Glial Expression of Amyloid Precursor Protein (APP) and its Processing Enzymes in the ANPC Mouse Model

by

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ABSTRACT

Alzheimer's disease (AD) is a complex and progressive neurodegenerative disorder believed to be triggered by the accumulation of β-amyloid (Aβ) peptides derived from the proteolytic processing of amyloid precursor protein (APP). The regions primarily affected in AD brains are the hippocampus and cortex, whereas the striatum and cerebellum are relatively spared. Although neurons are considered to be the major source of Aβ proteins in the brain, the activated astrocytes associated with neuritic plaques, the key neuropathological hallmark of AD brains, have also been shown to accumulate Aβ. Growing evidence over the last decades revealed that alterations in the levels and/or subcellular distribution of cholesterol can influence Aβ metabolism and development of AD pathology, but the underlying mechanisms remain unknown. Since cholesterol has been shown to influence Aβ generation, it is of interest to determine whether an accumulation of cholesterol within the endosomal-lysosomal system, the major site of Aβ production, can influence levels and/or processing of APP. Several recent studies have shown that AD exhibits some distinct similarities with Niemann-Pick Type C (NPC) disease – an autosomal recessive disorder caused primarily by loss-of-function mutations in the NPC1 gene. NPC disease, which is neuropathologically characterized by the intracellular accumulation of cholesterol, exhibits tau-positive neurofibrillary tangles and increased levels of Aβ peptides that are also the hallmarks of AD brains. To determine how activated astrocytes express APP and its processing enzymes and therefore may contribute to the overall Aβ burden we used mutant APP transgenic (APP-Tg) mice, mice lacking Npc1 protein (NPC1-null) required for intracellular cholesterol transport, and our recently developed bigenic ANPC mice (mutant APP-Tg mice in the absence of NPC1 protein) that overexpress mutant human APP in absence of Npc1 protein. Our results show that APP and its processing enzymes such as β-secretase BACE1 and components of the γ-secretase complex (Psen1andPen2) are expressed in a subset of reactive astrocytes in ANPC, APP-Tg and NPC1-null mice but not in age-matched wild-type control mice. The relative number of astrocytes expressing APP and its processing enzymes appear more in ANPC>APP-Tg>NPC1-null mice. To further test the significanceof cholesterol, we used mice that were treated with the sterol binding agent 2-hydroxypropyl-β - cyclodextrin (2-HPC) that has been shown to promote the removal of the sequestered cholesterol from lysosomes. We observed that reversal of cholesterol accumulation by 2-HPC treatment attenuates the observed glial pathological abnormalities

especially in ANPC mice and to a lesser extent in APP-Tg and NPC1-null mice. Additionally, our results indicate a functional interaction between APP and NPC1 genes that may connect both AD and NPC pathologies.

Collectively, our results indicate that reactive astrocytes may have an important role in the generation of Aβ-peptides in AD-related pathology. Additionally, accumulation of cholesterol within the endosomal-lysosomal system may influence APP levels/processing in the activated astrocytes of the three mutant mouse lines.

This thesis is dedicated to my children Rose, Frederick and Christian.

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ABC	ATP-binding cassette
Aβ	Amyloid β peptide
ACAT	Acyl-coenzyme-A cholesterol acetyltransferase
AD	Alzheimer's disease
ANPC	APP transgenic and NPC1-null
ApoE	Apolipoprotein E
APP	Amyloid precursor protein
BACE1	β-site APP cleaving enzyme 1
BBB	Blood-brain barrier
CNS	Central nervous system
CTF	Carboxy-terminal fragment
EL	Endosomal-lysosomal
ER	Endoplasmic reticulum
FAD	Familial Alzheimer's disease
GFAP	Glial fibrillary acidic protein
2-HPC	2-hydroxypropyl-β-cyclodextrin
Iba1	Ionizing calcium-binding adaptor molecule 1
NFTs	Neurofibrillary tangles
NPC	Niemann-Pick type C
PEN-2	Presenilin enhancer protein 2
PS1/PS2	Presenilin 1/Presenilin 2
sAPP	Soluble APP
Tg	Transgenic
WT	Wild-type

INTRODUCTION

1. Alzheimer's disease (AD)

Dementia is a syndrome characterized by failure of recent memory and other intellectual functions that is usually insidious in onset but progresses steadily. Alzheimer's Disease (AD) is the most common type of dementia, accounting for 60-70% of cases in the elderly (Hebert et al., 2003). AD is a progressive, multifactorial, and heterogeneous neurodegenerative disorder. The prevalence of AD increases with age, affecting approximately 1% to 3% of the population around the age of 60, 3% to 12% of the population between 70 and 80 years and up to 35% of the population older than 85 years (Walsh and Selkoe 2004). AD is the sixth leading cause of death in the United States of America. Since life expectancy is constantly rising, especially in industrial countries, it is predicted that the incidence and prevalence of AD will increase by three-fold over the next 50 years (Alzheimer's Association 2012). Therefore, AD is one of the most serious health problems of this century. Patients suffer from progressive loss of intellectual functions that include memory impairment, loss of language and visuospatial defects. Basic activities of daily living are progressively impaired as the neuropathology gradually worsens. Motor deficits and psychosis can often be found in the middle or later stages of the disease. In the end stages of AD, the patients often stop responding to their environment and they are mutant, incontinent, and bedridden. Death occurs on average 10 years after diagnosis, but the rate of progression is variable (Cummings et al., 2004). At autopsy, the AD brain shows a macroscopically severe cerebral atrophy involving brain regions associated with learning and memory processes, including the temporal, parietal, and frontal cortex. The hippocampus, entorhinal cortex and amygdala are noticeably reduced in volume, and total brain weight is usually reduced by over 35% in AD patients (Burns et al., 2009).

2. Familial *vs* Sporadic AD

Only 6 - 8% of all AD cases are inherited in an autosomal-dominant manner as the early-onset familia l form of AD (FAD) occurring prior to 65 years of age. Until now, mutations in the following three genes are known to be the cause of FAD: the amyloid precursor protein (*APP*) gene on chromosome 21, the presenilin 1 (*PSEN1*) gene on chromosome 14 and the *PSEN2* gene on chromosome 1 (Tanzi et al., 2005). But mutations in these 3 genes only account for 30 to 50% of all autosomal-dominant early-onset cases of AD. Mutations in regions that generate amyloid β (Aß) peptide, as seen in early-onset FAD, alter APP processing and lead to increased Aß production and/or misfolded Aß peptides, such as the “Swedish” (KM670/671NL) and “Indiana” (V717F) mutations (Chen et al., 2000). Mutations on *PSEN1/2* alter the preferred cleavage site of γ-secretase, leading to the production of the more toxic Aß1-42 isoform which is highly prone to aggregate (Holcomb et al., 1998). Studies of FAD further

support the amyloid cascade hypothesis, which focuses on the importance of APP-metabolism/Aß production in AD pathology.

Over 90% of all AD cases are sporadic, the late-onset form of AD occurring usually in individuals over 65 years of age. It is known that both genetic and environmental factors contribute to the development of sporadic AD. The genetic pattern of late-onset AD is more complex than of FAD. Data revealed that some genetic factors can influence the risk of developing late-onset AD. The best known is the Apolipoprotein E (ApoE) gene on chromosome 19, which has been shown to influence the risk of developing late-onset AD (Coon et al., 2007). This gene codes for a protein which is involved in cholesterol transport. Of the three distinct alleles of ApoE ε2, ε3 and ε4, ε4 has been shown to increase the risk of developing AD and decrease the age of onset in a gene-dose-dependent manner. Having two copies of ApoE ε4 increases the risk of developing AD by 50-90% (Reiss et al., 2012), whereas having ApoE ε2 is the least active in promoting Aß aggregation and has been shown to even be protective against AD (Corder et al., 1994). Also, data have shown that ApoE plays a role in Aß clearance, with ε4 as the least effective, resulting in higher accumulation of Aß peptides (Bell et al., 2007). Several other studies have identified certain genes involved in cholesterol metabolism can have an influence on AD pathogenesis. Other environmental and biological risk factors that can influence disease pathology include aging, female gender, diabetes, mild-cognitive impairment (MCI), prior head trauma, poor education, and history of depressive episodes (Chen et al., 2007)

3. Neuropathology of AD

Neuropathological hallmarks in both sporadic and familial AD include extracellular, parenchymal amyloid deposits, intracellular neurofibrillary tangles (NFTs) and loss of neurons and synaptic integrity, located predominantly in the temporal lobe, association cortex and to some extent in subcortical nucle i of AD brains. Noradrenergic neurons in the locus coeruleus and serotonergic neurons in the dorsal raphe are diminished (Querfurth et al., 2010). Cholinergic neurons in the basal forebrain are also affected, whereas cholinergic neurons in other regions of the brain such as the brainstem or striatum are not affected until later stages of the disease (Francis et al., 1999). NFTs consist of paired helical filaments (PHFs), which are abnormal hyperphosphorylated microtubules-associated tau proteins. Formation of PHF-tau leads to disruption of neuronal transport and finally death of affected neurons. Data revealed that the number of NFTs correlate positively with the severity of dementia in AD patients. However, NFTs are not an exclusive feature of AD, they also can be found in a variety of other neurodegenerative diseases, known as tauopathies, including frontotemporal dementia or Picks disease (Johnson et al., 1999).

Neuritic plaques are multicellular lesions and consist of a dense core of Aß peptides surrounded by activated microglia and astrocytes, as well as dystrophic neurites. Aß is derived from its precursor APP by successive proteolytic cleavage. The most damaged brain regions in AD, such as hippocampus, entorhinal cortex and neocortex, have been found to contain the highest amounts of plaques (Selkoe et al., 2001). Some data support the connection between loss of cognitive function and Aß levels in the brain (Naslund et al., 2000). Increased levels of Aß peptides and plaques appear before other neuropathological hallmarks of AD (Tanzi et al., 1996). Furthermore, Aß peptides can promote the formation of NFTs (Mawuenyega et. al., 2010) and soluble oligomeric/fibrillar Aß peptides can be neurotoxic (Selkoe et al., 2001). Together, these data back up the amyloid cascade hypothesis, which is the most predominant hypothesis today in AD pathology. According to this hypothesis Aß peptides are the main culprit driving the AD pathology - an abnormal built-up of Aß is considered to be the initia l step which leads to a cascade of events resulting in neurodegeneration and development of dementia associated with AD patients (Gandy et al., 2005). Increasing evidence supports the concept that altered processing of APP is one of the early events occurring in the pathogenesis of AD (Selkoe et al., 2003).

4. APP and Aß biosynthesis

APP is a member of the glycosylated transmembrane protein family, expressed in almost all regions of the brain. It has a large extracellular N-terminal, a single trans-membrane, and a short cytoplasmic C-terminal portion (Selkoe et al., 2008). The APP gene is localized on chromosome 21 and encodes for several isoforms, ranging in length from 639 to 770 amino acids. The APP695 is the most abundant in the brain and expressed mainly in neurons, whereas APP571 and APP770 are predominately expressed in glíal cells and other non-neuronal tissue. The protein is associated with various cell processes, including synaptogenesis, cell death, synaptic plasticity, neuronal excitability, calcium, and metal homeostasis (O'Brien et al., 2011). Interestingly, deletion of the APP gene in mice did not alter their phenotype nor their life expectancy (Zheng et al., 1995). Rather than being solely responsible for critical key functions within the cells, members of the APP gene family including amyloid precursor-like protein 1 and 2 (APLP1 and APLP2) seem to share critical key functions. This idea is supported by different studies. In a study by Anliker and Muller, 2006 knocking out APP/APLP2 and APLP1/APLP2 lead to early postnatal death in mice, whereas knocking out APP/APLP1 did not.

The precursor APP can undergo successive proteolytic cleavages involving 3 enzymes: α-secretase called a desintegrin and metalloprotease (ADAM 10 or ADAM 17); ß-secretase called ß-site APP cleaving enzyme 1 (BACE1) and γ-secretase. The γ-secretase is a multimeric protein complex, consisting of four subunits: the aspartyl protease, presenilin-1/2 (PS1/PS2) and three co-factors, [nicastrin, presenilin enhancer 2 (PEN2) and anterior pharynx-defective 1 (APH1)] (Wolfe et al., 2010). The role of PS1/PS2 as the catalytic subunit is supported by the fact that mutations on PS1/PS2 genes

are responsible for some FAD cases (Iwatsubo et al., 2004). APP is usually processed by two alternative pathways, the non-amyloidogenic pathway mediated by α- and γ-secretasesthat precludes the formation of Aß peptides and the amyloidogenic pathway mediated by ß- and γ-secretases that leads to the formation of Aß peptides (De Stroopert et al., 2012). The enzyme α-secretase cleaves APP within the Aß domain to yield a soluble APP fragment (sAPPα) and a membrane-bound C-terminal fragment α-CTF, which is cleaved further by γ-secretase to yield p3 instead of Aß peptide. Alternatively, cleavage by ß-secretase leaves the Aß sequence intact and forms a C-terminal fragment ß-CTF, which is then cleaved by γ-secretase complex within the transmembrane domain. This leads to Aß peptide and the amyloid precusor protein intracellular domain (AICD). The cleavage site for γ-secretase varies and results in Aß peptides containing 39-43 amino acids. Soluble Aß1-40 makes up about 90% of the Aß peptides and only slowly converts into an insoluble ß-sheet form (Chavez-gutierrez et al., 2012). Aß1- 42 is highly fibrillogenic and more toxic to the cells (Selkoe et al., 2001). Aß peptides are mainly synthesized along the endocytic pathway on endosomes but to some degree also synthesized in the plasma membrane, ER and Golgi apparatus (Greenfield et al., 1999).

5. Aß clearance and degradation

The amount of Aß within cells is a finely balanced ratio between synthesis and clearance/degradation. The half-life of APP, its proteolytic intermediates and Aß peptides varies depending on the cellular state and can be influenced by various external factors. Clearance and degradation of Aß is a more complex process and involves different mechanisms. The foremost enzymes that mediate proteolysis include Neprilysin (NEP) and Insulin Degrading Enzyme (IDE). NEP is a transmembrane zinc metallopeptidase of the M13 family and expressed in neurons. NEP can degrade monomeric as well as oligomeric forms of Aß peptides in a gene-dose dependent manner. Both pharmacologically and genetically induced overexpression of NEP could diminish Aß deposits in animal models of AD (Poirier et al., 2006). Huang et al., 2006 showed that after knocking out NEP, the cell load of Aß1-42 and Aß1-40 doubled. IDE is a zinc metallopeptidase but is only able to degrade Aß monomers and found primarily in the cytosol (Caccamo et al., 2005). Additionally, passive and active transport over membranes and cell mediated clearance, such as autophagy are involved in Aß clearance and degradation (Baranello et al., 2015). Autophagy is a way to degrade damaged or abnormal proteins and the bulk of cytoplasmic material by engulfing them into autophagosomes which subsequently fuse with the endosomal/lysosomal system. Data from Zare-shahabadi et al. (2015) revealed an active role of autophagy for APP turnover/Aß metabolism. Furthermore, autophagosomes are known to accumulate in various neurodegenerative disease, including AD (Nixon et al., 2007).

6. Astrocytes and AD

Astrocytes, specialized glia cells are the most abundant cell type in the brain. They outnumber neurons by over fivefold. For a long time, astrocytes were only seen as the connective tissue of the brain, but in the last few decades it has become clear that astrocytes participate in many essential central nervous system (CNS) functions. The functional unit of microcirculation in the brain is formed by astrocytes by integration of neurons, pericytes, endothelium cells and smooth muscle cells. Astrocytes have an important role in adjusting the local blood flow to the neuronal activity by regulating vasodilatation and vasoconstriction due to several signalling cascades. Astrocytic end-feet envelop brain capillaries to regulate transport of water, electrolytes, and glucose. Furthermore, direct contact between astrocytes and endothelial cells has an impact on the integrity and permeability of the blood-brain-barrier (BBB) (Zlokovic et al., 2008). Astrocytes are of fundamental importance for the function of neuronal networks, and astrocytic dysfunction or atrophy can have a tremendous impact on healthy brain function (Sofroniew et al., 2010).

