amount of Aβ42, or alters the primary sequence of Aβ such that it is more prone to self-aggregate (Nilsberth et al., 2001; Scheuner et al., 1996). Second, APOE4, a risk factor in LOAD, increases Aβ deposition (Holzman et al., 2000). Third, longer Aβ species, including Aβ42, deposit more rapidly than do shorter species (Jarret et al., 1993). Fourth, co-expression of *APP* and *PSEN1* FAD mutants in the brains of transgenic mice leads to accelerated Aβ deposition (Borchelt et al., 1997) and progressive cognitive impairment (Arendash et al., 2001). Fifth, Aβ can self-aggregate *in vitro*, and these aggregates can mediate neurotoxicity (Roher et al., 1991; Yankner et al., 1989; Yoshiike et al., 2003).

Thus, the single initiating event in AD is postulated to be the abnormal accumulation of neurotoxic Aβ in specific brain regions important in cognition and memory processes, which form the basis for our intellectual abilities. Elevated levels of neurotoxic Aβ lead

to injury of neurites within the plaques and in the surrounding tissue. Consequently, the lesions disrupt neuronal function and homeostasis, which results in neuronal death. The neuronal loss following the increase in the number of neuritic plaques in the progression of AD ultimately leads to dementia (Hardy and Selkoe, 2002; Hardy and Higgins, 1992).

Studies to dissect the role of Aβ as a mediator of neurotoxicity in AD have identified numerous biochemical pathways and mechanisms which are altered by elevated Aβ levels, suggesting that these may constitute downstream events in the amyloid cascade. There is strong evidence that chronic inflammation is central to AD pathogenesis. The presence of activated microglia and reactive astrocytes surrounding neuritic plaques are invariable features in the amyloidogenic process (Akiyama et al., 2000), and increases in various cytokines and components of the complement cascade further suggest that inflammatory responses have been provoked by the Aβ deposits (Akiyama et al., 2000; Emmerling et al., 2000). Defective energy metabolism, as a result of mitochondrial dysfunction, has also been implicated in AD (Castellani et al., 2002). Furthermore, inhibition of the mitochondrial electron transport chain may lead to excessive production of reactive oxygen species (ROS), which can alter proteins, lipids and nucleic acids, and cause oxidative stress, thus contributing to neuronal death (Bonilla et al., 1999; Smith et al., 2000). FAD mutations in *APP*, *PSEN1* and *2* disrupt calcium homeostasis, implicating dysfunctional calcium signalling as a significant factor in AD (LaFarla, 2002). There are some indications that the detrimental effect of Aβ on calcium homeostasis is caused by the ability of Aβ to form calcium permeable ion channels (Kagan et al., 2002). One important downstream event in the amyloid cascade appears to

be the development of neurofibrillary tangles. Mutations in the gene encoding tau protein cause **frontotemporal dementia and Parkinsonism linked to chromosome 17 (FTDP-17)** (Goedert and Spillantini, 2000). This disease is characterised by excessive formation of NFT in the brain, leading to neurodegeneration, and ultimately dementia and death. Thus, NFT formation alone is sufficient to cause severe and fatal brain disease, in the absence of amyloid plaque formation. Mice overexpressing FAD mutant APP together with mutant human tau show increased formation of NFT (compared with mice overexpressing tau alone), while amyloid plaque pathology is essentially unaltered (Lewis et al., 2001). Also, injection of Aβ42 into the brain of tau mutant mice have been shown to induce accelerated NFT formation (Goetz et al., 2001). This suggests that abnormal APP processing and generation of Aβ42 may induce NFT formation, leading to fatal neurodegeneration in AD brains. Thus, as illustrated above, it is likely that Aβ toxicity is mediated through various pathways and mechanisms, collectively acting to cause AD pathogenesis. However, it seems unlikely that all the identified effects of Aβ are associated with dementia.

1.4. Presenilins constitute the active site of γ-secretase

The human PSEN1 and PSEN2 proteins show a high degree of amino acid identity (67%) (Czech et al., 2000). However, although the high degree of similarity between these presenilin proteins suggests that they have related overlapping molecular activities, they are not functionally redundant (Donoviel et al., 1999; Steiner et al., 1999). Both proteins show approximately 50% identity to the single known *Drosophila* presenilin,

indicating that their function has been highly conserved throughout evolution (Ye and Fortini, 1999). The presenilin proteins are found in the endoplasmic reticulum and the Golgi apparatus consistent with a role in protein processing (De Strooper et al., 1997; Kovacs et al., 1996). However, they have also been detected in the plasma membrane, in the nuclear membrane, in centrosomes and interphase kinetochores (Li et al., 1997) and at the cell surface (Ray et al., 1999; Schwarzman et al., 1999). There is strong support for a role of cell surface presenilins in receptor-mediated signalling (see below).

Presenilins are transmembrane spanning proteins with, apparently, eight transmembrane domains (Li and Greenwald, 1998) although this is the subject of some debate (Dewji and Singer, 1997). Both PSEN1 and PSEN2 are subject to endoproteolysis to form more stable N- and C-terminal fragments (Ratovitski et al., 1997). Mutagenesis experiments have shown that two transmembrane aspartate residues in both PSEN1 and PSEN2 are required for their endoproteolysis (Kimberly et al., 2000; Leimer et al., 1999; Wolfe et al., 1999b). Interestingly, single aspartate point mutations abolish γ-secretase-dependent cleavage of APP in a manner indistinguishable from deletion of either PSEN1 or PSEN2 (Herreman et al., 2000; Zang et al., 2000), which suggests that those residues may be required for formation of the active site for proteolysis. The N- and C-terminal presenilin fragments are phosphorylated in a complex regulatory manner (Walter et al., 1998) and are associated after cleavage (Thinakaran et al., 1998).

As suggested above, the Presenilins are essential for cleavage of APP (De Strooper et al., 1998) and may be the catalytic subunit of γ-secretase, an intramembrane-cleaving

protease (Kimberly et al., 2000; Wolfe et al., 1999b). The γ-secretase multi-protein complex includes either Presenilin1 or Presenilin2, and a minimum of three other core components, Nicastrin, APH-1 and PEN-2 (**FIG. 1.2**) (Edbauer et al., 2003; Kimberly et al., 2003; Marlow et al., 2003; Takasugi et al., 2003). Presenilin-dependent γ-secretase activity is not only responsible for the cleavage of APP, but also for the proteolysis of Notch as well as other type 1 integral membrane proteins (De Strooper, 2003).

1.5. Presenilins are γ-secretases for Notch singalling

Notch receptors are large transmembrane receptor proteins that interact with numerous signal transduction pathways (Fortini, 2002). They are essential for many cell differentiation events, including the delineation of boundaries between tissues and inductive interactions between neighbouring tissues during development (Selkoe and Kopan, 2003). Recently, Notch signalling has been implicated in controlling enteroendocrine cell differentiation, e.g. insulin- and glucagon-secreting cells of the islets of Langerhans (Jensen et al., 2000). However, Notch receptors are best known for their effects on neurogenesis and segmentation, in particular the process of somitogenesis in vertebrates. In mouse embryos lacking *Notch1* gene activity, excess neurons differentiate and the formation of boundaries between somites becomes irregular (Conlon et al., 1995; de la Pompa et al., 1997). Interestingly, PSEN1-deficient mice show both decreased γ-secretase processing of APP and developmental abnormalities consistent with altered Notch signalling (Wong et al., 1997). Despite its apparently ubiquitous distribution in vertebrate embryos, *PSEN2* cannot compensate for the loss of *PSEN1* activity during embryogenesis (Donoviel et al., 1999).

How the Notch receptor transduces signals after activation remains poorly understood, but one model suggests that an intramembrane γ-secretase-like proteolytic cleavage of Notch results in the release of the intracellular domain, which translocates to the nucleus and associates with a DNA-binding adaptor protein, Suppressor of Hairless, to regulate transcription of target genes (Fortini, 2002).

Presenilin 1 and 2 are required for the release of the Notch intracellular domain (NICD) from the plasma membrane (De Strooper et al., 1999; Saga and Takeda, 2001; Steiner et al., 1999; Ye and Fortini, 1999). In *PSEN1*-deficient *Drosophila* embryos the Notch protein is not cleaved in its transmembrane domain and the NICD is not released. Consequently, the intracellular domain of Notch is prevented from entering the nucleus and Notch signal transduction is abolished (Struhl and Greenwald, 1999b). The protease responsible for NICD release apparently has γ-secretase-like properties, since γ-secretase inhibitors, which block APP processing also block Notch1 processing (De Strooper et al., 1999). The fact that both APP and Notch are cleaved in the transmembrane domains by a γ-secretase-like activity that requires PSEN1 and PSEN2 protein supports the idea that these two proteolytic activities are closely related. Thus, a shared mechanism of regulated proteolysis may be a key feature of both Notch signalling and Alzheimer's disease.

1.6. γ-secretase-mediated proteolysis in cell-surface-receptor signalling of APP and Notch

As indicated above, the normal function of APP still remains poorly understood. Disruption of *APP* in transgenic mice does not result in any prominent phenotype (Zheng et al., 1995). This may be due to genetic redundancy, with other members of the *APP* gene family substituting for the loss of APP function (Coulson et al., 2000; Heber et al., 2000; von Koch et al., 1997). However, recent studies provide compelling evidence for a role of APP in signal transduction (Fortini, 2002; Haass and Steiner, 2002). The amino acid sequence of APP suggests a structure of a cell-surface receptor (Kang et al., 1987). The fact that only a small fraction of APP is present at the cell surface, and is subjected to rapid turnover, further supports this idea (Selkoe, 2001).

Processing of APP and Notch is strikingly similar. Both proteins undergo ectodomain shedding, leaving a membrane-bound C-terminal fragment competent for γ-secretase-mediated intramembrane cleavage (Haass and Steiner, 2002). Recently, it has been demonstrated that Notch, like the corresponding cleavage of APP at position 40 and 42 of the Aβ domain, is cleaved in the middle of the membrane in a γ-secretase-dependent manner to release an Aβ-like peptide (Nβ) (Okochi et al., 2002). Also, γ-secretase-dependent cleavage of APP C-terminal fragments, to release AICD, takes place after position 50 (Aβ numbering) (**FIG. 1.1**). Thus, this cleavage is distinct from the cleavages at position 40 and 42 (Haass and Steiner, 2002). Interestingly, this cleavage is almost identical to the S3 cleavage of Notch, which leads to the liberation of the NICD (Fortini,

2002; Selkoe and Kopan, 2003). Similar to NICD, the AICD may translocate to the nucleus and participate in the transcriptional regulation of target genes (Cupers et al., 2001; Gao and Pimplikan, 2001; Gu et al., 2001; Kimberly et al., 2001). Taken together, these results strongly suggest that APP functions as a receptor in a similar fashion to Notch. It will be interesting to identify ligands that bind to APP and elicit the regulated cleavage of the protein, which ultimately may lead to the control of target gene expression. Thus, it is suggested that the molecular pathology of Alzheimer's disease may also involve dysfunctional regulation of APP target genes as a causative mechanism for the disease.

1.7. Fibroblast growth factor signalling and Alzheimer's disease

The possible involvement of perturbed receptor-mediated signalling in AD is not limited to APP. Previously, FGF signalling has been implicated in AD pathology. For instance, elevated levels of FGF2 protein have been reported in reactive astrocytes surrounding senile plaques (Cummings et al., 1993). Also, FGF2 is present within the plaques and has been found associated with neurofibrillary tangles (Gomez-Pinilla et al., 1990; Stopa et al., 1990). *In vitro* studies show that FGF2 enhances APP mRNA synthesis in astrocytes (Gray and Patel, 1993; Quon et al., 1990), and Aβ can stimulate FGF2 production in astrocytes and microglia (Araujo and Cotman, 1992).

A major mediator of FGF2 signalling is the fibroblast growth factor receptor 1 (FGFR1) (Ornitz et al., 1996). FGFR1 is expressed in neurons and astrocytes in non-demented and AD individuals (Ferrer and Marti, 1998; Takami et al., 1998) overlapping with

FGF2 expression (Stopa et al., 1990). Both FGF2 and FGFR1 were found to be up-regulated in reactive astrocytes surrounding senile plaques in AD brains (Cummings et al., 1993; Ferrer and Marti, 1998; Takami et al., 1998). This has also been observed in other pathological conditions. For example, in a rodent model, local injury to the brain cortex also leads to a coordinated increase in the expression of FGF2 and FGFR1 (Logan et al., 1992). The functional significance of this coordinated expression of FGF2 and its receptor is unknown.

FGF2-induced activation of the FGFR1 receptor is linked to the **Ras/mitogen-activated protein (MAPK) cascade**, which can modulate intracellular enzyme activity and regulate expression of target genes (Szebenyi and Fallon, 1999). Several enzymes of the MAPK cascade show elevated expression in neurons and reactive astrocytes of AD patients (Arendt et al., 1995), and the cascade is chronically activated in such cases (Pei et al., 2002; Zhu et al., 2003). This may be important, as it has been shown that activated MAPK can phosphorylate tau *in vitro* on sites that are hyperphosphorylated in AD (Roder et al., 1993). The MAPK cascade plays a central role in synaptic plasticity and learning (Sweatt, 2001). Thus, dysfunctional MAPK signalling may play an essential role in AD pathogenesis, contributing to the cognitive impairment and memory loss associated with this devastating disease. This proposition is further supported by the observation that overexpression of APP *in vitro* leads to hyperphosphorylation of MAPK in response to FGF stimulation, and enhanced nuclear translocation of MAPK (Grant et al., 1999). Excessive nuclear MAPK may lead to misregulation of target gene expression.

As indicated above, abnormal FGF signalling may be an essential causative mechanism leading to AD. Central to the signal transduction events mediated by FGFs is the activation of the FGFR1 receptor upon ligand binding. Thus, the mechanisms controlling FGFR1 signalling competence and receptor activation define crucial steps in the regulation of FGFR1-dependent signalling cascades, such as the MAPK cascade. Given this importance, the remaining part of this introduction will be dedicated to review our current understanding of the role of FGFR1-mediated signalling in vertebrates.

1.8. The general structure of FGFR1

The vertebrate fibroblast growth factor receptor (FGFR) gene family consists of four highly related genes, *FGFR1*, *FGFR2*, *FGFR3* and *FGFR4*, encoding polypeptides that are 55% to 72% identical in their amino acid sequence (Johnson and Williams, 1993). Recently a new member of this family (*FGFR5*) was identified in human and mouse (Kim et al., 2001; Sleeman et al., 2001). *FGFR5* is the most distantly related member of the FGFR family, showing approximately 30% amino acid identity to other FGFR proteins. FGF receptors are involved in many biological processes during embryo development and homeostasis of adult body tissues, and disruption of normal *FGFR* functions lead to pathological conditions in humans. For example, mutations in the *FGFR* genes are the cause of several human developmental disorders characterized by skeletal abnormalities such as achondroplasia (i.e. dwarfism) (Muenke and Schell, 1995; Passos-Bueno et al., 1999), and misregulation of *FGFR* mRNA splicing or upregulation

of *FGFR* expression may lead to cell transformation and cancer (Valve et al., 2001; Yamaguchi et al., 1994a).

The general structure of FGFR1 and other FGF receptors is highly conserved during evolution (Johnson and Williams, 1993). A schematic diagram of the longest single-pass transmembrane product encoded by the human *FGFR1* gene is shown **FIG. 1.5.** The extracellular region consists of a signal peptide followed by three immunoglobulin-like domains (Ig domains). Present between immunoglobulin-like domains II and II (IgII and IgIII) is the acidic box domain, including 8 consecutive acidic residues, termed the acidic box. A heparin-binding region important for interactions with extracellular matrix (ECM) components and a cell adhesion molecule (CAM) homology domain (CHD) are located downstream from the acidic box domain (Doherty and Walsh, 1996; Kan et al., 1991). Continuous with the extracellular region is a transmembrane (TM) domain followed by an intracellular region. The latter consists of a juxtamembrane domain next to the TM domain and a tyrosine kinase domain, which is split by a 14 amino acid long non-catalytic interkinase domain and followed by a short C-terminal tail (Johnson and Williams, 1993).

1.9. FGFR1 splice variants

The genomic organization of *FGFR1* is still not fully characterised. However, based on genomic comparison to other FGF receptor genes it is likely that the human *FGFR1* gene comprises 19 exons (**FIG. 1.5**) (Cote et al., 1997; Givol and Yayon, 1992; Johnson et al., 1991). Alternative splicing of *FGFR1* transcripts generates a diversity of isoforms,

which are differentially expressed in cells and tissues (Johnson and Williams, 1993). **FIG. 1.6** shows diagrams of some of the reported FGFR1 splice variants. These are mainly of human or murine origin, but isoforms isolated from frog and fish are also represented. The protein structure of each isoform is deduced from analysis of the cDNA sequence. Alternative sequence usage can generate mRNA isoforms encoding receptors truncated in the extracellular or intracellular region, lacking Ig-like domains, or derived from different exons encoding variants of particular Ig-like domains. For example, secreted receptor isoforms lacking the kinase domain have been shown to result from alternative splicing involving distinct sequences (Eisemann et al., 1991; Johnson et al., 1990; Werner et al., 1992). One such variant is generated by alternative splicing of mRNA from exon 3, which encodes IgI, to normally intronic sequences from upstream of exon 4 (**FIG. 1.6: 1**). This mRNA encodes IgI followed by 32 unique amino acids and a stop codon (Eisemann et al., 1991). Alternative splicing of exon 7 sequences to three different downstream sequences generates isoforms with a variable C-terminal half of IgIII (termed FGFR1IIIa, FGFR1IIIb and FGFR1IIIc). The latter two isoforms, produced by splicing exon 7 sequences to those of either exons 8 or 9, have been shown to generate transmembrane proteins with different ligand binding specificities (**FIG. 1.6: 5-12**) (Beer et al., 2000; Dionne et al., 1990; Eisemann et al., 1991; Gillespie et al., 1995; Hou et al., 1991; Johnson et al., 1990; Johnson and Williams, 1993; Lopez and Korc, 2000; Reid et al., 1990; Wang et al., 1996) (**see CHAPTER 5**). Splicing of mRNA from exon 7 to normally intronic sequences upstream of exons 8 and 9 generates another truncated and secreted FGFR1 product (FGFR1IIIa), which ends downstream of IgIII (**FIG. 1.6: 2 AND 3**) (Johnson et al., 1990; Werner et al., 1992).

Little is known about the biological functions of truncated soluble FGFR1 isoforms. The FGFR1IIIa isoform is expressed in postnatal and adult mouse tissues, and in human cell lines (Duan et al., 1992; Werner et al., 1992) and is present in the circulation (Hanneken, 2001). In the adult murine retina the truncated FGFR1IIIa is differentially expressed compared to full-length FGFR1IIIc, suggesting a specific role for this isoform in the retina (Guillonneau et al., 1998). Soluble fibroblast growth factor receptors have also been isolated that lack the transmembrane region (Givol and Yayon, 1992). In our laboratory we have isolated from zebrafish embryos a cDNA representing a FGFR1 variant that lacks the second half of IgIII as well as the transmembrane domain, thus representing a putative soluble isoform (**FIG. 1.6: 4**) (**see CHAPTER 5**). However, soluble FGFR1 receptors can also be generated by proteolytic cleavage, resulting in ectodomain shedding. The ectodomains from isoforms of FGFR1IIIb and FGFR1IIIc with either two or three Ig domains have been found in the extracellular matrix and in blood where they may function to regulate the biological activities of the FGFs during cell proliferation and development (Hanneken, 2001). Several groups have isolated cDNAs representing FGFR1 isoforms with either 2 or 3 Ig domains, termed FGFR1β and FGFR1α, respectively (**FIG. 1.6: 2 AND 3, 5-8**) (Beer et al., 2000; Dionne et al., 1990; Eisemann et al., 1991; Johnson et al., 1990; Johnson and Williams, 1993; Reid et al., 1990; Werner et al., 1992). The physiological significance of inclusion or exclusion of IgI is still largely unknown. However, it has been shown that a switch from FGFR1α to FGFR1β isoforms correlates with astrocyte malignancy (Yamaguchi et al., 1994a). This may be the result of changes in ligand affinity between these two forms of FGFR1 receptor, as it has been

shown that FGFR1β isoforms exhibit a 10-fold higher affinity for FGF1 and FGF2 than FGFR1α isoforms. This may lead to a growth advantage for tumourigenic cells, resulting in the development of brain tumours (Shi et al., 1993; Wang et al., 1995). FGFR1 is not only located at the cell surface but is also present in the nucleus (Kilkenny and Hill, 1996). However, only the FGFR1α form has been found in the nucleus, suggesting that IgI is important for nuclear targeting of this receptor (Prudovsky et al., 1996).

Subtle variations of structure have been found in the FGFR1α and β isoforms. These are inclusions or exclusions of a RM dipeptide downstream of the acidic box (**FIG. 1.6: 2 AND 3, 7 AND 8**) or of a VT dipeptide within the juxtamembrane domain (**FIG. 1.6: 9**). These isoforms are generated by alternative use of slightly different splice donor sites at the exon/intron boundaries of the exons encoding the acidic box and juxtamembrane regions (Eisemann et al., 1991; Gillespie et al., 1995). The VT dipeptide is part of a protein kinase C (PKC) phosphorylation consensus sequence within FGFR1 (Kennelly and Krebs, 1991). PKC-induced FGFR1 activity is strictly regulated during embryo development (Gillespie et al., 1995), and the relative expression levels of VT-containing and VT-lacking FGFR1 isoforms have been found to regulate mesoderm induction in *Xenopus* (Paterno et al., 2000).

Several kinase-deficient FGFR1 variants have been described. For example, (Wang et al., 1995) have isolated a cDNA which lacks codons for 37 amino acids in the kinase 2 domain (**FIG. 1.6: 11**). Hou et al. (1991) have characterized a transcript that, due to the use

of an alternative splice donor site in exon 14 of the *FGFR1* gene, leads to a shift in the reading frame that truncates the second kinase domain to 24 amino acid residues followed by 44 unique C-terminal residues (C-tail type 2) (**FIG. 1.6: 12**). The presence of kinase-deficient FGFR1 isoforms in cells that also express FGFR1 active forms is thought to be a mechanism for regulating the level of FGFR1 activity since kinase-deficient and active forms can heterodimerize leading to non-functional receptor dimers and down-regulation of ligand-induced signal transduction (Shi et al., 1993). Finally, an FGFR1 splice variant was isolated from human placental tissue that is derived by skipping exons 6 and 7 (encoding the C-terminal half of IgII and N-terminal half of IgIII) and directly splicing exons 5 and 9 together. This results in the generation of a product that can activate mitogenic signalling without phosphorylating FRS2α (FGF receptor substrate 2α) or phospholipase C_γ, key components of two mitogenic signalling pathways (**FIG. 1.6:10**).

The large number of different splice variants of FGFR1 isolated from various vertebrate tissues strongly suggests that FGFR1 isoforms have specific functions during development and in adult homeostasis. However, the number of different FGFR1 isoforms may, to a certain degree, be exaggerated for two reasons. First, many of the FGFR1 variants have been identified as cDNAs isolated from immortalised cell cultures. Thus, although they may well be important for processes in pathological cell states they may not be relevant for our understanding of *FGFR1* biology in the context of normal, healthy tissues. Secondly, RT-PCR, the central technique used to identify *FGFR1* isoforms, is highly sensitive and may amplify cDNAs that are mere by-products of

normal *FGFR1* RNA splicing and are present at concentrations too low to have any physiological effect.

1.10. FGFR1 ligands

The generation of multiple FGFR1 isoforms by alternative transcript splicing provides the basis for differential binding of ligands to the receptor resulting in specific cellular responses. FGFR1 has been found to interact with several different types of ligand, including fibroblast growth factors (FGFs), heparan sulphate proteoglycans (HSPGs) and neural cell adhesion molecules (CAMs). FGFR1 was originally identified as a high-affinity receptor for fibroblast growth factors (FGF). In human and mouse, 22 *FGF* genes have been identified (Ornitz and Itoh, 2001) with diverse functions during embryo development and adult tissue homeostasis, including the regulation of cell proliferation, differentiation, wound healing, angiogenesis and malignant transformation as well as migration and neurite outgrowth (for reviews see (Powers et al., 2000; Szebenyi and Fallon, 1999). *In vitro* assays with BaF3 cells show that FGFR1 isoforms have distinct FGF-binding affinities and only bind FGF1 and FGF2 with high affinity (Ornitz et al., 1996; Xu et al., 2000). The alternatively spliced C-terminal half of the IgIII domain has been shown to play an essential role in determining specificities for FGF binding (Beer et al., 2000; Duan et al., 1992; Ornitz et al., 1996; Werner et al., 1992), however a number of other sequence segments in the IgI and IgII domains and upstream of IgI may also significantly modulate ligand specificity (Chellaiah et al., 1999). The actual FGF ligand-binding region of FGFR1 has been located within IgII and IgIII (Chellaiah et al., 1999; Zimmer et al., 1993).

It is becoming increasingly evident that the *in vivo* binding of FGFs to FGFR1 is facilitated by HSPGs, which not only function as co-receptors, but may play a prominent role in determining the specificity of binding of FGFs to FGFR1. Thus, the results from *in vitro* binding assays may not reflect the binding affinity of FGFs to FGFR1 *in vivo*. This is illustrated by the developmental process of patterning of the brain where *FGF8* expression is highly restricted at the midbrain-hindbrain boundary (MHB) and certain other regions (Mason et al., 2000). Of the four known vertebrate *FGFR* genes, only *FGFR1* is expressed at the MHB, which suggests that the encoded protein may act as a receptor for FGF8-mediated signalling in this region. However, FGFR1 does not bind FGF8 in *in vitro* assays (Blunt et al., 1997; MacArthur et al., 1995; Ornitz et al., 1996), indicating that other factors, including endogenous HSPGs, may alter the binding affinity of FGF8 for FGFR1, leading to signal transduction *in vivo*.

HSPGs can be divided into cell surface and extracellular matrix (ECM) proteins (for reviews see (Bernfield et al., 1999; Iozzo, 1998). HSPG chains are linear polysaccharides made up of repeating disaccharide unit backbones, which are synthesised and attached to the HSPG core protein in the Golgi-complex. They are then modified further in several steps, including the addition of sulphate groups at various positions. This provides the basis for generation of structurally different HSPGs, contributing to the generation of differential binding affinities of FGFR1 for FGFs (Bernfield et al., 1999; Perrimon and Bernfield, 2000). For example, it has been shown that in the neural precursor cells of the developing murine nervous system, the

expression of specific HSPGs is highly regulated, leading to a shift in affinity from FGF2 to FGF1. This may have a significant effect on FGFR signalling at a critical phase during murine brain development where the differentiation of hundreds of different subclasses of neurons and glia is initiated (Nurcombe et al., 1993). HSPGs have been shown to be involved in a variety of biological processes during embryo development and in the adult organism, including cell proliferation, differentiation, wound healing, angiogenesis, regulation of blood coagulation, cell adhesion and malignant transformation (for review see (Tumova et al., 2000).

It is now well established that specific FGFs facilitated by HSPGs activate FGFR1 by inducing dimerization. Based on this, a general model has been proposed where two FGF molecules interact with one HSPG to facilitate and stabilise the binding of the FGFs to two FGFR1 molecules, leading to activation of the tyrosine kinase domain (**FIG. 1.7**) (Plotnikov et al., 1999).

FGFR1 has also been implicated in interactions with the neural cell adhesion molecules (CAMs) such as L1, NCAM and N-cadherin that play an important role during the development of the nervous system by mediating cell-cell interactions necessary for cell migration, neurite outgrowth and other physiological processes. CAMs have also been shown to be involved in the regeneration and synaptic plasticity of the neurons in the adult nervous system (Benson et al., 2000; Doherty and Walsh, 1996). Although no direct evidence has been established for the binding of FGFR1 to CAMs, several studies, using inhibitors against components of the PLCγ cascade and FGFR1 have

demonstrated, that in the presence of these inhibitors both CAM- and FGF-stimulated neurite outgrowth could be blocked. This suggests that FGFs and CAMs utilize a common PLCγ pathway via FGFR1 to initiate the neurite outgrowth response (Doherty and Walsh, 1996). Furthermore, a putative evolutionarily conserved CAM binding sequence (CAM homology domain, CHD) has been identified in FGFR1 and thought to interact directly with a CHD binding motif in the CAMs. Sharing of common binding motifs may lead to *cis* and *trans* homophilic/heterophilic binding between CAMs and FGFR1 and subsequent clustering of CAM/FGFR1 complexes followed by activation of FGFR1 and signal transduction (**FIG. 1.7**) (Doherty and Walsh, 1996). Co-clustering of N-cadherin and FGFR1 has recently been demonstrated, lending support for this model (Utton et al., 2001). It is worth pointing out, that even if both CAMs and FGFs interact with FGFR1, the cellular responses may be different, due to possible differences in the way the ligands interact and mediate dimerization and activation of FGFR1 (Doherty and Walsh, 1996).

1.11. FGFR1 signal transduction

After binding of the ligand to FGFR1, the signal must be transmitted across the plasma membrane and generate an appropriate biological response in the cell. Initiation of signal transduction is achieved by conformational changes in the receptor upon ligand binding, leading to dimerization and subsequent activation of FGFR1 by autophosphorylation of the intracellular domain (McKeehan et al., 1998). Seven tyrosine residues have been identified as potential substrates for phosphorylation and are important for kinase activity and receptor signalling (Mohammadi et al., 1996). Activation of the receptor

allows proteins containing Src homology (SH2) or phosphotyrosine binding (PTB) domains to bind to sequence recognition motifs in FGFR1, resulting in phosphorylation and activation of these proteins (Dhalluin et al., 2000; Forman-Kay and Pawson, 1999; Pawson et al., 1993). For example, phosphorylated FGFR1 tyrosine 766 is a high-affinity binding site for phospholipase C_γ (PLCγ) (Mohammadi et al., 1992). Activated PLCγ can hydrolyse phospatidylinositol-4,5-diphosphate (PIP_2) to inositol-1,4,5-triphosphate (IP_3) and diacylglycerol (DAG). IP_3 induces Ca^{2+} release from intracellular stores, whereas DAG is a PKC activator. Both CAM- and FGF2-stimulated neurite outgrowth has been suggested to rely on the same PLCγ cascade, involving conversion of DAG to arachidonic acid (AA) by DAG lipase and an AA-induced increase in calcium influx into neurons via calcium channels leading to activation of calcium-dependent proteins (**FIG. 1.8**) (Doherty and Walsh, 1996).

