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REVIEW

Non-steroidal anti-inflammatory drugs (NSAIDs) in Alzheimer's disease: old and new mechanisms of action

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Abstract

Alzheimer's disease (AD) is characterized by cerebral deposits of β-amyloid (Aβ) peptides and neurofibrillary tangles (NFT) which are surrounded by inflammatory cells. Epidemiological studies have shown that prolonged use of non-steroidal anti-inflammatory drugs (NSAIDs) reduces the risk of developing AD and delays the onset of the disease. It has been postulated that some NSAIDs target pathological hallmarks of AD by interacting with several pathways, including the inhibition of cyclooxygenases (COX) and activation of the peroxisome proliferator-activated receptor γ. A variety of experimental studies indicate that a subset of NSAIDs such as ibuprofen, flurbiprofen, indomethacin and sulindac also possess Aβ-lowering properties in both AD transgenic mice and cell cultures of peripheral, glial and neuronal origin. While COX inhibition occurs at low concentrations *in vitro* (nM-low μM range), the Aβ-lowering activity is observed at high concentrations (≤ 50 μM). Nonetheless, studies with flurbiprofen or ibuprofen in AD transgenic mice show that the effects on Aβ levels or deposition are attained at plasma levels similar to those achieved in humans at therapeutic dosage. Still, it remains to be assessed whether adequate concentrations are reached in the brain. This is a crucial aspect that will allow defining the dose-window and the length of treatment in future clinical trials. Here, we will discuss how the combination of anti-amyloidogenic and anti-inflammatory activities of certain NSAIDs may produce a profile potentially relevant to their clinical use as disease-modifying agents for the treatment of AD.

Keywords: Alzheimer's disease, β-amyloid, cyclooxygenase, γ-secretase, non-steroidal anti-inflammatory drugs, peroxisome proliferator-activated receptor γ.

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Alzheimer's disease (AD), the most common form of dementia in the elderly population, is characterized by an insidious onset with memory impairment and an inexorable progression of cognitive decline. Neuropathological examination of AD brain reveals extensive atrophy, accumulation of intraneuronal neurofibrillary tangles (NFT; Lee and Trojanowski 1992), and β-amyloid (Aβ) fibrillar deposits (Aβ plaques) (Glenner and Wong 1984) in vulnerable regions of the brain (e.g. cortex, hippocampus).

Another key hallmark of AD brain is the presence of chronic neuroinflammation, which is also common to other neurodegenerative disorders, including Parkinson's disease (PD) and Creutzfeld-Jacob disease (CJD) (Eikelenboom *et al*. 2002; Gao *et al*. 2003), and has some peculiar features. Neuroinflammation associated with AD and other neurodegenerative diseases is clinically 'silent'. In fact, AD, PD and CJD brains do not show the cardinal signs of Celsus: pain, swelling, heat, redness. In addition, the histopathological analysis shows that leukocyte infiltration is absent in AD brain. Nonetheless, at the cellular and molecular levels, a

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Abbreviations used: Aβ, β-amyloid; AD, Alzheimer's disease; βAPP, β-amyloid precursor protein; CHO, cells, Chinese hamster ovary cells; COX, cyclooxygenase; HEK293, cells, human embryonic kidney 293 cells; IC_{50}, concentration that elicits a 50% inhibition; N2a, cells, neuro-2A neuroblastoma cells; NFT, neurofibrillary tangles; NO, nitric oxide; NO-NSAIDs, NO-releasing derivatives of NSAIDs; NSAIDs, non-steroidal anti-inflammatory drugs; PPARγ, peroxisome proliferator-activated receptor γ; sβAPPα, soluble βAPPα.

variety of inflammatory processes are observed in AD brain, mainly associated to neuropathological lesions (i.e. Aβ plaques and NFT). In particular, inflammatory cells, i.e. astrocytes and microglia, are activated in areas of the brain affected by Aβ plaques and tau pathology; tissue levels of pro- and anti-inflammatory mediators including cytokines, chemokines, oxygen free radicals and reactive nitrogen species, are altered; inflammatory molecules and products of the complement system are associated with amyloid plaques (for extensive reviews see: The Neuroinflammation Working Group 2000; Eikelenboom *et al*. 2002; McGeer and McGeer 2003). Altogether, these data suggest that chronic inflammation has a crucial role in AD pathogenesis. This is also supported by recent genetic findings showing that polymorphisms in inflammatory genes, including four inflammatory cytokines [i.e. interleukin (IL)-1α; IL-1β; IL-6; tumour necrosis factors α, TNFα] and two acute phase reactants (i.e. α1-anti-chimotrypsin, ACT; α2-macroglobulin, A2M) enhance the risk of developing AD (McGeer and McGeer 2001).