In 1858 Rudolf Virchow first described neuroglia as the “glue” of the brain (Virchow 1858). But it was Michael von Lenhossek in 1893 who used the term astrocyte for the first time because of the stellate morphology of these cells (Lenhossek 1893). Golgi, who developed the first histological technique, the Golgi stain, had the first view of the morphological appearance of glia (Golgi, 1871), but it was Ramon y Cajal’s drawings which gave tremendous insights into the structure and functions of astrocytes (Cajal, 1897) (Figure 1). Furthermore, Andriezen subdivided astroytes into fibrous astrocytes of the white matter and protoplasmic astrocytes of the grey matter (Andriezen, 1893). Protoplasmic astrocytes have several stem branches and many fine processes with which they envelop synapses, while fibrous astrocytes have a few presynaptic processes which contact nodes of Ranvier. Besides these two groups of astrocytes there are other specialized types of astrocytes in different areas of the brain like the Müller glia in the retina or the Bergmann glia in the cerebellum. Staining with an antibody against intermediate glial fibrillary acid protein (GFAP) expressed in astrocytes is typically used to detect astrocytes in the CNS. GFAP has proven to be a reliable and sensitive marker for reactive astrocytes, but it has become clear that not all astrocytes express GFAP and not all cells that express GFAP are astrocytes (Kimelberg et al., 2004). Studies with injection of dyes have shown that GFAP only reveals 15% of the total volume of an astrocyte (Bushong et al., 2002). S100ß and glutamine synthetase (GS) are other molecula r markers but they are not exclusive for astrocytes either. Based on using a combination of astrocyte markers, Emsley and Macklis defined 9 different types of astrocytes in 2006. They also showed that protoplasmic astrocytes are organized in non-overlapping domains, and only the most distal ends of the processes are in interindividua l contact due to gap junctions. These are composed mainly of Connexin 43 and 30, which are four-pass transmembrane proteins which assemble to form gap junctions in vertebrates (Emsley and Macklis, 2006). The processes have contact with several hundreds of neuronal

dendrites and envelop thousands of synapses (Bushong et al. 2002). With evolution, the number, complexity, and diversity of astrocytes have increased. Astrocytes, unlike neurons, are not electrically excitable and do not conduct action potentials but rather they are chemically excitable. Astrocytes express potassium and sodium channels and can evoke inward currents and calcium waves. Calcium signals can occur within single cells or be propagated from one cell to the other; both forms occur independently from neuronal activity, as well as in response to different neurotransmitters (Sofroniew et al. 2010). Some experiments have shown that astrocytic calcium waves trigger the release of several gliotransmitters, including ATP, adenosine, glutamate, and D-serine which can then modulate the activity of surrounding cells such as neurons, microglia, and endothelial cells of the blood vessels. This led to the hypothesis of the "tripartite synapse" (Perea et al. 2009). Currently, it is unclear if the "tripartite synapse" is a normal physiological effect or occurs only under *in vitro* conditions.

Changes of astrocytes in AD were first described by Alois Alzheimer himself. He found astrocytes in an abundant amount around neuritic plaques (Alzheimer, 1910). Astrocytes become activated with hypertrophy and proliferation under pathophysiological conditions, including infection, injury and in neurodegenerative diseases such as AD. Interestingly, astrogliosis can also be found during the physiological aging process because of a proinflammatory shift. Various signals from damaged neurons and/or damaged glia cells can lead to the formation of reactive astrogliosis, which can be identified by elevated expression of GFAP. Furthermore, reactive astrocytes can form a glial scar in response to injury, leading to a separation of injured tissue and accompanying inflammatory processes (Sofroniew et al. 2009). There is evidence that reactive astrogliosis found regularly in the later stages of the disease is a hallmark of AD (Rodriguez et al., 2009). Aß plaques are usually infiltrated by microglia while reactive astrocytes are around the periphery. From there, reactive astrocytes project thick processes to envelop the plaque and thinner processes which infiltrate the plaque (Schwab et al., 2008). In AD brains, reactive astrocytes are found in the cortical molecular layer, as well as in the pyramidal cell layer (Sofroniew et al., 2010). A study from Kashon et al., 2004 reveals generalized astrogliosis, marked by cellular hypertrophy and increased expression of GFAP, in post-mortem brains of AD patients (Kashon et al., 2004). Simpson et al. (2010) have shown a correlation between severity of astrogliosis and cognitive decline in AD and non-AD patients. But this study did not show a direct correlation between astrogliosis and senile plaques. Astrogliosis can also be found in areas without senile plaques (Simpson et al., 2010).

At present, most of the scientific literature focuses on APP metabolism and Aß generation within the context of neurons. Although neurons are considered to be the major source of Aβ in the brain (Zhao et al., 2011), reactive astrocytes that are associated with neuritic plaques in AD brains have also been shown to contribute to the generation of Aβ-related peptides (Thal et al., 2000). Accumulation of Aß

peptides whether by overproduction and/or reduced clearance contributes to the development of NFTs, neuronal degradation and subsequent development of AD pathology (Selkoe et al., 2001). Reactive astrocytes surrounding plaques accumulate Aß in higher amounts, so that this material dominates the cytoplasmic volume (Nagele et al., 2003). Nagele et al. (2003) have also shown that Aß accumulation in reactive astrocytes is not dependent on the local presence of plaques since these cells exhibit Aß accumulation even in absence of plaques. Additionally, they provided evidence that accumulated Aß in reactive astrocytes is of neuronal origin because the Aß is co-localized with neuron-specific synapses, dendrites and neuron-specific enzymes including choline acetyltransferase. They suggested that this phenomenon may be a consequence of the debris-clearing properties *via* phagocytosis and endocytosis of activated astrocytes. Indeed, some reactive astrocytes which are in close contact to Aß plaques have been shown to express neprilysin, the amyloid degrading enzyme (Apelt et al., 2003). Furthermore, astrocytes are capable of phagocyting followed by degradation of Aß under *in vitro* conditions (Wyss-coray et al., 2003). However, only astrocytes from healthy brains showed a beneficial effect, whereas astrocytes from APP overexpressing mice did not show any beneficial effects (Whyss-Coray et al., 2003, Zhao et al., 2011).

AD pathology is also characterized by vascular defects and vascular pathology marked by an overall reduction of the brain blood flow. Aß plaques often surround brain capillaries, thus impairing microcirculation and vascular Aß clearance (Bell et al., 2009). To clear Aß from the brain and to prevent neurotoxicity, Aß must be transported across the BBB. Therefore, accumulation of Aß in vessel walls results in degeneration of cerebrovascular cells and disruption of the BBB (Zlokovic et al., 2003). Damage to the astrocytes at early stages of the disease could contribute in this way to cognitive abnormalities but further investigations are needed. Changes in glucose metabolic pathways in astrocytes due to Aß remain controversial. In a study with cultured astroglial cells, treatment with Aß increased glucose use in most of the glucose pathways and glycogenesis. Furthermore, Aß-pretreated neurons cultured with astrocytes showed a decreased neuronal survival compared with non-treated neurons (Allaman et al., 2010). Studies on enzymes involved in glucose metabolism have reported both increases (Soucek et al., 2003) and decreases (Liang et al., 2008) of metabolic enzymes in the presence of Aβ. These controversial data possibly reflect the changes induced by Aß at different stages of the AD (Allaman et al., 2010). The first histopathological signs of AD are synaptic dysfunction and synaptic loss (Coleman et al., 2004), which correlate positively with the severity of dementia (Terry et al., 1991). Recognition of the overall importance of astrocytes for synaptogenesis and synaptic function in AD and the hypothesis that synaptic loss is only associated with dysfunction of neurons need to be investigate d further. Activated astrocytes have reduced abilities to maintain homeostasis of ions and neurotransmitters and produce impaired performance of the neurovascular unit and decreased metabolic support of neurons. Furthermore, activated astrocytes can trigger microglial activation with release of

inflammatory and neurotoxic factors. These factors should be taken into consideration when the role of astrocytes in AD pathogenesis is evaluated.

Astrocytes in the aged human brain are marked by increased astrogliosis (Cotrina et al., 2002). Furthermore, astrocytes are capable of triggering the senescence-associated secretory phenotype (SASP) with increased levels of GFAP- and Vimentin filaments, which are cytoskeletal components, along with enhanced levels of various cytokines and accumulation of proteotoxic aggregates. Also, cultured astrocytes and isolated astrocytes from aged brains show a proinflammatory phenotype under *in vitro* conditions (Salminen et al., 2011). These activated astrocytes are involved in the inflammatory component of AD *via* release of proinflammatory factors such as cytokines and nitric oxide (Heneka et al., 2010). Cultured astrocytes have been reported to show spontaneous Ca^{2+} signals and oscillations upon exposure to Aß. Interestingly, spontaneous Ca^{2+} waves and oscillations were also observed *in vivo* with astrocytes which were associated with neuritic plaques (Abramov et al., 2004). Oddo et al. (2003) developed the triple transgenic mouse model of AD (3xTg-AD) which harbours the mutations for APP_{swe}, PS1 and tau protein. They show temporal and region-specific Aß and tau pathology (Oddo et al., 2003). At early stages of the AD pathology, 3xTg-AD mice show a reduced morphology of astrocytes. However, the appearance of senile plaques at around 12 months of age triggers astrogliosis only in astrocytes directly associated with plaques (Rodriquez et al., 2009).

Aß production requires the endoprotease BACE1 which is normally expressed only in neurons of the healthy brain. But under certain conditions such as AD or chronic stress, astrocytes are able to express BACE1 and contribute to Aß production. Expression of BACE1 in reactive astrocytes surrounding Aß plaques has also been reported in various mouse models of AD (Rossner et al., 2005). Whether this phenomenon is triggered by Aß plaque formation or is a general feature of astrocytic activation remains inconclusive. Hartlage-Rubsamen et al. (2003) have also shown expression of BACE1 in reactive astrocytes after different stimuli, including middle cerebral artery occlusion, induction of experimenta l autoimmune encephalomyelitis and infection with Borna disease virus. These results suggest that BACE1 expression by reactive astrocytes may increase the production of amyloidogenic fragments and potentially contribute to Aβ production. Reactive astrocytes in AD brains are also shown to express BACE1, possibly triggering amyloidogenic processing of APP. Since they vastly outnumber neurons, it is likely that astrocytes may represent a significant but unappreciated source of Aß under pathologic al conditions. A study by our lab has revealed that kainic acid-induced epileptic activity in the hippocampus triggers the extensive proliferation of reactive astrocytes which overexpress APP. This process occurs together with the loss of APP-positive neurons, indicating that reactive astrocytes supersede degenerating neurons as the primary source of APP processing under epileptogenic conditions (Kodam et al., 2019). Chronic inflammation is also known to trigger Aß formation in

astrocytes by triggering BACE1 expression following the release of proinflammatory cytokines by microglia (Whyss-Coray et al., 1997). As AD progresses, a new population of plaques is formed, which are smaller in size with intensive GFAP immunoreactivity. Nagele et al., (2003) demonstrated that this new population of plaques is derived from death and lysis of Aß-overburdened astrocytes. These typical plaques are only located in areas where astrocytes contain large intracellular deposits of Aß peptides (Nagele et al., 2003). However, the mechanism by which astrocytes regulate AD pathogenesis remains elusive.

7. Cholesterol and AD

Cholesterol is a crucial molecule within the body. It is the principal sterol synthesized by all animals, necessary for membrane integrity and fluidity. Within the plasma membrane it controls ionic homoeostasis and endocytosis. Cholesterol also serves as a precursor for the biosynthesis of steroid hormones, bile acid and vitamin D. In the nervous system it is responsible for neuronal development and outgrowth, neurotransmitter release and synaptic plasticity. Twenty-five percent of the total body cholesterol can be found in the brain primarily in myelin (~70-80%) and to a lesser extent in neuronsas well as glia (Martins et al., 2009). The entry of peripheral cholesterol into the CNS is prevented by the BBB. During development neurons synthesize most of their cholesterol but this ability is lost in mature neurons and the astrocytes start synthesizing cholesterol (Dietschy and Turley, 2004). Cholesterol levels are closely regulated by a negative feedback loop. The initial step of cholesterol synthesis is the conversion of acetyl CoA into 3-hydroxy-3-methylglutaryl-CoA (HMG-CoA) by HMG-CoA synthase. This is then converted into mevalonate by the rate limiting enzyme HMG-CoA reductase. Mevalonate is subsequently phosphorylated into isopentyl pyrophosphate and then converted into squalene. Consequently, squalene is catalysed into lanosterol which is finally converted into cholesterol (Maulik et al., 2013). Cholesterol is then transported from astrocytes to neurons *via* an ApoE-dependent mechanism and stored in the endosomal-lysosomal system following internalization. Once released from the endosomal-lysosomal system by Niemann-Pick type C1 (NPC1) and NPC2 proteins cholesterol is trafficked to other cellular compartments such as the ER and plasma membrane to mediate its functions (Poirier et al., 2003). Neurons and glial cells do not have the ability to degrade cholesterol and therefore excess amounts of cholesterol are transformed to 24-hydroxycholesterol and then eliminated *via* the BBB (Kim et al., 2007).

A disturbance in cholesterol metabolism has also long been demonstrated to play an important role in the development of AD pathology. This is supported by the evidence that i) inheritance of the ε4 isoformof ApoE, a protein carrier for cholesterol, enhances the risk of AD (Poirier et al., 1993; Strittmatter et al., 1993), ii) neurons bearing neurofibrillary tangles in AD brains exhibit higher levels of free cholesterol than adjacent tangle-free neurons (Distl et al., 2001) iii) some early epidemiological data

suggest statins (popular cholesterol lowering drugs which are used to treat cardiovascular disease) that block cholesterol biosynthesis, reduced the prevalence of AD (Jick et al., 2000; Wolozin et al., 2000, Reiss et al., 2012), iv) elevated cholesterol levels increase Aβ production (Sparks et al., 1994; Zatta et al., 2002), whereas inhibition of cholesterol synthesis lowers Aβ levels (Simons et al., 1998), (v) high-cholesterol diets can increase Aβ deposits (Refolo et al., 2000), whereas low-cholesterol diets can decrease Aβ deposits in mutant APP Tg mice (Refolo et al., 2001), (vi) a massive increase in cholesterol levels has been suggested to increase tau phosphorylation and degeneration of neurons (Ohm and Meske, 2006) and (vii) intracellular accumulation of cholesterol within cells following treatment with the class II amphiphilic drug U18666A can lead to increased Aβ production, phosphorylation of tau and loss of neurons under *in vitro* culture conditions (Koh et al., 2006; Chung et al., 2018). Some data showed that high midlife cholesterol levels are a risk factor for developing mild-cognitive impairme nt and AD. A study of post-mortem brain tissues and animal models of AD showed changes in many different lipids in AD-affected areas including the prefrontal and entorhinal cortex (Chan et al., 2012). Matsuzaki et al., 2011 showed higher levels of cholesterol in neurons containing NFTs. This is supported by the evidence that accumulation of free cholesterol within neurons observed in fatal autosomal recessive NPC disease, caused by a mutation in either *NPC1* or *NPC2* genes, can trigger hyperphosphorylation of tau protein, increased Aβ production (but no Aβ-containing neuritic plaques) and loss of neurons in selected brain regions (Vanier and Millat, 2003; Paul et al., 2004; Vance 2006; Pacheco and Lieberman, 2008). Interestingly, *Balb/cNctr-npc1*^{N} mice which lack NPC1 protein expression due to a spontaneous deletion/insertion mutation in the *NPC1* gene have been shown to recapitulate most of the pathological features associated with NPC disease. However, there are contradictory studies which have found Aß levels are decreased after a high cholesterol diet in animals (George et al., 2004) or there are unaffected Aß deposits after statin treatment (Höglund et al., 2004). This uncertainty emphasizes the necessity for further investigations to better understand the role of cholesterol in AD pathology.

8. Niemann-Pick Type C (NPC) Disease

NPC is a neurovisceral, lyosomal-lipid storage degenerative disorder. The disease pathology is characterized by an accumulation of unesterified cholesterol and glycolipids, including spingomye lin, sphingosine and ganglioside within the endosomal-lysosomal system. A mutation in the *NPC1* or *NPC2* gene leads to a disruption of transporting cholesterol from the endosomal-lysosomal system to the ER, Golgi apparatus and plasma membrane of various tissues including the CNS and thus disturbing cells' ability to maintain homeostasis (Walkley et al., 2004; Kwon et al., 2009). As a result, the cell senses a lack of cholesterol in the ER and upregulates cholesterol synthesis, but the "traffic jam" of lipids in the endosomal-lysosomal system disrupts severely the transport of a variety of molecules *via* the endocytic pathway. The lipid disturbances caused in neurons are also linked to structural changes, including

ectopic dendritogenesis, formation of meganeurites and NFTs in the hippocampus, medial temporal lobes, cingulate gyrus, and entorhinal cortex of NPC-affected brains without any evidence of amyloid deposition (Saito et al., 2002).