The Ras/mitogen activated protein kinase (MAPK) pathway is another important signalling cascade utilised by FGF-mediated activation of FGFR1. The adaptor protein FRS2α has been shown to link FGFR1 activation to this cascade. The PTB domain of FRS2α interacts with the juxtamembrane region of FGFR1 (Ong et al., 2000), and following tyrosine phosphorylation by FGFR1 it binds the adaptor protein Grb2 (Growth factor receptor-bound protein 2), which can form a complex with Sos (Son of sevenless), a guanine nucleotide exchange factor. The proximity of the Grb2/Sos complex to the cell surface allows Sos to activate the G protein Ras by GDP for GTP exchange, leading to activation of the Ras/MAPK signalling pathway (**FIG. 1.8**) (Hadari et al., 2001; Kouhara et al., 1997; Ong et al., 2000). Activated MAPK can phosphorylate cellular proteins (e.g.

Sos), and also migrate into the nucleus and activate transcription factors by phosphorylation (e.g. JUN and FOS) (for a review see (Karin and Hunter, 1995). Several groups have demonstrated the importance of FRS2α in FGFR1-mediated signal transduction in various biological processes such as cell proliferation, migration and embryo development (Hadari et al., 2001; Ong et al., 2000). It is interesting that the FRS2α binding site overlaps with a phosphorylation consensus sequence, which allows for PKC regulation of FGFR1 signalling (Gillespie et al., 1995). An alanine substitution for valine in the VT dipeptide significantly diminishes FRS2α binding to FGFR1 and phosphorylation of FRS2α by FGFR1, leading to a reduction in MAPK activity (Ong et al., 2000). In addition, FRS2α-deficient mice die early during embryo development (Hadari et al., 2001). Taken together these findings suggest that FRS2α and PKC interactions with FGFR1 play an important role during early vertebrate development.

A more detailed understanding of the role of the FGFR1 pathway in certain aspects of brain development is slowly emerging due to recent work by Faux et al. (2001) who provide evidence of cross-talk between the FGFR1 signalling pathway and the Notch signalling pathway (another important developmental signalling system). It has long been known that stimulation of FGFR1 signalling by FGF1 or FGF2 inhibits neuronal differentiation (Murphy et al., 1990). Cell-cell interactions mediated by the Notch signalling pathway are essential for regulation of neuron differentiation by maintaining precursor cells in an undifferentiated and proliferative state and, thus, inhibiting neurogenesis (Lewis, 1998). However, the downstream developmental cascade responsible for transduction of the FGF signal that inhibits neurogenesis remained

elusive. The neurons and glia, which make up the intricate neuronal network of the nervous system, originate from neuroepithelial precursor (NEP) cells found within the neural tube (Faux et al., 2001). A connection between FGFR1 and Notch signalling was established using murine forebrain NEP cells *in vitro* to demonstrate that FGF1- and FGF2-mediated FGFR1 signalling regulates the expression of Notch and Delta, key components of the Notch pathway, which subsequently leads to inhibition of neurogenesis (Faux et al., 2001).

1.12. The importance of nuclear FGFR1

FGFR1 is generally recognized as a protein of the plasma membrane, transmitting signals to the nucleus via the Ras/MAPK pathway (Karin and Hunter, 1995). However, recent findings suggest that FGFR1 may function after internalisation by receptor-mediated endocytosis, which is the principal mechanism for inactivation of the receptor (Reilly and Maher, 2001; Sorokin et al., 1994). A fraction of the internalised FGFR1 is targeted to the nucleus as FGF/FGFR1 complexes where it may be involved in the induction of cell proliferation and differentiation (Karesztes and Boonstra, 1999). Nuclear translocation of FGF2/FGFR1 complexes is mediated through interaction of FGFR1 with importin β, a critical component of multiple nuclear import pathways (Reilly and Maher, 2001). Nuclear FGFR1 shows kinase activity (Maher, 1996) and has been implicated in regulation of the cell cycle by inducing the expression of c-Jun, which subsequently leads to the induction of cyclin D1 expression (the principal G_1-phase cyclin, followed by cell proliferation) (Reilly and Maher, 2001). It is tempting to speculate that the interaction between the FGFR1 and Notch signalling pathways

demonstrated by Faux et al. (2001) could be accomplished by nuclear FGF/FGFR1 complexes regulating *Notch* and *Delta* gene expression.

1.13. The role of *FGFR1* during development

Vertebrates share a common evolutionary origin, which can be dated back more than 500 million years (Holland and Chen, 2001). Comparative analysis of *FGFR1* expression patterns between vertebrates may help to gain insights into the evolution of vertebrate *FGFR1* function during development. Expression analyses of *FGFR1* in different vertebrates demonstrate a high degree of evolutionary conservation during vertebrate embryo development. For example, *FGFR1* is expressed during the period of mesoderm induction in chick, mouse and frog (*Xenopus*) (Golub et al., 2000; Walshe and Mason, 2000; Yamaguchi et al., 1992). Embryos from both *FGFR1*-deficient mice and from *Xenopus* overexpressing a dominant negative FGFR1 exhibit defects in mesoderm formation, suggesting an important role for *FGFR1* in this developmental process (Amaya et al., 1991; Yamaguchi et al., 1994b). *FGFR1* is ubiquitously expressed during development of the brain in chick, mouse and *Xenopus*, with high levels of expression reported in the forebrain and midbrain-hindbrain boundary (MHB) in mouse and *Xenopus* (Golub et al., 2000; Walshe and Mason, 2000; Yamaguchi et al., 1992). Interestingly, chick embryos do not show any distinct high-level *FGFR1* expression domains during early neural development while in Medaka fish (*Oryzias latipes*) *FGFR1* expression is excluded from the anterior region of the developing brain (Carl and Wittbrodt, 1999; Walshe and Mason, 2000). We have isolated *FGFR1* cDNAs from another teleost, the zebrafish (*Danio rerio*) (**see CHAPTER 5**). In contrast to

observations in Medaka, the expression pattern of zebrafish *fgfr1* is similar to that of *Xenopus*, showing ubiquitous expression during early neural development with high level expression domains in the forebrain and MHB. The MHB, also termed the isthmus organizer, produces signalling molecules such as FGF8 that induce the specification and patterning of adjacent brain structures (Reifers et al., 1998). *FGFR1* is expressed at the MHB in all of the above vertebrates except Medaka. In chick and mouse, *FGFR1* is the only known FGF receptor expressed at the MHB (Wilke et al., 1997). Furthermore the elevated FGFR1 expression in this region in zebrafish and *Xenopus* suggests that FGFR1 is the receptor for FGF8-mediated signal transduction at the MHB in these vertebrates. In Medaka neither *FGFR1* nor any of the other three highly related *FGFR* genes (*FGFR2*, *FGFR3* and *FGFR4*) show expression overlapping with that of *FGF8* at the MHB. Thus, FGF8 may transduce its signal at the MHB via a yet unidentified receptor in this species. The expression patterns of *FGFR1* during somite formation share many similar features among amphibians, fish, birds and mammals (Carl and Wittbrodt, 1999; Golub et al., 2000; Walshe and Mason, 2000; Yamaguchi et al., 1992) (**see CHAPTER 5**). *FGFR1* is expressed in the presomitic mesoderm just posterior to newly formed somites and in the first 2-5 somites but is absent from older somites. A more detailed analysis of *FGFR1* expression has been performed in mouse and zebrafish, showing that the expression is restricted to the anterior half of the somites, whereas in Medaka *FGFR1* expression is found in the lateral areas of somites (Carl and Wittbrodt, 1999; Yamaguchi et al., 1992) (**see CHAPTER 5**).

In situ hybridisation studies to localise *FGFR1* expression during embryo development and in the adult body have mainly been performed using probes covering the kinase domain. Thus, these analyses show the cumulative expression of all transcripts not encoding intracellularily-truncated receptors. However, the few studies based on Northern blots, RNase protection assays and immunological staining that have included isoform-specific probes show that the isoforms are differentially expressed (Beer et al., 2000; Guillonneau et al., 1998; Reid et al., 1990; Werner et al., 1992). The lack of detailed knowledge about the expression patterns of the various *FGFR1* splice forms is surprising considering the large number of isoforms that have been isolated (**FIG. 1.6**), indicating that a driving force in the evolution of *FGFR1* function has been the generation of isoforms to perform specific functional roles during embryo development and adult homeostasis. In the adult body, the FGFR1IIIc isoforms are preferentially expressed compared to FGFR1IIb isoforms (Beer et al., 2000; Werner et al., 1992). In addition, mice with targeted deletions of exon 8 or 9 (required for isoforms FGFR1IIIb or IIIc, respectively) have been generated. The developmental defects of FGFR1IIIc-isoform deficiency were severe and these mouse embryos died early during development while mice lacking FGFR1IIIb isoforms were viable and fertile with few developmental abnormalities. This indicates that the FGFR1IIIc isoforms are critical for embryo development, whereas splice variants encompassing the FGFR1IIIb specific sequence apparently are not (Partanen et al., 1998). Also, targeted disruption in mice of exon 3 that encodes IgI, a domain found exclusively in FGFR1α isoforms, has demonstrated that these splice variants play a pivotal role during mesoderm induction and other early

developmental processes, but may not be essential during somitogenesis (Xu et al., 1999).

1.14. Zebrafish as a vertebrate model organism

Direct investigation of human biology by genetic manipulation is only possible in cultured human cell lines. However, recent studies of gene expression in cultured human cells using gene array technology have demonstrated that their genetic programme can be dramatically altered due to lack of normal cell-cell interactions and/or immortalisation (Iyer et al., 1999). Thus, cell lines can represent a poor or even misleading substitute for study of gene function *in vivo*. Therefore, whole animal model systems can be of great utility in investigation of questions relevant to human biology and health.

The mouse is a widely used organism to study the functional roles of genes implicated in human disease and vertebrate embryogenesis. The mouse is evolutionarily closely related to humans and shares a similar mammalian biology (Emes et al., 2003). This generally suggests that results obtained from the study of mouse pathogenesis may be directly relevant to our understanding of related human conditions. The availability of advanced methods for genetic manipulation of the mouse, a short generation time of approximately 2 months, and elaborate breeding schemes greatly facilitates such investigations. Recently, it has been realised that genes responsible for pathogenic conditions in humans and other vertebrates also play important roles during embryo development (Stern and Zon, 2003). This suggests that the study of developmental

pathways may reveal important clues about normal and dysfunctional genetic interactions in the adult vertebrate body. Embryo development is internal in placental animals making embryonic manipulation difficult and *in vivo* observation impossible. Thus, while potentially important insights may be gained from the study of mouse embryo development in relation to disease processes the analysis is necessarily laborious and complex in nature.

In recent years, the zebrafish (*Danio rerio*) has become one of the most important vertebrate organisms for biological research. The zebrafish has many characteristics that make it a very applicable organism for studying embryogenesis. Fertilisation is external and the subsequent embryo development occurs synchronously among eggs. The embryos are relatively large and the eggs are completely transparent throughout the first 24 hours of development, allowing easy observation of developing organ systems. Moreover, embryo development is rapid. Within 48 hours all major vertebrate body plan features are present, including a compartmentalised brain, eyes, ears and all internal organs. In addition, in comparison to higher vertebrates, zebrafish organs are relatively simple, which greatly facilitates the dissection of their function. (Fishman, 2001; Stern and Zon, 2003). The zebrafish has a second important advantage as a vertebrate model organism. The fish can be easily mutated and inbreeding schemes have been developed to facilitate genetic analysis and gene identification. The generation time of 2-4 months is not short. However, the large number of offspring compensates for this disadvantage, making genetic analysis more readily accessible compared to, for example, the mouse (Kimmel et al., 1995; Stern and Zon, 2003).

1.15. CNS development in zebrafish

The CNS of zebrafish and other vertebrates show an awe-inspiring high degree of structural complexity, consisting of hundreds of different cell types. The formation of the vertebrate nervous system is evolutionarily highly conserved and divided into temporally distinctive phases. First, an epidermal region of the gastrulating embryo is induced to become neuroectoderm and form the neural plate. Second, the neural plate forms the neural tube, providing the structural basis for the subsequent development of the complex three-dimensional organization of the CNS. Third, the neural tube is regionalised to form the forebrain, midbrain, hindbrain and spinal cord with all its distinctive neuronal and non-neuronal cell types positioned appropriately along the anteroposterior (AP) and dorsoventral (DV) axes. Forth, once the neurons are specified, they initiate processes, both axons and dendrites, which extend for some distance to make synaptic connections with appropiate target cells.

Below we will present a brief description of some of the key principles, with importance in the context of this thesis, which have helped us to gain mechanistic insights into the formation, regionalisation and patterning of the CNS.

1.15.1. Neural induction and signalling centres

The neuronal complexity of the CNS arises progressively during its development from the neural plate, an ectodermal epithelium on the dorsal side of the embryo. In a

combination of signalling events and morphogenetic convergence movements the neural plate is transformed into the hollow neural tube (Kimmel et al., 1994; Raible et al., 1992; Schmitz et al., 1993). Following the induction of neural tissue, it must be specified to form different regions along the anteroposterior and dorsoventral axes. This is accomplished by molecular signals eminating from non-neuronal tissues and local regions within the CNS, subsequently, leading to a subdivision of the developing CNS into inceasingly finer domains (for review see Appel, 2000). The signals orchestrating the developmental program of induction and patterning of the CNS are produced by discrete groups of embryonic cells termed signalling centres. The Spemann organiser is one such signalling centre with a pivotal role in the induction of the neuroectoderm and AP patterning. When cells from the organiser are transplanted to the ventral side of an embryo, they induce the formation of a secondary axis, which includes neural tissue derived from the host (Appel, 2000). The organiser is found in the shield of zebrafish and in homologous tissues of amphibians, birds and mammals (Kimmel et al., 1995).

A two-step model has been proposed to explain neural induction and AP patterning (for review see Sasai and De Robertis, 1997). In the first step, the organiser expresses and secretes the two morphogens Chordin and Noggin, which induce the formation of neural tissue from dorsal ectoderm. These two molecules antagonise the action of bone morphogenetic proteins, which are required for promotion of epidermal fate during gastrulation (Wilson and Hammati-Brivanlou, 1995). It has been proposed that the default developmental state of the embryonic neural plate has forebrain characteristics (for review see Moens and Prince, 2002). Consequently, in the second step, the organiser

produces molecules required to posteriorise the neural plate, leading to the specification of posterior CNS regions with distinct posterior identities, including midbrain, hindbrain and spinal cord. A complex set of signalling events, including the FGF, Wnt and retinoic acid signal transduction pathways, have been implicated in this process (reviewed in Moens and Prince, 2002).

The DV patterning of the neural tube is also under the control of signalling centre activities (for review see Tanabe and Jessell, 1996). For example, the notochord, which is positioned adjacent to the prospective ventral neuroectoderm, is the source of the morphogen sonic hedgehog (Shh), which promotes development of ventral neural tube fates. In the spinal cord, high levels of Shh protein, induce the development of motorneurons, whereas lower levels of the protein induce interneurons (reviewed in Lewis and Eisen, 2003).

1.15.2. Neurogenesis and Delta/Notch-mediated lateral inhibition

While long-range inductive signals eminating from signalling centres are important for the specification of distinctive neuroectodermal domains (Woo and Fraser, 1995), short-range local signals from within the neural plate are responsible for the further refinement of this intial neural regionalisation and patterning. Many of the cells in the neural plate have the potential to assume a neural fate and differentiate into neurons, but only a subset of the cells are finally committed to this fate. A key component in this selection mechanism is the cell-cell interactions between the Notch receptor and its ligand Delta (for review see Baron, 2003). In the most simplistic view of this process, termed lateral

inhibition, cells destined to become neural cells express Delta, which interact and activate Notch on neighbouring cells, thereby preventing them from becoming commited to neural differentiation (for review see Artavanis-Tsakonas et al., 1995). For example, neural crest cells (migratory cells giving rise the neurons and glia of the peripherial nervous system, pigment cells and other derivatives) and primary sensory Rohon-Beard (RB) neurons are generated from the same pool of precursor cells present in the lateral neural plate (Haddon et al., 1998; Inoue et al., 1994; Korzh et al., 1993; Raible et al., 1992). In embryos defective in Delta/Notch signalling supernumerary RB cells are formed and a concomitant reduction in the number of neural-crest-derived melanocytes is observed in the the trunk (Appel and Eisen, 1998; Cornell and Eisen, 2000). Thus, in the absence of inhibitory signals the cells adopt a neural fate.

1.15.3. The segmented vertebrate brain

Insects and vertebrates represent organisms with highly distinctive animal body plans and morphologies. Surprisingly, comparative studies have shown that the developmental control genes and their expression patterns, as well as the complex network of interactions of the genes and pathways, are evolutionarily highly conserved between insects and vertebrates (for reviews see Kammermeier and Reichert, 2001; Pires-daSilva and Sommer, 2003). These results suggest that the genetic mechanism responsible for the segmentation of the insect and vertebrate body may share a common origin predating the divergence of the protostome and deuterostome lineages over 600 million years ago (De Robertis and Sasai, 1996; Kimmel, 1996). This idea has received further support by the recent findings in spider of the involvement of the Notch signalling pathway in

segmentation processes (Patel, 2003; Stollewerk et al., 2003). An evolutionarily highly conserved genetic programme has also been shown to be involved in the control of regionalisation and patterning in the embryonic brains of insects and vertebrates (for reviews see Arndt and Nubler-Jung, 1996; Kammermeier and Reichert, 2001). This indicates that the basic architecture of the segmented brains of insects and vertebrates evolved only once during evolution and was already established in our common bilaterian ancestor (Hirth et al., 2003; Rubenstein et al., 1994).

As a consequence of the preservation of the key components of the genetic machinery regulating brain development and segmentation during evolution, the fundamental ground plan for the formation of the vertebrate brain exhibit a strikingly high degree of similarity at the morphological, neuroanatomical and molecular levels among the members of the vertebrate phylum (for reviews see Appel, 2000; Lewis and Eisen, 2003; Moens and Prince, 2002). This implies that results obtained from studying zebrafish brain development may be directly relevant for our understanding of human brain development and organization.

The vertebrate brain is traditionally divided into three major regions: the forebrain (proencephalon), midbrain (mesencephalon) and hindbrain (rhombencephalon). The forebrain is subdivided into the anterior forebrain (telencephalon) and posterior forebrain (diencephalon). The hindbrain is subdivided into 7-8 neural segments termed rhombomeres (**FIG. 1.9**) (Kimmel et al., 1995; Lumsden, 1990). From this simple segmental division of the early embryonic brain all the major components constituting

an adult brain will have developed within 5 days following fertilisation, making this model organism especially useful for dissecting the molecular mechanisms underlying vertebrate brain formation (Kimmel et al., 1995).

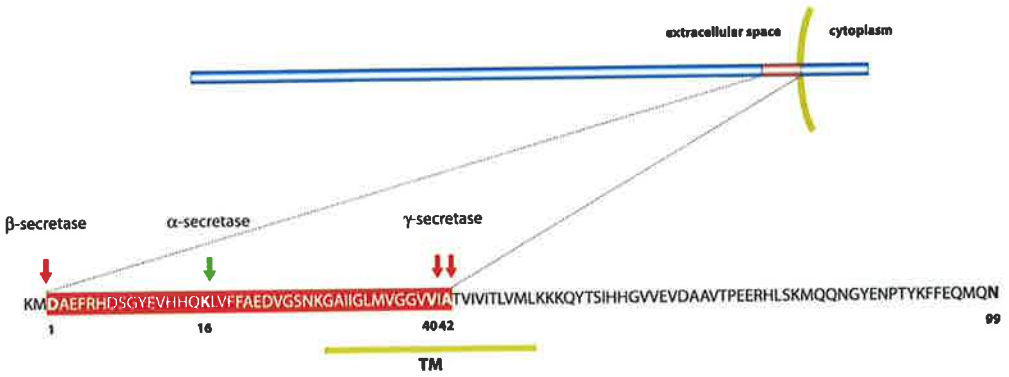
1.16. Aims

Despite the identification of a number genes and risk factors involved in the pathogenesis of AD, a mechanistic understanding of how these factors act together *in vivo* to generate AD pathology remains largely unknown. Recently it has been realised that genes associated with Alzheimer's disease, and other degenerative diseases, also play important functions during embryogenesis. Developmental pathways and networks are evolutionarily highly conserved. This suggests that investigation of the genetic mechanisms controlling developmental processes in various embryonic structures expressing AD-related genes may be a relevant approach in order to gain insight into their normal biological functions, and, ultimately, the cause of neurodegeneration in the adult. This thesis aims to further our understanding of some of the genes and mechanisms involved in AD by studying the function of AD-related genes in embryonic zebrafish. The first part of the thesis describes the isolation and characterisation of the zebrafish orthologues of the human familial AD genes *presenilin 1* and *2,* and *amyloid precursor protein* during early embryogenesis. A new approach to validate the biological side effects of AD drug candidates by looking at the developmental consequences of inhibition of γ-secretase activity in zebrafish embryos is presented. In order to get a thorough understanding of the molecular pathology of AD we need to identify the pathways and networks affected in AD in order to devise stratagies for

prevention and, ultimately, intervention in the progression of the disease. Therefore, in the second part we look beyond the central components in AD pathogenesis and describe the isolation and the analysis of the expression pattern of the zebrafish orthologue of the human gene encoding the FGF signalling cascade component FGFR1 since misregulation of the FGFR1 signalling pathway has been implicated in the AD.

FIGURE 1.1. Cleavage sites in APP subjected to α-, β- and γ-secretase processing. (A) APP processing is initiated by cleavage of the APP ectodomain by either α- or β-secretase. This proteolytic step is followed by γ-secretase-dependent cleavage at position 40/42 (Aβ numbering), leading to the generation of Aβ and p3 peptides, respectively. (B) γ-secretase can also cleave APP at a position C-terminal to position 40/42, resulting in the generation of a small C-terminal fragment (AICD), which may translocate to the nucleus and regulate the transcription of target genes. AICD, APP intracellular domain; TM, transmembrane domain.

A


extracellular space
cytoplasm
β-secretase
α-secretase
γ-secretase
KMDAEFRHDSGYEVHHQKLVFFAEDVGSNKGAIIGLMVGGVVIATVIVITLVMLKKKQYTSIHHGVVEVDAAVTPEERHLSKMQQNGYENPTYKFFEQMQN
1
16
4042
99
TM

B

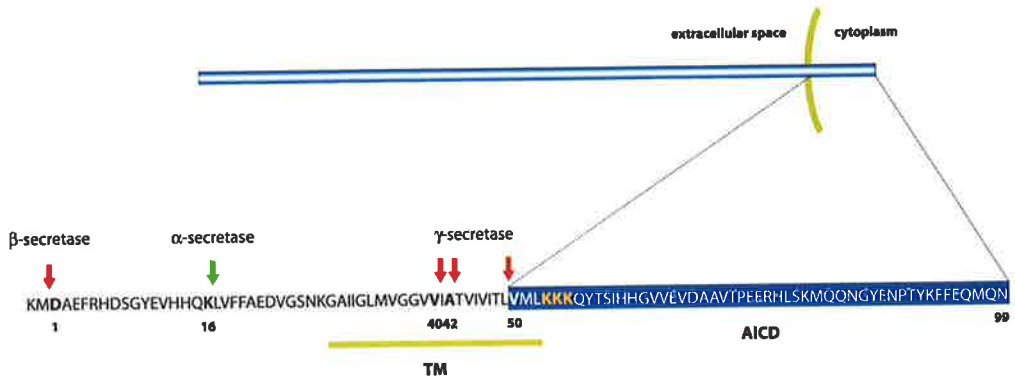
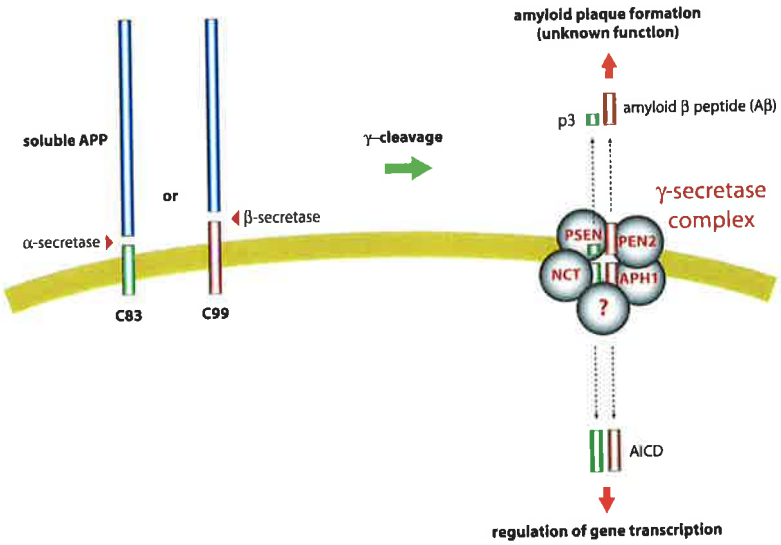

extracellular space
cytoplasm
β-secretase
α-secretase
γ-secretase
KMDAEFRHDSGYEVHHQKLVFFAEDVGSNKGAIIGLMVGGVVIATVIVITLVMLKKKQYTSIHHGVVEVDAAVTPEERHLSKMQQNGYENPTYKFFEQMQN
1
16
4042
50
99
AICD
TM

FIGURE 1.2. APP and Notch are both substrates for γ-secretase-dependent regulated intramembrane cleavage. APP and Notch are both type 1 integral membrane proteins regulated by γ-secretase-dependent proteolysis. A primary cleavage leads to ectodomain shedding. A secondary cleavage occurs within the membrane to liberate the intracellular domain (AICD or NICD) allowing it to translocate to the nucleus and regulate target gene transcription. At the same time a small peptide is released into the extracellular space (p3, Aβ or Nβ). (A) Regulated intramembrane processing of APP. (B) Regulated intramembrane processing of Notch depends on three proteolytic events. S1 cleavage is mediated by a furin-like enzyme, which generates a heterodimer competent to bind its ligands. Ligand binding induces the cleavage of Notch at site 2 (S2) by members of the ADAM family of metalloproteases. This cleavage generates a small membrane-bound fragment termed NEXT. This fragment can be cleaved at the S3 by γ-secretase to release the Notch intracellular domain (NICD) (Selkoe and Kopan, 2003). NEXT, Notch extracellular truncation.

A


amyloid plaque formation
(unknown function)
p3
amyloid β peptide (Aβ)
soluble APP
γ–cleavage
γ-secretase
complex
or
β-secretase
α-secretase
PSEN
PEN2
NCT
APH1
?
C83
C99
AICD
regulation of gene transcription

B

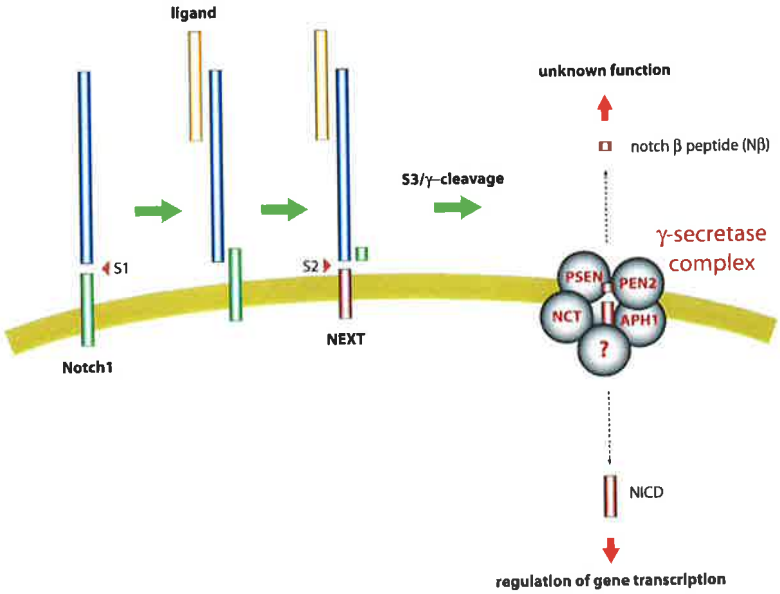

ligand
unknown function
notch β peptide (Nβ)
S3/γ–cleavage
γ-secretase
complex
S1
S2
PSEN
PEN2
NCT
APH1
?
Notch1
NEXT
NICD
regulation of gene transcription

FIGURE 1.3. *APP* is alternatively spliced to generate different APP isoforms. Four major isoforms of APP. APP695 (Kang et al., 1987). APP714 containing a 19 aa insert with homology to the MRC OX-2 antigen (Golde et al., 1990; Weidemann et al., 1989). APP751 containing a 56 aa insert with homology to the Kunitz-type protease inhibitor (KPI) family (Ponte et al., 1988). APP770 containing both OX-2 and KPI inserts (Kitagushi et al., 1988; Tanzi et al., 1988).

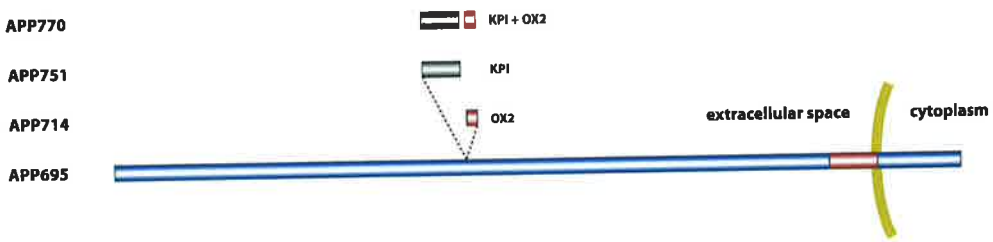

APP770
APP751
APP714
APP695
KPI + OX2
KPI
OX2
extracellular space
cytoplasm

FIGURE 1.4. The amyloid cascade hypothesis. The sequence of pathogenic events associated with AD according to the amyloid cascade hypothesis. Three potential sources have been suggested to contribute to the aggregation and accumulation of Aβ peptides: 1) APP misprocessing, 2) subtle changes in cellular processes implicating polymorphic genetic risk factors (e.g. APOE), and 3) age-related detrimental changes in normal biological processes. Abnormal Aβ peptide accumulation triggers downstream pathological processes leading to toxicity, neuronal death and dementia, and, ultimately, death.