A number of factors can trigger inflammation in neurodegenerative diseases, including protein aggregates, accumulation of abnormally modified cellular components, molecules derived from injured neurons and synapses or dysregulation of inflammatory control mechanisms (Wyss-Coray and Mucke 2002). A wealth of data indicates the extracellular deposition of Aβ in AD brain as one of the triggers of inflammation. For example, Aβ activates microglia by binding to the receptor for advanced glycation end products (RAGE; Yan *et al*. 1999) or other scavenger receptors (El Khoury *et al*. 1996; Paresce *et al*. 1996). Aβ fibrils also bind to RAGE on neurons, causing cellular stress and the release of inflammatory factors (Yan *et al*. 1999). Furthermore, aggregated Aβ activates the complement system through the classical and alternate pathways by binding C1q and C3b, respectively, and sets off numerous immune and inflammatory processes (Shen and Meri 2003). Neurofibrillary tangles and cellular distress signals derived from injured neurons and synapses represent other triggering factors that contribute in maintaining the inflammatory reaction in AD brain (Shen *et al*. 2001; Wyss-Coray and Mucke 2002). It is worth noting that Aβ effectively binds and sequesters endogenous anti-inflammatory reactants, including apolipoprotein E (ApoE), ACT and A2M and, consequently, also interferes with the resolution of inflammation (Janciauskiene *et al*. 2002).

In addition to its central role in AD neuroinflammation, Aβ is a key player in the neurodegenerative processes (Kawahara and Kuroda 2000). Within this context, reducing AD amyloidosis represents one of the main therapeutic strategies under investigation for AD. Examples of this approach include the Aβ vaccine (which reduces brain amyloidosis by several mechanisms), inhibitors of secretases, Aβ disaggregants, cholesterol-lowering compounds and estrogen (Xu *et al*. 1998; Citron 2002). Lately, novel clinical and biological evidence has drawn renewed interest on strategies targeting the inflammatory processes and, in particular, on the potential use of non-steroidal anti-inflammatory drugs (NSAIDs) in AD. NSAIDs are among the most widely prescribed drugs for the treatment of pain, fever and inflammation. Their effects are largely attributed to the inhibition of the enzymatic activity of cyclooxygenase (COX)-1 and -2, leading to the suppression of prostaglandin synthesis. The selectivity for COX-1 and COX-2 differs among different NSAIDs, ranging from COX-unselective compounds (e.g. ibuprofen, naproxen) to COX-2 selective drugs (e.g. celecoxib, rofecoxib, diclofenac, nimesulide) (Frolich 1997; Flower 2003). Epidemiological studies indicate that prolonged usage of NSAIDs diminishes the risk of AD, delays dementia onset, slows its progression and reduces the severity of cognitive symptoms (In't Veld *et al*. 2002; Etminan *et al*. 2003). Nonetheless, given the failure of recent clinical trials, the potential of NSAIDs for the treatment of AD is still a matter of debate (Aisen *et al*. 2003; Reines *et al*. 2004). This failure could be due to a number of factors, e.g. the selection of patients which already had mild to moderate AD, the pharmacological characteristics of the drugs, their brain penetration properties and the dosing schedule.

Here, we will review which cellular and molecular mechanisms might underlie the beneficial actions of certain NSAIDs in AD brain. We will highlight novel findings indicating that some, but not all, NSAIDs can directly influence AD amyloid pathology, and we will discuss how these anti-amyloidogenic effects and the classical anti-inflammatory activities of NSAIDs might provide clinical benefits in AD and could explain current clinical results (Aisen *et al*. 2003; Reines *et al*. 2004).

Anti-amyloidogenic properties of NSAIDs: a novel mechanism of action potentially relevant for AD

NSAIDs and Aβ aggregation

Amyloid plaques contain an aggregated population of heterogeneous Aβ peptides derived from β-amyloid precursor protein (βAPP). Full-length βAPP undergoes proteolytic cleavages by β- and γ-secretase activities to generate Aβ40, the predominant Aβ species, and Aβ42 peptides, a less abundant but highly amyloidogenic variant. In addition to these amyloid-generating activities, full-length βAPP undergoes alternative processing by an enzymatic activity termed 'α-secretase' which cleaves within the Aβ region. This cleavage releases a soluble fragment (sβAPPα) extracellularly and precludes Aβ formation (Greenfield *et al*. 2000) (Fig. 1).

The aggregation of Aβ into β-sheet fibrils is believed to be critical to make Aβ peptides toxic to neurons (Harkany *et al*.