NPC is a rare disease with a prevalence of 1:120000-150000 live births. The mode of inheritance is autosomal recessive with a loss of function mutation in the NPC1 gene in approximately 95% and only 5% in NPC2 gene (Chang et al., 2005). The NPC1 gene is located on chromosome 18, whereas the NPC2 gene is located on chromosome 14 (Pentchev et al., 1997). These genes encode for NPC1 and NPC2 proteins, which are ubiquitously expressed hydrophobic-polytopic transmembrane proteins responsible for the efflux of low-density lipoprotein-derived unesterified cholesterol from the endosomal-lysosomal system. Interestingly, mutations in either gene lead to identical phenotypes indicating that the same pathway in regulating cholesterol efflux from late-endosomes/lysosomes is affected. The clinical spectrum is wide and ~50% of cases present before 10 years of age, but manifestations are occasionally seen as late as the sixth decade of life. The progressive neurodegenerative phenotype includes cerebellar ataxia, vertical supranuclear gaze palsy, dysphagia , dysarthria and dementia. Seizures, dystonia and cataplexy also occur frequently, whereas psychiatric disturbances may often appear in the later stages of the disease. Infiltration of lipid-loaded macrophages, called foam cells, in the liver, spleen and lung are part of the visceral symptoms of NPC disease (Vanier et al., 2010).

NPC and AD neuropathology exhibit some similarity, including NFTs, neuroinflammation, altered cholesterol levels, endosomal-lysosomal abnormalities, and increased levels of Aß in the affected brain regions (Jin et al., 2004). NFTs found in NPC and AD pathologies are very similar in terms of structure and immunology. Interestingly, extracellular Aß deposits could be found in NPC patients carrying two copies of ApoE ε4 (Saito et al., 2002). Conversely, altered levels of NPC1 mRNA were detected in the affected regions of the AD brain (Kagedal et al., 2010). NPC mutations in animal models of AD showed that intracellular cholesterol accumulation can exacerbate behavioural and pathological hallmarks associated with AD (Maulik et al., 2013). Additionally, astrocytes and microglia are activated, and the white matter is found to be progressively demyelinated. Degeneration of neurons is evident especially in the cerebellar Purkinje cell layer and to some extent also in thalamus and cortex (Maulik et al., 2013, 2015; Walkley et al., 2004). At present, there is no known cure or effective treatment for NPC disease. Only supportive care can improve the quality of life for people affected by this disease (Pacheco and Lieberman., 2008). Approaches to alter the disease pathology or the clinical outcome with statins or a low cholesterol diet failed (Patterson et al., 2004). A reason could be that peripheral and brain cholesterol are two distinctive pools and cholesterol lowering drugs are hardly penetrating the BBB. However, studies showed that oral or intrathecal application of cyclodextrin can mobilize cholesterol and therefore could be an approach for an effective treatment for NPC (Maulik et al., 2013).

Cyclodextrins are cyclic oligosaccharides which are able to lodge the large hydrophobic cholesterol molecule inside the cyclodextrin ring and therefore can be removed from the plasma membrane of the cell. Cyclodextrins, made from starch by enzymatic conversion, are relatively non-toxic and have been approved by FDA for use in the food and pharmaceutical industry (Zidovetzki et al., 2007). A study showed that a single subcutaneous injection of 2-hydroxypropyl-ß-cyclodextrin (2-HPC) into 7-day old NPC1-null mice reduced cholesterol accumulation in the endosomal-lysosomal system, delayed neurodegeneration and prolonged the life expectancy, results were even better after repeated injections over several weeks (Liu et al., 2009). However, single treatment of 49 day old NPC1-null mice with 2-HPC were found to be less effective, possibly due to the progressive nature of the disease or the development of mature BBB (Davidson et al., 2009). Evidence suggests that 2-HPC enters the endocytic pathway of NPC deficient cells through bulk phase endocytosis and releases cholesterol and glycolipids from the endosomal-lysosomal system to the metabolically active pool of the ER (Rosenbaum et al., 2010). By some yet unknown mechanism, 2-HPC seems to bypass the NPC1-NPC2 interaction and release cholesterol from the endosomal-lysosomal system in NPC1-deficient cells. An earlier study from our lab demonstrated that 2-HCP administration can attenuate the NPC pathology and the life spanof NPC1-deficient mice as well as the NPC1-deficient AD mouse model (Maulik et al., 2013).

9. Animal models of AD

APP is the precursor for the potentially cytotoxic Aß peptide. Located on chromosome 21, the APP gene was the first gene to be associated with the familial form of AD (St George-Hyslop et al., 1987). Deletion of the APP gene does not alter the phenotype or the life expectancy of mice, despite its potential involvement in a variety of functions (Zheng et al., 1995). To study the role of Aß peptides in AD pathogenesis, several lines of mutant APP transgenic (Tg) mice have been developed with various promoters on different genetic backgrounds. Many lines of Tg mice with APP mutations express high levels of mutant human APP and develop many of the pathological hallmarks of AD, including extracellular Aß plaques, reactive gliosis, and synaptic loss in brain regions similar to those observed in human AD brains (Chen et al., 2000). Additionally, most of these Tg-mice exhibit cognitive impairments in a variety of behavioural paradigms (Eriksen and Janus, 2007). By crossing mutant APP-Tg mice with over-expressing PS1 mice, an increase in Aß1-42 production and extracellular deposition were detected in double mutant Tg mice (Borchelt et al., 1997). However, none of the APP or APP/PS1-Tg mouse models develop the entire spectrum of AD pathology (McGowan et al., 2006). In 2003, Oddo et al. developed a line of triple Tg mice which harbour mutant APP, PS1 and Tau transgenes (3xTg-AD; Oddo et al., 2003). The NFTs found in this mouse model develop months after amyloid plaque formation; thus supporting the amyloid cascade hypothesis. A more recent mouse model (rTg3696AB) of AD is able to show significant neuronal loss in the CA1 and CA2 area of the hippocampus and in the pyramidal layer of the cortex, in addition to increased levels of human Aß and tau protein (Paulson et

al., 2008). The differences in expressing AD-related pathology in various mouse models possibly relate to the expression levels of mutant proteins, promoters, and the nature of FAD mutations (McGowan et al., 2006).

The most well-studied and used animal model of NPC disease is the BALB/*c-npcl*^{nih} mouse (NPC1-null) which lacks the Npc1 protein due to a spontaneous mutation in the NPC1 gene (Lofts et al., 1997). These mice exhibit an accumulation of unesterified cholesterol in the endosomal-lysosomal system, activation of astrocytes and microglia, loss of myelin in the CNS and neuronal loss in various brain regions including the cerebellum but not in the hippocampus. Behaviourally, these NPC1-null mice exhibit cognitive deficits, tremor, ataxia and a shortened lifespan compared to age-matched controls (Walkley et al., 2004). However, intracellular NFTs, which are a pathological hallmark in human disease are not detected in NPC1-null mice (Vanier et al., 2003). A study from Jin et al. (2004) reportedthat these mice exhibit higher levels of intracellular Aß peptides in parts of the brain but its significanc e in the development of NPC pathology remains unclear. Our lab engineered a new line of bigenic mice by crossing mutant human APP-Tg mice (APP-Tg) with heterozygous NPC1-deficient mice to evaluate the potential role of Aß-peptide in NPC pathology. These bigenic mice showed that the NPC1-null phenotype with APP overexpression increases the neuropathological and behavioural abnormalities and drastically reduces the lifespan (Maulik et al., 2012).

10. Hypothesis and objectives

Compelling evidence suggests that increased levels of Aβ peptides derived from APP contribute to the development of AD pathogenesis. The regions severely affected in AD brains are hippocampus and cortex, whereas striatum and cerebellum are relatively spared. Although neurons are considered to be the major source of Aβ in the brain, the activated astrocytes associated with neuritic plaques, the key neuropathological hallmark of AD brains, have also been proven to accumulate Aβ, which correlates positively with the severity of AD-associated tissue damage. A number of earlier studies (Hartlage-Rubsamen et al. 2003; Rossner et al., 2005) as well as data obtained from our own lab have provided evidence that Aß peptides derived from reactive astrocytes may have an important role in AD pathology (Ourdev et al., 2015, 2019; Yang et al., 2017). Additionally, accumulation of cholesterol within the endosomal-lysosomal system may influence APP levels/processing in the activated astrocytes. Many studies, although contradictory, have suggested that altered neuronal levels/distribution of cholesterol may have an important role in modifying APP metabolism in favour of increased Aß synthesis. These results, together with the evidence of striking similarities between AD and NPC disease, led us to hypothesize that cholesterol plays an important role in regulating amyloidogenesis in both disease phenotypes. Thus, it is our objective to determine how accumulation of cholesterol within the endosomal-lysosomal system, the major site of Aβ production, can influence levels and/or processing

of APP in activated astrocytes. To address this issue, we used mutant APP-Tg mice, NPC1-deficient mice and the bigenic ANPC mice that overexpress mutant human APP in the absence of NPC1 protein. The bigenic ANPC mice were generated by crossing mutant APP-Tg mice with heterozygous NPC1-deficient mice. Using 7-week-old ANPC and littermate APP-Tg, NPC1-deficient and wild-type control mice, we evaluated the expression of APP and its processing enzymes in reactive astrocytes in the hippocampal and cerebellar regions of the brain.

MATERIALS AND METHODS

1. Materials

Prolong Gold Antifade and Alexa Fluor488/594 conjugated secondary antisera were purchased from Life Technologies Corp. (Burlington, ON, Canada). The enhanced Chemiluminescence (ECL) kit is from Thermo Fisher Scientific (Montreal, QC, Canada), whereas 2-HPC (product H107) was obtained from Sigma-Aldrich, Inc. Vectashield mounting medium is from Vector Laboratories, (Burlingame, Canada). Sources of all primary antibodies used in the study are listed in Table 1. The associated horseradish peroxidase-conjugated secondary antibodies were purchased from Santa Cruz (Pasa Robles, CA, USA). All other reagents were from either Sigma-Aldrich or Fisher Scientific.

2. Generation of Transgenic mice

Mutant human APP transgenic mice maintained on a C3H/C57BL6 background were obtained from Dr. David Westaway's group (Centre for Prions and Protein Folding Diseases, University of Alberta) and heterozygous Npc1 gene knock-out ($Npc1^{-/-}$) mice maintained on BALB/c strain background were obtained from Dr. Jean E. Vance (Department of Medicine, University of Alberta). The APP transgenic mice carry the APP695 isoform with Swedish (K670M/N671L) and Indiana (V717F) mutations. These two parental mice lines were crossed to generate $APP^{+/-}Npc1^{+/-}$ and $APP^{-/-}Npc1^{+/-}$ F1 progeny on a mixed C3H/C57BL6/BALB/c strain background which were subsequently inter-crossed to produce all six genotype combinations of which four lines of mice (WT, APP-Tg, NPC1-KO and ANPC) were usedin this study. All animals were bred and housed in our own colony maintained on a 12 h light/dark cycle and access to food and water ad libitum. The maintenance of the colony and experiments included in the thesis were performed in accordance with University of Alberta and Canadian Council of Animal Care guidelines. All transgenic mice were identified by a unique ear notching pattern and genotyped by PCR analysis of tail DNA as described earlier (Loftus et al., 1997; Chishti et al., 2001).

3. 2-HPC Treatment

Subsets of WT, NPC1-null, APP-Tg and ANPC mice were administered a single subcutaneous injection of either 2-HPC (4000mg/kg body weight; 20% wt./vol. in saline solution; n = 6-8 per genotype) or normal saline (n = 4-6 per genotype) at 7 days of age at the scruff of the neck as described recently (Maulik et al., 2012). Following treatment, saline- and 2-HPC-treated mice from the various groups (i.w., WT, NPC1-null, APP-Tg and ANPC) were processed for immunohistochemistry at 7-weeks of age.

4. Immunohistochemistry

We transcardially perfused WT, NPC1-null, APP-Tg and ANPC mice at 4, 7 or 10 weeks (n = 3-5 animals/group) of age. The mice were deeply anesthetized prior to perfusion with PBS, pH 7.2 followed by 4% paraformaldehyde. After perfusion, brains were post-fixed overnight in the same fixative and then stored at 4°C in 30% PBS/sucrose. Brain tissues were coronally sectioned (20µm) on a cryostat and then processed for either enzyme-linked immunoperoxidase or by a double immunofluorescence method in a free-floating manner as described earlier (Maulik et al., 2012). For the enzyme-linked procedure, sections were first washed with PBS, boiled for 20min in a 10mM sodium citrate buffer (pH 6.0) and then treated with 1% H_2O_2 for 30min prior to incubation with either Y188 (1:1000), BACE1 (1:500), PS1 (1:500), Nicastrin (1:500) or Pen2 (1:1000) antibodies overnight at 4°C. Subsequently brain sections were exposed to appropriate HRP-conjugated secondary antiserum (1:400) for 2hr at room temperature and developed using a glucose-oxidase diaminobenzidine tetrahydrochloride-nickel enhancement method as described earlier (Maulik et al., 2012). Sections were then mounted using Vectashield mounting medium (Vector Laboratories, Burlingame, CA) on poly-L-lysine coated glass coverslips. Immunostained sections were examined under a Zeiss Axioskop-2 fluorescence microscope and the photomicrographs were taken with a Nikon 200 digital camera.

5. Double labelling

To determine the potential localization of APP and its processing enzymes on reactive astrocytes, brain sections are being treated with Y188 (1:500), BACE1 (1:250), PS1 (1:250), Nicastrin (1:250) and Pen2 (1:500) antibodies overnight at 4°C in combination with either anti-GFAP (1:1000) or anti-Iba1 (1:1000) antibodies at 4°C overnight. After incubation with primary antibodies, sections were rinsed with PBS, exposed to Alexa Fluor 488/594 conjugated secondary antibodies (1:1000) for 2hr at room temperature, washed and mounted with Prolong Gold antifade. Immunostained sections were examined under a Zeiss Axioskop-2 fluorescence microscope and the photomicrographs were taken with a Nikon 200 digital camera.

6. Astrocyte cell counting

For astrocyte quantification, every sixth section from the hippocampus of 7-week-old mice of different genotypes were used (n = 4 per genotype). Hippocampal sections were double labelled with Y188, BACE1 or PS1 together with GFAP as described earlier and then used for quantification.

7. Data analysis

All data obtained from three to four experiments were expressed as mean ± SEM. Statistical significance was determined by one-way ANOVA followed by Bonferroni's *post-hoc* analysis for multiple comparisons, with significance set at $p < 0.05$. All analysis was performed using GraphPad Prism.

Table 1. Details of the primary antibodies used in this study

Antibody	Type	IHC/IF dilution	Source
APP (clone Y188)	Monoclonal	1:1000	Abcam Inc.
BACE1	Polyclonal	1:500	Abcam Inc.
Glial fibrillary acidic protein (GFAP)	Polyclonal	1:1000	Dako
Nicastrin	Polyclonal	1:500	Santa Cruz
Pen2	Polyclonal	1:1000	Dr. G. Thinakaran
PS1	Polyclonal	1:500	Dr. G. Thinakaran

RESULTS

Since cholesterol has been shown to influence Aß production, it is of interest to determine whether accumulation of cholesterol within the endosomal- lysosomal system, which is the major site of Aß production, can influence levels and/or processing of APP (Greenfield et al., 1999). To address this issue, we used a well-characterized mutant APP transgenic mouse (APP- Tg), Niemann-Pick Typ C1 protein (NPC1)-null mice which exhibit cholesterol accumula t io n with the EL system and a novel bigenic ANPC *(APP-Tg and Npc1-null: APP+/0Npc1-/-)* mouse model generated in our lab that overexpresses mutant human APP in the absence of Npc1 protein and age-matched wild-type (WT) control mice (Maulik et al., 2012). Since a subset of reactive astrocytes in NPC1-null mice, in contrast to WT control mice, have been shown to express APP and its processing enzymes BACE1 and components of the γ-secretase complex (i.e., presenilin 1, nicastrin, APH1 and PEN2) (Kodam et al., 2010), we wanted to investigate if overexpression of mutant APP in NPC1-null mice can enhance the expression of APP or its processing enzymes in reactive astrocytes. Furthermore, to establish the significa nce of cholesterol in APP metabolism, we evaluated the effects of the sterol binding agent 2-HPC that has been shown to promote movement of the sequestered cholesterol from lysosomes to the metabolically active pool in various experimental paradigms (Davidson et al., 2009; Liu et al., 2009; Ramirez et al., 2010). Thus, WT, NPC1-null, APP and ANPC mice were injected with 2-HPC or saline at postnatal day 7 and then we evaluated their behavioural and pathological features at 4 or 7 weeks of age. In an earlier study, we reported that 2-HPC treatment can lead to significant improvement in the longevity and behavioural deficits of ANPC and NPC1-null mice compared with the respective saline-treated genotypes. At the cellular level, we observed that 2-HPC treatment was able to sequester filipin- labe lled cholesterol accumulation in most neurons in 4-week-old ANPC and NPC1-null mice (Maulik et al., 2012). In this study, we evaluated the potential effects of 2-HPC on the astrocytic expression of APP and its processing enzymes in NPC1-null, APP and ANPC mice.