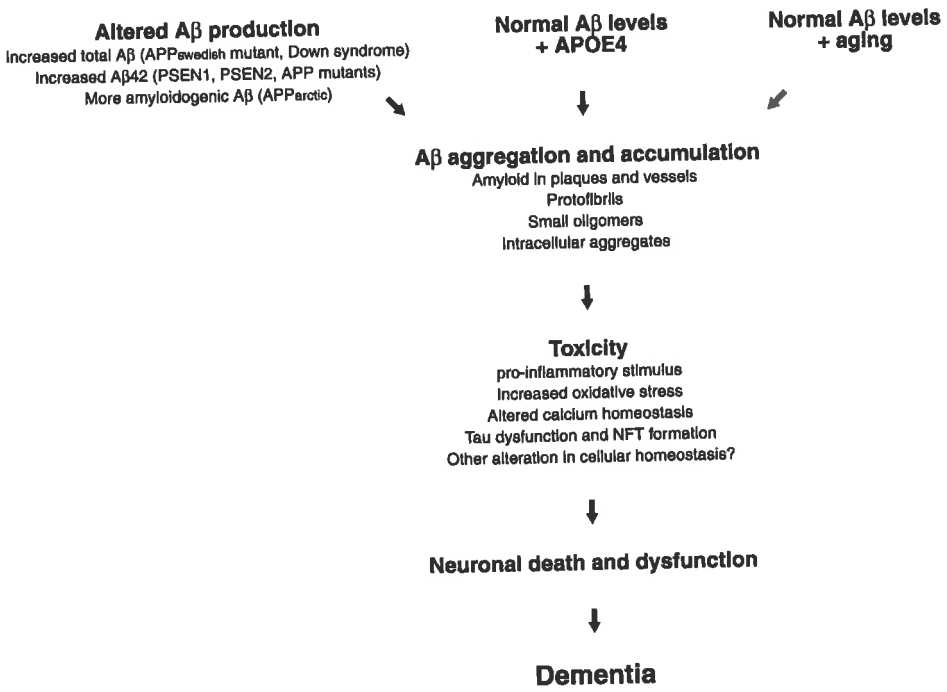

Altered Aβ production
Increased total Aβ (APPswedish mutant, Down syndrome)
Increased Aβ42 (PSEN1, PSEN2, APP mutants)
More amyloidogenic Aβ (APParctic)
Normal Aβ levels + APOE4
Normal Aβ levels + aging
Aβ aggregation and accumulation
Amyloid in plaques and vessels
Protofibrils
Small oligomers
Intracellular aggregates
Toxicity
pro-inflammatory stimulus
Increased oxidative stress
Altered calcium homeostasis
Tau dysfunction and NFT formation
Other alteration in cellular homeostasis?
Neuronal death and dysfunction
Dementia

FIGURE 1.5. Human FGFR1 protein and genomic structure. A) The domain structure of a 3 Ig domain FGFR1 protein is shown (modified from Johnson and Williams, 1993). A type 1 C-tail is shown, which is found in isoforms that have kinase activity. Truncated, kinase-deficient isoforms have been isolated with a different "type 2" C-tail. For further detail on domain structure see the text. B) The first exon encodes a non-translated region, and exons 8 and 9 encode the alternatively spliced C-terminal half of IgIII. Domain sizes shown are not to scale.

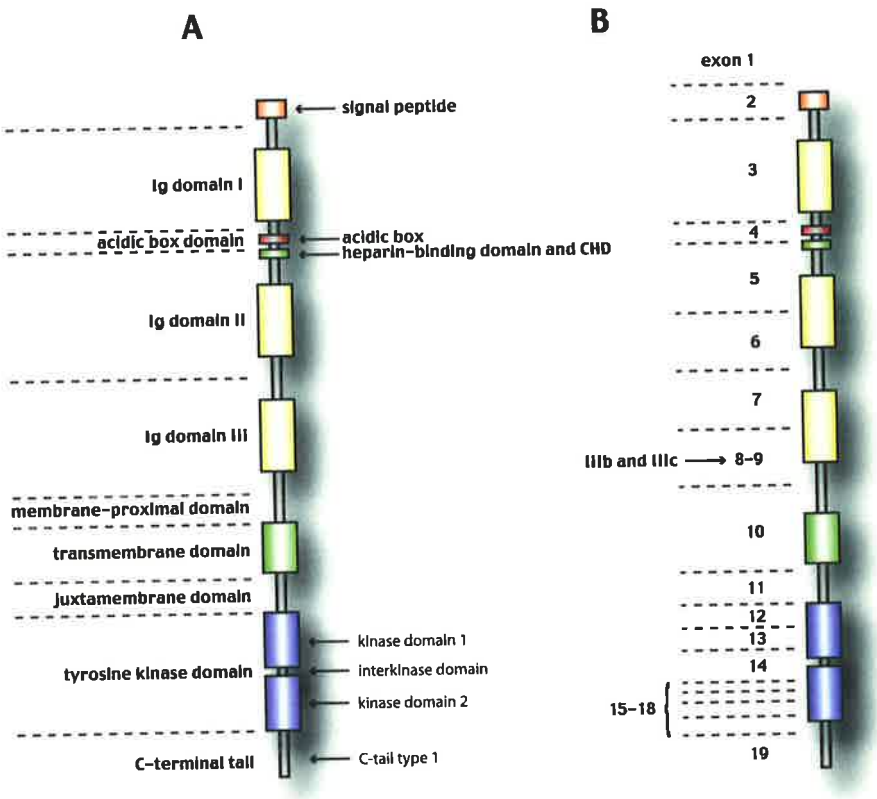

A
signal peptide
Ig domain I
acidic box domain
acidic box
heparin-binding domain and CHD
Ig domain II
Ig domain III
membrane-proximal domain
transmembrane domain
juxtamembrane domain
tyrosine kinase domain
kinase domain 1
interkinase domain
kinase domain 2
C-terminal tail
C-tail type 1
B
exon 1
2
3
4
5
6
7
IIIb and IIIc
8-9
10
11
12
13
14
15-18
19

FIGURE 1.6. FGFR1 isoform structures. Alternative splicing of the *fgfr1* primary transcript leads to the generation of multiple transcripts encoding numerous receptor isoforms. Isoforms 1-4 are soluble receptors. 5-12 are transmembrane variants. Isoforms 11 and 12 are transmembrane proteins but are truncated in the kinase domain and therefore represent kinase-deficient isoforms. Alternative sequences are indicated by the colour code above the figures. The various isoforms were compiled from the following sources: (1) (Eisemann et al., 1991), (2) (Werner et al., 1992), (3) (Johnson et al., 1990; Werner et al., 1992), (4) (see Chapter 5), (5) (Johnson and Williams, 1993), (6) (Beer et al., 2000), (7) (Dionne et al., 1990; Eisemann et al., 1991; Reid et al., 1990), (8) (Eisemann et al., 1991; Johnson et al., 1990; Reid et al., 1990), (9) (Eisemann et al., 1991; Gillespie et al., 1995; Johnson et al., 1990; Reid et al., 1990), (10) (Lopez and Korc, 2000), (11) (Wang et al., 1996), (12) (Hou et al., 1991). RM, arginine and methionine dipeptide; VT, valine and threonine dipeptide.

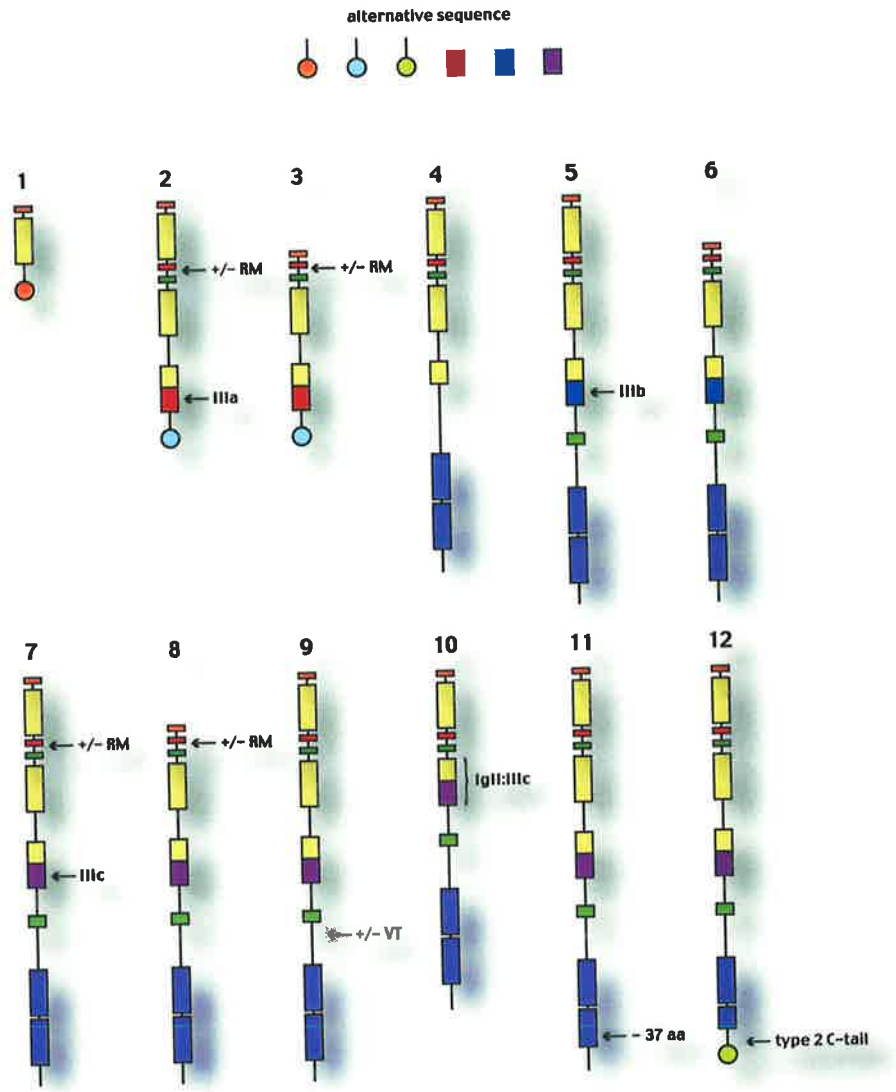

alternative sequence
1
2
+/- RM
IIIa
3
+/- RM
4
5
IIIb
6
7
+/- RM
IIIc
8
+/- RM
9
+/- VT
10
IgII:IIIc
11
- 37 aa
12
type 2 C-tail

FIGURE 1.7. Putative FGF- and CAM-mediated FGFR1 activation complexes. Signal transduction through FGFR1 can be activated by interaction with various ligands and/or extracellular matix components. A) HSPG facilitated binding of FGFs to FGFR1 leads to receptor activation. B) The receptor may also be activated through interactions with CAM molecules.

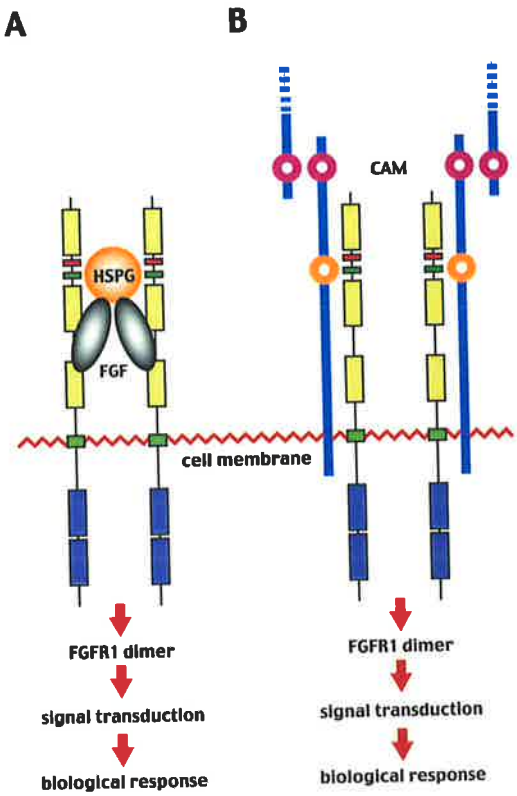

A
B
CAM
HSPG
FGF
cell membrane
FGFR1 dimer
signal transduction
biological response
FGFR1 dimer
signal transduction
biological response

FIGURE 1.8. Intracellular signalling pathways activated by FGFR1. Two major signal transduction pathways, Ras/MAPK and PLCγ, are shown. The positions of the potential phosphotyrosine sites in human FGFR1 are indicated. FGFR1 signalling may activate the Notch signalling pathway via the Ras/MAPK cascade or, possibly, via a direct interaction of nuclear FGF/FGFR1 complexes.

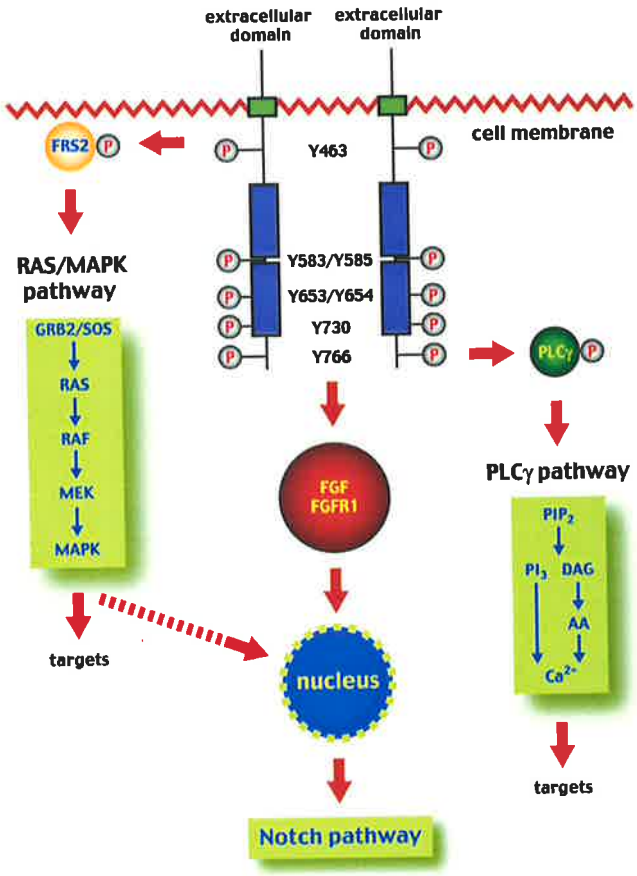
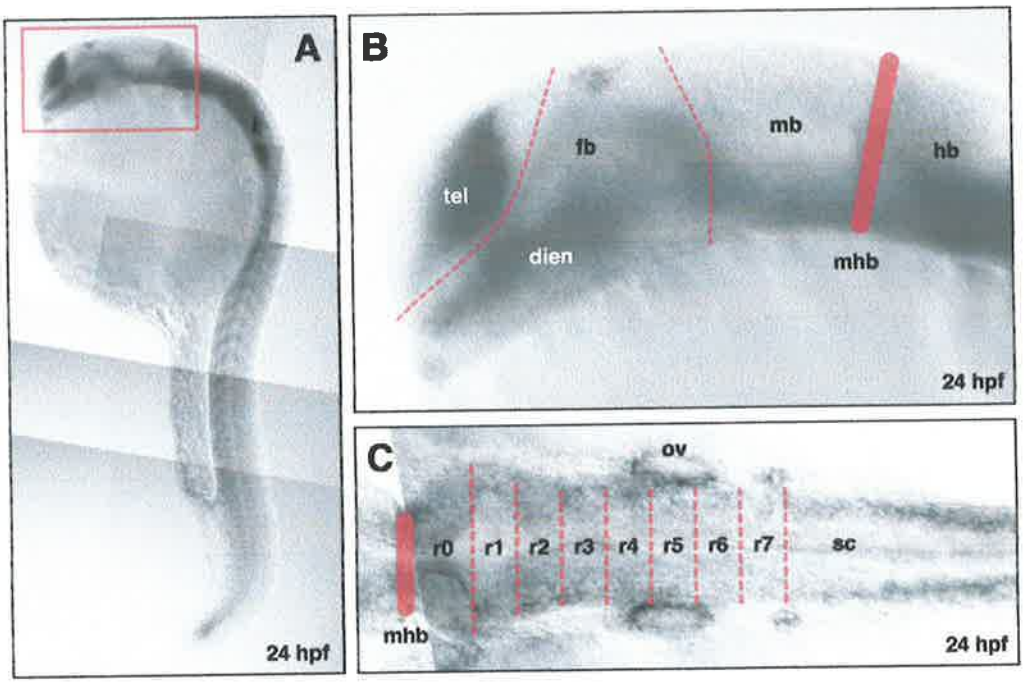

extracellular domain
extracellular domain
cell membrane
FRS2
P
Y463
Y583/Y585
Y653/Y654
Y730
Y766
RAS/MAPK pathway
GRB2/SOS
RAS
RAF
MEK
MAPK
targets
FGF
FGFR1
nucleus
Notch pathway
PLCγ
PLCγ pathway
PIP2
PI3
DAG
AA
Ca2+
targets

FIGURE 1.9. Division of the embryonic zebrafish brain. (A) Dorsal view of embryo at 24 hpf. (B) Magnification of boxed region in (A) showing its division into the three major brain regions: forebrain, midbrain and hindbrain. (C) Lateral view of embryo in (A) showing the division of the hindbrain into 8 rhombomeric segments. dien, diencephalon; fb, forebrain; hb, hindbrain; mb, midbrain; mhb, midbrain-hindbrain boundary; ov, otic vesicle; r, rhombomere; sp, spinal cord; tel, telencephalon.


A
24 hpf
B
fb
mb
hb
tel
dien
mhb
24 hpf
C
ov
r0
r1
r2
r3
r4
r5
r6
r7
sc
mhb
24 hpf

Chapter 2

Overexpression of human Alzheimer's disease presenilin1 mutants in zebrafish embryos does not cause severe disruption of embryogenesis

2.1. ABSTRACT

Presenilins **play prominent roles in the molecular pathogenesis of Alzheimer's disease (AD) and during embryo development. A recent study reported the cloning of zebrafish *presenilin1* (*psen1*). We have now identified zebrafish *psen2*. Analysis of their embryonic expression patterns show that both genes are expressed in overlapping domains in the brain and eyes. Abundant and distinct *psen1* expression is found in the otic vesicles and primordial germ cells while *psen2* shows unique expression in the neural crest and neural-crest-derived melanocytes. Thus, our results suggest both overlapping and distinct functions for the *presenilins* in zebrafish development. To analyse whether mutations in *PSEN1* associated with Alzheimer's disease alter the functions of *PSEN1* during embryogenesis we overexpressed human wild-type and mutant *PSEN1* in zebrafish embryos. Our results indicate that pathogenic *PSEN1* mutations do not significantly affect the developmental roles of *PSEN1* during embryogenesis.**

2.2. INTRODUCTION

Alzheimer's disease (AD) is a common, devastating neurodegenerative disorder of the brain. Accumulation of different forms of β-amyloid peptide (Aβ) in extracellular plaques is an early step in the pathogenesis of AD. Dominant mutations in the genes encoding amyloid precursor protein (APP), and the Presenilins (PSEN1 and PSEN2) have been suggested to cause early-onset familial AD (FAD) by altering the proteolytic processing of APP, resulting in the overproduction of the highly amyloidogenic 42-residue form of Aβ (Selkoe, 1996; Selkoe and Wolfe, 2000). Presenilins have also been linked to the control of apoptosis. Overexpression of Presenilin2 was shown to drive apoptosis in rat pheochromocytoma derived (PC12) cells and overexpression of a mutated FAD-associated PS2 enhanced apoptosis in these cells. This suggests that

overexpression of Presenilins may be part of the pathological processes leading to neuronal death in observed FAD cases (Wolozin et al., 1996). *Presenilin* genes are ubiquitously expressed in mammalian embryonic and adult tissues, including the developing and adult brain (Berezovska et al., 1999; Kovacs et al., 1996; Lee et al., 1996). During embryo development they have been shown to have important functions during somite formation and neurogenesis (Handler et al., 2000; Wong et al., 1997). Interactions between Presenilins and Notch transmembrane receptor proteins, that control numerous signal transduction pathways and mediate embryonic cell fate decisions in both invertebrates and vertebrates (Greenwald, 1998), appear to be central for the regulation of these Presenilin-dependent developmental processes (Donoviel et al., 1999; Handler et al., 2000; Lee et al., 1996; Shen et al., 1997; Steiner et al., 1999; Wong et al., 1997).

The Presenilins are highly conserved hydrophobic multipass transmembrane (TM) proteins. Previous analyses suggest that they possess eight TM domains (Li and Greenwald, 1998). They are found predominately in the endoplasmic reticulum and Golgi apparatus as cleaved heterodimers, which constitute the biologically active form of the proteins (De Strooper et al., 1997; Kovacs et al., 1996; Ratovitski et al., 1997). Presenilins have also been detected in the plasma membrane, in the nuclear envelope and in centrosomes and kinetochores (Li et al., 1997; Petanceska et al., 2000; Ray et al., 1999; Schwarzman et al., 1999). One *presenilin* gene (*Dps*) has been identified in *Drosophila*, whereas two orthologues have been found in *Caenorhabditis elegans* (*Sel-12* and *Hop-1*), zebrafish (*zf-PS1* here called *psen1,* (Leimer et al., 1999) and, *psen2*, this

study) and mammals (*PSEN1* and *PSEN2*), respectively (for reviews see Chan and Jan, 1999; Fraser et al., 2000).

We have cloned the zebrafish *presenilins* and characterised their expression patterns. Furthermore we demonstrate that pathogenetic *PSEN1* mutations associated with familial Alzheimer's disease when overexpressed in zebrafish embryos during early development do not cause an increased rate of abnormality relative to overexpression of normal *PSEN1*.

2.3. MATERIALS AND METHODS

2.3.1. Zebrafish *presenilin* cDNA

A full-length zebrafish *psen1* cDNA was isolated by RT-PCR using primers based on the published cDNA sequence (GenBank accession number AJ132931), and used as a hybridisation probe to screen cDNA sublibraries (Lardelli, 1997) made from a 9-16 hours post fertilisation (hpf) embryonic randomly primed zebrafish cDNA library in the lambda-ZAP vector (kindly provided by R. Riggleman, K. Helde and D. Grunwald). PCR components were: 0.5 μM primer, 0.25 mM dNTP, standard PCR buffer (final concentrations were: 50 mM KCl; 1.5 mM $MgCl_2$; 10 mM Tris-HCl, pH 8.3), 1 Unit *Taq* polymerase. Primers used were: forward, zfPS1001F, 5'-GAGTTCCCGCT GTTTCTATGATCC-3' and reverse, zfPS1002R, 5'-GAGATGTGCTCTGCGTTCTCT CC-3'. PCR conditions were: 94 °C for 30 sec, 67 °C for 45 sec, 0.5 °C/sec to 72 °C, 72 °C for 3 min, 35 cycles. The *psen1* PCR fragment containing the entire open reading

frame of *psen1* was isolated and used as a probe to isolate a *psen1* cDNA clone (in pBSSK) from a positive sublibrary. Sequencing confirmed that the clone included the entire open reading frame of *psen1*. The cDNA of zebrafish *psen2* in pBSSK was kindly provided by M. Lardelli. The sequence of this cDNA has been deposited in GenBank (accession number AJ271795).

2.3.2. Embryos and whole mount *in situ* transcript hybridisation

Zebrafish embryos were staged according to (Kimmel et al., 1995). Stages are given as equivalent to hours of development post fertilisation (hpf) at 28.5 °C. The transcript expression patterns of zebrafish *psen1* and *psen2* were analysed by whole mount *in situ* transcript hybridisation on fixed zebrafish embryos at various stages of development up to 24 hpf as previously described (Kimmel et al., 1995). In order to make antisense *psen1* and *psen2* riboprobes we amplified by PCR the *psen1* and *psen2* inserts from their respective vectors using M13 (-20): 5'-GTAAAACGACGGCCAGT-3' and M13 reverse: 5'-GGAAACAGCTATGACCATG-3' primers (Stratagene, USA). The PCR conditions were: 94 °C for 30 sec, 52 °C for 45 sec, 0.5 °C/sec to 72 °C, 72 °C for 3 min, 35 cycles. The PCR fragments were subsequently isolated using the QIAquick Gel Extraction Kit (Qiagen, USA). The riboprobes were transcribed using T3 RNA polymerase and the Digoxigenin (DIG) RNA labelling Kit (Boehringer Mannheim, Germany). Unincorporated nucleotides were removed using RNase-free Micro Bio-Spin Columns P-30 (BIO-RAD, USA) or Nanosep 500 devices (Pall Filtron, USA).

2.3.3. Sequence and phylogenetic analysis of Psen1 and Psen2

Human and zebrafish Presenilin protein sequences were aligned using Clustal W (Thompson et al., 1994) and prepared for publication using Boxshade by Hofmann K. and Baron M.D. and BioManager by ANGIS (Australian National Genomic Information Service). The evolutionary relationship between Presenilin protein sequences was analysed using the Phylip phylogeny inference package (Felsenstein, 1989) and BioNavigator by ANGIS. The distance measure for the protein sequences was computed using the BLOSUM62 matrix and the Neighbor-Joining method was used to generate the phylogenetic tree. The tree was prepared for publication using Phylodendron by Gilbert D.G. and BioManager by ANGIS. GenBank accession numbers: Human PSEN1 (L42110), mouse PSEN1 (AF149111), *Xenopus PSα* (D84427), zebrafish Psen1 (AJ132931), human PSEN2 (L44577), mouse PSEN2 (AF038935), *Xenopus* PSβ (D84428) and zebrafish Psen2 (this study). *Drosophila melanogaster* DPS (U77934) was chosen as the outgroup.

2.3.4. Overexpression of human *PSEN1* during zebrafish embryo development

The human wild-type *PSEN1*, the Alzheimer's disease mutant *PSEN1* M146I and the artificial *PSEN1* mutant M146R expression constructs, cloned into pCS2+, were kindly provided by Arne Lund Jorgensen (Aarhus University, Denmark). RNA for injections was prepared in duplicate for each of the three different human *PSEN1* cDNAs using the mMessage mMachine SP6 Kit (Ambion, USA). The *PSEN1* RNAs were injected into 1-cell stage embryos, at a concentration of 100 nanogram per microlitre, and the

morphology of live embryos were examined at 12 hpf. Only embryos with no obvious unilateral morphological defects were included in the analysis. This was done to ensure that the effects of RNA overexpression analysed are the result of global distribution of RNA and translated protein rather than a local phenomenon caused by the restriction and concentration of RNA into a limited number of cells early in development. All injections and subsequent morphological analyses were carried out double blind. For the purpose of the statistical analysis we divided the embryos into two categories: normal phenotype at 12 hpf (normal) and defective embryo development at 12 hpf (defects). The latter category includes embryos with cell adhesion defects, localised necrosis and body axis defects. We used the injection of wild-type *PSEN1* RNA as a baseline and compared the distribution of the two categories, normal and defects, between wild-type and mutant *PSEN1* constructs. To determine if the distribution of the two morphological categories were significantly different between embryos injected with wild-type and mutant *PSEN1* RNAs we used the contingency χ^2 test.

2.4. RESULTS

2.4.1. Sequence analysis of zebrafish Psen1

Human and zebrafish are distantly related species, belonging to vertebrate lineages that diverged from the last common ancestor 400 million years ago (Carroll, 1988). Given the time since the divergence of the human and zebrafish lineages conservation of specific regions in Presenilin homologs may reflect regions of functional importance. We therefore compared the zebrafish Psen1 sequence with human PSEN1 and PSEN2 in

order to identify putative regions important for PSEN1 function. Psen1 and human PSEN1 are 74% identical at the amino acid level, whereas zebrafish Psen1 is 67% identical to human PSEN2 (**FIG. 2.1**). Differences in the protein sequences of zebrafish Psen1 and human PSEN1 and PSEN2 are predominantly found in the N-terminal region and the C-terminal half of the loop domain. Disregarding these two highly variable regions amino acid sequences between zebrafish and human Presenilins are very similar, implicating functional importance of these regions.

2.4.2. Expression of *psen1* in zebrafish

The biochemical aspects of zebrafish Psen1 processing and degradation and its role in APP metabolism have been characterised (Leimer et al., 1999). However, the expression of zebrafish *psen1* during embryo development has only received limited attention. We therefore decided to perform a detailed analysis of *psen1* expression during zebrafish embryogenesis. The developmental expression pattern of zebrafish *psen1* was analysed by whole mount *in situ* RNA transcript hybridisation (**FIG. 2.2**) (Jowett, 1997). Staining of *psen1* was evident in blastomeres, demonstrating maternal inheritance of *psen1* RNA transcripts (**FIG. 2.2A**). *psen1* expression was found to be ubiquitously expressed until 16 hpf (**FIG. 2.2A-E**). Especially the region of the prospective brain showed prominent staining, but weak *psen1* expression was also present in the otic placodes (**FIG. 2.2D**). At 24 hpf elevated levels of *psen1* mRNA was detected in the brain, eyes and otic vesicles (**FIG. 2.2F, G**). Distinctive *psen1* staining was present in the primordial germ cells at 24 hpf (**FIG. 2.2H, I**) consistent with observations in rodents (Dirami et al., 2001; Nilsberth et al., 1999; Suzuki et al., 1996; Yamaguchi et al., 2000).