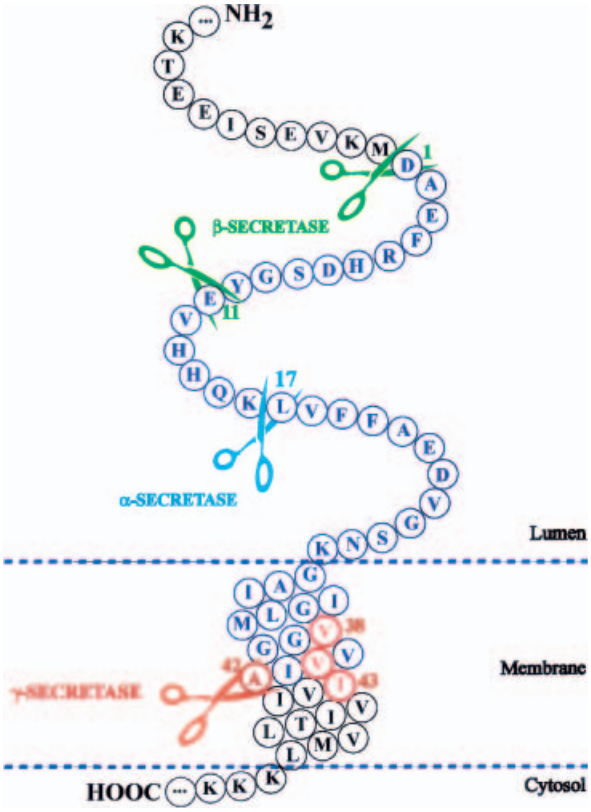

Fig. 1 Schematic representation of βAPP metabolism by secretases. βAPP is a type I transmembrane glycoprotein with a large extracellular N-terminal domain and a short C-terminal cytoplasmic tail. βAPP undergoes proteolysis by means of three different secretase activities (Greenfield *et al.* 2000), i.e. α-, β- (i.e. BACE; Vassar *et al.* 1999) and γ-secretase activities, leading to the production of a variety of different fragments. Sequential cleavage by β- (green) and γ-secretases (red) generates several secretable Aβ peptides (i.e. Aβ1–38/40/42/43) composed of 38/40/42/43 amino acids (red circles represent main γ-cleavage sites), the N-terminal truncated species Aβ11–40/42, derived from BACE alternate cleavage at position 11 (Gouras *et al.* 1998; Vassar *et al.* 1999) and soluble βAPPβs (sβAPPβ1 and sβAPPβ11). Alternatively, βAPP undergoes non-amyloidogenic cleavage via α- (light blue) and γ-secretases. α-secretase activity cleaves at amino acid 17 within the Aβ region, leading to the formation and secretion of soluble βAPPα (sβAPPα) and p3 (Aβ17–40/42). Blue circles represent the Aβ peptides sequence.

2000; De Felice and Ferreira 2002). In particular, it has been recently proposed that intermediates of the aggregation process, i.e. Aβ oligomers, are the main neurotoxic species (Podlisny *et al.* 1998; El-Agnaf *et al.* 2000). Thus, one drug discovery strategy to identify an effective AD therapy relies on the inhibition of the formation of Aβ fibrils and oligomers (Talaga 2001; De Felice and Ferreira 2002). In the last few years, evidence has emerged that NSAIDs may alter the conformation of Aβ peptides exerting anti-aggregation activity *in vitro* (Thomas *et al.* 2001; Agdeppa *et al.* 2003). For example, aspirin significantly reverses the β-sheet conformation of the aggregated Aβ25–35, Aβ40 and Aβ42 peptides in the test tube. This inhibitory effect on amyloid β-sheet conformation is not restricted to aspirin alone. Indomethacin, naproxen, ketoprofen, ibuprofen, which inhibit both COX-1 and COX-2, and two COX-2 selective compounds, i.e. rofecoxib and celecoxib, also have anti-aggregation properties with the following order of potency: indomethacin > naproxen > ketoprofen > ibuprofen > aspirin > celecoxib > rofecoxib (Thomas *et al.* 2001). Similar results were obtained using the positron emission tomography (PET) probe, 2–1-{6-[2-[^{18}F]fluoroethyl)(methyl)amino]-2-naphthyl}ethylidene)malonnitrile or [^{18}F]FDDNP, that binds Aβ fibrils *in vitro* and *in vivo*. Naproxen and, to a lesser extent, ibuprofen concentration-dependently decrease the binding of [^{18}F]FDDNP to Aβ40 fibrils formed in the test tube, probably through dissolution of aggregated Aβ40 fibrils. Importantly, naproxen and ibuprofen also compete for the binding of [^{18}F]FDDNP to Aβ aggregates in AD brain specimen incubated *in vitro* with both the probe and the NSAIDs. Conversely, diclofenac, congo red and thioflavin T do not show anti-aggregation properties (Agdeppa *et al.* 2003).