***1. Aggravated glial activation in WT, NPC1-null, APP and ANPC mice*:** Reactive glios is defines when astrocytes get activated or undergo specific modifications resulting from diseases or injuries. This is chracterized by hypertrophy of cellular processes and upregulation of intermediate filament proteins such as GFAP. Key roles for maintaining neuronal homeostasis are found to be compromised by activation of astrocytes. Furthermore, activated astrocytes are involved in inflammatory processes which play an important role in the progression of various

neurodegenerative diseases including AD and thus can negatively affect disease progression and neuronal viability (Rodriguez et al., 2009; Batarseh et al., 2016; Li et al., 2019). At the cellular level, ANPC mice showed a profound activation of GFAP-labeled astrocytes in both hemispheres of the hippocampus (Fig. 1G, H). This is evident not only by an increase in the number of astrocytes but also by hypertrophy of their somas and processes compared to WT (Fig. 1A, B), NPC1-null (Fig. 1C, D) and APP-Tg mice (Fig. 1E, F) at 7 weeks of age. The relative number of reactive astrocytes appears to be highest in ANPC mice, followed by APP- Tg mice and then NPC1-null mice. Only occasionally were reactive astrocytes found in age-matched WT mice (Fig. 1A-H).

***2. Expression of APP in GFAP-labelled astrocytes in NPC1-null, APP and ANPC mice*:** To determine the association between reactive astrocytes and APP-related peptides in NPC1-null mice with or without overexpression of mutant APP, we first examined the expression of APP in the hippocampus of 7 week old NPC1-null, APP-Tg, ANPC and age-matched WT control mice. In normal conditions, neurons of the hippocampus are the primary source of APP, and astrocytes, which express only very little APP, are usually responsible for the clearance and degradation of Aβ-related peptides (Osborn et al., 2016). At the cellular level, the majority of APP immunoreactivity in the hippocampus of WT control mice at 7 weeks of age was visib le in the CA1-CA3 pyramidal neurons and granule cells of the dentate gyrus but not in glial cells. A few APP-immunoreactive neurons were also visible in the hilus area of the dentate gyrus (Fig. 2A, B)(Beeson et al., 1994). In NPC1-null, APP-Tg and ANPC mice the overall expression of APP appears to be only modestly increased in CA1-CA3 region pyramidal neurons of the hippocampus (Fig. 2C-H). In APP-Tg mice with or without Npc1 protein as well as NPC1-null mice, we found APP expression in a subset of glial cells of both hippocampi (Fig. 2A-H). To determine further if the expression of APP is on the astrocytes or in microglia, we performed double immunolabelling of APP in the presence of the astrocyte marker GFAP and the microglia marker Iba1 (Fig. 3A-O). Interestingly, immunoreactive APP is found to be expressed in a subset of GFAP-labelled astrocytes but not in microglia (Fig. 3D-O). The relative number of astrocytes expressing APP appears to be the highest in ANPC mice followed by APP-Tg and NPC1-null mice (Fig. 5). Additionally, we observed that 2-HPC treatment substantially decreased the number of GFAP-labelled astrocytes expressing APP in mutant APP-Tg and ANPC mice but not in NPC1-null mice compared to respective saline-treated control mice at 7 weeks of age (Figs. 2I-P, 4A-L, 5).

3. Expression of BACE1 in GFAP-labelled astrocytes in NPC1-null, APP and ANPC mice: To determine if the expression of APP in a subset of reactive astrocytes was associated with a parallel increase in APP processing enzymes, we first examined the expression of BACE1 in the hippocampus of 7 week old NPC1-null, APP-Tg, ANPC and age-matched WT mice (Fig. 6A-H). Consistent with the results for APP expression, immunoreactivity for the ß-secretase BACE1 in the hippocampus of WT control mice was evident mostly in neurons of the pyramidal layer of the CA1-CA3 region and the granule cells of the dentate gyrus (Fig. 6A, B). Besides neuronal BACE1 expression in ANPC and APP-Tg mice, several glial cells showed profound expression of BACE1 in stratum radiatum and stratum oriens of the hippocampus. The same results were evident in NPC1-null mice but to a lesser extent than in APP-Tg or ANPC mice (Fig. 6C-H). The WT mice did not exhibit BACE1 expression in glial cells (Fig. 6A, B). To evaluate if the expression of BACE1 is on the astrocytes or in microglia, we subsequently performed double labelling of BACE1 in the presence of GFAP and Iba1. As observed with APP, immunoreactive BACE1 is found to be expressed in a subset of GFAP- labelled astrocytes but not in Iba1-labelled microglia in NPC1-null, APP-Tg and ANPC mice (Fig. 7A-O). The relative number of astrocytes expressing BACE1 appears to be the highest in ANPC mice compared to APP-Tg and NPC1-null mice (Fig. 9). In contrast to immunoreact ive APP, our immunohistochemical analysis of BACE1 revealed significant reduction in its astrocytic expression profile after 2-HPC treatment in ANPC and APP-Tg mice compared to their untreated counterparts. NPC1-null mice, on the other hand, did not show any signific ant alteration in the expression profile of BACE1 in astrocytes following 2-HPC treatment (Figs. 6I-P, 8A-L, 9).

4. Expression of γ-secretase complex in GFAP-labelled astrocytes in NPC1-null, APP and ANPC mice: In addition to APP and BACE1, we evaluated the expression of three components of the γ-secretase complex (PS1, nicastrin and Pen2) which are involved in the processing of APP (Figs. 10-15). WT mice express PS1, nicastrin and Pen2 in the CA1-CA3 pyramidal cell layer and to some extent in scattered neurons in the stratum radiatum and the stratum oriens of the hippocampus. Occasionally granule cells of the dentate gyrus also express PS1 (Fig. 10A, B), nicastrin (Fig. 14A, B) and Pen2 (Fig. 15A, B) in WT mice. Similar to APP and BACE1, we did not observe any expression of the PS1 (Fig. 10A, B) or other gamma-secretase complex components, i.e, nicastrin (Fig. 14A, B) and Pen2 (Fig. 15A, B) in glial cells of WT control mice at 7 weeks of age. At a cellular level, APP-Tg and ANPC mice showed expression for PS1 (Fig. 10E-H), nicastrin (Fig. 14E-H) and Pen2 (Fig. 15E-H) at a similar level in a subset

of glial cells in the hippocampus of both hemispheres. PS1- (Fig. 10C, D), nicastrin- (Fig. 14C, D) and Pen2- (Fig. 15C, D) expressing glial cells were less obvious in Npc-null mice compared to APP-Tg and ANPC mice. Our double immunolabeling of PS1 with GFAP and Iba1 revealed that a subset of reactive astrocytes, but not microglia, expresses immunoreactive PS1 in the hippocampus of 7 week old NPC1-null, APP-Tg and ANPC mice (Fig. 11A-L). The age-matched WT control mice rarely express PS1 in GFAP-labelled astrocytes (Fig. 11A-L). Additionally, we observed a significantly decreased expression of PS1 in astrocytes of 2-HPC treated NPC1-null, APP-Tg and ANPC mice compared to respective saline-treated control mice (Figs. 10I-P, 12A-L, 13).

Figure - 1 : Expression of GFAP

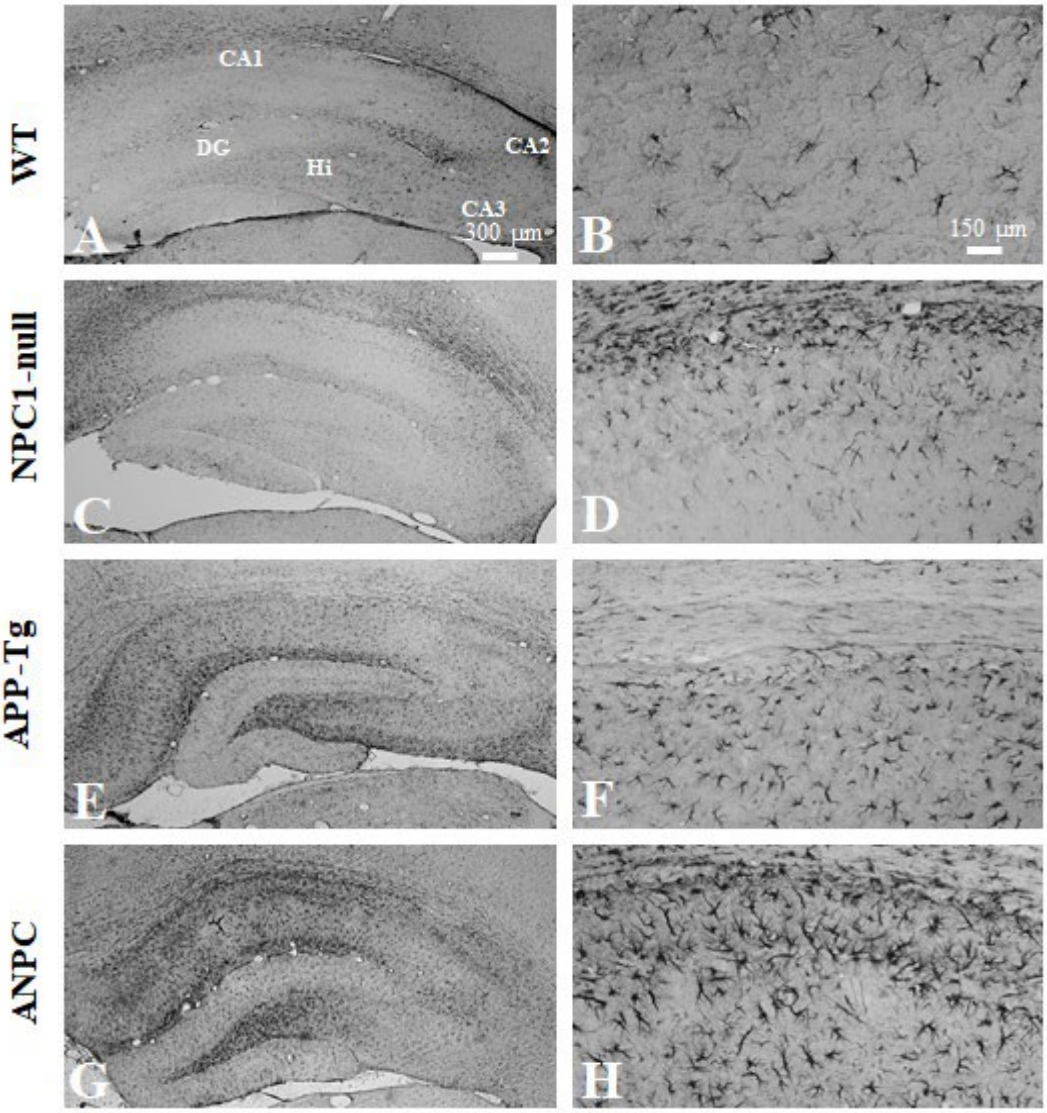

Figure - 1: Bright- field photomicrographs showing GFAP-positive astrocytes in the hippocampus of 7-week-old WT (A, B), NPC1-null (C, D), APP-Tg (E, F) and ANPC (G, H) mice. Note the increased expression of GFAP immunoreactivity in the hippocampus of NPC1-null (C, D) APP-Tg (E, F) and ANPC (G, H) mice compared to WT (A, B) mice.

Figure - 2 : Expression of APP

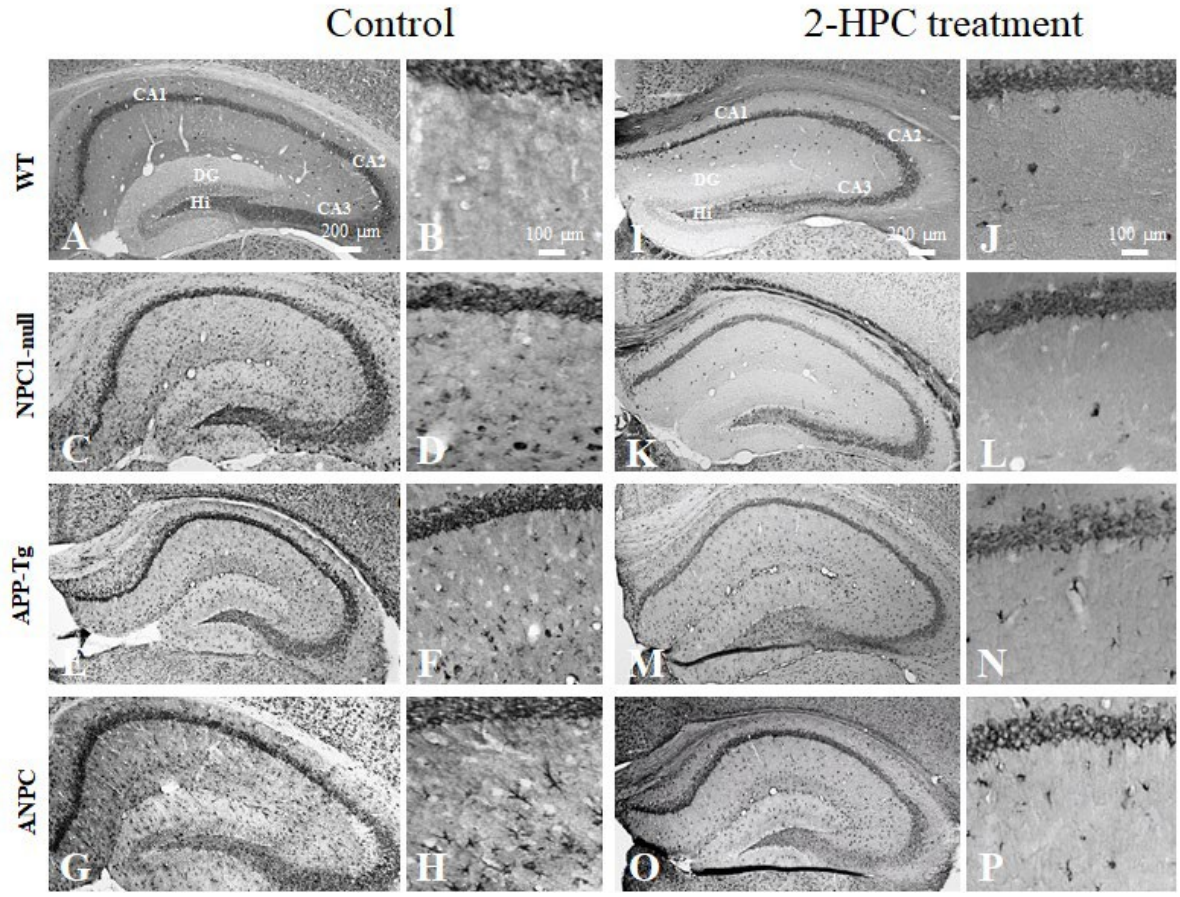

Figure - 2: A-H; Bright- field photomicrographs showing APP-positive glial cells in the hippocampus of 7-week-old WT (A, B), NPC1-null (C, D), APP-Tg (E, F) and ANPC (G, H) mice. Note the increased expression of APP immunoreactivity in the hippocampus of ANPC and APP-Tg mice compared to WT and NPC-null mice. I-P; Bright-fie ld photomicrographs showing the effect of 2-HPC treatment on APP-positive glial cells in the hippocampus of 7-week-old WT (I-J), NPC1-null (K, L), APP-Tg (M, N) and ANPC (O, P) mice. Note the attenuation in the proliferation/activation of APP-labelled astrocytes in the hippocampus of 2-HPC-treated NPC1-null (K, L), APP-Tg (M, N) and ANPC (O, P) mice compared to respective control (A-H) mice. CA1-CA3, Cornu Ammonis areas1-3 of the hippocampus; DG, Dentate gyrus; Hi, Hilus.