2.4.3. Sequence and phylogenetic analysis of zebrafish Psen2

Putative protein sequence comparison of zebrafish Psen2 to known vertebrate Presenilins revealed 74% amino acid residue (aa) identity to human PSEN2 (**FIG. 2.3**), 69% aa identity to human PSEN1 and 70% aa identity to zebrafish Presenilin Psen1 (Leimer et al., 1999), suggesting that the cDNA represents an orthologue of human *PSEN2*. To investigate this in more detail we performed a phylogenetic analysis using various metazoan Presenilin protein sequences (**FIG. 2.4**). The resultant phylogenetic tree shows that our translated zebrafish *presenilin* cDNA confidently groups with other PSEN2 orthologues, whereas zebrafish Psen1 groups with PSEN1 orthologues. This demonstrates that the zebrafish genome includes at least two *presenilin* genes, a *PSEN2*-like gene (this study) which we have named *psen2* and a *PSEN1*-like gene, *psen1* (Leimer et al., 1999). The *psen2* gene encodes a protein of 441 amino acids with a predicted molecular weight of approximately 49 kDa. Kyte-Dolittle hydrophobicity plots of Psen2 and human PS2 are very similar (not shown), indicating that zebrafish Psen2 and human PSEN2 share similar membrane topology (Li and Greenwald, 1998). This suggests that Psen2 contains eight transmembrane domains with a comparatively large cytoplasmic loop domain between TM domain 6 (TM6) and TM7 (**FIG. 2.3**). Alignment of the zebrafish Psen2 and human PSEN2 protein sequences shows that the primary structure is highly conserved between them with only the N-terminal region and the C-terminal half of the cytoplasmic loop domain being highly variable (**FIG. 2.3**). A putative endoproteolytic site between amino acid residues 297 and 298 is indicated based on similarity to the principal processing site of human PSEN2 (Jacobsen et al., 1999).

Aspartate residues 262 in TM6 and 360 in TM7 are found at positions, which have been shown to be conserved in all Presenilins and to be obligatory for their postulated intramembrane proteolytic activity (Kimberly et al., 2000; Leimer et al., 1999).

2.4.4. Expression of *psen2* in zebrafish

Analysis of the pattern of transcription of *psen2* by whole mount *in situ* transcript hybridisation (**FIG. 2.5**) using antisense digoxigenin-labelled RNA probes (Jowett, 1997) showed that *psen2* expression is ubiquitous during early embryogenesis, but becomes more restricted by 24 hpf (hours post fertilisation at 28.5°C). A sense probe did not give any staining (not shown). Maternally derived *psen2* mRNA is present in cleavage stage blastomeres, (i.e. prior to the onset of zygotic genome transcription, **FIG. 2.5A, B**). The concentration of *psen2* transcript is visibly reduced by the onset of gastrulation at 6 hpf (**FIG. 2.5C**). Interestingly, we could not detect Psen2 protein by Western blotting during these early developmental stages (**FIG. 2.6** was kindly provided by S. Nornes). This suggests that Psen2 activity during these stages may be restricted by translational repression or rapid protein degradation. However, we observed increased levels of *psen2* transcripts and the presence of Psen2 protein at 12 hpf (**FIG. 2.5D; 2.6**). Interestingly, an antibody developed against the N-terminal end of zebrafish Psen2 recognised primarily the uncleaved holoprotein whereas the proteolytic N-terminal fragment of Psen2 (NTF) was a minor species (**FIG. 2.6**; S. Nornes, personal communication). This conflicts with results from cell culture studies demonstrating that the holoprotein is rapidly cleaved into two enzymatic active proteolytic N- and C-terminal fragments (Thinakaran et al., 1998). *psen2* transcription is, apparently, ubiquitous during early somitogenesis (**FIG.**

2.5D, E) but, by 24 hpf, shows variation in different tissues. For example, we could not detect *psen2* transcript in the notochord or tailfin primordium at 24 hpf (FIG. 2.5F, H), while relatively high levels could be detected in the anterior developing central nervous system (CNS) and the eye (FIG. 2.5H, K), the midline of the developing spinal cord (FIG. 2.5I) and in the cells derived from the neural crest (FIG. 2.5F, I). These cells include the pigment-containing melanocytes (FIG. 2.5G, J) although we could not determine whether high-level *psen2* transcription is restricted only to this cell type.

2.4.5. Overexpression of human *PSEN1* during zebrafish embryo development

PSEN1 mutations are the most common cause for familial Alzheimer's disease, with more than 50 mutations described so far (Czech et al., 2000; Fraser et al., 2000). Substitution of methionine 146 of the PSEN1 protein for valine, leucine or isoleucine leads to Alzheimer's disease with an age of onset in the late thirties to early forties (Group, 1995; Jorgensen et al., 1996; Sherrington et al., 1995). All three missense mutations in methionine codon 146 lead to a conservative amino acid substitution of one hydrophobic amino acid with another. The fact that no mutations have been reported leading to a replacement of the hydrophobic methionine 146 with a hydrophilic amino acid raises the possibility that a non-conservative amino acid substitution at this codon position would severely affect the function of the protein. PSEN1 has been shown to play an important function during development (Shen et al., 1997; Wong et al., 1997). Thus major distortion of its protein structure by non-conservative substitutions at critical codon positions may be prohibitive and lead to embryonic lethality. We wanted to test this hypothesis and therefore overexpressed human wild-type *PSEN1*, the Alzheimer's

disease *PSEN1* M146I mutant and the artificial *PSEN1* M146R mutant during zebrafish embryo development. Results from cell culture studies have shown that overexpression of exogenous PSEN1 or PSEN2 proteins lead to the replacement of all endogenous PSEN1 or PSEN2 proteins (Thinakaran et al., 1997). The mechanism responsible for this phenomenon is unknown, but may involve competition between newly produced presenilin proteins for limiting cellular factors, resulting in the stabilisation of the excessively expressed exogenous presenilin proteins and the concomitant degradation of endogenous proteins (Thinakaran et al., 1997). Consequently, we expected that overexpression of human PSEN1 in zebrafish embryos would lead to the replacement of endogenous zebrafish presenilin proteins, thus allowing the analysis of the effect of introducing mutant human PSEN1 proteins during zebrafish embryonic development.

RNA injection into developing zebrafish embryos normally produces moderate levels of non-specific perturbations. Therefore, we expected to see a certain degree of variability in the distribution of normal and affected embryos as the result of the two RNA injections of each *PSEN1* construct. However, statistical analysis showed no significant difference in the outcome of the RNA injections ($0.10 = P = 0.20$) (not shown), demonstrating that each specific RNA injection affects zebrafish embryogenesis in a reproducible manner.

Overexpression of the human *PSEN1* constructs caused similar phenotypic effects upon examination of zebrafish embryos at 12 hpf. 30-50% of injected embryos, irrespective of type of *PSEN1* construct injected, showed some degree of developmental aberrations,

including deformed head, localised cell-death in the head region and split axis. However, the predominant developmental defects (70-90% of affected embryos) included various degrees of reduced cell adhesion, mainly in the head region (**FIG. 2.7**), which in fewer cases also extended to affect the whole body. Apparently, the majority of the cell-adhesion defects were non-lethal and transient as most embryos survived to 24 hpf, showing normal phenotype. Some of the embryos, which showed severe local cell-aggregation and -detachment from the main body during the segmentation stage (**FIG. 2.7**) were missing these areas at 24 hpf but looked otherwise normal (not shown). Overexpression of the unrelated zebrafish *amyloid precursor protein a* (*appa*) did not cause any developmental abnormalities at 12 hpf (not shown), indicating that the observed phenotypic effects are specific to *PSEN1* overexpression rather than a non-specific phenomenon due to excessive amount of RNA in the embryo following injection.

We have shown that overexpression of wild-type human and mutant *PSEN1* in embryonic zebrafish perturb development (see above). To answer the question whether the injection of the different human *PSEN1* constructs affect embryo development with different severity (observed at the gross morphological level) we performed contingency χ^2 tests on the morphological data compiled (**TABLE 2.1**). Injection of wild-type *PSEN1* compared to mutant *PSEN1* (M146I or M146R) did not cause significant different distribution of normal and defective phenotypes, $0.20 = P = 0.30$ and $0.10 = P = 0.20$, respectively. This indicates that the mutant PSEN1 proteins do not cause enhanced

susceptibility to developmental disruptions, compared to overexpressed normal PSEN1 protein, as a consequence of modifications of normal presenilin function.

2.5. DISCUSSION

2.5.1. Zebrafish *presenilins* have both overlapping and distinct expression domains during embryogenesis

Previously, a human *PSEN1*-like zebrafish gene was isolated (zebrafish *psen1*) (Leimer et al., 1999). In this study we report the isolation of a second *presenilin* gene in zebrafish with similarity to human *PSEN2* (zebrafish *psen2*) (Groth et al., 2002). Thus, like other vertebrates, zebrafish possesses two different genes encoding highly similar proteins (Groth et al., 2002). Whereas significant efforts have been devoted to study their roles in the pathophysiological processes leading to Alzheimer's disease, their normal biological functions during development and in the adult are still largely unknown (Fraser et al., 2000; St George-Hyslop, 2000). We have performed a detailed analysis of the embryonic expression patterns of the zebrafish *presenilin* genes to provide a basis for further studies of their developmental functions. Both genes were ubiquitously expressed at high levels in the brain as well as in distinct regions in the rest of the developing embryo (**FIG. 2.2; 2.5**). Whole mount *in situ* RNA transcript hybridisation indicated that the level of *psen2* mRNA was considerable lower than that of *psen1* mRNA, as the detection of *psen2* transcripts in embryos were only evident after an extended period of staining. Both genes were widely expressed in the paraxial mesoderm during the segmentation stage, consistent with an important function in somite formation (**FIG. 2.2;**

2.5). This is supported by results showing that inhibition of all Presenilin-dependent γ-secretase activities in zebrafish lead to interference of segmentation (**see CHAPTER 3**). Transcripts of both *presenilins* were found widely expressed in the brain at increased levels, consistent with essential roles during zebrafish brain development (**FIG. 2.2; 2.5**). Outside the brain, *psen1* transcripts were abundant in the eyes, the otic placodes and in the primordial germ cells (**FIG. 2.2F, H, I**). Abundant expression of *psen2* in non-neuronal tissues was detected in the eyes, neural crest cells and neural-crest-derived melanocytes (**FIG. 2.5F-K**). We found that both presenilins were ubiquitously expressed in the neural plate during the segmentation stage where they may participate in the regulation of cell fate decisions under the control of the Notch pathway, for example, neural crest specification (**see CHAPTER 3**). However, at 24 hpf, *psen2* was the only *presenilin* found to be expressed in the dorsal neural crest cells and migrating melanocytes (**FIG. 2.5F, G, H-K**). To our knowledge this is the first study to demonstrate that a *presenilin* is specifically expressed in neural crest cells, and in migrating and differentiating neural-crest-derived melanocytes during later stages of development (**FIG. 2.5F, G, H-K**). It will be interesting to learn about the specific role of *psen2* in neural crest and melanocyte development. High levels of presenilin1 mRNA and protein expression have been reported in the developing and adult testis in mouse and rat (Dirami et al., 2001; Nilsberth et al., 1999; Suzuki et al., 1996; Yamaguchi et al., 2000), suggesting that *presenilin1* may play an important role in testis morphogenesis and spermatogenesis in adulthood. The abundant expression of *psen1* transcripts in the primordial germ cells of presumptive male zebrafish may indicate a similar function of *psen1* in gonad development in fish. In conclusion, the *presenilins* were expressed in the brain and in

distinct peripheral tissues at high level, suggesting that they may have diverse functions during organogenesis in addition to their pivotal roles in brain development.

2.5.2. Overexpression of human wild-type or mutant *PSEN1* in zebrafish embryos has similar effects on development

The molecular mechanisms leading to neurogeneration and the manifestation of familial Alzheimer's disease remains unclear. However, in the case of the Presenilins, the mutations associated with Alzheimer's disease are predominantly located within or adjacent to the TM domains, suggesting that distortion of the precise conformation of the molecule within the membrane, may cause slight changes to normal Presenilin protein function (Selkoe, 2002). Over time, these minor functional changes may lead to aberrant APP processing and generation of detrimental amyloid plaques (Hardy and Selkoe, 2002). The deleterious changes in the biological activities of Presenilin could be the result of mutations leading to loss-of-function or, alternatively, gain-of-function. The first idea has gained support from studies of *C. elegans* development. Wild-type human *PSEN1* was shown to complement an egg-laying defect in *C. elegans* lacking the *PSEN1*-like *sel-12*, while familial-AD-linked *PSEN1* variants exhibited a reduced rescue activity (Baumeister et al., 1997; Levitan et al., 1996). The second proposition was advanced as a result of experiments with *PSEN1*-deficient mice. Human wild-type *PSEN1* and mutant *PSEN1* was shown to rescue the developmental phenotypes of *PSEN1*-null mouse embryos (Davis et al., 1998; Qian et al., 1998). This obvious dichotomy in rescue activity in *C. elegans* and mice is difficult to resolve. However, the possibility remains that the differential effects on development are the consequence of

variable functional roles of the *presenilins* in regulation of distinct developmental processes. In terms of AD pathogenesis, the fact that all *presenilin*-mediated familial AD mutations are missense mutations, except for two exon-skipping mutations, suggests that major structural restraints exist to prevent the loss of complete protein function (Czech et al., 2000; Fraser et al., 2000). Thus it appears that the AD phenotype results from gain-of-function mutations. We overexpressed human wild-type and mutant *PSEN1* in zebrafish to further explore the role of *presenilin1* during embryogenesis.

Overexpression of PSEN1 proteins caused a transient loss-of-cell-adhesion phenotype in the cranial region of the developing embryo (**FIG. 2.7**). Presenilin1 has been shown to be an integral component of cadherin/catenin cell adhesion complexes (Baki et al., 2001; Georgakopoulos et al., 1999; Singh et al., 2001). *In vitro* overexpression of *PSEN1* in cell cultures promotes the assembly of cadherin/catenin cell-adhesion complexes and enhances cell-cell adhesion. Our observation of a transient reduction in intercellular adhesion *in vivo* is obscure and warrants further investigations.

In cell culture experiments the overexpression of exogeneous PSEN1 or PSEN2 leads to replacement of endogenous presenilin proteins, but the steady-state level of the proteins are apprarently preserved (Thinakaran et al., 1997). Our observation that both wild-type and mutant human PSEN1 overexpression result in similar developmental aberrations suggests that the steady-state level of presenilin proteins is elevated in favour of the excessively expressed PSEN1 species. Indeed, it has recently been shown that overexpression of zebrafish Psen2 in developing embryos leads to an increased level of

Psen2 protein without reducing the Psen1 protein level (S. Nornes, personal communication). This may suggest that the described presenilin replacement mechanism is a cell culture specific artefact. Alternatively, the precisely regulated and coordinated expression of presenilins could be relaxed during early zebrafish embryogenesis and only operative during later stages of the zebrafish life cycle. The overexpression of human PSEN1 RNA in zebrafish embryos represents initial experimental trials. Although the observation of developmental malformations, following human PSEN1 RNA injections, may be interpreted as indirect evidence that human PSEN1 protein is expressed in these embryos, this needs to be shown. This can be achieved by Western analysis using an antibody specific for human PSEN1. Analysis of presenilin functions can also be studied by the inhibition of RNA translation using morpholino oligonucleotides (Nasevicius and Ekker, 2000). The mRNA sequences of the zebrafish *presenilin* orthologues, including the 5' UTR regions, have been deposited in the sequence databases making *presenilin* 'knock down' experiments seem highly desirable as next steps in our analysis of their functions.

FIGURE 2.1. Sequence alignment of zebrafish Psen1 to human PSEN1 and PSEN2 proteins. (A) zebrafish Psen1 versus human PSEN1. (B) zebrafish Psen1 versus human PSEN2. Identical amino acids are shaded in gray and gaps that were introduced for optimal alignment are indicated by dashes. Bars indicate the putative transmembrane domains I, II, VI and VII of human PSEN1 (Leimer et al., 1999). Orange shading indicate amino acid substitutions occurring at similar positions in human PSEN1 and PSEN2 in comparison to zebrafish Psen1. Red shading indicates the conserved aspartate residues important for intramembrane proteolytic activity (Kimberly et al., 2000; Leimer et al., 1999). Blue shading in (A) and (B) represents a polymorphic site at amino acid position 317 in zebrafish Psen1 (Alanine/Proline) (Leimer et al., 1999) (Groth and Lardelli, unpublished data). Green shading represents human PSEN1 methionine 146, which is replaced with valine, leucine or isoleucine in familial Alzheimer's disease mutants.

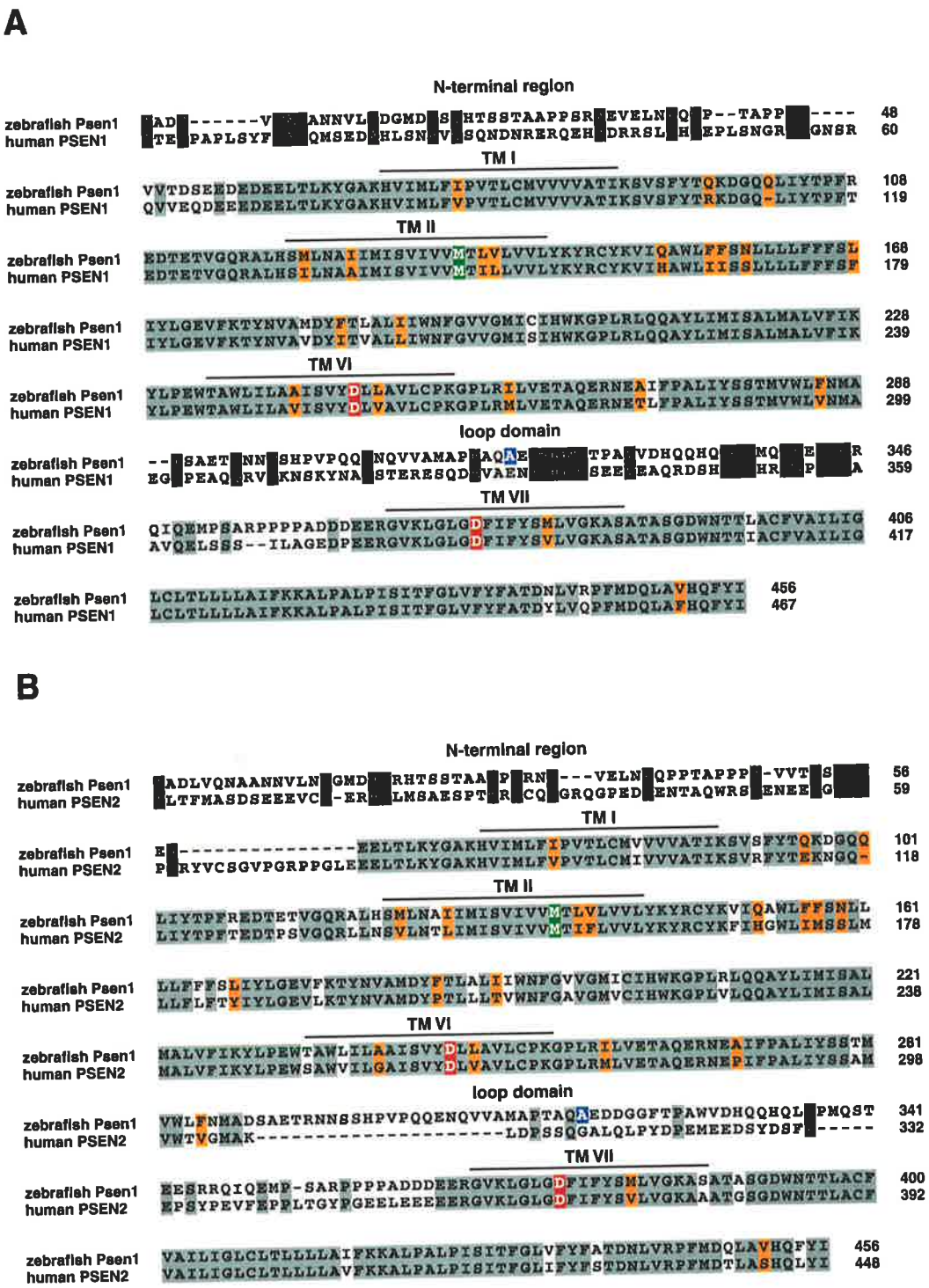

A
N-terminal region
zebrafish Psen1
human PSEN1
TM I
TM II
TM VI
loop domain
TM VII
B
N-terminal region
zebrafish Psen1
human PSEN2
TM I
TM II
TM VI
loop domain
TM VII

FIGURE 2.2. Expression of *psen1* during embryogenesis. (A-C) Lateral view of embryos at the 16-cell stage, 2, 6 and 12 hpf. *psen1* shows ubiquitous expression. (D) Dorsal view of 16 hpf embryo. Expression of *psen1* is widespread with relatively high levels in the brain. (E) Transverse section through the hindbrain of 16 hpf embryo. (F-I). Different views of 24 hpf embryos. (F, G) The brain and eyes show strong *psen1* staining. (H, I) The primordial germ cells express *psen1*. hb, hindbrain; no, notochord; nr, neural rod; ov, otic vesicle; pgc, primordial germ cells. Scale bars indicate 100 μm, except for (A-C), 200 μm.

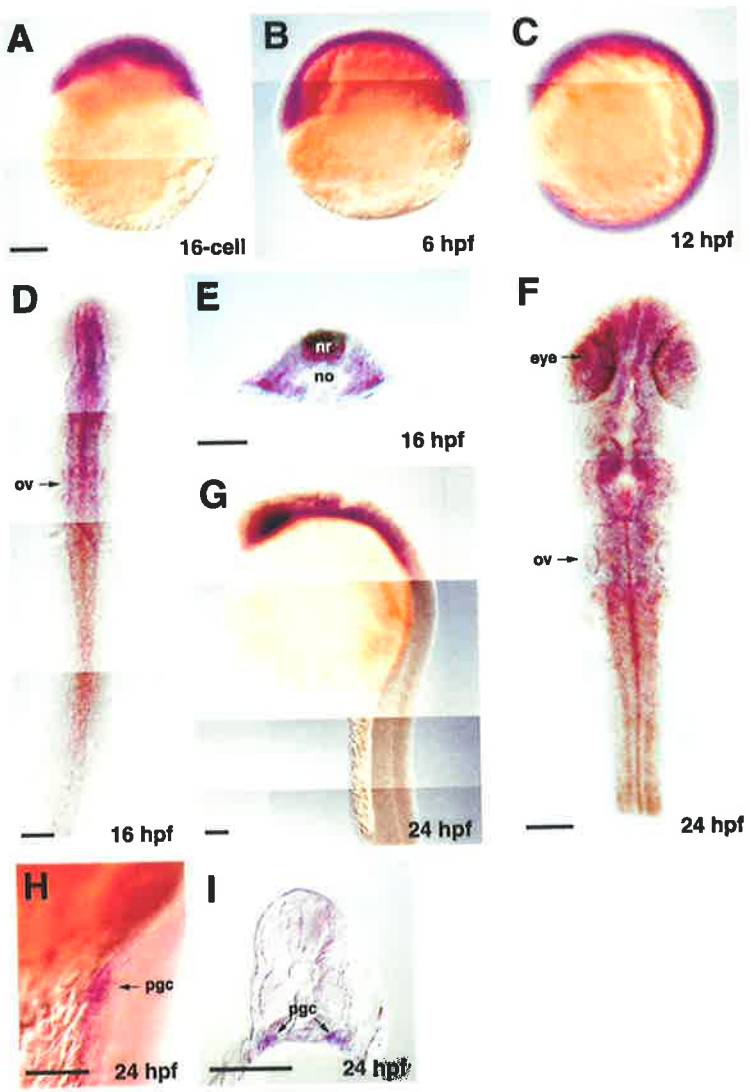

A
16-cell
B
6 hpf
C
12 hpf
D
ov
16 hpf
E
nr
no
16 hpf
F
eye
ov
24 hpf
G
24 hpf
H
pgc
24 hpf
I
pgc
24 hpf

FIGURE 2.3. Sequence alignment of the Presenilin2 protein from human and zebrafish. Identical amino acids are boxed and shaded and gaps that were introduced for optimal alignment are indicated by dots. Bars indicate the putative transmembrane domains of human PSEN2 (Fraser et al., 2000). Arrowhead indicates a putative endoproteolytic site. Red shading indicates the conserved aspartate residues important for intramembrane proteolytic activity (Kimberly et al., 2000; Leimer et al., 1999).

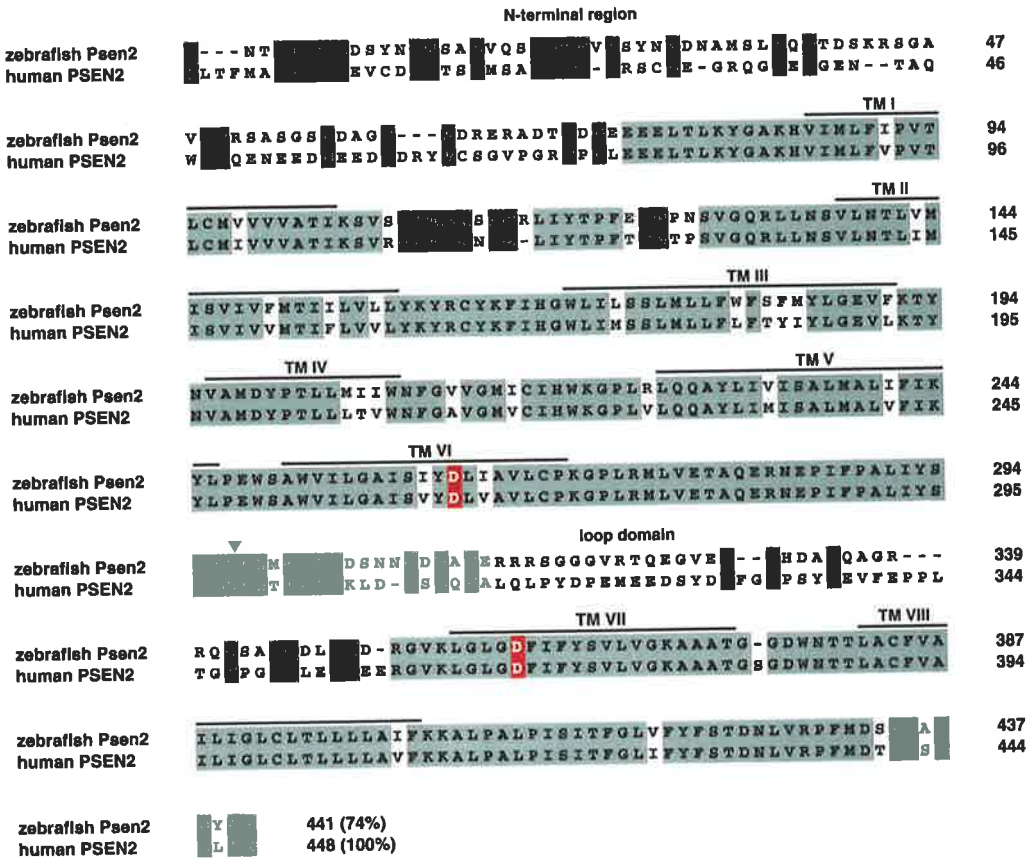

N-terminal region
TM I
TM II
TM III
TM IV
TM V
TM VI
loop domain
TM VII
TM VIII
zebrafish Psen2
human PSEN2
47
46
94
96
144
145
194
195
244
245
294
295
339
344
387
394
437
444
441 (74%)
448 (100%)

FIGURE 2.4. Phylogenetic analysis of Presenilin proteins. Tree based on metazoan Presenilin protein sequences. The scale bar represents 10 base substitutions per 100 nucleotide positions and the numbers refer to bootstrap percentages.

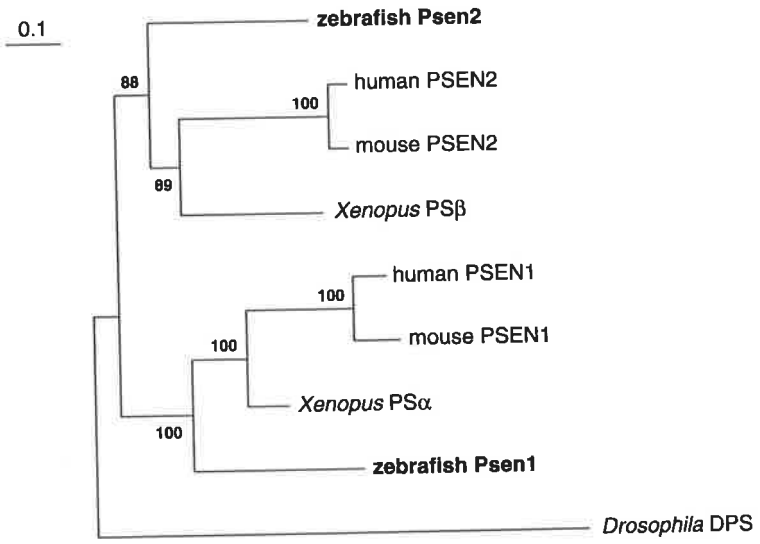

0.1
zebrafish Psen2
human PSEN2
mouse PSEN2
Xenopus PSβ
human PSEN1
mouse PSEN1
Xenopus PSα
zebrafish Psen1
Drosophila DPS
88
100
89
100
100
100

FIGURE 2.5. Distribution of *psen2* transcript at various developmental stages. In all images, animal pole or anterior is to the top and dorsal is to the right unless otherwise indicated. A-E and H, Lateral views of embryos at the 1-cell stage and at 2, 6, 12, 16 and 24 hpf respectively. F, G and J, Transverse sections through the hindbrain (G, J) and midway along the yolk extension (F) of 24 hpf embryos with dorsal to the top. I and K, dorsal views of 24 hpf embryos. Anterior is to the left in I. Images J and I are magnifications of the boxed regions in images G and K, respectively. hb, hindbrain; m, melanocytes; ml, midline of the spinal cord; n, notochord; nc, neural crest; tfp, tailfin primordium. Scale bars indicate 100 μm, except for in F (50 μm) and J (25 μm).