Figure - 3 : Colocalization of APP and GFAP

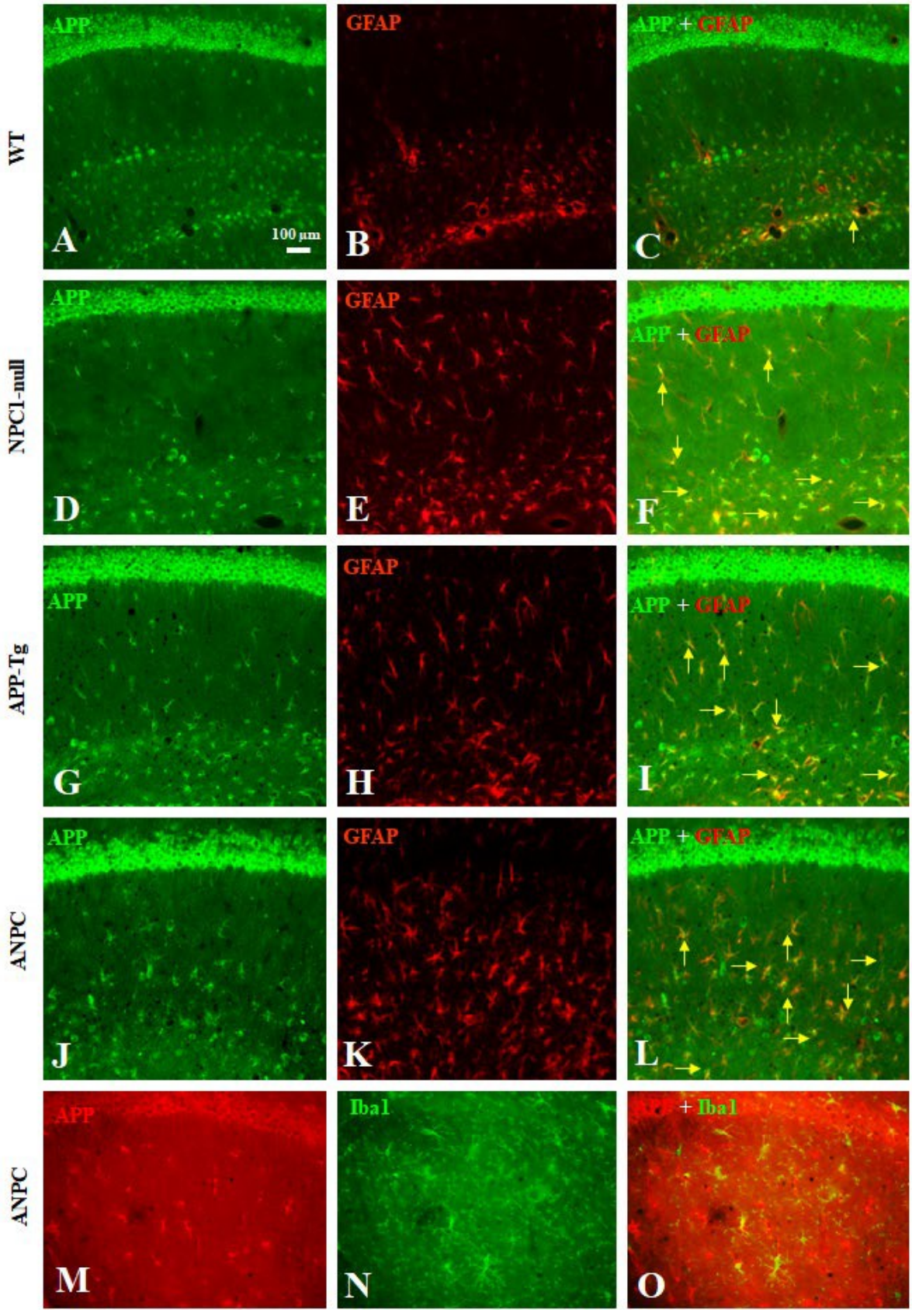

Figure - 3: A - L, Photomicrographs showing fluorescence-double labelling of APP and GFAP in the hippocampus of 7-week-old saline treated WT (A-C), NPC1-null (D-F), APP-Tg (G-I) and ANPC (J-L) mice. Colocalization of APP and GFAP (arrows) is evident in all genotypes. Note the increased colocalization of GFAP-labelled astrocytes and APP expression in the hippocampus of NPC1-null (F), APP-Tg (I) and ANPC (L) mice compared to WT (C) mice. M-O, Photomicrographs showing APP-labelled astrocytes (M) and Iba1-labelled microglia (N) in the hippocampus of ANPC mice. No localization of APP in Iba1-labelled microglia was found (O).

Figure - 4 : Colocalization of APP and GFAP in 2-HPC-treated mice

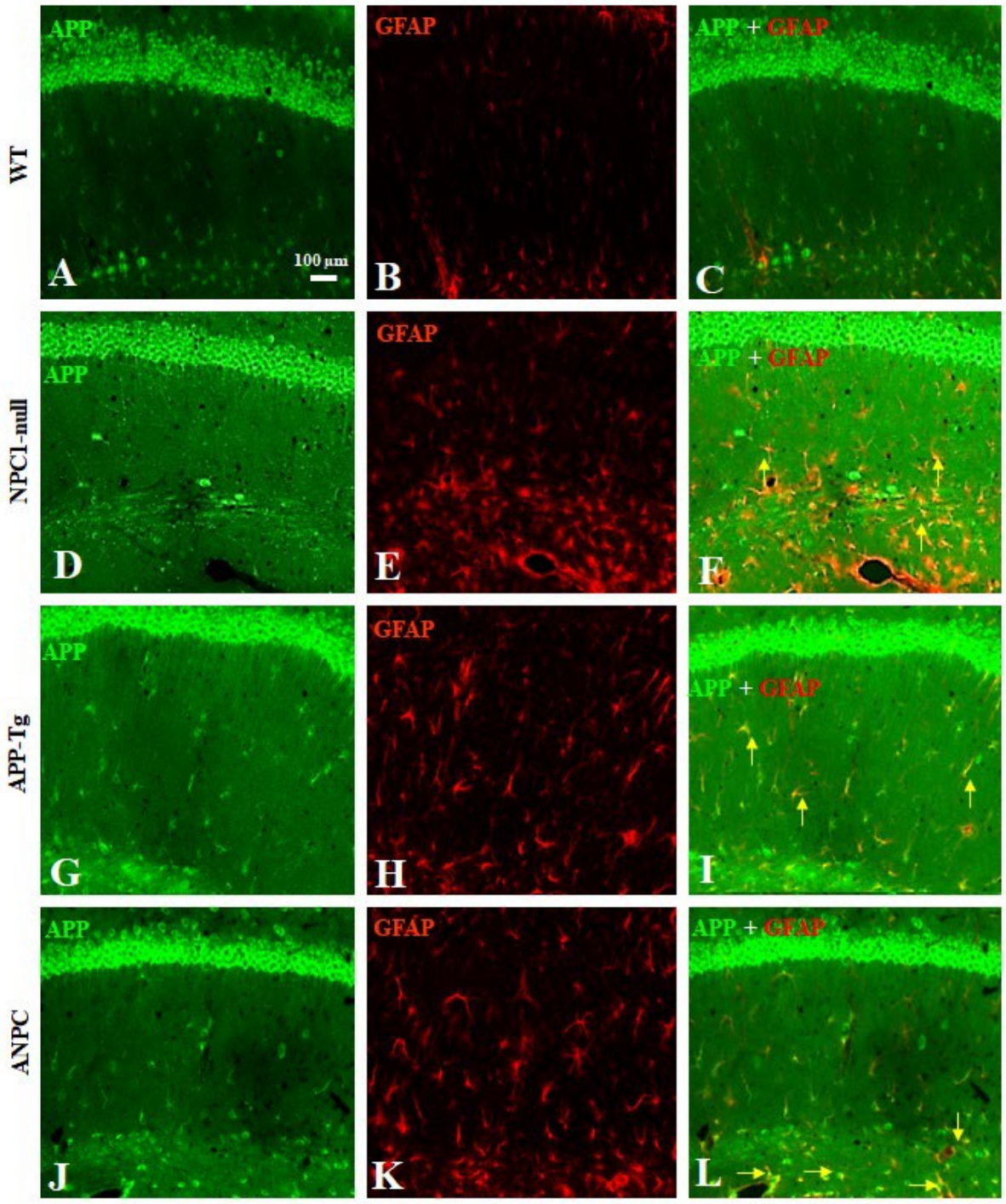

Figure - 4: Photomicrographs showing the effect of 2-HPC treatment on WT (A-C), NPC1-null (D-F), APP-Tg (G-I) and ANPC (J-L) mice. APP labelling (A, D, G, J), GFAP labelling (B, E, H, K) and double labelling of APP and GFAP (C, F, I, L) of the hippocampus of 7-week-old mice reveals colocalization of APP and GFAP (arrows) is evident in NPC1-null (F), APP-Tg (I) and ANPC (L) mice.

Figure - 5 : Number of GFAP-labelled astrocytes expressing APP

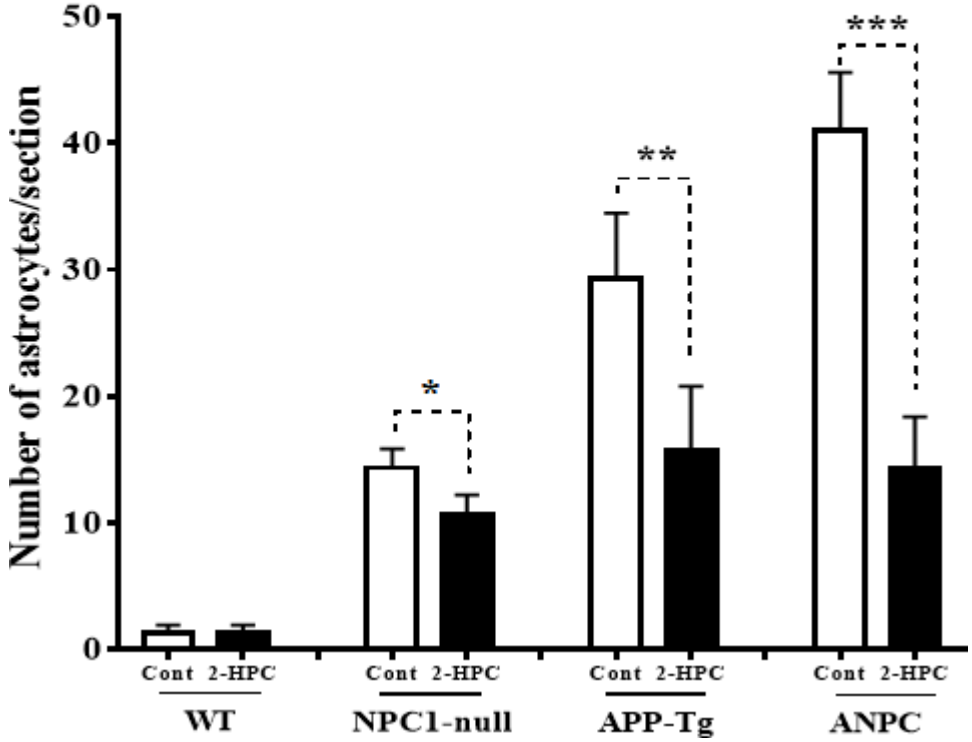

Figure - 5: Histogram shows the effect of 2-HPC treatment on the number of GFAP-labelled astrocytes expressing APP in the hippocampus of 7-week-old WT, NPC1-null, APP-Tg and ANPC mice. Quantitative analysis revealed a significant decrease in the number of GFAP-labelled astrocytes expressing APP in 2-HPC-treated NPC1-null, APP-Tg and ANPC mice compared to respective control mice. Values are means ± SEM, with n = 5 animals for each group. * $p<0.05$; ** $p<0.01$, *** $p<0.001$.

Figure - 6 : Expression of BACE1

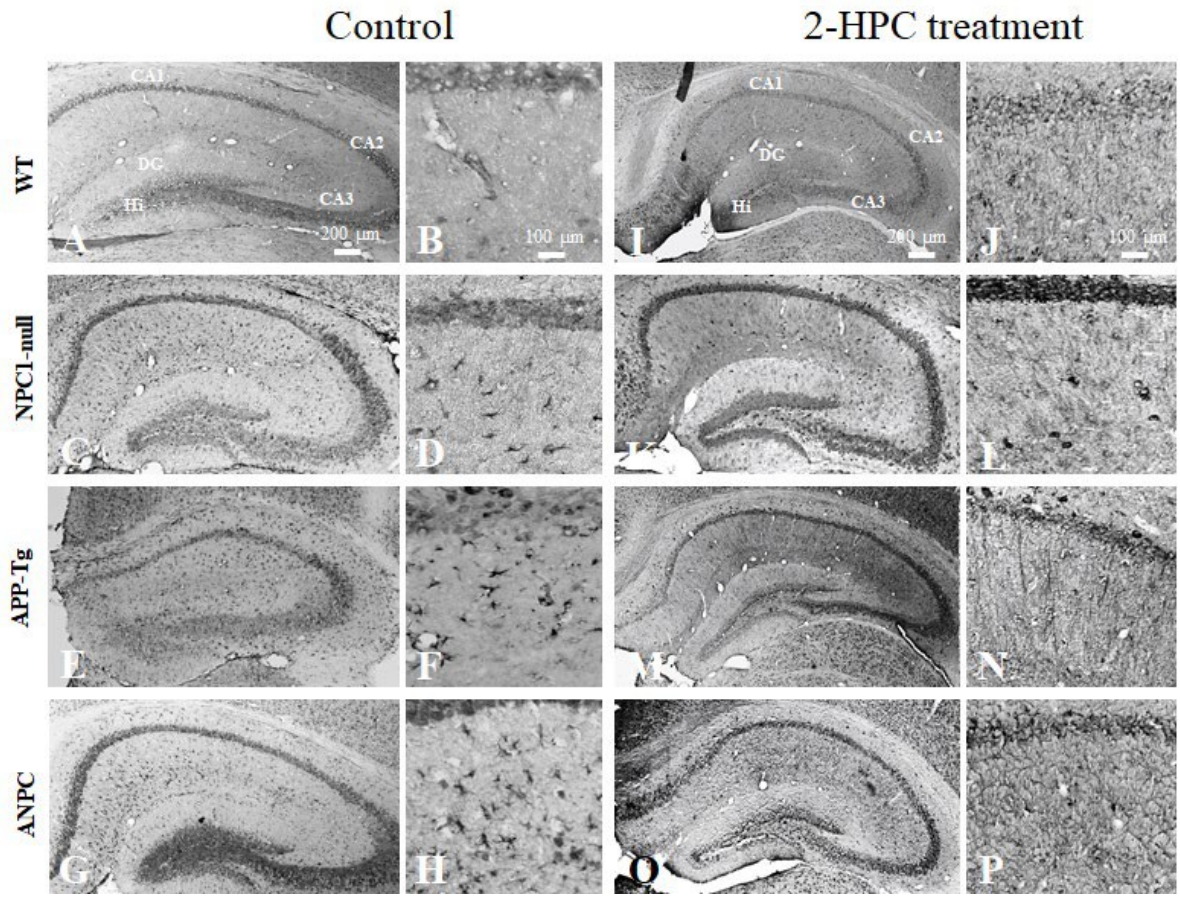

Figure - 6: A-H; Bright-field photomicrographs showing BACE1-positive glial cells in the hippocampus of 7-week-old WT (A, B), NPC1-null (C, D), APP-Tg (E, F) and ANPC (G, H) mice. Note the increased expression of BACE1 immunoreactivity in the hippocampus of APP-Tg (E, F), AnPC (G, H) and to some extent in NPC1-null (C, D) mice compared to WT (A, B) mice. I-P; Bright- field photomicrographs showing the effect of 2-HPC treatment on BACE1-positive glial cells in the hippocampus of 7-week-old WT, NPC1-null, APP-Tg and ANPC mice. Note the attenuation in the proliferation/activation of BACE1-labelled astrocytes in the hippocampus of 2-HPC-treated NPC1-null (K, L), APP-Tg (M, N) and ANPC (O, P) mice compared to respective control (A-H) mice. CA1-CA3, Cornu Ammonis areas1-3 of the hippocampus; DG, Dentate gyrus; Hi, Hilus.

Figure - 7 : Colocalization of BACE1 and GFAP

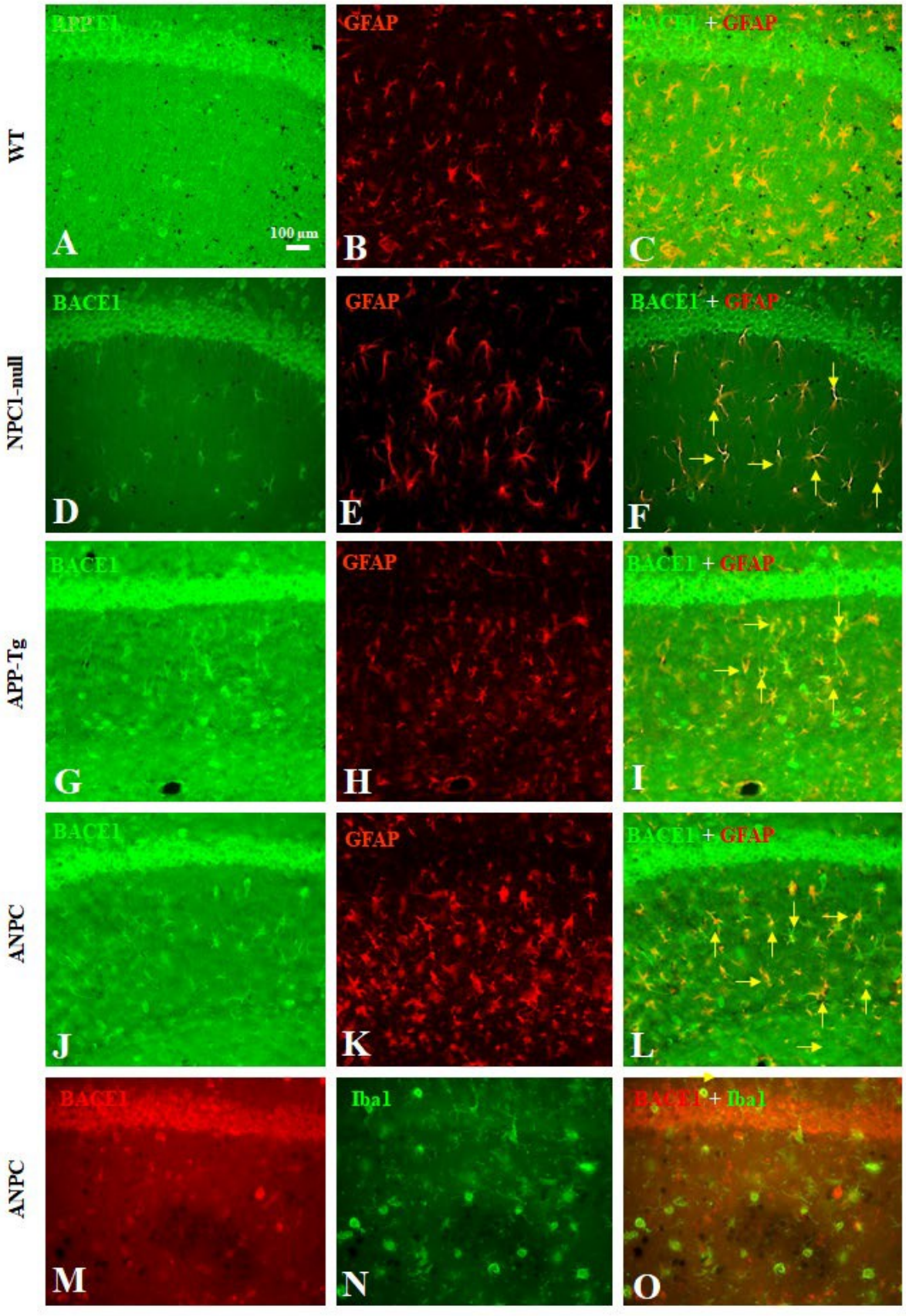

Figure - 7: A - P, Photomicrographs showing Fluorescence-double labelling of BACE1 and GFAP in the hippocampus of 7-week-old saline treated WT (A-C), NPC1-null (D- F), APP-Tg (G-I) and ANPC (J-L) mice. Colocalization of BACE1 and GFAP is evident in all genotypes. Note the increased colocalization of GFAP-labelled astrocytes and BACE1 expression (arrows) in the hippocampus of NPC1-null (F), APP-Tg (I) and ANPC (L) mice compared to WT (C) mice. M-O, Photomicrographs showing BACE1- labelled astrocytes (M) and Iba1-labelled microglia (N) in the hippocampus of ANPC mice. No localization of BACE1 in Iba1-labelled microglia was evident (O).

Figure - 8 : Colocalization of BACE1 and GFAP in 2-HPC-treated mice

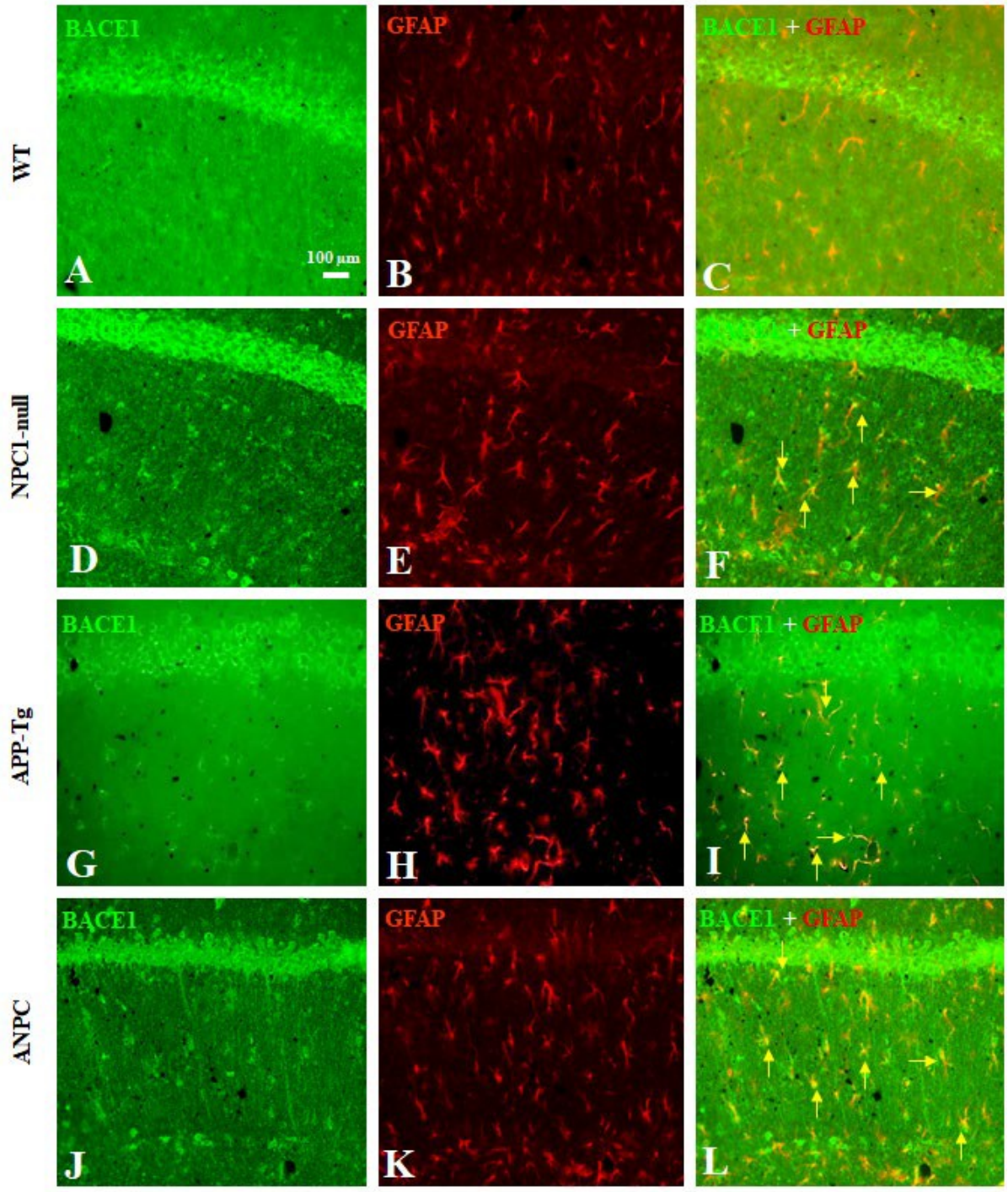

Figure - 8: A – P, Photomicrographs showing the effect of 2-HPC treatment on WT (A- C), NPC1-null (D-F), APP-Tg (G-I) and ANPC (J-L) mice. BACE1 labelling (A, D, G, J), GFAP labelling (B, E, H, K) and double labelling of BACE1 and GFAP (C, F, I, L) of the hippocampus of 7-week-old mice reveals colocalization of BACE1 and GFAP (arrows) is evident in NPC1-null (F), APP-Tg (I) and ANPC (L) mice.

Figure – 9 : Number of GFAP-labelled astrocytes expressing BACE1

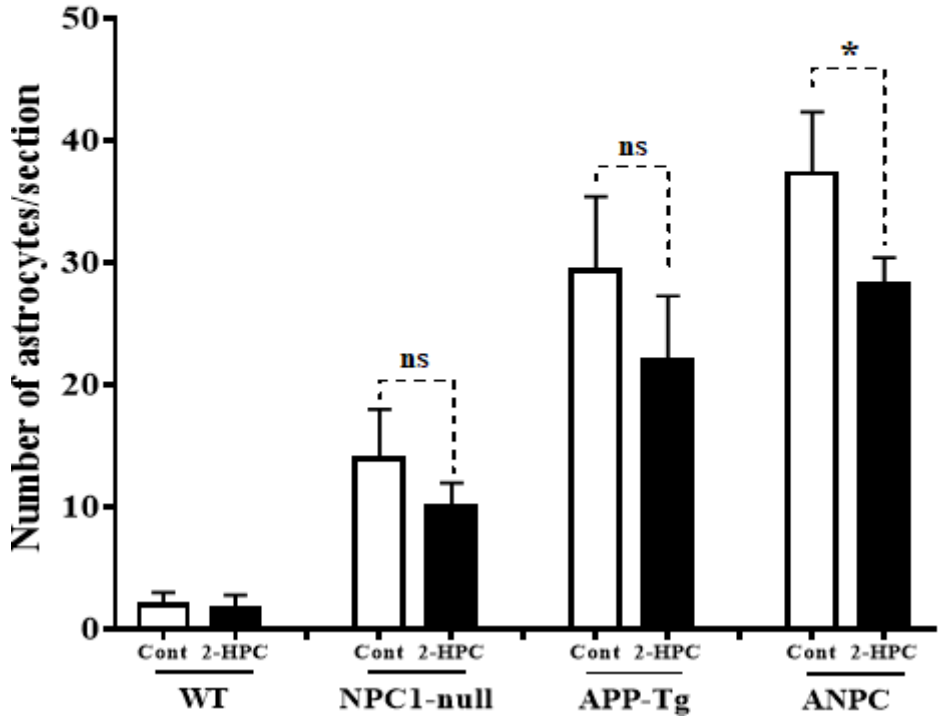

Figure - 9: Histogram shows the effect of 2-HPC treatment on the number of GFAP-labelled astrocytes expressing BACE1 in the hippocampus of 7-week-old WT, NPC1-null, APP-Tg and ANPC mice. Quantitative analysis revealed a significant decrease in the number of GFAP-labelled astrocytes expressing BACE1 in 2-HPC-treated ANPC mice compared to respective saline-treated control mice. Values are means ± SEM, with n = 5 animals for each group. * $P<0.05$.

Figure - 10 : Expression of PS1

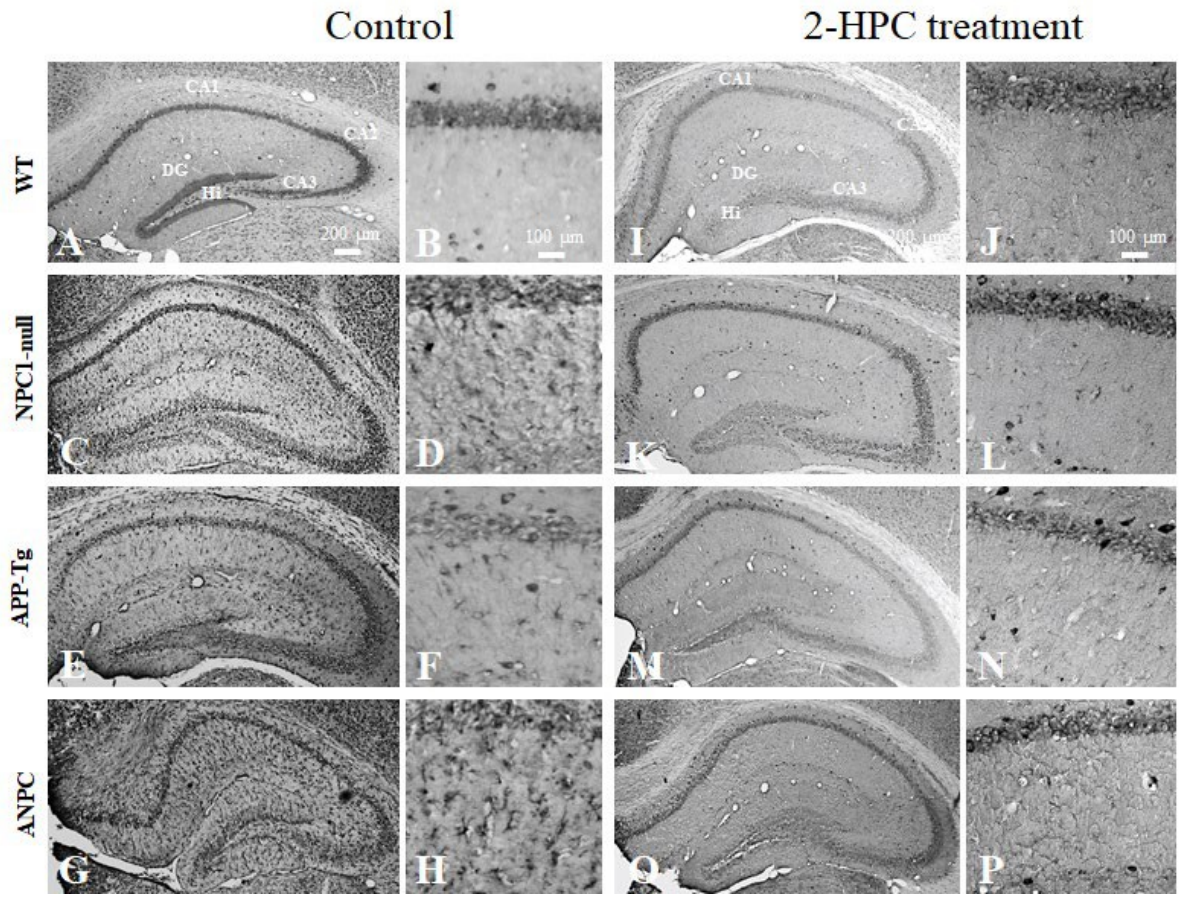

Figure - 10: A-H; Bright- field photomicrographs showing PS1-positive glial cells in the hippocampus of 7-week-old WT (A, B), NPC1-null (C, D), APP-Tg (E, F) and ANPC (G, H) mice. Note the increased expression of PS1 immunoreactivity in the hippocampus of ANPC and to some extent in NPC1-null as well as APP-Tg mice compared to WT mice. I-P; Bright- field photomicrographs showing the effect of 2-HPC treatment on PS1-positive glial cells in the hippocampus of 7-week-old WT, NPC1-null, APP-Tg and ANPC mice. Note the attenuation in the proliferation/activation of PS1-labelled astrocytes in the hippocampus of 2-HPC-treated NPC1-null (K, L), APP-Tg (M, N) and ANPC (O, P) micecompared to respective control (A-H) mice. CA1-CA3, Cornu Ammonis areas1-3 of the hippocampus; DG, Dentate gyrus; Hi, Hilus.