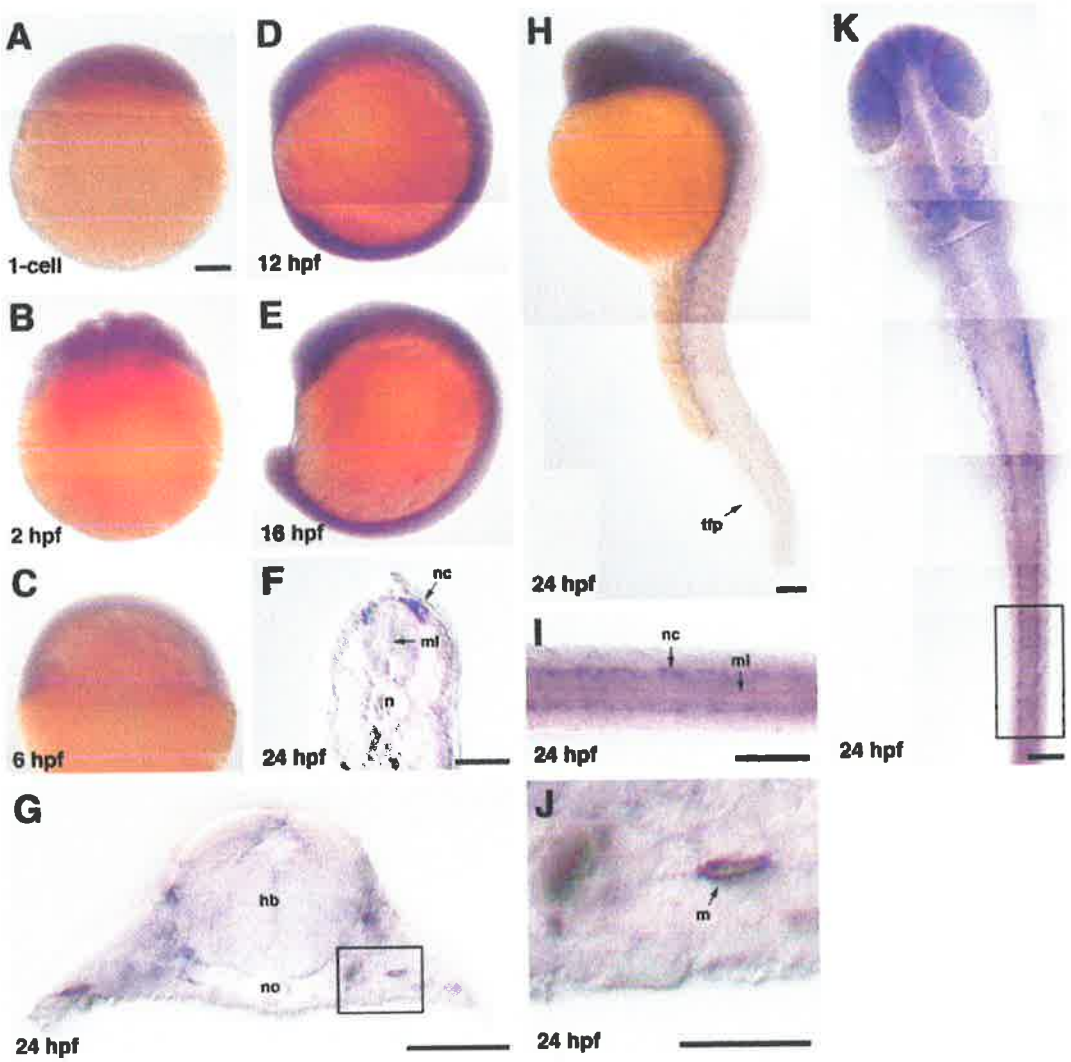

A
1-cell
B
2 hpf
C
6 hpf
D
12 hpf
E
16 hpf
F
nc
ml
n
24 hpf
G
hb
no
24 hpf
H
tfp
24 hpf
I
nc
ml
24 hpf
J
m
24 hpf
K
24 hpf

FIGURE 2.6. Psen2 holoprotein is the predominant species during early embryogenesis. Western blot analysis of Psen2 protein levels (upper panels) and β-tubulin (protein level control, lower panel) at an early cleavage stage (left), 6 hpf (centre) and 12 hpf (right). The anti-Psen2 antibody was raised against a synthetic peptide derived from the N-terminal proteolytic fragment of zebrafish Psen2 (Nornes et al., 2003). The bands corresponding to the Psen2 holoprotein ($Psen2_{holo}$), N-terminal fragment ($Psen2_{NTF}$) and β-tubulin bands are indicated. Psen2 protein could not be detected at the 64-cell stage or at 6 hpf.

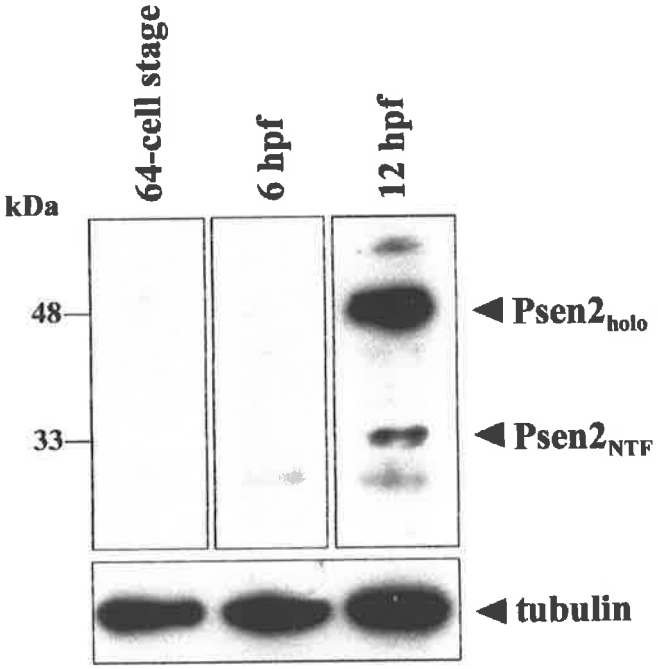

64-cell stage
6 hpf
12 hpf
kDa
48
33
Psen2holo
Psen2NTF
tubulin

FIGURE 2.7. Overexpression of human *PSEN1* in zebrafish embryos perturbs development. (A-E) Lateral view of 12-14 hpf embryos. (A) normal embryo. (B-E) Embryos showing reduced cell-adhesion phenotypes generated by human *PSEN1* overexpression. In (E), red arrowhead shows region with massive cell detachment. Injection of control RNA did not produce these defects (not shown). Scale bars indicate 100 μm.

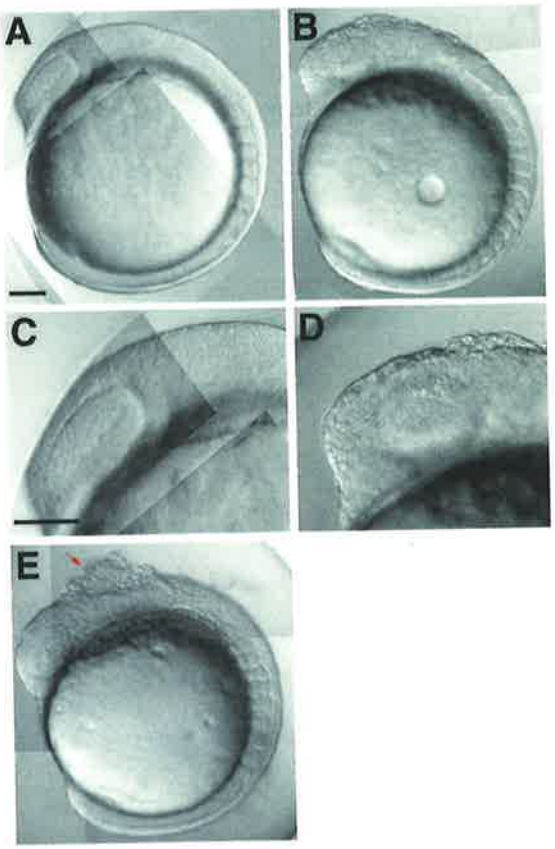

A
B
C
D
E

TABLE 2.1. Distribution of phenotypes following overexpression of wild-type or human *PSEN1* in zebrafish embryos. (A) Comparison between wild-type and M146I AD mutant *PSEN1*. (B) Comparison between wild-type and M146R mutant *PSEN1*.

A

PSEN1	NORMAL	DEFECTS	TOTAL
WILD-TYPE	**56**	**72**	**128**
M146I	**33**	**30**	**63**
TOTAL	**89**	**102**	**191**

χ^2: 1.52, 0.20 =P =0.30

B

PSEN1	NORMAL	DEFECTS	TOTAL
WILD-TYPE	**56**	**72**	**128**
M146R	**30**	**36**	**66**
TOTAL	**86**	**108**	**194**

χ^2: 0.17, 0.10 =P =0.20

Chapter 3

gamma-secretase activity controls Notch-dependent and Notch-independent neural crest development

3.1. ABSTRACT

γ-secretase is a multi-component protein complex, including Presenilins, responsible for the final cleavage of APP to release the neurotoxic amyloid β-peptide (Aβ). Inhibition of Aβ accumulation by blocking γ-secretase activity is considered a promising way to intervene in the progression of Alzheimer's disease (AD). γ-secretase inhibitors apparently work by blocking the function of the Presenilins. However, the realisation that γ-secretase interacts with Notch and other signalling pathways, important during development and in adult homeostasis, suggests that prolonged inhibitor treatment of AD patients would cause severe side effects. We demonstrate that treatment of zebrafish with DAPT, a well-characterised γ-secretase inhibitor, phenocopies the abnormalities seen in mutants with defects in the Notch pathway during zebrafish embryogenesis. This further supports the proposition that disruption of the Notch pathway during γ-secretase inhibitor therapy of AD patients, would result in adverse side effects. Previously, it has been reported that the development of trunk neural crest is dependent on the Notch pathway, while cranial neural crest specification is regulated by Notch-independent signals. In this report we show that γ-secretase activity is essential for the development of both cranial and trunk neural- crest-derived cells. Thus γ-secretase-mediated control of neural crest differentiation involves the regulation of Notch-dependent, and possibly Notch-independent pathways.

3.2. INTRODUCTION

Accumulation of amyloid β peptide (Aβ) is an early event in the cascade of neurogenerative processes leading to Alzheimer's disease (Hardy and Selkoe, 2002). Aβ is generated by the enzymatic activity of two sequentially acting proteases, β-secretase and γ-secretase, which liberates Aβ from its precursor, the amyloid precursor protein (APP) (Esler and Wolfe, 2001). Aberrant processing of the APP by γ-secretase has been suggested to lead to increased formation of a neurotoxic Aβ variant and its deposition in

amyloid plaques, consequently leading to Alzheimer's disease (Hardy and Selkoe, 2002). Presenilin is essential for cleavage of APP (De Strooper et al., 1998) and may be the catalytic subunit of γ-secretase, an intramembrane-cleaving protease (Kimberly et al., 2000; Wolfe et al., 1999b). γ-secretase is a multi-protein complex which, in addition to Presenilin1 or Presenilin2, comprises three other core components, Nicastrin, APH-1 and PEN-2 (Edbauer et al., 2003; Kimberly et al., 2003; Marlow et al., 2003; Takasugi et al., 2003). Presenilin-dependent γ-secretase activity is not only responsible for the cleavage of APP, but also the proteolysis of Notch and other type 1 integral membrane proteins (De Strooper, 2003). Notch proteins act as receptors controlling important cell fate decisions during embryogenesis and adulthood (Selkoe and Kopan, 2003). γ-secretase cleavage of Notch leads to the release of its intracellular domain (NICD), which is free to translocate to the nucleus and initiate transcription of target genes (De Strooper et al., 1999; Struhl and Greenwald, 1999a). The realisation that Notch and other components of the Notch signalling pathway are functionally dependent on γ-secretase activity raises concern about the safety of using γ-secretase inhibitors to reduce Aβ production as a treatment for Alzheimer's disease (Bland et al., 2003; Ikeuchi and Sisodia, 2003). For example, it has been demonstrated that Notch signalling is important for differentiation of T lymphocytes and in hematopoiesis (Hadland et al., 2001; Varnum-Finney et al., 1998). Moreover, Notch1 ablation in mammalian skin leads to tumour growth and cancer, suggesting that Notch1 may act as a tumour suppressor (Nicolas et al., 2003). Taken together, these findings implicate that prolonged treatment with general γ-secretase inhibitors most likely would cause severe side effects. In order to overcome these putative problems, it will be important to discover drug candidates

which selectively inhibits Aβ generation but do not affect the Notch pathway. However, whereas γ-secretase inhibitors have been extensively studied *in vitro* only limited knowledge is available on their effects in animals *in vivo* (Geling et al., 2002; Micchelli et al., 2003). Therefore, taking advantage of the accessibility and manipulability of the zebrafish as a model system for the study of developmental biology (Kimmel et al., 1995), we decided to perform a detailed analysis of the effects of a typical potent γ-secretase inhibitor (DAPT) on zebrafish embryogenesis.

3.3. MATERIALS AND METHODS

3.3.1. Embryos and whole mount *in situ* transcript hybridisation

Zebrafish embryos were staged according to (Kimmel et al., 1995). Stages are given as equivalent to hours of development post fertilisation (hpf) at 28.5°C. The transcript expression patterns of zebrafish *her1*, *islet1*, *myoD* and *notch6* were analysed by whole mount *in situ* transcript hybridisation on fixed zebrafish embryos at various stages of development up to 24 hpf as previously described (Kimmel et al., 1995). The preparation of cDNA templates and RNA transcription were performed as detailed below. *her1* and *myoD* cDNAs cloned into pBSSK (kindly provided by S. Wells and R. McCarthy, respectively) were amplified by PCR from their respective vectors using M13 (-20): 5'-GTAAAACGACGGCCAGT-3' and M13 reverse: 5'-GGAAACAGCTAT GACCATG-3' primers (Stratagene, USA). The PCR conditions were: 94 °C for 30 sec, 52 °C for 45 sec, 0.5 °C/sec to 72 °C, 72 °C for 3 min, 35 cycles. The PCR fragments were subsequently isolated using the QIAquick Gel Extraction Kit (Qiagen, USA). *her1*

was transcribed using T3 RNA polymerase, while *myoD* was transcribed using T7 RNA polymerase. *islet1* cloned into pBSSK (kindly provided by S. Wells) was linearised with *Xba*I and transcribed using T3 RNA polymerase. *notch6* cloned into pBSSK (Westin and Lardelli, 1997) was linearised with *Bam*I and transcribed using T3 RNA polymerase. All riboprobes were labelled using the Digoxigenin (DIG) RNA labelling Kit (Boehringer Mannheim, Germany). Unincorporated nucleotides were removed using RNase-free Micro Bio-Spin Columns P-30 (BIO-RAD, USA).

3.3.2. Treatment of zebrafish embryos with the γ-secretase inhibitor DAPT

A 10 mM stock of DAPT (γ-secretase inhibitor IX, Calbiochem, USA) in DMSO was diluted in embryo medium (Westerfield, 1995) and applied to zebrafish embryos at 28.5°C from 6 hpf until the stage of analysis (16, 24, 48 or 72 hpf). Control embryos were mock treated with embryo medium with 1% DMSO. All experiments were performed in a volume of 1 ml of embryo medium containing 15 embryos. DAPT treatment of dechorionated zebrafish embryos has previously been reported (Geling et al., 2002). However, mechanical dechorionation of live zebrafish embryos is a laborious procedure, consequently limiting the numbers of embryos available for subsequent inhibitor treatment. DAPT is a small cell-permeable inhibitor molecule (Dovey et al., 2001), which may be able to cross the zebrafish chorion. Therefore, we decided to investigate whether effective DAPT treatment of embryos could be accomplished without prior mechanical dechorionation, allowing more embryos to be processed and treated with the inhibitor. To this aim we performed a dose-response analysis and

established that a minimal concentration of 50 μM of DAPT was required to affect melanogenesis and somitogenesis at the morphological level. This concentration is similar to that needed to affect somitogenesis in dechorionated embryos (Geling et al., 2002). This demonstrates that DAPT can permeate the chorion and gain access to the embryo with similar efficacy as in dechorionated embryos treated with DAPT. As a consequence all analyses in this study were carried out on embryos with chorions. Analyses at the molecular level were performed with a dose of 100 μM DAPT. Analyses at the morphological level were performed using DAPT doses of 25, 50 and 100 μM, respectively.

3.4. RESULTS

3.4.1. Blockage of γ-secretase causes severe developmental defects

The zebrafish, as a model organism, has the advantage of producing transparent eggs and embryos during early embryogenesis, allowing simple visual inspection of morphological changes (Kimmel et al., 1995). We therefore decided to investigate if γ-secretase inhibitor treatment caused morphological defects associated with perturbed Notch signalling. We also looked for developmental aberrations not generally attributed to the impairment of the Notch pathway, thus indicating disruption of other γ-secretase-dependent pathways following inhibitor treatment.

Treatment of zebrafish with 25 μM DAPT did not cause significant morphological defects at 24 hpf. However, after prolonged exposure to DAPT (72 hpf) the embryos showed curved spinal cords and slightly reduced numbers of melanocytes in the trunk and tail (75%, n = 20) (not shown). Increasing the γ-secretase inhibitor concentration to 50 μM led to more severe developmental defects (98%, n = 305). By 48 hpf, embryos showed curved spinal cords and the number of melanocytes were significantly reduced, especially in the trunk and tail (not shown). Brain haemorrhages were found in 29% of the embryos, suggesting that vasculogenesis is compromised by DAPT treatment. Treating zebrafish embryos with 100 μM DAPT exacerbated the morphological aberrations. At 16 hpf, posterior somite formation was interrupted whereas the first 6-8 anterior-most somites formed normally (not shown). This phenotype is similar to that of zebrafish Notch signalling mutants such as *deadly-seven* (*des*) and *after-eight* (*aei*) (van Eeden et al., 1996). At 24 hpf, somite boundaries were misshapen and separated somites of irregular size (**FIG. 3.1**). By 48 hpf, melanogenesis was markedly affected (100%, n = 347). Cranial neural-crest-derived melanocytes were visibly reduced in number, with the occurrence of abnormal spot-like melanocytes (**FIG. 3.2C, D**), while neural-crest-derived melanocytes of the trunk and tail exhibited a dramatic reduction in number upon DAPT treatment (**FIG. 3.2A, B**). Typically, neural-crest-derived melanocytes were few or absent in the trunk and tail. Brain haemorrhages were found in 35% (n = 347) of embryos, which indicates that there was no obvious further deterioration in the integrity of the vascular system following a two-fold increase in inhibitor concentration. The curved spinal cord phenotype together with the observation that DAPT treated embryos showed

severe locomotor impairment, with fast, but restricted and static, movements of the tail, suggests that the embryos have widespread defects in their neuronal circuitry.

3.4.2. Anteroposterior polarity is lost in the paraxial mesoderm when γ-secretase activity is blocked

Notch signalling is involved in the control of somite anteroposterior polarity and the cyclic expression of segmentation clock genes in the presomitic mesoderm (Pourquie, 2001). To extend our morphological analysis, we looked at the molecular level to access the effect of DAPT treatment on these two Notch-dependent developmental processes.

The segment prepatterning gene *her1* is a downstream target of the Notch pathway, which regulates its cyclic expression in the presomitic mesoderm (Holley et al., 2002; Muller et al., 1996; Sawada et al., 2000). In DAPT treated embryos the dynamic expression of *her1* was lost (86%, n = 42) (**FIG. 3.1A, B**), demonstrating that γ-secretase inhibition blocks Notch signalling *in vivo*.

notch6 and *myoD* have been shown to be expressed in the presomitic mesoderm and in complementary patterns in the anterior and posterior portions of somites, respectively (Weinberg et al., 1996; Westin and Lardelli, 1997). Normally, *notch6* is up-regulated in the anterior-half of the 4-5 most recent forming somites and down-regulated as somitic differentiation proceeds (**FIG. 3.1C**) (Westin and Lardelli, 1997). However, upon DAPT treatment, the expression pattern of *notch6* became ubiquitous in the posterior-most somites and was prematurely lost in more advanced, differentiating somites (86%, n =

28) (**FIG. 3.1D**). In DAPT treated embryos *myoD* expression was normal in the 7-8 anterior-most somites but became ubiquitous in later forming somitic mesoderm (**FIG. 3.1E, F**) (100%, n = 19). This phenotype is similar to that of *des* and *aei* mutants, which show deficiencies in the Notch pathway genes *notch1a* and *deltaD*, respectively (Gray et al., 2001; Holley et al., 2000).

We next asked whether DAPT treatment would impair neurogenesis. Primary neurons are a class of early-born neural cells, which include Rohon-Beard spinal primary sensory (RB) neurons in the lateral neural plate, and primary motorneurons and primary interneurons in the intermediate and medial neural plates, respectively (Kimmel et al., 1991). Because primary neurons are born early during zebrafish embryogenesis examination of their distribution is readily accessible using RNA transcript *in situ* hybridisation. We used the primary neuronal marker *islet1* (Inoue et al., 1994; Korzh et al., 1993) to analyse the effect of DAPT treatment on primary neurogenesis. By 16 hpf, we reproducibly observed *islet1*-positive primary neuronal hyperplasia following γ-secretase inhibitor treatment (**FIG. 3.2E-J**) (100%, n = 18), showing that γ-secretase activity regulates neurogenesis *in vivo*. Strikingly, RB neurons showed a 2-3 fold increase in number after inhibitor treatment (**FIG. 3.2E, F, I, J**).

3.5. DISCUSSION

3.5.1. γ-secretase inhibitor treatment and Alzheimer's disease

The effectiveness of Aβ-lowering drugs is dependent on their ability to gain unrestricted access to areas of the brain affected by Alzheimer's disease-specific metabolic lesions. Therefore, as a prerequisite, they must be small and highly cell-permeable pharmacological agents. Recently, Geling et al. (2002) have demonstrated that zebrafish embryogenesis is a suitable *in vivo* test system for the validation of Aβ-lowering drugs. It would be advantageous, in terms of using zebrafish development for high-throughput drug screening, if such agents would be permeable to the zebrafish chorion. Indeed, in this study we demonstrate that a potent γ-secretase inhibitor (DAPT) (Dovey et al., 2001) perturbs Notch signalling in zebrafish embryos subjected to drug treatment with intact chorions in a manner indistinguishable from embryos that had their chorions removed prior to DAPT treatment. If this proves to be a general characteristic of Aβ-lowering drug candidates, it would promise to accelerate the *in vivo* testing of these therapeutic molecules in zebrafish.

DAPT and other γ-secretase inhibitors were developed as Aβ-lowering drugs for long-term treatment of patients suffering from Alzheimer's disease (Dovey et al., 2001; Wolfe et al., 1999a). However, the realisation that the blockage of Presenilin-dependent γ-secretase activity interferes with the Notch pathway (De Strooper et al., 1999; Saxena et al., 2001) challenges the validity of this approach. Notch activity is required during

embryogenesis to regulate many cell fate decisions (Selkoe and Kopan, 2003). We confirm and extend the findings of Geling et al. (2002), and show that disruption of normal Notch signalling, by inhibition of γ-secretase, affects somitogenesis and neurogenesis in zebrafish. However, the Notch pathway has also been implicated in the developmental control of the embryonic vasculature. Disruption of Notch1 and Notch1/Notch4 function in mice results in impairment of angiogenesis, leading to severe vascular defects (Krebs et al., 2000). In zebrafish embryos with mutations in the *mindbomb* (*mib*) gene, that encodes a RING ubiquitin ligase required for normal Notch function (Itoh et al., 2003), arterial-venous differentiation is defective (Lawson et al., 2001). Wild-type embryos expressing a dominant negative form of Suppressor of Hairless (*Su(H)*) (an intermediary of the Notch pathway) which blocks Notch signalling, show similar defects in vasculogenesis as *mib* mutant embryos (Lawson et al., 2001; Zong et al., 2001). Furthermore, *mib* embryos exhibit cranial haemorrhaging, indicating that the integrity of the vasculature is compromised in these embryos (Lawson et al., 2001). Taken together, these studies show that Notch signalling is essential for development of the embryonic vasculature. We demonstrate that DAPT treatment of zebrafish embryos causes substantial cranial haemorrhage; supporting the idea that vascular morphogenesis is severely affected in embryos with inhibited Notch signalling (**FIG.3.3G, H**).

The Notch pathway is not only essential during embryo development, but also plays important roles in adults where it controls diverse cell differentiation events in the immune system, blood and adult brain (Hadland et al., 2001; Hitoshi et al., 2002;

Varnum-Finney et al., 1998). Also, the Notch pathway may be involved in vascular homeostasis in the adult. Mutations in the *NOTCH3* gene cause **CADASIL (Cerebral Autosomal Dominant Arteriopathy with Subcortical Infarcts and Leucoencephalopathy)**, a degenerative vascular disease, with symptomatic recurrence of strokes, progressive cognitive impairment, dementia and premature death (Joutel et al., 1996). Thus prolonged treatment of Alzheimer's disease patients with general γ-secretase inhibitors would most likely lead to adverse side effects (Lewis et al., 2003). Consequently, efforts in this area must be focused on finding drugs which selectively inhibit aberrant Aβ generation but, simultaneously, do not interfere with the Notch and other γ-secretase/presenilin-dependent pathways. The zebrafish promises to be a valuable system for *in vivo* testing of Aβ-lowering drug candidates.

3.5.2. γ-secretase activity controls embryo development

Genetic manipulations have been widely used to perturb specific protein and signalling pathways in order to dissect their function in embryo development. In contrast, studies involving the use of pharmacological compounds to change specific signalling pathways during development have been rare. An example has been the use of the FGFR inhibitor SU5402, which disrupts FGFR kinase activity (Mohammadi et al., 1997), to study the role of FGF signalling in zebrafish (Furthauer et al., 2002; Furthauer et al., 2001; Sawada et al., 2001). In this report we have analysed the role of γ-secretase-dependent pathways in development by pharmacologically blocking its activity using the chemical inhibitor DAPT. We show that γ-secretase activity regulates segmentation and

neurogenesis in zebrafish. We also observed that in absence of this activity development of the vasculature is disrupted.

??5????γ–SECRETASE MAY REGULATE BOTH CRANIAL AND TRUNK NEURAL CREST DEVELOPMENT

The neural crest is a population of cells that originate in the dorsal neural tube from where they segregate and migrate, and differentiate into various cell types, including neurons and glia of the peripheral and enteric nervous systems, head and neck cartilage, and pigment cells (Knecht and Bronner-Fraser, 2002). In the zebrafish trunk and tail, neural crest cells arise from lateral neural plate precursor cells (Raible et al., 1992), which also have the developmental potential to produce RB neurons (Haddon et al., 1998; Inoue et al., 1994; Korzh et al., 1993). Delta/Notch signalling is essential for the regulation of these two alternative fates. In embryos defective in Delta/Notch signalling supernumerary RB cells are formed and a concomitant reduction in the number of neural-crest-derived melanocytes is observed in the trunk and tail (Appel and Eisen, 1998; Cornell and Eisen, 2000). Interestingly, the number of cranial neural-crest-derived melanocytes is not affected by disruption of the Notch/Delta pathway (Cornell and Eisen, 2000) suggesting that cranial and trunk neural crest cells are under the regulatory control of different pathways. Our observations confirmed that the Notch pathway performs an essential role in the specification of trunk neural crest cells. DAPT-mediated chemical inhibition of γ-secretase activity mimicked the hypoplasia of trunk and tail melanocytes seen in Notch/Delta-deficient embryos (**FIG. 3.2A, B**) (Cornell and Eisen, 2000). Importantly, in contrast to the Notch/Delta phenotypes, the number of

cranial neural-crest-derived melanocytes was significantly reduced (**FIG. 3.2C, D**). Thus it appears that γ-secretase-mediated intramembrane proteolysis may regulate a Notch/Delta-independent signalling event important for cranial neural crest development. It will be interesting to learn the nature of this γ-secretase-dependent process.

?5???. γ–SECRETASE ACTIVITY CONTROLS THE ANTEROPOSTERIOR POLARITY OF THE PARAXIAL MESODERM

During embryogenesis, somite formation is the first manifestation of the segmented vertebrate body plan. Somite-derived tissues include the axial skeleton, the dermis of the back and all striated muscle of the adult body (Stickney et al., 2000). It is postulated that the segmented somites are generated through the action of a segmentation clock, operating in the immature presomitic mesoderm (Holley and Takeda, 2002; Saga and Takeda, 2001). The clock generates a distinct temporal pattern of cyclic gene expression, creating the basis for the spatial periodicity of the somites. The identity of the prospective somitic cells is finally established in the anterior PMS, where a maturation wavefront arrests the clock and initiates somite differentiation (Pourquie, 2001). So far, a limited number of genes have been implicated in the molecular mechanisms governing somitogenesis, including *Notch* and *Delta* (Conlon et al., 1995; Hrabe de Angelis et al., 1997), *Presenilin* (Donoviel et al., 1999; Herreman et al., 1999; Koizumi et al., 2001; Shen et al., 1997), *tbx24* (Nikaido et al., 2002) and *FGFR1* (Yamaguchi et al., 1994b). To examine the function of presenilin-dependent processes in somitogenesis we used the pharmacological compound DAPT to inhibit presenilin-dependent γ-secretase activity

(Dovey et al., 2001). We show that the normal segmental expression of *notch6* and *myoD* (Weinberg et al., 1996; Westin and Lardelli, 1997), two genes expressed in a complementary manner in each anterior and posterior half-somite, respectively, were lost in inhibitor treated embryos (**FIG. 3.1C-F**). Instead, transcripts were seen throughout the somitic region of the paraxial mesoderm. A similar phenotype was reported for *paraxial protocadherin* (*papc*), a marker of nascent anterior half-somites (Yamamoto et al., 1998), in DAPT-treated embryos (Geling et al., 2002). This loss-of-polarity phenotype is typical for Notch signalling mutants, such as *des* and *aei*, when examined for *myoD* expression (Holley et al., 2000; van Eeden et al., 1996). Thus we argue that γ-secretase-mediated intramembrane cleavage of Notch regulates zebrafish somite formation. Furthermore, our findings corroborate the hypothesis that disruption of Notch signalling leads to desynchronisation of the segmentation clock (Jiang et al., 2000), causing progressive failure in somite segmentation.

The Notch receptor is only one of several cell surface receptors identified as substrates for γ-secretase-mediated-intramembrane-cleavage to release an intracellular domain, which may be important for transcriptional activation of target genes (Fortini, 2002). This group of diverse receptors also include APP. The strong similarity between the *notch1a* KO phenotype of *des* mutant zebrafish and embryos subjected to DAPT-mediated γ-secretase inhibition suggests that the major developmental aberrations observed are caused by disruption of Notch signalling. However, it is very likely that changes in regulatory networks and processes dependent on *app* and other γ-secretase-dependent proteins would also be affected by γ-secretase blockage. Such changes may

be subtle and not lead to gross morphological malformations during early embryonic development. Thus they would not easily be identified by visual inspection. In fact an important goal in the future would be to identify potential target genes, which may be regulated by the App intracellular domain. A two-step procedure involving zebrafish DNA microarray technology could be employed to achieve this. In the first step *app* specific morpholino oligonucleotide knock down of App activity, during a specific embryonic stage, would identify genes potentially regulated by *app* in general. In the second step embryos treated with a γ-secretase inhibitor would be subjected to microarray analysis. Comparative analysis of the two array experiments would identify genes, which are regulated in a similar manner under both experimental paradigms. Such genes may be regulated by γ-secretase-mediated-intramembrane-proteolysis of APP and could subsequently be selected for detailed analysis.

FIGURE 3.1. DAPT treatment interferes with somite formation. (A-H) Dorsal axial view of 16 hpf embryos. Anterior is to the top. (A, C, E, G) Control embryos. (B, D, F, H) Embryos treated with DAPT. (A and B) *her1* expression. (C and D) *notch6* expression. (E and F) *myoD* expression. (G and H) Lateral views of trunk somites of segmentation stage embryos (16 hpf). (G) Control embryo. (H) Embryo treated with DAPT shows severe disruption of somite boundary formation. Red arrowheads indicate somite boundaries. Scale bars indicate 100 μm.

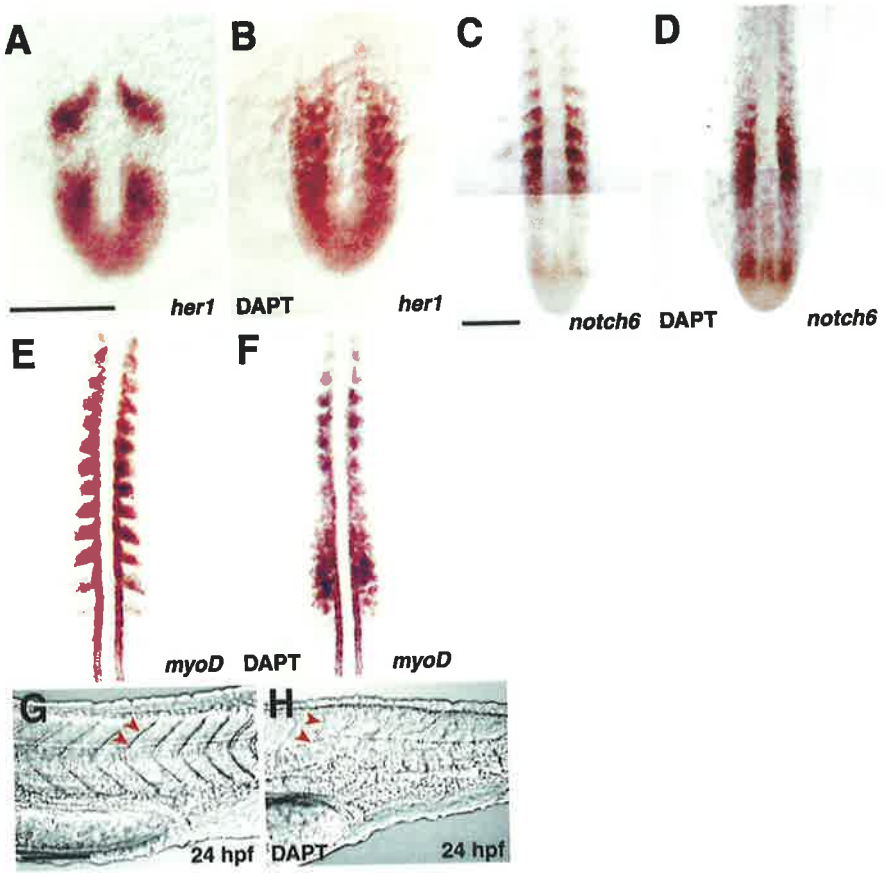
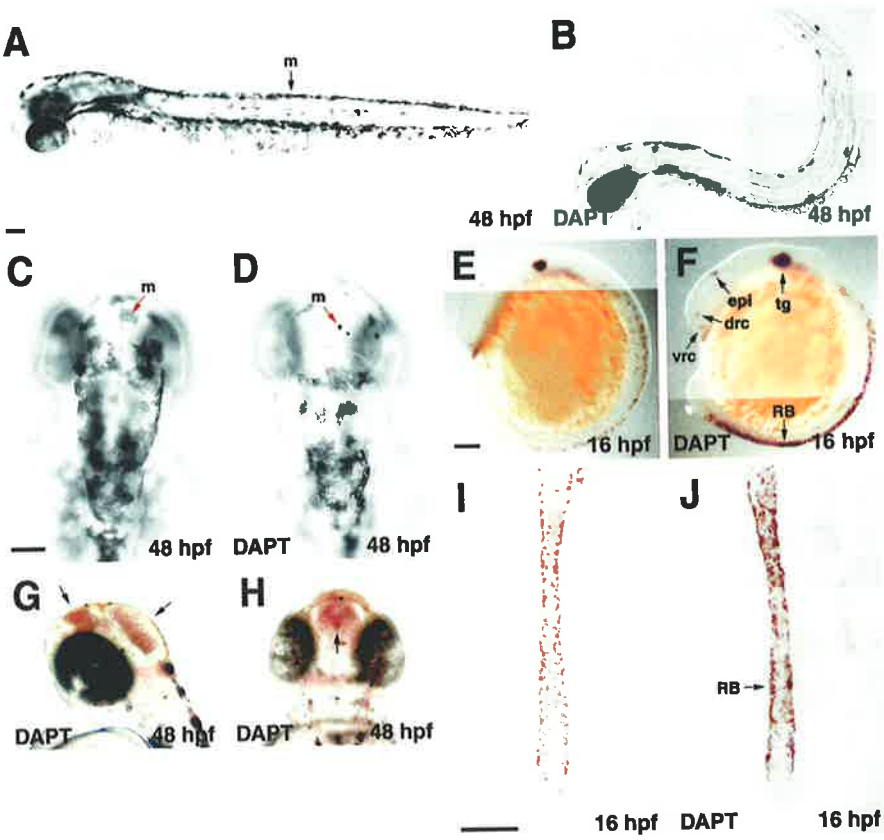

A
B
C
D
her1
DAPT
her1
notch6
DAPT
notch6
E
F
myoD
DAPT
myoD
G
H
24 hpf
DAPT
24 hpf

FIGURE 3.2. Inhibition of γ-secretase activity results in a neurogenic phenotype and disrupts neural crest development. (A-D, G, H) 48 hpf embryos. (E, F, I, J) 16 hpf embryos. (A and B) Lateral view of 48 hpf embryos, (A) control and (B) embryo treated with DAPT. Anterior is to the left. The number of trunk and tail neural-crest-derived melanocytes is dramatically reduced following DAPT treatment. (C, D) Dorsal view of head region. Anterior is to the top. Note the marked reduction in the number of cranial neural-crest-derived melanocytes after DAPT treatment. (E, F, I, J) *islet1* expression is shown by red staining. (E, F) Lateral view. (I, J) Dorsal view. DAPT treatment leads to an expansion of a number of neural clusters in the brain (F) and increased spinal Rohon-Beard neurons (F, J). (G and H) Lateral and dorsal view of a live DAPT treated embryo, showing brain haemorrhages. drc, dorso-rostral cluster; epi, epiphysis; m, melanocyte; RB, Rohon-Beard neurons; tg, trigeminal ganglion; vrc, ventro-rostral cluster. Scale bars indicate 100 μm except in (A and B), 200 μm.


A
m
B
48 hpf
DAPT
48 hpf
C
m
48 hpf
D
m
DAPT
48 hpf
E
16 hpf
F
epi
tg
drc
vrc
RB
DAPT
16 hpf
G
DAPT
48 hpf
H
DAPT
48 hpf
I
J
RB
16 hpf
DAPT
16 hpf

Chapter 4

The Kunitz-type protease inhibitor domain is evolutionarily conserved and is retained in two zebrafish orthologues of the human Alzheimer's disease gene amyloid precursor protein

4.1. ABSTRACT

Aberrant processing of the amyloid precursor protein plays a pivotal role in the pathogenesis of Alzheimer's disease. A previous analysis identified two duplicate orthologues of *APP* in zebrafish lacking the conserved Kunitz-type protease inhibitor (KPI) domain. We have isolated cDNA from two zebrafish *amyloid precursor protein* orthologues (*appa* and *appb*) encoding proteins that include the alternatively spliced KPI domain. The retention of the KPI domain in both duplicate zebrafish *app* orthologues suggests that this domain may play an important role in normal *app* biology. We have performed a detailed analysis of the expression pattern of *appa* in segmentation stage embryos. *appa* is expressed in distinct domains of the CNS and PNS. Outside the developing nervous system, *appa* is expressed in the lenses, otic vesicles, somites, pronephric ducts and the tail bud. As embryogenesis progresses somitic *appa* expression becomes restricted to the medial region of each somite.

4.2. INTRODUCTION

The deposition of amyloid plaques is an early and invariant pathological event in the progression of Alzheimer's disease (AD) (Selkoe, 2001). The major component of amyloid plaques is the amyloid β peptide (Aβ), which is derived from the amyloid precursor protein (APP) by multiple proteolytic events. The APP is a type I membrane glycoprotein, consisting of a relatively large extracellular and a short intracellular domain (De Strooper and Annaert, 2000). The event(s) leading to the initiation of the APP proteolytic cascade is unknown. However, once initiated, amyloidogenic processing of the APP protein, by the two-step process of regulated intramembrane proteolysis (RIP), follows with β-secretase-dependent shedding of the extracellular domain as the first step. This is leading to a second γ-secretase-dependent

intramembrane cleavage and subsequent release of the Aβ into the extracellular space (Haass and Steiner, 2002). γ-secretase is a multi-component protein complex, the activity of which is dependent on the incorporation into the complex of Presenilin protein (De Strooper, 2003). Mutations in the Presenilins, leading to misprocessing of APP, are the most common cause of familial AD (Selkoe, 2002).

The normal physiological function of APP is unclear, however roles in cell adhesion (Fossgreen et al., 1998) or signal transduction have been suggested (Cao and Sudhof, 2001). The idea that APP may function in signal transduction has gained support by the demonstration of striking similarities between the processing of APP and Notch (Selkoe, 2002). Notch is a cell surface receptor that plays a pivotal role in many cell fate decisions during embryogenesis and in adults (Weinmaster, 2000). Notch signalling is accomplished by a RIP event similar to that of APP which, upon ligand binding, results in the shedding of the extracellular domain followed by γ-secretase-mediated intramembrane proteolysis and release of the Notch intracellular domain (NICD). The NICD then translocates to the nucleus where it can regulate the transcription of target genes (Kopan, 2002). Recently, a NICD analogous APP intracellular domain (AICD) has been identified and shown to translocate to the nucleus and to initiate gene transcription (Cupers et al., 2001; Gao and Pimplikan, 2001; Gu et al., 2001; Kimberly et al., 2001). The fact that both NICD and AICD are generated by Presenilin-dependent γ-secretase activity further supports a link between APP and a role in signal transduction.

Alternative splicing of human *APP* generates multiple mRNAs. These include species encoding APP isoforms consisting of 695 and 751 amino acids (APP695 and APP751) (Kang et al., 1987; Ponte et al., 1988). APP751 differs from APP695 by the insertion of a 56 amino acid (aa) long domain with similarity to Kunitz-type protease inhibitor (KPI) domains (Ponte et al., 1988; Tanzi et al., 1988). APP751 has been demonstrated to have protease inhibitory activity (Kitagushi et al., 1988). However, the significance of this finding in relation to its physiological roles remains unclear. The soluble, extracellular domains, generated by ectodomain shedding of KPI domain-containing APP isoforms, have been shown to be increased in AD brains (Moir et al., 1998) and may be more amyloidogenic (Ho et al., 1996), suggesting that APP751 mis-regulation may be a contributing factor for amyloid deposition in sporadic AD. In a previous study two zebrafish *app* orthologues (*appa* and *appb*) similar to the human *APP695* transcript variant were isolated (Musa et al., 2001). However, the presence of other alternatively spliced zebrafish *app* mRNAs during embryogenesis has not been demonstrated. In this report, we describe the cloning and characterisation of full-length cDNAs from zebrafish *appa* encoding isoforms similar to human APP695 and APP751. We have also isolated and characterised a partial cDNA from zebrafish *appb* encoding a KPI domain. Thus we demonstrate that KPI domain-containing isoforms have been retained in the duplicate zebrafish *app* orthologues, suggesting evolutionarily conserved functions for this domain in *app* physiology.

4.3. MATERIALS AND METHODS

4.3.1. Isolation of *appa* and *appb* cDNA

Full-length zebrafish *appa* cDNA was isolated by plaque hybridisation screening of cDNA sublibraries (Lardelli, 1997) made from a 9-16 hours post fertilisation (hpf) embryonic randomly primed zebrafish cDNA library in lambda-ZAP vector (a kind gift of R. Riggleman, K. Helde and D. Grunwald). The template for the hybridisation probe was generated by RT-PCR (see below) from 12 hpf stage embryos based on the published partial cDNA sequence of *appa* (GenBank accession number AF257742). PCR components were: 0.5 µM primer, 0.25 mM dNTP, standard PCR buffer (final concentrations were: 50 mM KCl; 1.5 mM $MgCl_2$; 10 mM Tris-HCl, pH 8.3), 1 Unit *Taq* polymerase. Primers used were: forward, APP001F 5'-GAAGAGCCGCA AGCAGTGCC-3' and reverse, APP002R 5'-CGTCTGAATCGTCCTCCTCC-3'. PCR conditions were: 94 °C for 30 sec, 60 °C for 45 sec, 0.5 °C/sec to 72 °C, 72 °C for 1 min, 35 cycles.. A positive cDNA clone of approximately 2.3 kb was isolated and confirmed by sequencing to include the entire open reading frame of *appa*. The protein sequence of our zebrafish cDNA showed similarity to the 751 aa human APP isoform APP751, which includes the alternatively spliced Kunitz-type protease inhibitor domain (Ponte et al., 1988).

Next we amplified by RT-PCR variant cDNAs containing the complete open reading frame of different *appa* isoforms. These were cloned using the Zero Blunt TOPO PCR Cloning Kit (Invitrogen, USA). The isolation of RNA from 12 hpf stage embryos was

performed using the RNeasy Mini Kit (Qiagen, USA) and cDNA was generated using the Omniscript Reverse Transcriptase Kit (Qiagen, USA). PCR components were: 0.5 μM primer, 0.25 mM dNTP, *pfu* buffer (final concentrations were: 10 mM KCl; 2 mM $MgSO_4$; 10 mM $(NH_4)_2SO_4$; 20 mM Tris-HCl, pH 8.3; 0.1 mg/ml BSA, 0.1% Triton X-100), 2 Units *pfu* DNA polymerase (Promega, USA). Primers used were: forward, APP010F 5'-GCATGCGGTCGAGGGAGC-3' and reverse, APP004R 5'-CTCTGA TTGCTCATTTCTGACC-3'. PCR conditions were: 94 °C for 30 sec, 57 °C for 45 sec, 0.5 °C/sec to 72 °C, 72 °C for 4 min, 35 cycles. In addition to cDNA encoding zebrafish *appa751* we also isolated a cDNA with similarity to the 695 aa human APP isoform APP695, termed zebrafish *appa695*. The *appa* sequences are deposited at GenBank under the accession numbers AY271746 (*appa695*) and AF3894019 (*appa751*).

The cloning of an *appb* transcript variant with similarity to the human *APP695* has previously been reported (Musa et al., 2001). To clarify whether the *appb* gene produces transcript variants encoding the Kunitz-type protease inhibitor domain we screened 12 hpf cDNA by RT-PCR. PCR components were: 0.5 μM primer, 0.25 mM dNTP, *pfu* buffer (final concentrations were: 10 mM KCl; 2 mM $MgSO_4$; 10 mM $(NH_4)_2SO_4$; 20 mM Tris-HCl, pH 8.3; 0.1 mg/ml BSA, 0.1% Triton X-100), 2 Units *pfu* DNA polymerase (Promega, USA). Primers used were: forward, APP0017F 5'-ACCAGTGAAC AGACCTCC-3' and reverse, APP0018R 5'-ATCAGCAGGGCTCGGGG-3'. PCR conditions were: 94 °C for 30 sec, 52 °C for 45 sec, 0.5 °C/sec to 72 °C, 72 °C for 1 min, 35 cycles. Two PCR fragments corresponding to the predicted size for the selected region of *appb695* and *appb751* were amplified.

DNA isolation of the putative *appb751* PCR product was performed with the MinElute Gel Extraction Kit (Qiagen, USA) and the following sequencing confirmed that it represents an *appb* transcript variant encoding a Kunitz-type protease inhibitor domain. The partial *appb751* sequence is deposited at GenBank under the accession number AY271744.

4.3.2. Whole mount *in situ* transcript hybridisation

The transcript expression pattern of the zebrafish *appa* gene was analysed by whole mount *in situ* transcript hybridisation on fixed zebrafish embryos from 12 to 24 post fertilisation (hpf) as previously described (Kimmel et al., 1995). *appa751* cDNA cloned into the pCR-BluntII-TOPO vector was linearised with *Not*I and a riboprobe was subsequently produced using SP6 RNA polymerase and the Digoxigenin (DIG) RNA Labelling Kit (Boehringer Mannheim, Germany). Unincorporated nucleotides were removed using RNase-free Micro Bio-Spin Columns P-30 (BIO-RAD, USA).

4.3.3. Sequence and phylogenetic analysis

Phylogenetic and sequence analyses were performed using software available on BioNavigator by ANGIS (Australian National Genomic Information Service). The protein sequence of the human 695 amino acid isoform of APP was aligned to similar sequences from zebrafish Appa and Appb proteins using Clustal W (Thompson et al., 1994) and prepared for publication using Boxshade by Hofmann K. and Baron M.D. The evolutionary relationships between APP protein sequences lacking the KPI domain were

analysed using the Phylip phylogeny inference package (Felsenstein, 1989). The distance measure for the protein sequences was computed using the Dayhoff PAM matrix and the Neighbor-Joining method was used to generate the phylogenetic tree. The tree was prepared for publication using Phylodendron by Gilbert D.G. GenBank accession numbers: Human APP (Y00264), mouse App (NM007471), rat APP (X07648), guinea pig APP (X97631), chick APP (AF289218), *Xenopus* APPA (AJ298150), *Xenopus* APPB (AJ298151), zebrafish Appa (this study), zebrafish Appb (AJ315639). *Drosophila* APPL (J04516) was chosen as the outgroup. The scale bar represents 10 base substitutions per 100 nucleotide positions and the numbers refer to bootstrap percentages.

4.4. RESULTS

4.4.1. Sequence and phylogenetic analysis of zebrafish Appa and Appb

Full-length zebrafish *appa* transcript variants were isolated by screening an embryonic cDNA library or by RT-PCR. Two zebrafish *appa* variants were identified with similarity to the 695 and 751 aa encoding variants of human *APP* (*APP695* and *APP751*) (Kang et al., 1987; Ponte et al., 1988). Zebrafish *appa695* and *appa751* encode putative proteins of 678 and 734 aa, respectively (**FIG. 4.1** and **4.3**). Alignment of the APP695 protein sequences from human and zebrafish revealed that zebrafish Appa695 is 74% identical to human APP695. A second zebrafish *app* gene has been isolated, termed *appb* (Musa et al., 2001). The *appb* was shown to encode a splice variant similar to human APP695, and is designated Appb695. The amino acid sequence of Appb695 is

71% identical to human APP695. The two zebrafish App695 protein sequences are 77% identical (**FIG. 4.1**). The Appa695 protein sequence is highly conserved in the C-terminal part of the protein subjected to γ-secretase-dependent cleavages, whereas regions known to be subject to α- and β-secretase-dependent proteolysis are less well conserved between human and zebrafish APP sequences (**FIG. 4.1**).

KPI-domain-containing APP isoforms have been found widely among the tetrapods (Coulson et al., 2000). The conservation of *APP* genes encoding KPI domains in the two teleosts, pufferfish (Villard et al., 1998) and zebrafish (this study), and in the evolutionarily divergent tetrapods (Coulson et al., 2000), suggests that this domain is involved in important biological functions in vertebrates. To clarify whether this domain has been retained in the duplicate *appb* gene we analysed, using RT-PCR, embryonic cDNA for the presence of *appb* mRNAs encoding the KPI domain. A PCR fragment of the predicted size was amplified and confirmed by sequencing to contain an Appb KPI domain. The Appb KPI domain is 57 aa long compared to 56 aa from zebrafish Appa and the human APP (**FIG. 4.3**). An alignment of the KPI domains from zebrafish and human showed that the Appa and Appb KPI domains are 68% and 61% identical to the human KPI domain and 66% identical to each other (**FIG. 4.3**). Subsequently, we have obtained full-length cDNA clones of *appb695* and *appb751* mRNAs (not shown).

To further investigate the evolutionary relationship between zebrafish App and other vertebrate APP proteins we performed a phylogenetic analysis (**FIG. 4.2**). This analysis was performed using protein sequences encoded by APP695 transcript variants. As

expected zebrafish Appa and Appb form a group separate from the tetrapod clade. The divergence of the two zebrafish App proteins is considerably greater than for any of the other sequences compared consistent with their creation in an duplication event early in vertebrate evolution and, possibly, with reduced selective pressure for sequence conservation due to subfunctionalisation (Force et al., 1999).

4.4.2. Spatial and temporal expression of *appa*

The expression pattern of *appa* during zebrafish embryo development was analysed by whole mount *in situ* hybridisation (**FIG. 4.4**). We first detected ubiquitous *appa* expression between 6 and 8 hpf and this continued until 10 hpf (Musa et al., 2001). Since the *appa* expression pattern in later stage embryos has been incompletely characterised we performed a detailed analysis of the expression of this gene from 12 to 24 hpf. Relatively high levels of expression were found in the presomitic and segmented paraxial mesoderm of 12 and 16 hpf embryos (**FIG. 4.4A-F**). Adaxial cells showed especially high expression levels in both presomitic and segmented paraxial mesoderm (**FIG. 4.4D-F**). *appa* was also weakly expressed as a pair of narrow bilateral stripes in the lateral plate mesoderm at 12-16 hpf (not shown). High levels of *appa* expression were observed in the otic vesicles between 12 and 24 hpf (**FIG. 4.4A, B, H**). Expression in the CNS was weak and limited to the presumptive forebrain at 16 hpf (**FIG. 4.4D**). At 24 hpf *appa* expression was strongly up-regulated in the telencephalon, diencephalon and olfactory placodes (**FIG. 4.4I**). *appa* also showed significant expression in the lenses of the eyes (**FIG. 4.4F, H**) and in the epiphysis (**FIG. 4.4G**). *appa* exhibited strong and restricted ventrolateral expression in the hindbrain (**FIG. 4.4J**). It was also expressed in the

trigeminal and posterior lateral line ganglia of the PNS (**FIG. 4.4H, J**). Outside of the nervous system, *appa* was expressed in somites with strongest expression in the ventromedial portion of each somite (**FIG. 4.4K, M**). *appa* also showed high level of expression in the pronephric ducts (**FIG. 4.4K, L**) and tail bud (**FIG. 4.4G**). Note the absence of any *appa* expression in the outer surface epithelia (**FIG. 4.4J, K, M**).

4.5. DISCUSSION

Analysis of the zebrafish genome has indicated that the extant genome is a result of a whole genome duplication event that occurred in the zebrafish lineage after it diverged from the lineage of tetrapods 100-450 million years ago (Postletwait et al., 1998; Taylor et al., 2001). An important mechanism for the retention of duplicate genes in the course of evolution is the diversification and subdivision of the functions (subfunctionalisation) of the ancestral gene, resulting in partly complementary expression patterns and conservation of functions of the duplicates (Force et al., 1999). This evolutionary process may involve the degeneration of alternative exons, regulatory elements and promoters (Altschmied et al., 2002). For example, there is evidence that two functionally, different alternative transcripts of the ancestral vertebrate *mitf* gene have been replaced by two distinct genes in zebrafish, *mitfa* and *mitfb* (Lister et al., 2001). This involved the loss of one ancestral alternative exon in one of the duplicate *mitf* genes (Altschmied et al., 2002).

Duplicate *app* genes have been identified in the zebrafish genome (zebrafish *appa* and *appb*) (Musa et al., 2001). While superficial analyses of the developmental expression patterns of both *appa* and *appb* has been published (Musa et al., 2001), no detailed analysis of their expression patterns exist. We have previously isolated the complete cDNA of the *appa* gene and so we decided to provide a comprehensive description of the expression pattern of *appa* from 12 to 24 hpf (**FIG. 4.4**). In good agreement with Musa et al. (2001) we showed that *appa* is widely expressed in the CNS. In addition, we provided the novel observations that *appa* is expressed in the pronephric ducts and medial somites. The role of APP outside the CNS has received relatively little attention. For example, APP is expressed in muscle tissues throughout the human body, and misregulation of APP has been implicated in the pathogenesis of **INCLUSION BODY MYOSITIS (IBM)**, the most common muscle disorder to inflict the elderly (Sugarman et al., 2002). Aβ deposition occurs in both IBM and AD, suggesting that similar pathogenic events may be of central importance in both diseases. The presence of *appa* expression in presomitic and somitic mesoderm (**FIG. 4.4D, 4.4K**), suggests that muscle progenitor cells may also express this gene. This question could be answered by double *in situ* hybridisation using a myogenic precursor cell specific marker such as *myoD* (Weinberg et al., 1996) in combination with *appa*. If this turns out to be the case, the use of embryonic zebrafish to study the genetic interactions relevant to IBM would be an invaluable new model system available in the fight against this devastating disease.

We have also isolated the full-length cDNA of zebrafish *appb*. *In situ* hybridisation analysis using the entire *appb* as a riboprobe replicated the results of Musa et al. (2001).

However, we consistently demonstrated very weak levels of expression of *appb* relative to that of *appa* (not shown). Because of the absence of any detailed description of the *in situ* hybridisation conditions in the analysis of Musa et al. (2001) direct comparison of our results is precluded. Thus, it remains possible that the duplicate *app* genes exhibit markedly different levels of expression during early embryogenesis in zebrafish.

An intriguing aspect of the expression of the *app* duplicates is the occurrence of distinct, complementary expression domains. For example, unique and distinctive expression of *appa* was found in the lenses, the otic vesicles and somites (**FIG 4.4**; Musa et al., 2001) and *appb* was uniquely expressed in a set of repetitive nuclei within the hindbrain, the ventral mesencephalon and the spinal cord (Musa et al., 2001). The combined developmental expression patterns of *appa* and *appb* (this study; Musa et al., 2001) are very similar to that of mouse *App* (Sarasa et al., 2000), a prototypical vertebrate *APP* gene. This indicates that subfunctionalisation may have played a role in the evolution of the duplicate zebrafish *app* genes and their retention in the zebrafish genome. We went on to explore if the implied subfunctionalisation of the ancestral *APP* gene may have involved the degeneration/loss of the alternative exon encoding the KPI domain in the zebrafish duplicates. We showed that transcripts of the duplicate *app* genes show alternative splicing, and both genes encode transcripts encompassing the KPI domain (**FIG. 4.1; 4.3**). Thus, it appears that the KPI domain regulates important functional aspects of normal *app* biology in zebrafish since it has been retained in the duplicates for more than 100 million years (Postletwait et al., 1998; Taylor et al., 2001). Inhibition of translation using morpholino oligonucleotides against the translation start codons of

zebrafish mRNAs has been shown to be an efficient way to knock down gene expression (Araki and Brand, 2001). We could use this method to specifically knock down the expression of *appa* and/or *appb* during embryogenesis. It would also be very interesting to dissect the functions of the alternatively spliced transcripts. It has recently been demonstrated that it is possible to inhibit the translation of alternatively spliced zebrafish transcripts by blocking pre-mRNA splicing using morpholino oligonucleotides (Draper et al., 2001). Thus, it is possible to selectively block the expression of the various alternatively spliced *appa* and *appb* transcripts. Morpholino oligonucleotide studies promise to provide very important clues about the role of the *app* duplicates during embryo development.

The two *app* duplicates are highly conserved genes encoding proteins with approximately 80% protein sequence identity (**FIG. 4.2**). This raises the possibility that RNA transcript *in situ* hybridisation analysis would be obscured by cross-reactivity between the two *app* transcripts. However, the distinct and complementary expressions patterns observed between *appa* and *appb* argue against this proposition (This study; Musa et al., 2001).

FIGURE 4.1. Sequence alignment of APP695 proteins from zebrafish and human. Identical amino acids are shaded. Gaps introduced for optimal alignment are indicated by dashes. The 40-42 aa amyloid β peptide (Aβ) is boxed. The cleavage sites of the three secretase activities are indicated by arrows. A? numbering starts from the β-secretase clevage site. The insertion site for the alternatively spliced Kunitz-type protease inhibitor domain is indicated (KPI). The transmembrane domain is indicated with a dashed bar. γ-secretase-dependent proteolytic processing of the C-terminal of *APP* leads to the liberation of the AICD and subsequent translocation to the nucleus followed by initiation of target gene transcription.