Figure - 11 : Colocalization of PS1 and GFAP

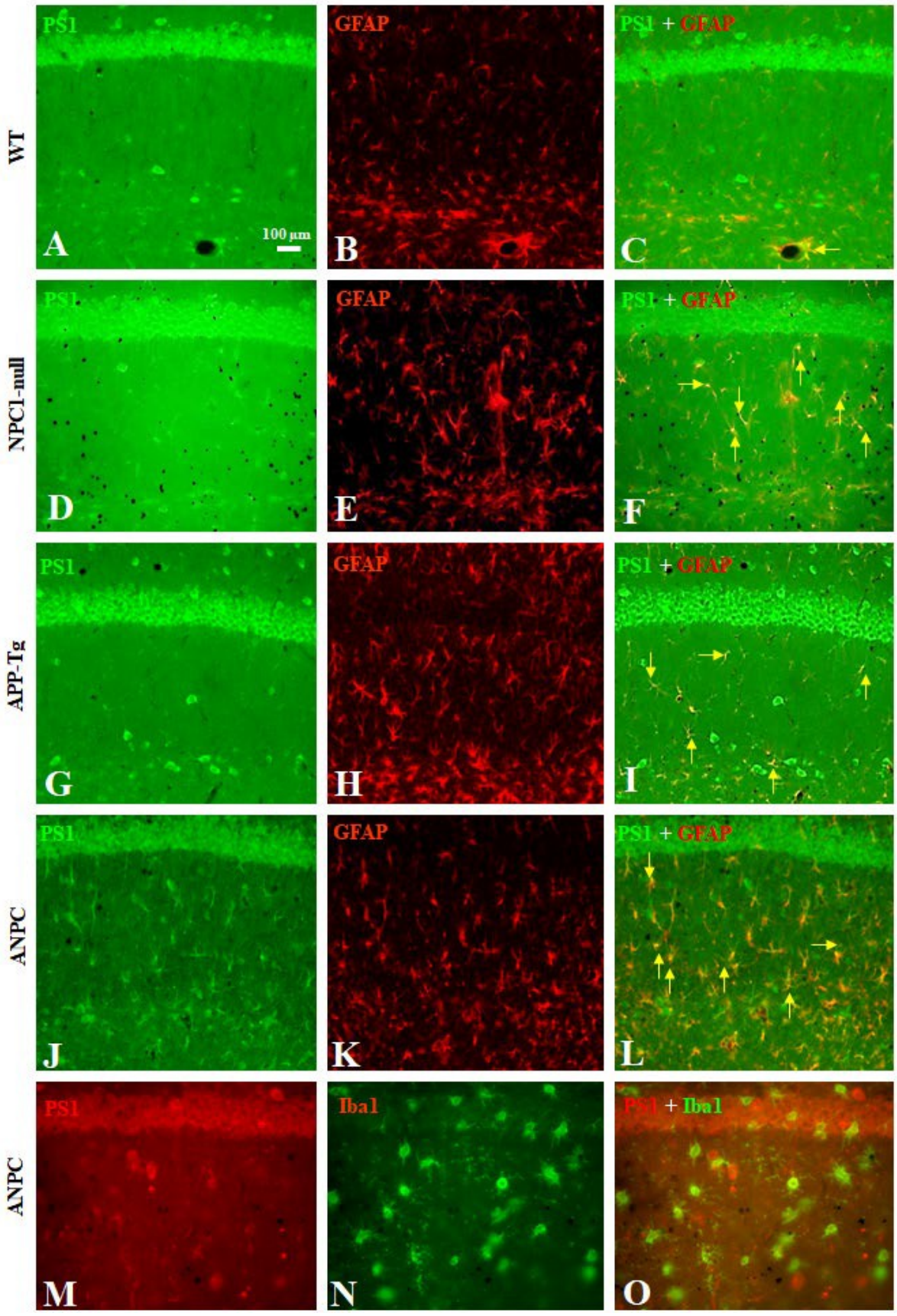

Figure - 11: A - P, Photomicrographs showing labelling of PS1 (A, D, G, J, M), GFAP (B, E, H, K) and double labelling with PS1 and GFAP (C, F, I, O) in the hippocampus of 7-week- old saline-treated WT (A-C), NPC1-null (D-F), APP-Tg (G-I) and ANPC (J-L) mice. Colocalization of PS1 and GFAP is evident in all genotypes to various levels. Note the increased colocalization of GFAP-labelled astrocytes and PS1 expression (arrows) in the hippocampus of ANPC (L) mice compared to APP-Tg (I), NPC1-null (F) and WT (C) mice. M – O, Photomicrographs showing PS1-labeled astrocytes (M) and Iba1-labeled microglia (N) in the hippocampus of ANPC mice. No colocalization of PS1 positive cells and Iba1-labeled microglia was found (O).

Figure - 12 : Colocalization of PS1 and GFAP in 2-HPC-treated mice

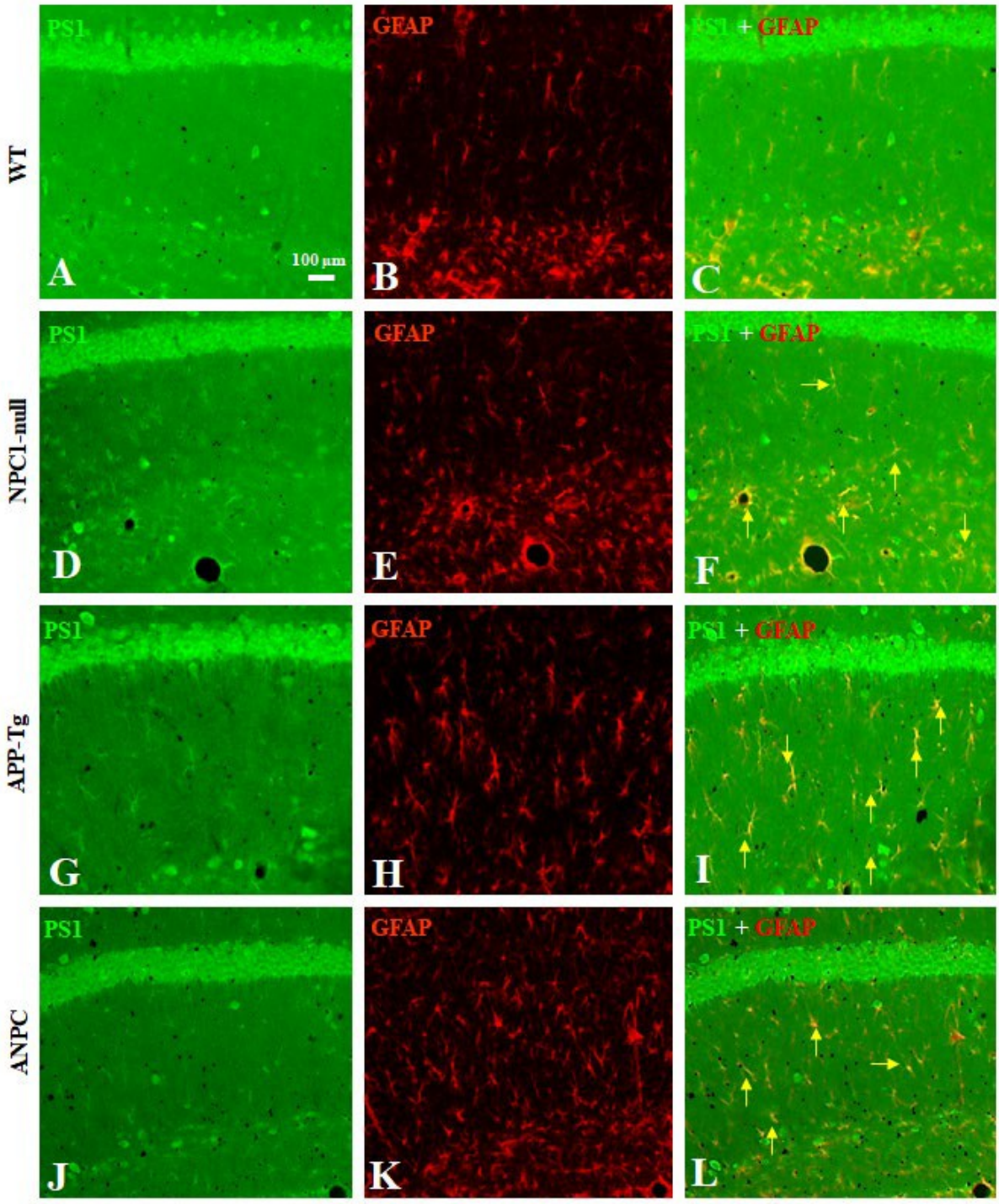

Figure - 12: A – P, Photomicrographs showing the effect of 2-HPC treatment on WT (A-C), NPC1-null (D-F), APP-Tg (G-I) and ANPC (J-L) mice. PS1 labelling (A, D, G, J), GFAP labelling (B, E, H, K) and double labelling of PS1 and GFAP (C, F, I, L) in the hippocampus of 7-week-old mice reveals colocalization of PS1 and GFAP primarily in NPC1-null (F), APP-Tg (I) and ANPC (L) mice.

Figure - 13 : Number of GFAP-labelled astrocytes expressing PS1

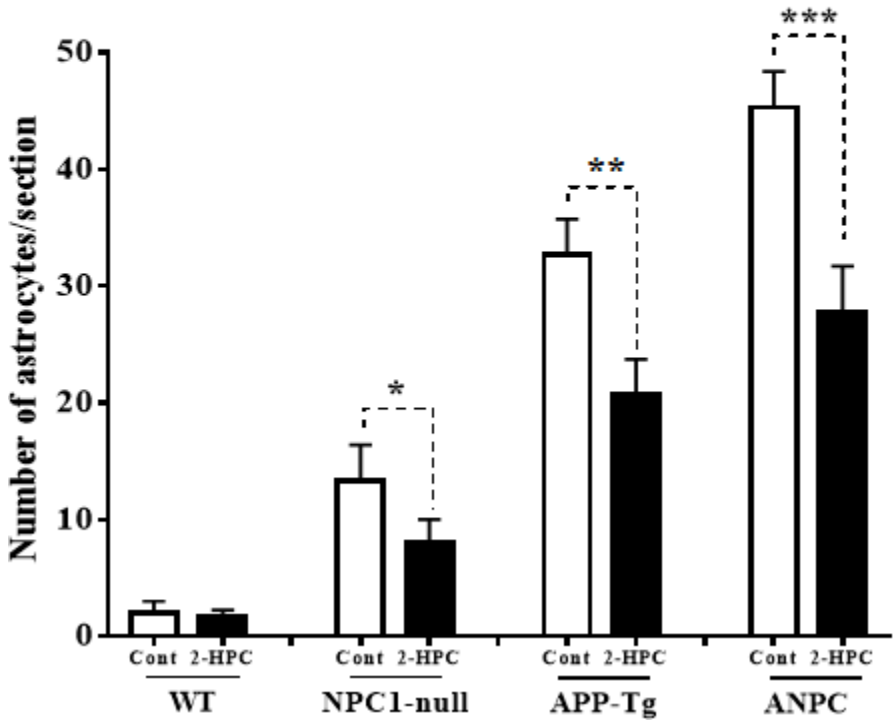

Figure - 13: Histogram shows the effect of 2-HPC treatment on the number of GFAP-labelled astrocytes expressing PS1 in the hippocampus of 7-week-old WT, NPC1-null, APP-Tg and ANPC mice. Quantitative analysis revealed a significant decrease in the number of GFAP-labelled astrocytes expressing PS1 in 2-HPC-treated NPC1-null, APP-Tg and ANPC mice compared to respective control mice. Values are means ± SEM, with n = 5 animals for each group. * $p<0.05$; ** $p<0.01$, *** $p<0.001$.

Figure - 14 : Expression of Nicastrin

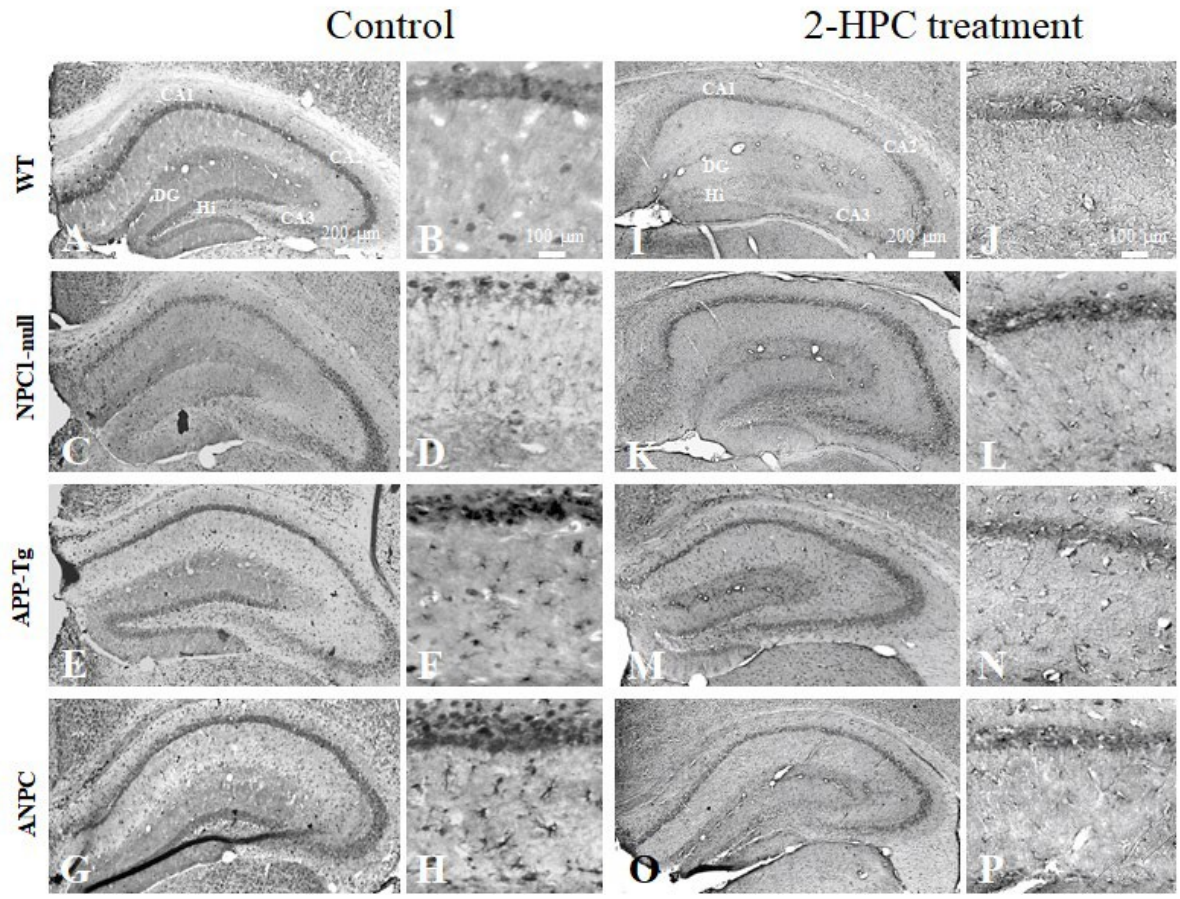

Figure - 14: A-H; Bright-field photomicrographs showing Nicastrin-positive glial cells in the hippocampus of 7-week-old Wild-type (WT, A, B), NPC1-null (C, D), APP-Tg (E, F) and ANPC (G, H) mice. Note the increased expression of Nicastrin immunoreactivity in the hippocampus of primarily ANPC and APP-Tg and to some extent NPC1-null mice compared to WT control mice. I-P; Bright- field photomicrographs showing the effect of 2-HPC treatment on Nicastrin-positive glial cells in the hippocampus of 7-week-old WT, NPC1-null, APP-Tg and ANPC mice. Note the attenuation in the proliferation/activat io n of Nicastrin-labelled astrocytes in the hippocampus of 2-HPC-treated NPC1-null (K, L), APP-Tg (M, N) and ANPC (O, P) mice compared to respective control (A-H) mice. CA1-CA3, Cornu Ammonis areas1-3 of the hippocampus; DG, Dentate gyrus; Hi, Hilus.

Figure - 15 : Expression of Pen2

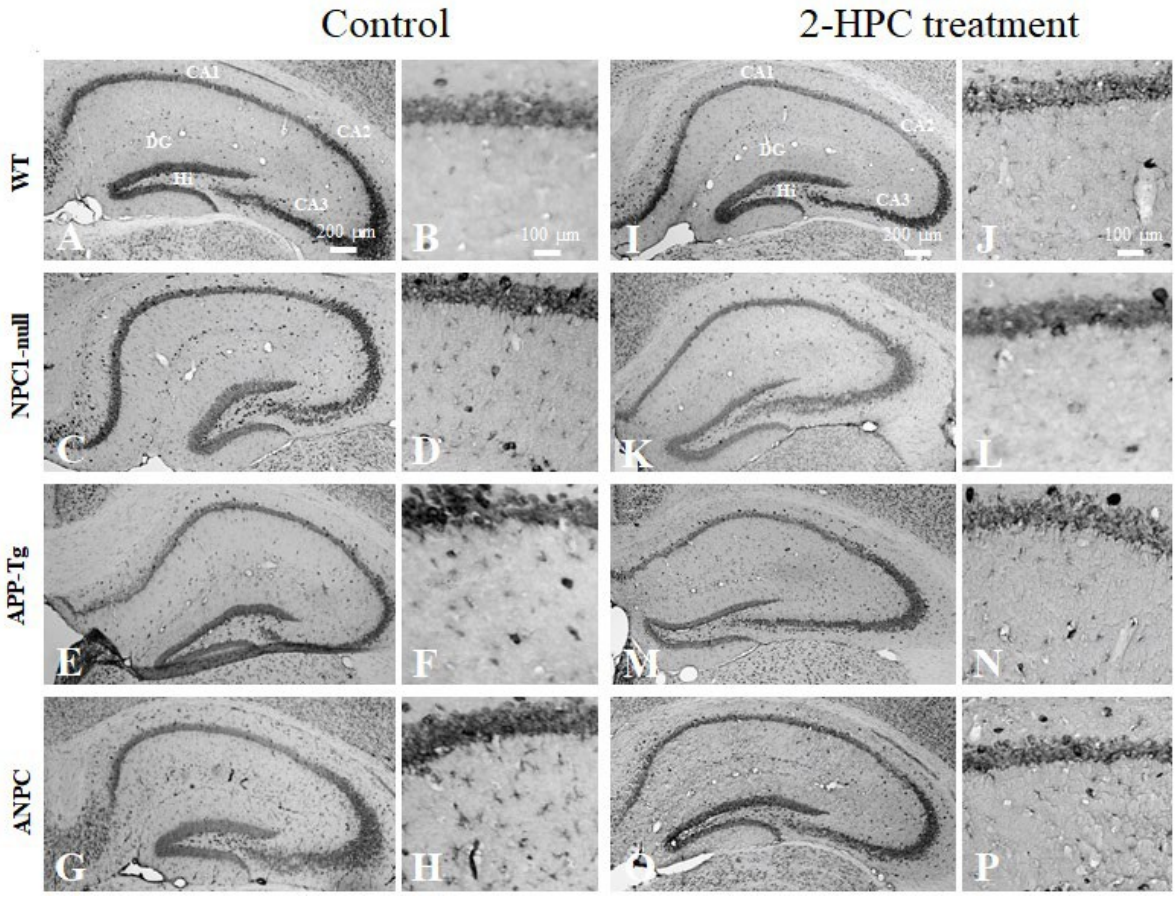

Figure - 15: A-H; Bright- field photomicrographs showing Pen2-positive glial cells in the hippocampus of 7-week-old wild-type (WT, A, B), NPC1-null (C, D), APP-Tg (E, F) and ANPC (G, H) mice. Note the increased expression of Pen2 immunoreactivity in the hippocampus of NPC1-null, APP-Tg and ANPC mice compared to WT mice. I-P; Bright- field photomicrographs showing the effect of 2-HPC treatment on Pen2-positive glial cellsin the hippocampus of 7-week-old WT, NPC1-null, APP-Tg and ANPC mice. Note the attenuation in the proliferation/activation of Pen2-labelled astrocytes in the hippocampus of 2-HPC-treated NPC1-null (K, L), APP-Tg (M, N) and ANPC (O, P) mice compared to respective control (A-H) mice. CA1-CA3, Cornu Ammonis areas1-3 of the hippocampus; DG, Dentate gyrus; Hi, Hilus.