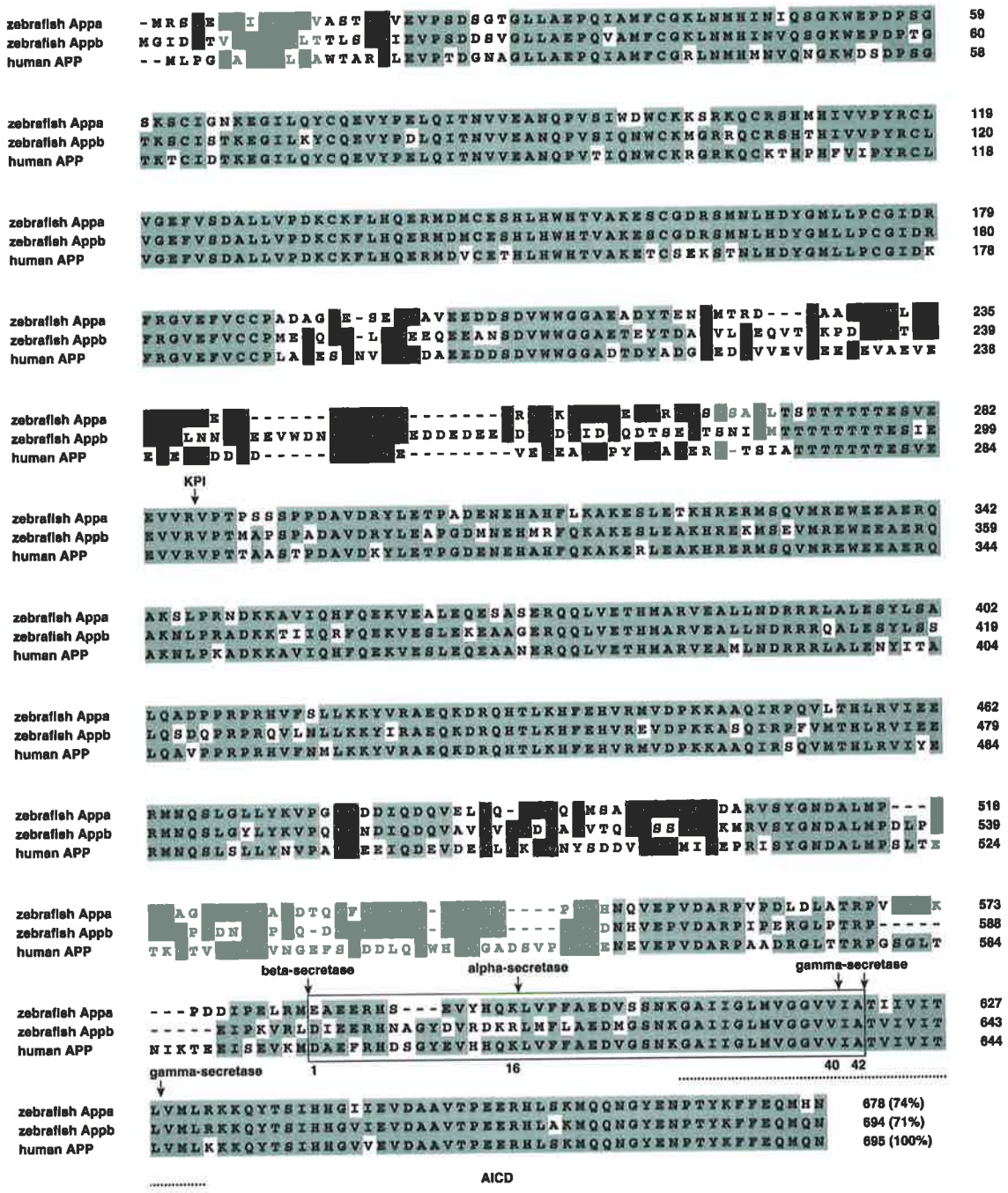

zebrafish Appa
zebrafish Appb
human APP
KPI
beta-secretase
alpha-secretase
gamma-secretase
gamma-secretase
1
16
40 42
678 (74%)
694 (71%)
695 (100%)
AICD

FIGURE 4.2. Phylogeny of zebrafish App duplicates. The evolutionary relationships of zebrafish Appa and Appb to other vertebrate APP proteins (lacking the Kunitz-type protease inhibitor domain) was established by comparison to selected vertebrate APP protein sequences and the construction of a phylogenetic tree. *Drosophila melanogaster* APPL was chosen as the outgroup. Bootstrap values shown are based on 100 replications.

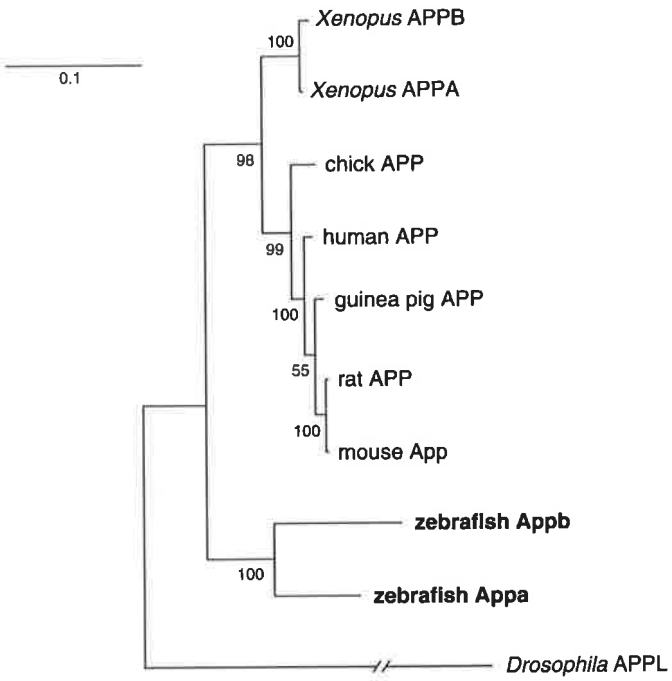

0.1
Xenopus APPB
100
Xenopus APPA
98
chick APP
99
human APP
100
guinea pig APP
55
rat APP
100
mouse App
zebrafish Appb
100
zebrafish Appa
Drosophila APPL

FIGURE 4.3. Sequence alignment of the alternatively spliced Kunitz-type protease inhibitor (KPI) domain from zebrafish and human. Identical amino acids are shaded. Gaps introduced for optimal alignment are indicated by dashes. The KPI domain is boxed. The alternatively spliced KPI domain is inserted between aa 288 and 289 (**see FIGURE 4.1**), with numbers referring to the positions of the flanking amino acids of the human APP695 isoform.

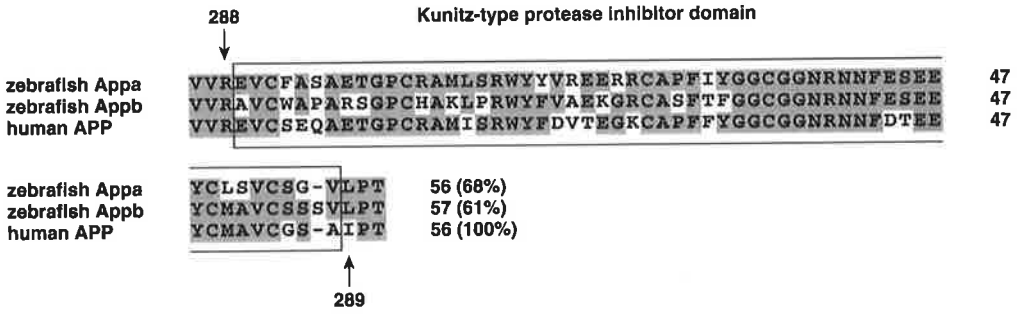
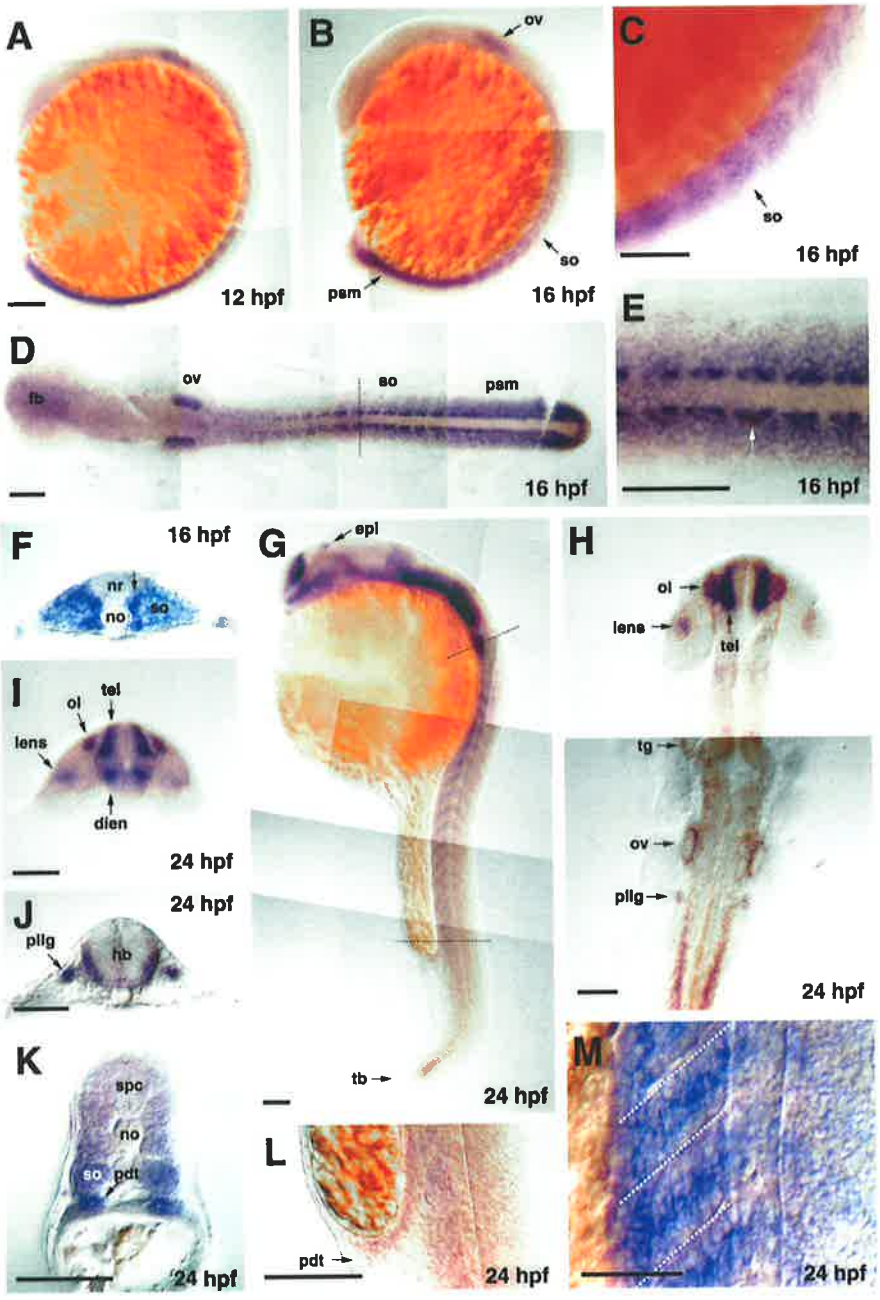

288
Kunitz-type protease inhibitor domain
zebrafish Appa
zebrafish Appb
human APP
VVREVCFASAETGPCRAMLSRWYYVREERRCAPFIYGGCGGNRNNFESEE
VVRAVCWAPARSGPCHAKLPRWYFVAEKGRCASFTFGGCGGNRNNFESEE
VVREVCSEQAETGPCRAMISRWYFDVTEGKCAPFFYGGCGGNRNNFDTEE
47
47
47
zebrafish Appa
zebrafish Appb
human APP
YCLSVCSG-VLPT
YCMAVCSSSVLPT
YCMAVCGS-AIPT
56 (68%)
57 (61%)
56 (100%)
289

FIGURE 4.4. The developmental expression pattern of zebrafish *appa*. (A, B) Lateral view of embryos at 12 hpf (A) and 16 hpf (B). (B-F) Different views of the same 16 hpf embryo. (C) Magnification of the somitic region in (B). *appa* is strongly expressed in the otic vesicle/placode, somites and presomitic mesoderm. (D) Dorsal view showing weak *appa* expression in the forebrain region. (E) Magnification of (D) showing widespread *appa* staining in the segmented paraxial mesoderm with particularly strong staining in the adaxial cells (encircled area). (F) Transverse section through the trunk region in (D). Note the strong expression of *appa* in the adaxial cells (arrow) and absence of staining in the neural rod and notochord. (G-M) Different views of 24 hpf embryos. (G) Lateral view showing *appa* expression in the epiphysis and tail bud. (H) Dorsal view. (I) Frontal view of the head shows that *appa* is strongly expressed in the telencephalon, diencephalon and the olfactory placodes. (J) Transverse section through posterior lateral line ganglia, showing strong expression of *appa* in the ventro-lateral region of the hindbrain. (K) Transverse section through the trunk at the posterior yolk extension. (L) Magnification of the tip of the posterior yolk extension in (G). (M) Magnification of the posterior trunk region in (G) showing medial somitic expression of *appa*. dien, diencephalon; fb, forebrain; epi, epiphysis; hb, hindbrain; no, notochord; ol, olfactory placode; ov, otic vesicle; pllg, posterior lateral line ganglion; prd, pronephric duct; psm, presomitic mesoderm; so, somite; spc; spinal cord; tb, tail bud; tel, telencephalon; tg, trigeminal ganglion. Scale bars indicate 100 μm, except (M), 50 μm.


A
12 hpf
B
ov
so
psm
16 hpf
C
so
16 hpf
D
fb
ov
so
psm
16 hpf
E
16 hpf
F
16 hpf
nr
no
so
G
epi
tb
24 hpf
H
ol
lens
tel
tg
ov
pllg
24 hpf
I
ol
tel
lens
dien
24 hpf
J
24 hpf
pllg
hb
K
spc
no
so
pdt
24 hpf
L
pdt
24 hpf
M
24 hpf

Chapter 5

Zebrafish fgfr1 is a member of the fgf8 synexpression group and is the only fgfr gene expressed at the midbrain-hindbrain boundary

5.1. ABSTRACT

Human FGFR1 is an important signalling molecule during embryogenesis and in adulthood. Disruption of its normal functions may lead to developmental defects and pathological conditions, including cancer and Alzheimer's disease. We have now cloned zebrafish *fibroblast growth factor receptor 1* (*fgfr1*) and characterised its expression pattern. *fgfr1* is expressed in the adaxial mesoderm with transcripts distinctly localised to the anterior portion of each half-somite. *fgfr1* is also strongly expressed in the otic vesicles, branchial arches and the brain, especially at the midbrain-hindbrain boundary (MHB). The expression patterns of *fgfr1* and *fgf8* are strikingly similar. Thus *fgfr1* is a new member of the *fgf8* synexpression group. *fgfr1* is the only *fgfr* gene expressed at the MHB, suggesting that it is the receptor responsible for the transduction of Fgf8 signals at the MHB.

5.2. INTRODUCTION

Fibroblast growth factors (FGFs) comprise a large protein family of more than 20 members important in regulating cell proliferation, survival and differentiation during embryogenesis and adult homeostasis (Ornitz and Itoh, 2001; Szebenyi and Fallon, 1999). The cellular events controlled by FGFs are mediated through their interactions with fibroblast growth factor receptor proteins (FGFRs) belonging to the receptor tyrosine kinase superfamily (Schlessinger, 2000). Four closely related *FGF receptor* genes (*FGFR1-4*) have been found in vertebrates (Groth and Lardelli, 2002). *FGFR1* has been shown to play an important role during embryo development as well as in the adult animal. For example, *FGFR1* dysfunction has been implicated in Pfeiffer's syndrome (Passos-Bueno et al., 1999), a developmental disorder characterised by craniofacial

abnormalities, and in pathological conditions including cancer and Alzheimer's disease (Takami et al., 1998; Valve et al., 2001).

The canonical view of the biological role of FGFR1 and other FGF receptors is that of cell surface receptors which, upon binding of their ligand, dimerise, leading to phosphorylation at specific cytoplasmic tyrosine residues and activation of the receptor. The activated kinase can then phosphorylate and activate the membrane-bound FRS2 protein, allowing the recruitment of other adapter proteins to the complex and subsequent activation of the Ras/mitogen-activated protein kinase (MAPK) signalling cascade. MAPK, the last member of this cascade, can translocate into the nucleus and phosphorylate and activate transcription factors, leading to the transcription of target genes (Schlessinger, 2000).

One of the FGF family members, *FGF8*, is an important signalling molecule expressed in the forebrain and at the midbrain-hindbrain boundary (MHB), a region involved in the patterning of the developing vertebrate brain. Outside the brain, *FGF8* expression is found in the otic vesicles, heart, limb buds, presomitic mesoderm and somites (Reifers et al., 1998). Overexpression of zebrafish *fgf8* affects dorsoventral patterning, leading to dorsalisation of embryos (Furthauer et al., 1997) and this effect is mimicked by overexpression of a constitutively activated form of zebrafish *fgfr1* (Furthauer et al., 2001). This suggests that FGFR1 may be a key mediator of FGF8-dependent signalling during embryo development.

In this study we report the isolation and characterisation of a zebrafish orthologue of human *FGFR1* (Dionne et al., 1990). Zebrafish *fgfr1* shows a strikingly similar expression pattern to *fgf8* during early zebrafish development, and thus represents a novel member of a growing group of genes with expression similar to *fgf8* (the *fgf8* synexpression group).

5.3. MATERIALS AND METHODS

5.3.1. Cloning of zebrafish *fgfr1* cDNA

A cDNA revealing a restricted pattern of embryonic gene expression similar to *fgf8* and a high degree of sequence similarity to the kinase domain of known Fgf receptor genes was isolated as part of an *in situ* transcript hybridisation screen (Tamme et al., 2001). This cDNA was used as a template for PCR screening of cDNA sublibraries (Lardelli, 1997) made from a 9-16 hpf embryonic randomly primed zebrafish cDNA library in the lambda-ZAP vector (kindly provided by R. Riggleman, K. Helde and D. Grunwald) to identify positive sublibraries. Overlapping cDNA clones were isolated by plaque hybridisation from positive sublibraries using this cDNA as probe. Successive screenings using the 5' end of isolated positive cDNA clones as probes allowed the isolation of overlapping cDNA clones covering the complete open reading frame of the Fgf receptor. Subsequently, full-length *fgfr1* transcript variants were isolated from 12 hpf cDNA by RT-PCR as followed: The isolation of RNA from 12 hpf stage embryos was performed using the RNeasy Mini Kit (Qiagen, USA) and cDNA was generated using the Omniscript Reverse Transcriptase Kit (Qiagen, USA). PCR components were:

0.5 μM primer, 0.25 mM dNTP, *pfu* buffer (final concentrations were: 10 mM KCl; 2 mM $MgSO_4$; 10 mM $(NH_4)_2SO_4$; 20 mM Tris-HCl, pH 8.3; 0.1 mg/ml BSA, 0.1% Triton X-100), 2 Units *pfu* DNA polymerase (Promega, USA). Primers used for RT-PCR of full-length *fgfr1* were: forward, FGFR021F 5'-TGGAGTTCAGATGTAGAGG-3' and reverse, FGFR022R 5'-CAGCTG TATGTGTTTCTCC-3'. PCR conditions were: 94 °C for 30 sec, 51 °C for 45 sec, 0.5 °C/sec to 72 °C, 72 °C for 4 min, 35 cycles. The generated PCR products were gel extracted using the MinElute Gel Extraction Kit (Qiagen, USA) and cloned into the pCRII-Blunt-TOPO vector using the Zero Blunt TOPO PCR Cloning Kit (Invitrogen, USA). Subsequent sequencing confirmed the identity of the *fgfr1* cDNA clones.

5.3.2. Embryos and whole mount *in situ* transcript hybridisation

Zebrafish were staged according to (Kimmel et al., 1995). Stages are given as equivalent to hours of development post fertilisation (hpf) at 28.5 °C. The transcript expression pattern of the zebrafish *fgfr1* was analysed by whole mount *in situ* hybridisation on fixed embryos at various stages of development up to 28 hpf as previously described with the modification that prehybridisation and hybridisation were performed at 70 °C (Kimmel et al., 1995). A riboprobe recognising the kinase domain of *fgfr1* was synthesised by *in vitro* transcription from a full-length *fgfr1* cDNA (GenBank accession number AF389400) cloned into the pCRII-Blunt-TOPO vector. The insert was PCR amplified and subsequently isolated using the QIAquick Gel Extraction Kit (Qiagen, USA). PCR components were: 0.5 μM primer, 0.25 mM dNTP, standard PCR buffer (final concentrations were: 50 mM KCl; 1.5 mM $MgCl_2$; 10 mM Tris-HCl, pH 8.3), 1 Unit

Taq DNA polymerase. The PCR conditions were: 94 °C for 30 sec, 65 °C for 45 sec, 0.5 °C/sec to 72 °C, 72 °C for 3 min, 35 cycles. Primers used were: forward, Kami01 5'-CCTGCGCATATCAGGTGGC-3' and reverse, FGFR035R 5'-TAATACGACTCACTA TAGGGAGGCAGCTGTATGTGTTTCTCC-3'. A T7 RNA polymerase promoter was included in the 5' end of the reverse primer allowing antisense transcription using T7 RNA polymerase and the Digoxigenin RNA Labelling Kit (Boehringer Mannheim, Germany).

5.3.3. Sequence and phylogenetic analysis

Human FGFR1 and zebrafish Fgfr1 protein sequences were analysed using programs available through BioManager on ANGIS (Australian National Genomic Information Service). The sequences were aligned using Clustal W (Thompson et al., 1994) and prepared for publication using Boxshade by Hofmann K. and Baron M.D. The evolutionary relationship between metazoan *FGFR* gene sequences was analysed using Clustal W (Thompson et al., 1994) for sequence alignment and the maximum likelihood method of DNAML for generating the phylogenetic tree using the Phylip phylogeny inference package by Felsenstein (1989). The tree was prepared for publication using Phylodendron by Gilbert D.G. The following *FGFR* gene sequences were included in the phylogenetic analysis (accession numbers are given in parentheses): Human *FGFR1* (M55614), human *FGFR2* (31373), human *FGFR3* (13112046), human *FGFR4* (13112051), mouse *Fgfr1* (6753855), mouse *Fgfr2* (6753857), mouse *Fgfr3* (6679786), mouse *Fgfr4* (6679788), chick *CEK1* (M24637), chick *CEK3* (M35196), chick *CEK2* (M35195), *Xenopus FGFR1* (U2449), *Xenopus FGFR2* (64694), *Xenopus FGFR3*

(2425187), *Xenopus FGFR4* (AF288453), *P. waltl FGFR1* (64250), *P. waltl FGFR2* (396744), *P. waltl FGFR3* (414683), *P. waltl FGFR4* (64252), zebrafish *fgfr1* (this study), zebrafish *fgfr3* (AF157560), zebrafish *fgfr4* (U23839). *Drosophila DFGFR* (285753) was chosen as the outgroup.

5.3.4. Mapping zebrafish *fgfr1*

Zebrafish *fgfr1* was mapped using the LN54 mouse/zebrafish radiation hybrid panel (Hukriede et al., 1999). PCR components were: 0.5 µM primer, 0.25 mM dNTP, standard PCR buffer (final concentrations were: 50 mM KCl; 1.5 mM $MgCl_2$; 10 mM Tris-HCl, pH 8.3), 1 Unit *Taq* polymerase. 5' UTR primers used were: forward, FGFR030F 5'-CACGAGCGCAAACAAAACC-3' and reverse, FGFR031R 5'-GCACTTTCTGAAGCAAATCC-3'. The PCR conditions were: 94 °C for 30 sec, 52 °C for 45 sec, 0.5 °C/sec to 72 °C, 72 °C for 2 min, 35 cycles. The PCR experiment was replicated twice producing identical results. The RH panel was scored according to Hudson et al. (1995): 10110100000000000200011101000011100000000000000000000000000010001000020010010000100000010000. 1 indicates a positive hybrid, 0 indicates a negative hybrid and 2 indicates missing or ambigous hybrid data: Results were analysed with RHMAPPER and placed on the zebrafish radiation hybrid map provided by ZFIN (http://zfish.uoregon.edu/ZFIN).

5.3.5. RT-PCR analysis

The isolation of RNA from 8, 10, 12, 16, 24, 36 and 48 hpf embryos was performed using the RNeasy Mini Kit (Qiagen, USA) and cDNA was generated using Omniscript Reverse Transcriptase (Qiagen, USA) according to the manufacturer's instructions. Primers used to identify transcript variants including or missing the exons encoding the second half of the IgIII domain and the TM domain were: forward, FGFR028F 5'-CTGCCTGCAAACCGTACC-3' and reverse, CG003R 5'-GACGGACCAACATCCC AC-3. Primers used to identify *fgfr1IIIb* variants were: forward, FGFR0025F 5'-AACTCTGGGGTCAACAGCTC-3' and reverse, FGFR0024R 5'-TAATACGACTCAC TATAGGGAGGGTGTTTAACCACGGTGAGCC-3'. Primers used to identify *fgfr1IIIc* variants were: forward, FGFR027F 5'-ACGGCAGGCGTCAACACC-3' and reverse, FGFR026R 5'-TAATACGACTCACTATAGGGAGGTTATAGACAGTCAACCATGC AG-3'. PCR components were: 0.5 µM primer, 0.25 mM dNTP, standard PCR buffer (final concentrations were: 50 mM KCl; 1.5 mM $MgCl_2$; 10 mM Tris-HCl, pH 8.3), 1 Unit *Taq* polymerase. PCR conditions were identical for all three PCR experiments: 94 °C for 30 sec, 65 °C for 45 sec, 0.5 °C/sec to 72 °C, 72 °C for 3 min, 35 cycles.

5.4. RESULTS

5.4.1. Isolation of zebrafish *fgfr1*

Zebrafish *fgfr1* cDNA was first isolated from an *in situ* hybridisation screen to identify developmental control genes (Tamme et al., 2001). An approximately 800 bp cDNA showed expression similar to *fgf8* during zebrafish embryogenesis and its amino acid sequence was highly related to that of known Fgfr kinase domains (Johnson and

Williams, 1993), suggesting it encoded a region of a putative Fgf receptor. The open reading frame of our *fgfr* gene was assembled from the sequences of overlapping cDNA clones isolated from screening of zebrafish sublibraries (Lardelli, 1997). A phylogenetic tree of full-length vertebrate *FGFR* genes showed that our zebrafish *fgfr* cDNA groups with *vertebrate FGFR1* genes, and thus represents a zebrafish *fgfr1* gene (**FIG. 5.1**). Like other FGFR1 proteins the amino acid sequence of zebrafish Fgfr1 has been highly conserved during evolution, showing 81% similarity and 74% identity to human FGFR1 (**FIG. 5.2**). Alternative splicing of vertebrate *FGFR1* is extensive with approximately 20 transcript variants described so far (Groth and Lardelli, 2002). We isolated full-length zebrafish *fgfr1* cDNAs by RT-PCR using primers specific for the 5' and 3' UTRs of the assembled sequence representing four transcript variants. The open reading frame of the longest full-length cDNA (transcript variant 1) is 2418 bp in length encoding a putative 806 amino acid (type I integral) protein (**FIG. 5.2; 5.3**). The ectodomain of the predicted protein is 359 aa and includes three immunoglobulin-like domains (IgI-IgIII). The endodomain is 426 aa long and includes a tyrosine kinase domain split by a short interkinase region, a characteristic feature of Fgf receptor proteins (Johnson and Williams, 1993). The kinase domain is preceded by the juxtamembrane domain, a region important for Fgfr-mediated signal transduction (Gillespie et al., 1995; Paterno et al., 2000). A six nucleotide insert encoding a VT dipeptide is found in the juxtamembrane domain of the longest cDNA, but is absent in all other isolated cDNAs (**FIG. 5.2; 5.3**). Only the VT+ isoform has been found to interact with the FRS2 adaptor protein subsequently leading to activation of the Ras/MAPK signalling pathway (Burgar et al., 2002). In *Xenopus*, the relative expression level of VT+ and VT- FGFR1 isoforms has

been found to regulate mesoderm induction (Paterno et al., 2000). Transcript variant 2 is identical to transcript variant 1 except for the absence of the VT dipeptide (**FIG. 5.3**). Alternative splicing of two exons that encode the C-terminal half of IgIII, generating different isoforms (FGFR1IIIb and FGFR1IIIc) with different binding-affinities for Fgfs has been widely reported in vertebrates (Johnson and Williams, 1993). Zebrafish transcript variants 1 and 2 both encode Fgfr1IIIc isoforms. Transcript variant 3 is missing any of the alternatively spliced exons encoding the C-terminal half of IgIII while transcript variant 4 lacks the exon encoding the TM domain as well as any of the exons encoding the C-terminal half of IgIII (**FIG. 5.3**). The sequences of the full-length *fgfr1* transcript variants have been deposited in GenBank with the accession numbers AF389400, AY197498 to AY197500. We also isolated a partial cDNA representing a fifth transcript variant encoding a putative Fgfr1IIIb isoform. The cDNA sequence of this transcript variant has been deposited in GenBank with the accession number AY197501.

5.4.2. Mapping of *fgfr1*

Zebrafish *fgfr1* was mapped using the LN54 radiation hybrid panel (Hukriede et al., 1999) to linkage group 8 at 59.97 cR from the marker Z1052 with a LOD of 11.0 (**FIG. 5.3**). The best marker in the second-best linkage group (linkage group 12) showed apparent linkage to *fgfr1* with a LOD of 5.7.

5.4.3. Developmental expression of *fgfr1* transcripts

The expression pattern of *fgfr1* during zebrafish embryo development was analysed by whole-mount *in situ* transcript hybridisation (**FIG. 5.4**). The riboprobe used was specific for the kinase domain and, consequently, the pattern shown represents the sum of all transcript variants that include this region. The kinase domain regions of *fgfr* transcripts are highly conserved (Johnson and Williams, 1993). This points to a potential problem with cross-reactivity between the different transcripts and our riboprobe. However, the unique and distinctive expression domains demonstrated for *fgfr1* (see below) strongly argue against this possibility. Maternally derived *fgfr1* mRNA is found in cleavage stage blastomeres (**FIG. 5.4A, B**). Strong ubiquitous expression is present during gastrulation (**FIG. 5.4C**). During the segmentation stage high-level expression of *fgfr1* is found in the forebrain, in the region of the midbrain-hindbrain boundary, presomitic mesoderm and somites (**FIG. 5.4D-G**). However, widespread weak expression is also present at this stage and is mainly restricted to ventral mesoderm (**FIG. 5.4F, G**). At 12 hpf expression in the MHB region is present as one broad stripe (**FIG. 5.4D**), which by 15 hpf is clearly split into two separate domains (**FIG. 5.4E**). Expression is found in the cranial sensory ganglia at 15 hpf (**FIG. 5.4F, G**). During late segmentation and early pharyngeal stage strong expression is found in domains of the telencephalon and ventral diencephalon including the olfactory placode and optic stalk (**FIG. 5.4H-J**). In the midbrain region the tegmentum shows a high level of *fgfr1* transcription. Expression in the MHB region is especially strong. In the hindbrain *fgfr1* transcripts are found in the otic vesicles and branchial arches (**FIG. 5.4H-M**). A weaker but distinct metameric *fgfr1* pattern is present in the hindbrain (**FIG. 5.4I, M**). Expression is also present in the pectoral fin buds and the migrating primordia of the posterior lateral line (**FIG. 5.4H, I**). In order to determine the

temporal developmental expression pattern of some of the isolated transcript variants we performed variant-specific RT-PCR on cDNA isolated from different developmental stages ranging from 8 hpf to 48 hpf. (**FIG. 5.3**). We showed that the predominant transcript species contains the C-terminal half of IgIII, i.e. *fgfr1IIIb* or *fgfr1IIIc* variantsb. *fgfr1IIc* was expressed during all stages examined whereas *fgfr1IIIb* expression was first evident from 12 hpf and onwards (**FIG. 5.3**).