DISCUSSION

In this study, we have shown that over-expression of APP in the absence of functional Npc1 protein in our recently developed bigenic ANPC mouse model exacerbates glial abnormalities. In addition to accumulation of cholesterol, the ANPC mice show marked activation of glial cells, both astrocytes and microglia, in all major brain regions including the cortex, hippocampus and cerebellum. Interestingly, only reactive astrocytes in the hippocampus of 7 week old ANPC mice exhibit enhanced levels of APP and its processing enzymes. Additionally, reversal of cholesterol accumulation following 2-HPC treatment was found to attenuate the astroglial abnormalities in ANPC mice, thereby suggesting a role of altered cholesterol homeostasis in reactive astrogliosis observed in AD-related pathology.

Astrocytes are the most abundant glial cells in the central nervous system and outnumber neurons by five-fold. Organized in territorial domains, astrocytes are connected to the vasculature, wrap synapses, provide trophic and metabolic support to neurons and therefore are essential for maintaining neuronal homeostasis. Astrocytes are key regulators for blood-brain barrier integrity, synaptic activity, neurotransmitter milieus, as well as synapse remodeling and formation (Pekny et al., 2014). Following any injury to the CNS, astrocytes go through specific modifications, resulting in "reactive gliosis" which is characterized by hypertrophy of cellular processes and upregulation of intermediate filament proteins including GFAP. In AD, reactive astrocytes are found to surround amyloid-β plaques and contribute to altered calcium signaling and local inflammatory response (Rodriguez et al., 2009, Olabarria et al., 2010). Additionally, activated astrocytes are involved in inflammatory processes which contribute to various pathological changes and therefore can negatively affect disease progression and neuronal viability (Batarseh et al., 2016). Reactive astrocytes might be neurotoxic by releasing proinflammatory cytokines and reactive oxygen species. Elevated levels of IFNγ, TNFα, and IL-1β have been found in human AD brain samples and transgenic mouse models (Sofroniew et al., 2010). Furthermore, increased amounts of IL-1β, a key regulator of the inflammatory response, are evident near the sites of amyloid plaques (Licastro et al., 2000). IL-1β has been found to upregulate APP in human astrocytes (Rogers et al., 1999). Stimulation with a combination of AD-specific proinflammatory mediators leads to increased APP and BACE1 expression in primary mouse astrocytes, resulting in 20-40% more $A\beta_{40}$ production (Zhao et al., 2011). The neuro-neglect hypothesis states that reactive astrocytes neglect their protective role and become key players in neurodegenerative processes (Fuller et al., 2010). Astrocytes in

AD brain undergo region-specific changes. While astrocytes close to amyloid plaques become reactive due to the presence of Aβ peptides in later stages of the disease, the majority of the astroglial populations undergo atrophy (Rodriguez et al., 2014). Interestingly, the degree of astrogliosis in AD is correlated with the severity of cognitive decline (Kashon et al., 2004).

In this study, we show that astrogliosis as part of the phenotype is by far more profound in ANPC mice compared to either APP-Tg or NPC1-null littermates at 7 weeks of age. Furthermore, we see expression of APP and its processing enzymes in reactive astrocytes of APP-Tg mice with or without functional NPC1 protein. At the cellular level, APP immunoreactivity in WT control mice is distributed in neurons. The hippocampal formatio n exhibit intense immunoreactive APP expression primarily in CA1-CA3 pyramidal neurons and granule cells of the dentate gyrus. Occasionally, APP-immunoreactive neurons are apparent as well in the hilus region of the dentate gyrus. Previous data from our laboratory has shown thata subset of reactive astrocytes express increased levels of APP and its processing enzymes in the hippocampus and cerebellum of NPC1-null mice (Kodam et al., 2010). The number of these astrocytes increased with the progression of disease pathology and was more evident in the cerebellum than in the hippocampus of NPC1-null mouse brains. We confirmed these previous findings in our study showing that a subset of reactive glial cells expresses high levels of APP in the hippocampus of 7-week-old ANPC, APP-Tg, and NPC1-null mouse brains with the expression being highest in ANPC mice. Double-labeling experiments confirmed the hypothesis that APP-expressing reactive glial cells are GFAP-positive astrocytes. Activated microglia did not express APP in brains of ANPC, APP-Tg or NPC1-null mice at 7 weeks of age.

Earlier studies showed that activated astrocytes surrounding Aß-containing neuritic plaques in AD brains and mutant APP-Tg mouse models express increased levels of APP and its processing enzymes (Rossner et al., 2001, Hartlage-Rubsamen et al., 2003, Nagele et al., 2003, Simpson et al., 2010). Additionally, there is evidence that activated astrocytes express APP, BACE1, and PS1 under specific conditions such as traumatic brain injury, kainic acid-induced excitotoxicity, cerebral ischemia, or cholesterol sequestration (Nadler et al., 2008, Kodam et al., 2010, Avila-Munoz and Ariaz, 2015; Kodam et al., 2019). In physiological conditio ns, neurons are the primary source of Aß and astrocytes only express very little APP and its processing enzymes, such as BACE1 and components of the ɣ-secretase complex. In a healthy brain, astrocytes are responsible for the clearance and degradation of Aβ peptides (Osborn et

al., 2016). However, increasing evidence shows that under certain circumstances, in particular cellular stress, expression of APP and its processing enzymes in astrocytes are upregulated and therefore secretion of Aβ is elevated (Zhao et al., 2011). By keeping in mind that astrocytes substantially outnumber neurons in the brain, under certain pathological conditions it might be possible that activated astrocytes serve as a significant source of neurotoxic Aß peptides. A study from Veeraraghavalu et al. showed increased Aβ production in both astrocytes and microglia after the inactivation of neurons in the FAD PS1 mutant mouse model (Veeraraghavalu et al., 2014). Grolla and colleagues also demonstrated that astrocytes express all components for the amyloidogenic and nonamyloidogenic pathway in primary rat hippocampal astrocyte cultures (Grolla et al., 2013). Taken together, these results indicate that reactive astrocytes, by way of pronounced APP expression, may have an important role in AD-related neurodegeneration due to increased generation of neurotoxic Aβ-peptides.

Cholesterol is a major component of the brain. It plays a key role in membrane fluid ity, maintaining lipid rafts, neuronal repair, and remodeling. Various lines of evidence show a potential role for increased cholesterol levels to influence APP metabolism and AD pathology (Simons et al., 1998; Refolo et al., 2001; Martins et al., 2009; Maulik et al., 2013). Previous data from our laboratory showed that filipin- labeled cholesterol was evident in almost all neurons of the hippocampus and cerebellum in NPC1-null and ANPC mice at 7 weeks of age. By contrast, no cholesterol accumulation was evident in WT and APP-Tg littermates of same age group. In contrast to the cerebellum which displays a significant loss of Purkinje cells in ANPC and NPC1-null mice, the hippocampus does not exhibit any major neuronal loss in either APP-Tg, ANPC or NPC1-null mouse brains compared to the WT littermates (Maulik et al., 2013). Consistent with these data, we observed no significant neuronal loss in the hippocampus of the different mouse lines. The expression of APP and its processing enzymes is moderately increased in hippocampal neurons of mutant ANPC and NPC1-null mice compared with WT control and APP-Tg mice. Studies have shown that treatment with 2-HPC can decrease cholesterol accumulation in NPC1-null mice. 2-HPC can bind cholesterol and promotes the removal of cholesterol from lysosomes even in the absence of functional NPC protein (Rosenbaum et al., 2011). Furthermore, administration of 2-HPC either as a single or repetitive injection, markedly improved neuronal degeneration and glial pathology (Ramirez et al., 2010). 2-HPC treatment was able to sequester filipin- labeled cholesterol accumulation in most neurons in 4-week old ANPC and NPC1-null mice in a previous study from our laboratory (Maulik et al., 2013). Consequently, ANPC mice treated with 2-HPC showed an increased

lifespan and slower progression of motoric and object recognition deficits. Here we show that reversal of cholesterol build-up following a single injection of 2-HPC at postnatal day 7 was able to decrease the proliferation/activation of GFAP-labeled astrocytes in the hippocampus of ANPC mice, APP-Tg, and NPC1-null mice compared with the respective saline-treated animals. Consistent with the reduction of glial activation, expression of APP and its processing enzymes in reactive astrocytes of 2-HPC-treated animals also declined.

Thus, a feed-forward mechanism is possible in which Aβ, initially neuron-derived, triggers proinflammatory cytokine release from reactive astrocytes which then increases APP, BACE1, and γ-secretase expression, resulting in astrocytic Aβ generation. Increased Aβ levels then further induce neuroinflammation and Aβ secretion. Recent studies showed that the aging process itself, which is the major risk factor for late-onset AD, is associated with proliferation and reactivity of astrocytes in certain parts of the brain, mainly the CA1 region of the hippocampus. Therefore, neuroinflammation induced by aging could be an initiating event for late-onset AD (Rodriguez et al., 2014). It is possible that factors released from activated glial cells and/or damaged neurons trigger the astrocytic expression of APP and its processing enzymes (Rossner et al., 2005). Interestingly, 60% of Aβ-peptides from astrocytes are N- truncated, compared to 20% from neurons. N-Truncated peptides seem to be more prone to aggregation, and the percentage of N-truncated Aβ in senile plaques increases with the Braak stage (Oberstein et al., 2015). Similar to astrocytes, microglia respond to Aβ peptides with activation and can release proinflammatory cytokines which contribute to further generation of astrocytic Aβ generation (Block et al., 2007). Although we observed a strong microglial activation in ANPC, APP-Tg, and NPC1-null mice, we could not detect any expression of APP, BACE1, or PS1 in this glial cell type. It also remains unclear to what extent reactive astrocytes can accumulate Aß-related peptides or contribute to the increased production/levels of Aß peptides in ANPC mouse brains.

APP is ubiquitously expressed, but amounts vary among different cell types. All isoforms of APP mRNA have been detected in rat astrocytes and other non-neuronal human brain cells (LeBlanc et al., 1997). Various proinflammatory cytokines upregulate APP expression and therefore reactive astrocytes in the neuroinflammatory state of AD express higher amounts of APP than resting astrocytes. Treatment with neuroinflammation- inducing lipopolysaccharides (LPS) has been shown to increase the amount of GFAP-positive astrocytes along with a 2-fold increase of APP expression in APP_{swe} mice. Furthermore, β-CTF levels rose by 18-fold, and

finally there was a 3-fold increase in $A\beta_{40}$ and $A\beta_{42}$. The higher levels of β-CTF are presumably due to an increase in BACE1 activity (Sheng et al., 2003). Nonreactive astrocytes express only insignificant amounts of BACE1 (Rossner et al., 2005). Our data clearly showed BACE1 expression in reactive astrocytes of APP-overexpressing mice in the absence or presence of functional NPC1 protein. There was markedly less astrocytic BACE1 expression in NPC1-null mice. Bettegazzi and colleagues reported that resting astrocytes express BACE1 mRNA but not the protein (Bettegazzi et al., 2011). Interestingly, the activation of glial cells due to various chronic stressors leads to increases in BACE1 expression in GFAP-positive astrocytes (Hartlage-Ruebsamen et al., 2003). High amounts of BACE1 expression have been found in reactive astrocytes surrounding amyloid plaques (Leuba et al., 2005). Proinflamma tory cytokines such as IFNγ and TNFα seem to be able to upregulate APP and BACE1 (Rossner et al., 2005). Chao et al. 2007 showed that this enhanced expression is mediated through JAK2 and ERK1/2 pathways (Chao et al., 2007). Aβ itself has proinflammatory properties and also stimulates cytokine secretion from astrocytes and therefore can upregulate astrocyte BACE1 expression in AD *via* an inflammatory response (Maezawa et al., 2011). Other studies found altered calcium homeostasis in glial cells due to Aβ neurotoxicity, leading to enhanced BACE1 expression and inducing Aβ generation (Cho et al., 2008). Furthermore, pretreatment with a calcineurin inhibitor was able to prevent enhanced BACE1 expression (Jin et al., 2012). It seems that Aβ-peptides generated by neurons can stimulate Aβ production in reactive astrocytes. Expression of PS1 mRNA and protein has been found in astrocytes (Lah et al., 1997). Besides neuroinflammation, elevated glucocorticoid levels due to activation of the hypothalamic-pituitary-adrenal axis seem to upregulate APP and BACE1 expression and Aβ production from reactive astrocytes. Treatment with dexamethasone was able to increase APP, BACE1 expression, and Aβ generation in 9-month old mice, specifically in reactive astrocytes (Wang et al., 2011). Tissue damage is another stressor that is related to increased APP expression in reactive astrocytes. Traumatic brain injury is also associated with increased AD risk (Guo et al., 2000). PS1, as part of the γ- secretase complex, is increased in reactive astrocytes similar to APP and BACE1. A strong immunoreactivity for PS1 has been reported in reactive astrocytes and neurofibrillary tangles of AD brains (Huynh et al., 1997). Increased levels of reactive astrocytes along with PS1 and Nicastrin have also been reported in differe nt head trauma modalities (Nadler et al., 2008). However, γ-secretase activity is not correlated with the levels of γ-secretase protein, and therefore assumptions about Aβ-generation mediated by the γ-secretase complex in reactive astrocytes are often difficult to make. Thus, the increased expression of APP and its processing enzymes in a subset of reactive astrocytes observed in the

present study might not be specific to NPC and/or AD pathology, but could be a general phenomenon that results from or accompanies neurodegenerative events and/or chronic gliosis.

Further understanding of neuroinflammation which triggers astrocytic Aβ production/secretion is critical for defining the role of reactive astrocytes in AD-related pathology. Future experiments are necessary to detect the underlying mechanisms by which reactive gliosis leads to APP expression and if it serves as a significant source of Aß peptides which could lead to pathological developments such as neuronal loss and/or deposition of extracellular Aβ peptides. Given the fact that the cerebellum experiences a more severe neuronal loss than the hippocampus and that levels of APP and BACE1 are increased earlier in the cerebellum than in the hippocampus of NPC1-null mice (Amritraj et al., 2009; Kodam et al, 2010), it would be of interest to examine the pathologic expression of APP and its processing enzymes in the cerebellum of ANPC mouse brains and its relation to loss of Purkinjee cells, if any. Moreover, considering the marked reduction of reactive gliosis and expression of APP and its processing enzymes after a single systemic administration of 2-HPC in ANPC mice in our study, it would be worth looking into the effect of repeated 2-HPC injections on the glial cell pathology. Maybe repetitive 2-HPC treatment will be able to completely prevent glial dysfunction in ANPC mice. We think that reactive astrogliosis in ANPC mice may lead to increased inflammatory response, oxidative stress, and elevated Aβ production. Thus, this study highlights the contribution of reactive astrocytes to the increasing amyloid burden in the brain and AD-related neurodegeneration. Further, it establishes a role of altered brain cholesterol level/distribution in the activation of astrocytes and increased Aβ generation.

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