5.5. DISCUSSION

5.5.1. Evolution of *fgfr1*

We have isolated a zebrafish fibroblast growth factor receptor with very high structural similarity to other vertebrate FGFR proteins. Phylogenetic analysis (**FIG. 5.1**) suggested that the zebrafish *fgfr* gene is probably an orthologue of human *FGFR1*. Consequently, we named this zebrafish fibroblast growth factor receptor *fgfr1*. Human FGFR1 shows 74% amino acid identity and 81% similarity to zebrafish Fgfr1, and the kinase domain is 94% identical between these two distantly related species (**FIG. 5.2**), suggesting that FGFR1 controls important functions preserved during the evolution of the divergent lineages leading to extant fish and mammals. This idea is also supported by our results, which demonstrate significant similarity of embryonic *fgfr1* expression patterns in zebrafish and other vertebrates. For example, elevated *fgfr1* expression at the MHB is found in zebrafish and *Xenopus* (Golub et al., 2000). In addition, metameric expression of *FGFR1* in the presomitic mesoderm and somites has also been reported in mouse and chick (Walshe and Mason, 2000; Yamaguchi et al., 1992). We also found that *fgfr1* was

expressed in the pectoral fin buds (**FIG. 5.4**), indicating that zebrafish *fgfr1* may play a role in limb bud development similar to that shown in mouse (Partanen et al., 1998; Peters et al., 1992). A prominent feature of FGFR1 biology is the extensive usage of alternative splicing to generate transcripts encoding proteins, which may have distinct cellular functions (Groth and Lardelli, 2002). In this study we have identified 5 different zebrafish *fgfr1* transcript variants (**FIG. 5.2; 5.3**), showing that, like mammals, the zebrafish uses complex *fgfr1* regulatory mechanisms in order to control various aspects of Fgfr1 function.

5.5.2. *fgfr1* is the only *fgfr* gene expressed at the MHB

The expression patterns of *fgf8* and *fgfr1* are strikingly similar (**FIG. 5.4**) (Reifers et al., 1998), which may suggest that *fgfr1* is a receptor for *fgf8*-mediated signalling in zebrafish. Several genes of the *fgf8* synexpression group have been demonstrated to function in the FGF signalling pathway and are often found to be co-regulated. For example, the expression of the FGF signalling antagonists, *sef* and *sprouty4*, are both positively regulated by *fgf8* (Furthauer et al., 2002; Furthauer et al., 2001; Tsang et al., 2002). We have shown that *fgfr1* is abundantly expressed at the MHB (**FIG. 5.4**), while none of the three other zebrafish *fgfr* genes show expression at the MHB (Slepsova-Friedrich et al., 2001; Thisse et al., 1995; Tsang et al., 2002). This strongly supports that Fgfr1 is the receptor that is responsible for the transduction of Fgf8 signals at the MHB. It will be interesting to study the details of this putative interaction.

FIGURE 5.1. Phylogeny of zebrafish *fgfr1*. (A) The evolutionary relationship of zebrafish *fgfr1* to other members of the *FGFR* multigene family was established by comparison to selected metazoan *FGFR* gene sequences and the construction of a phylogenetic tree. *Drosophila melanogaster DFGFR* was chosen as the outgroup. Bootstrap values shown are based on 100 replications. (B) The map position of zebrafish *fgfr1* was assigned to LG8 using the LN54 radiation hybrid panel (Hukriede et al., 1999).

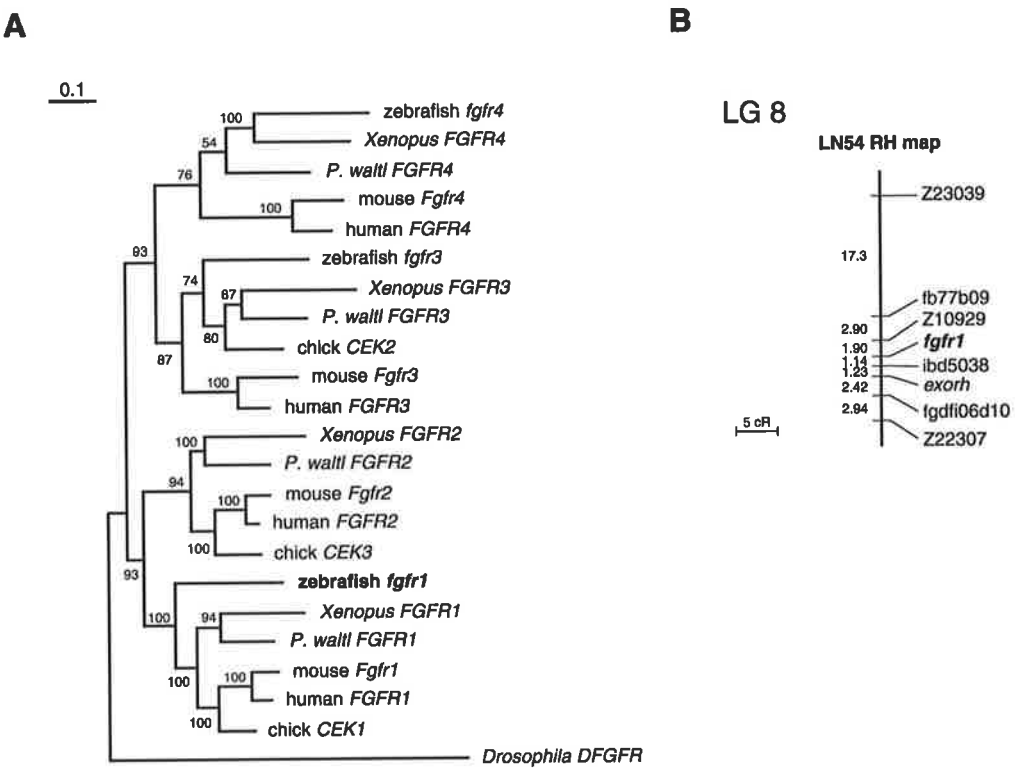

A
0.1
zebrafish fgfr4
Xenopus FGFR4
P. waltl FGFR4
mouse Fgfr4
human FGFR4
zebrafish fgfr3
Xenopus FGFR3
P. waltl FGFR3
chick CEK2
mouse Fgfr3
human FGFR3
Xenopus FGFR2
P. waltl FGFR2
mouse Fgfr2
human FGFR2
chick CEK3
zebrafish fgfr1
Xenopus FGFR1
P. waltl FGFR1
mouse Fgfr1
human FGFR1
chick CEK1
Drosophila DFGFR
100
54
76
100
93
74
87
80
87
100
100
94
100
100
93
100
94
100
100
100
B
LG 8
LN54 RH map
Z23039
17.3
fb77b09
Z10929
2.90
fgfr1
1.90
1.14
ibd5038
1.23
exorh
2.42
fgdfi06d10
2.94
Z22307
5 cR

FIGURE 5.2. Sequence alignment of FGFR1 protein from zebrafish and human. Identical amino acids are shaded. Gaps introduced for optimal alignment are indicated by dashes. Structural domains identified in human FGFR1 (Johnson and Williams, 1993) are marked by lines. Arrowheads with numbers indicate the exon boundaries of human FGFR1 (Cote et al., 1997; Givol and Yayon, 1992; Johnson et al., 1991). IgI-IgIII, immunoglobulin-like domain I to III; TM, transmembrane domain; K1, kinase domain 1; IK, interkinase domain; K2, kinase domain 2.

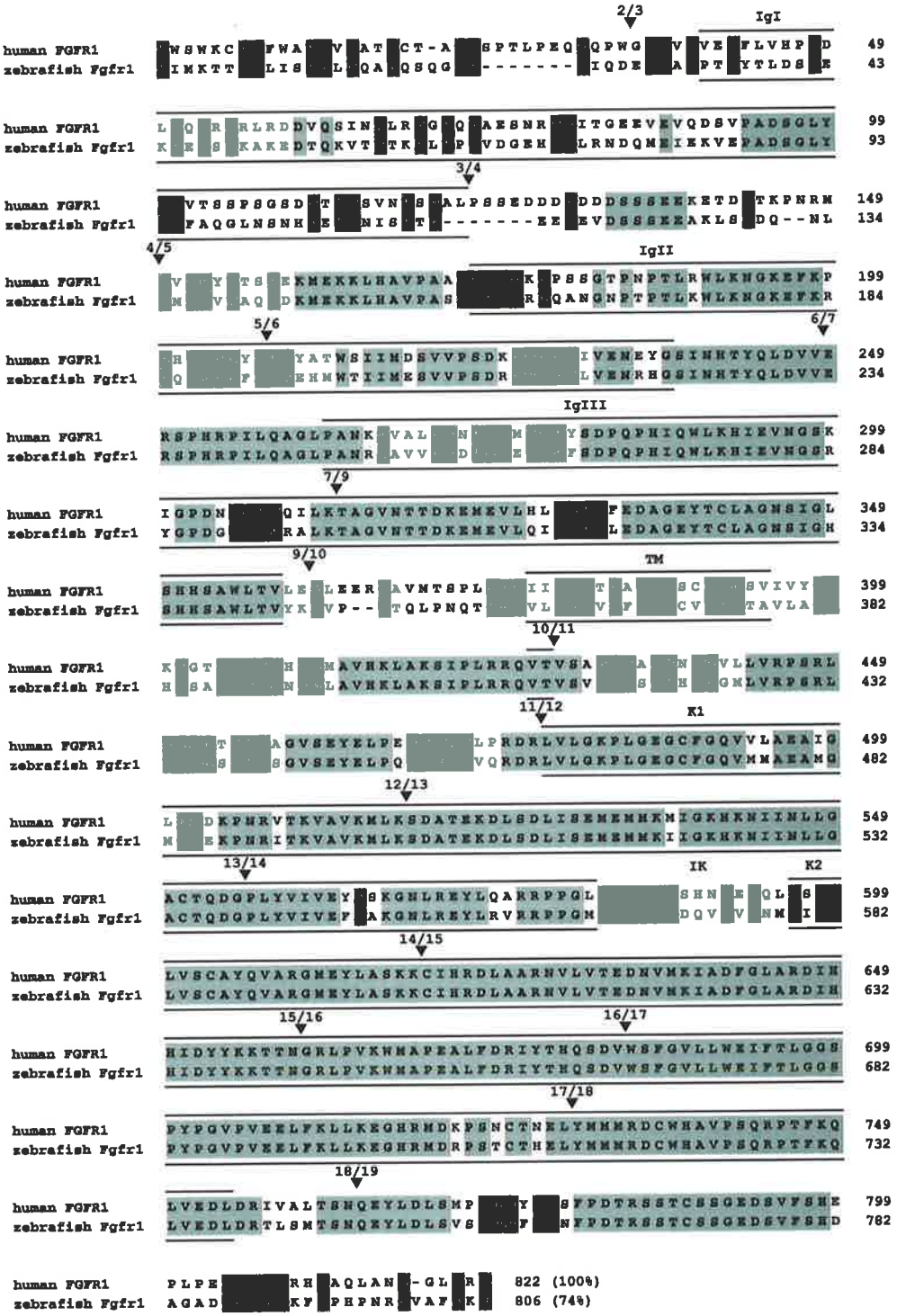

2/3
IgI
human FGFR1
zebrafish Fgfr1
49
43
99
93
3/4
149
134
4/5
IgII
199
184
5/6
6/7
249
234
IgIII
299
284
7/9
349
334
9/10
TM
399
382
10/11
449
432
11/12
K1
499
482
12/13
549
532
13/14
IK
K2
599
582
14/15
649
632
15/16
16/17
699
682
17/18
749
732
18/19
799
782
822 (100%)
806 (74%)

FIGURE 5.3. Alternative splicing of zebrafish *fgfr1*. (A) Schematic drawing showing the general structure of Fgfr1 and some of the predicted Fgfr1 isoforms (numbered 1 to 4). Isoforms 1 and 2 both represent Fgfr1IIIc isoforms, while isoforms 3 and 4 are missing the C-terminal half of IgIII (red box). Isoform 4 lacks that the TM domain. Yellow bars indicate regions amplified by RT-PCR. Primers used are also shown. (B) RT-PCR of the temporal developmental expression of *fgfr1* transcript variants from 8-48 hpf (indicated by numbers). Top panel, the majority of transcripts encode putative isoforms that include the C-terminal half of IgIII. Middle panel, *fgfr1IIIb* transcript variants. Bottom panel, *fgfr1IIIc* transcript variants.

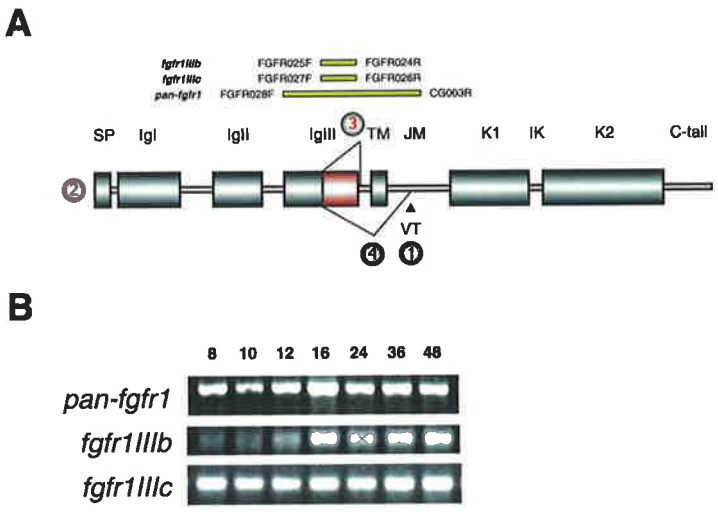
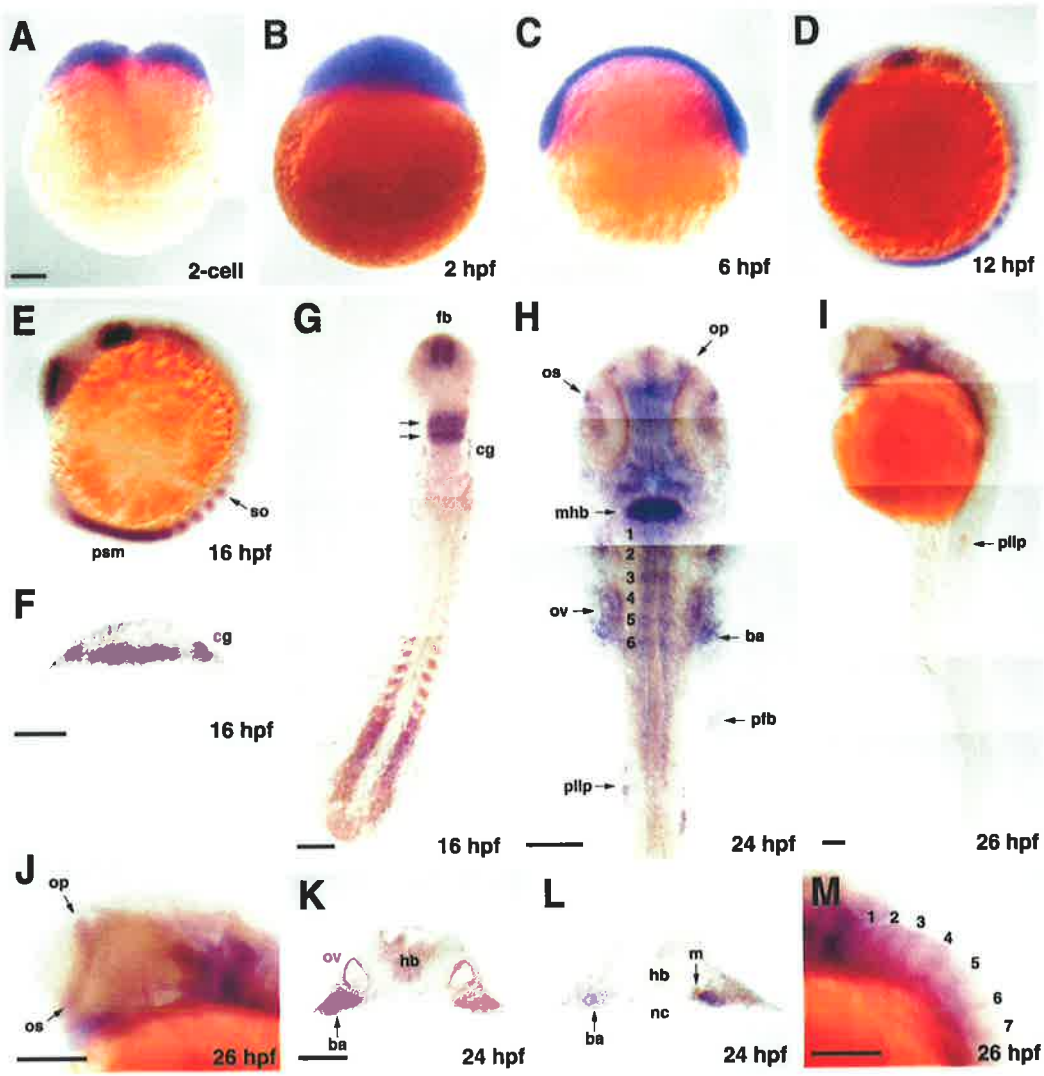

A
fgfr1IIIb
fgfr1IIIc
pan-fgfr1
FGFR025F
FGFR024R
FGFR027F
FGFR026R
FGFR028F
CG003R
SP
IgI
IgII
IgIII
TM
JM
K1
IK
K2
C-tail
VT
B
8
10
12
16
24
36
48
pan-fgfr1
fgfr1IIIb
fgfr1IIIc

FIGURE 5.4. The spatial expression of zebrafish *fgfr1* during embryogenesis. (A-C) Lateral view of embryos at 2-cell stage (A), 512-cell stage (B), 6 hpf (C) and 12 hpf embryos (D). (E-G) Different views of the same 15 hpf embryo. (E) lateral view. (F) Transverse section through the presumptive hindbrain region. Note that *fgfr1* is predominantly expressed in ventral mesoderm and cranial ganglia. (G) Dorsal view. Two stripes of *fgfr1* expression are observed in the midbrain-hindbrain region (indicated by arrowheads). (H, K, L) Different views of the same 24 hpf embryo. (H) Dorsal view showing strong *fgfr1* expression at the MHB. (K) Transverse section through the otic vesicles. (L) Cross-section through the post-otic region. (I, J, M) Lateral views of the same 28 hpf embryo. (I) *fgfr1* staining in the migrating primordium of the posterior lateral line. (J) Magnification of the head region in (I). (M) Magnification of the hindbrain region in (I) showing weak expression of *fgfr1* in a segmental pattern in rhombomeres. cg, cranial ganglion; ba, branchial arches; fb, forebrain; hb, hindbrain; mhb, midbrain-hindbrain boundary; m, melanocyte; nc, neural crest; op, olfactory placode; os, optic stalk; ov, otic vesicle; pfb, pectoral fin bud; pllp, posterior lateral line primordium; psm, presomitic mesoderm; so, somite. Scale bars indicate 100 μm, except (F), 50 μm.


A
2-cell
B
2 hpf
C
6 hpf
D
12 hpf
E
so
psm
16 hpf
F
cg
16 hpf
G
fb
cg
16 hpf
H
op
os
mhb
1
2
3
4
5
6
ov
ba
pfb
pllp
24 hpf
I
pllp
26 hpf
J
op
os
26 hpf
K
ov
hb
ba
24 hpf
L
m
hb
nc
ba
24 hpf
M
1 2 3 4 5 6 7
26 hpf

Chapter 6

Conclusions

The overall aim of this thesis was to isolate and characterise zebrafish orthologues of human genes implicated in the pathogenesis of Alzheimer' disease. Essential genetic factors associated with AD are also found to play important functions during embryogenesis. The zebrafish is easily amenable to experimentation during embryo development, allowing *in vivo* studies of its developmental biology to help dissect the pathways and mechanisms involved in AD. This does not necessarily mean that studies need to be restricted to the development of the zebrafish CNS. We have to keep in mind that many of the developmental pathways controlling neurogenesis are evolutionarily highly conserved and play important functions during the development of other tissue and organ systems. Consequently, the study of these conserved pathways in non-neuronal tissues may provide important clues and insights relevant to AD research. For example, the knowledge that presenilin-dependent γ-secretase activity controls Notch signalling during somitogenesis has been used to validate a new class of γ-secretase inhibitors *in vivo*. These inhibitors specifically inhibit γ-secretase-mediated clavage of APP but do not interfere with somite formation in zebrafish, i.e. Notch signalling is unaffected in inhibitor-treated embryos (Petit et al., 2003).

The use of zebrafish to study AD-related issues *in vivo* is still in its infancy. The isolation of the zebrafish presenilin and app genes promise to advance the use of this *in vivo* system significantly in the future in order to answer questions of relevance to AD and developmental biology.

6.1. *app* and *presenilin in situ* RNA hybridisation and alternatively spliced transcripts

As a first step to develop the zebrafish as a model system in AD research, we have cloned and analysed the expression patterns of *presenilin* and *app* genes during embryogenesis. We have demonstrated that the developmental expression patterns of zebrafish and mammalian *presenilin* and *app* genes are evolutionarily highly conserved. These results were obtained using whole mount *in situ* hybridisation analyses with riboprobes recognising all transcripts present in the developing embryos. However, alternatively spliced transcripts are found to be numerous during embryo development and in adults (Coulson et al., 2000; Sarasa et al., 2000; Suh and Checler, 2002). The identification of regions expressing the AD genes provides some indications about their role during development. Therefore, to appreciate the functional complexity and to advance our understanding of the biology of *app* and *presenilins* knowledge about the spatiotemporal expression patterns of the various alternatively spliced transcripts is needed. This is complicated by the fact that the exons encoding alternatively spliced regions are often small (less than 200 base pairs), making their detection difficult using *in situ* RNA hybridisation methods. This problem has been partially overcome by using small radioactively labelled riboprobes and cross-sections of whole embryos to detect the spatiotemporal expression patterns of the four major *APP* transcripts during rat development (Sarasa et al., 2000). It would be a further advantage if it would be possible to determine the three-dimentional expression patterns of alternatively spliced transcripts. We have attempted this by modifying established DIG-based whole mount *in situ* transcript hybridisation protocols and used small riboprobes, which specifically recognise alternatively spliced transcripts, and *in situ* hybridisations on 12-24 hpf

zebrafish embryos under various experimental conditions. Our preliminary results suggest that it may be possible to use riboprobes as small as 100 base pairs to show diffential expression of e.g. *appa* transcript variants (not shown). However, we did experience problems with high non-specific background staining. This problem needs to be resolved in order to develop a robust and easily reproducible method for the use of very small DIG-labelled riboprobes to detect alternatively spliced transcripts.

6.2. The γ-secretase complex and presenilin function

The presenilins are central components of the γ-secretase complex, mediating the intramembrane cleavage of type I integral proteins, including APP and Notch (Fortini, 2002). The two presenilins apparently form similar but independent γ-secretase complexes (Yu et al., 1998). *PSEN1*-deficient mice exhibit severe developmental defects and perinatal lethality (Shen et al., 1997; Wong et al., 1997), whereas *PSEN2*-deficient mice are viable and fertile and develop only age-related mild pulmonary fibrosis and hemorrhage (Herreman et al., 1999). This suggests that the presenilins may have different biological roles. The different developmental phenotypes following disruption of *PSEN1* or *PSEN2* gene function in mice may partially be a consequence of differential biochemical activities of the γ-secretase complexes incorporating the two presenilins. This hypothesis has found support by the finding that the PSEN1 and PSEN2 γ-secretase complexes show different affinities to a potent γ-secretase inhibitor, indicating that they have distinct enzymatic properties (Lai et al., 2003). To complicate the matter even more, a recent report suggests that the catalytic core of the γ-secretase

complex contains a presenilin dimer (Schroeter et al., 2003). It can be hypothesised that the incorporation of proteins encoded by different *PSEN* splice variants would be able to subtle modulate γ-secretase activity to serve specific cellular processes. Thus, the recombination of different PSEN isoforms would add an extra level of regulatory control to the function of the complex. Dissecting the role of presenilin in γ-secretase-dependent procceses will necessarily be difficult. A good start would be to to inhibit all and/or specific *psen* transcripts during zebrafish embryogenesis using morpholino oligonucleotides and then analyse the effects on a developmental process known to be γ-secretase-dependent, such as somite formation (this study).

One of the most enigmatic aspects of γ-secretase is that only a small fraction of the cellular pool of presenilin1 and presenilin2 is incorporated into the active complex (Ratovitski et al., 1997). One might propose that the rapid degradation of most newly synthesised presenilin is not a reflection of the tightly restricted assembly of the γ-secretase complex with limiting factors or available substrates but, instead, is a consequence of the demands of an unknown presenilin function. Intriguingly, presenilin1 holoprotein has been found in the inner membrane of the nuclear envelope but not the cleaved heterodimer (Honda et al., 2000). What is the significance of this? Do the presenilin holoprotein and heterodimer have different functions? Could full-length presenilin proteins be involved in transcriptional regulation? One way to address these questions would be to overexpress full-length presenilin1 or 2 in embryonic zebrafish by mutating the major endoproteolytic site in the *presenilins*, prohibiting post-translational cleavage of the presenilin holoprotein into heterodimers. Alternatively, it

should be possible, by using splice-blocking morpholino oligonucleotides, to inhibit the pre-mRNA splicing of exon 9 in *presenilin 1* (and the corresponding exon in *presenilin2*), which encodes the region encompassing the endoproteolytic site (Fraser et al., 2000; Hutton et al., 1996). This would generate transcript variants missing exon 9 and, consequently, produce uncleavable presenilin1 isoforms. It is noteworthy that a *presenilin1* FAD mutation has been identified where in the acceptor site for exon 9 is mutated, causing the splicing out of exon 9 and generation of a highly stable presenilin holoprotein not subjected to endoproteolysis (Perez-Tur et al., 1995; Lee et al., 1997; Zang et al., 1998).

6.3. γ-secretase, regulated Intramembrane proteolysis and gene regulation

The most significant insight in recent years in AD research is the discovery that the presenilin-dependent γ-secretase complex is a central component in an evolutionarily highly conserved signalling mechanism involving the intramembrane proteolysis of cell-surface receptor proteins and release of an intracellular fragment with potential gene regulatory functions (Fortini, 2002). The Notch signalling cascade and its γ-secretase generated NICD fragment is the prototypical representative of this signal transduction mechanism (Baron, 2003). Whereas the role of Notch and NICD is well-characterised, the analogous roles of γ-secretase-generated intracellular fragments from several other potential signal transduction pathways, including APP and CD44, need to be confirmed and their physiological functions assessed (Gao and Pimplikan, 2001; Lammich et al., 2002). In order to produce appropriate cellular responses to intrinsic and extrinsic

biological requirements the various signalling pathways and events need to be coordinated. An example of such coordination or cross-talk between γ-secretase, Notch, APP and presenilin has recently been shown *in vitro* using mouse and human cell lines (Lleo et al., 2003). Activation of the Notch signalling pathway by the overexpression of a γ-secretase-dependent truncated Notch construct or the NICD was shown to lead to down-regulation of presenilin1 expression at both transcriptional and protein levels. Consequently, down-regulation of presenilin1 resulted in the reduction of γ-secretase activity and a concomitant reduction in proteolysis of APP to generate Aβ. This illustrates the existence of a complex set of feedback interactions controlling γ-secretase activity. It will be important to confirm these results *in vivo*. For example, as a first step we could overexpress NICD by RNA microinjection in embryonic zebrafish and then analyse the expression of presenilin1 at the transcriptional and protein levels using *in situ* RNA hybridisation and Western blotting (zebrafish NICD and presenilin1 cDNA, as well as presenilin1 antibodies are available from the Lardelli lab).

The past several years of research on the γ-secretase complex have provided fascinating insights into intramembrane proteolysis and its role in cell-surface receptor signalling, and future work will no doubt continue to uncover more secrets of γ-secretase and its associated signalling pathways.

6.4. Is the *fgfr1*-dependent signalling pathway regulated by AD-related genes?

The main focus in AD research has been aimed at understanding the details of the pathological processing of APP and the proteolytic machinery responsible for this process. Knowledge about the biological pathways and networks affected by the central components in AD pathogenesis is very limited or non-existent. FGF-mediated MAPK signalling plays a central role in synaptic plasticity and learning, and misregulation of components of this pathway has also been implicated in AD pathogenesis (Ferrer and Marti, 1998; Sweatt, 2001; Takami et al., 1998). Therefore, we decided to look at this pathway and study the zebrafish orthologue of the human *FGFR1* gene during embryo development, since misregulation of FGFR1 signalling has been associated with AD (Takami et al., 1998). We have cloned and analysed the developmental expression pattern of zebrafish *fgfr1* and shown that it was strongly expressed in the brain, especially at the midbrain-hindbrain boundary, as well as in somites outside the brain. The next step will be to investigate potential interactions between the central AD components and *fgfr1*. One way would be to investigate if presenilin-dependent γ-secretase activity controls *fgfr1* expression. This question can be addressed by performing γ-secretase inhibitor treatment and *fgfr1 in situ* RNA hybridisation analysis on zebrafish embryos. If misregulation of *fgfr1* is identified after blocking the activity of γ-secretase, then morpholino-mediated knock down of the function of *presenilin1* and/or *presenilin2* function and *fgfr1 in situ* RNA hybridisation analysis could be employed to study the further details of this putative interaction. The reverse experimental approach can also be suggested, i.e. blocking Fgfr1 signalling with Fgfr

inhibitors and then study the expression patterns of the central components involved in AD pathogenesis using *in situ* RNA hybridisation analysis on embryonic zebrafish.

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