It has also been shown that NSAIDs could interfere with Aβ aggregation by inducing the expression of amyloid-binding proteins, e.g. transthyretin (TTR). TTR plays an important role in sequestering Aβ peptides and preventing their aggregation (Schwarzman *et al.* 1994; Mazur-Kolecka *et al.* 1995; Ray *et al.* 1998). Moreover, TTR facilitates the transport of Aβ from CNS to plasma across the choroid plexus (Carro *et al.* 2002). Several studies show that the levels of TTR are reduced in the CSF of AD patients (Riisoen 1988; Palha *et al.* 1996; Merched *et al.* 1998). Recently, it has been reported that NSAIDs can increase the levels of TTR in HepG2 cell cultures in a time- and concentration-dependent manner, indicating an alternative mechanism by which NSAIDs may affect Aβ levels and aggregation (Nie and Goldgaber 2003).

NSAIDs and cellular βAPP/Aβ metabolism

The effects of NSAIDs on Aβ are rather complex. Compelling evidence indicates that some NSAIDs directly modulate the metabolism and the secretion of βAPP and Aβ. Skovronsky *et al.* (2001) showed that indomethacin and aspirin inhibit the collagen- and arachidonic acid-stimulated secretion of sβAPP, but not that of Aβ, in human platelets. Furthermore, it has been reported that some NSAIDs modulate the secretion of sβAPPα from SHSY-5Y and PC12 cells according to a U-shaped concentration-response curve. In fact, indomethacin, ibuprofen and nimesulide stimulate sβAPP secretion at concentration up to 0.1–1 μM and decrease sβAPP levels at 10–100 μM (Avramovich *et al.* 2002). Indomethacin and other COX inhibitors also enhance sβAPP secretion in neuroglioma cells (Kinouchi *et al.* 1995), astrocytes and neurons (Lee and Wurtman 2000).

Conversely, sulindac sulfide and flurbiprofen do not alter sβAPPα secretion in cell lines transfected with wild-type or mutated human βAPP (Weggen *et al*. 2001) and in primary cultures of rat cortical neurons (Gasparini *et al*. 2004). However, only NSAID concentrations above 1 μM (i.e. the descending part of the U-shaped concentration-response curve of sβAPP secretion) were analyzed in the latter studies, thus possibly explaining the apparent discrepancies with previous findings. The effects of NSAIDs on sβAPP secretion seem to be mediated by a metalloprotease similar to α-secretase via activation of protein kinase C (PKC) and Erk mitogen-activated (MAP) kinase (Avramovich *et al*. 2002). However, the mechanisms by which NSAIDs affect sβAPP secretion remain to be thoroughly explored.

It has been reported that some NSAIDs (Table 1), including sulindac, indomethacin, flurbiprofen and ibuprofen, selectively reduce Aβ42 levels in cultured cells from peripheral or glial origins, i.e. CHO, HEK293 and H4 cells, while others (e.g. naproxen or aspirin) do not (Weggen *et al*. 2001; Morihara *et al*. 2002; Eriksen *et al*. 2003a). However, this striking Aβ42 selectivity is not observed in neuronal cultures. In fact, the COX-unselective NSAIDs flurbiprofen and sulindac sulfide decrease the secretion of both Aβ40 and Aβ42 in N2a neuroblastoma cells (Takahashi *et al*. 2003) and primary cultures of rat cortical neurons (Gasparini *et al*. 2004). Only a modest selectivity of sulindac sulfide in reducing Aβ42 over Aβ40 secretion was observed in N2a cells (Takahashi *et al*. 2003; Gasparini *et al*. 2004). In contrast, no significant selectivity in lowering Aβ42 over Aβ40 was observed for flurbiprofen in N2a cells and primary neurons, as indicated by the unchanged Aβ42/Aβ40 ratio (Gasparini *et al*. 2004). The use of different cellular models, i.e. neuronal versus peripheral or glial cells, may account for the discrepancies between these findings (Takahashi *et al*. 2003; Gasparini *et al*. 2004) and previous ones (Weggen *et al*. 2001; Morihara *et al*. 2002; Eriksen *et al*. 2003a). Neuronal cells differently metabolize βAPP (Gouras *et al*. 1998) and may also have a different sensitivity to NSAIDs, especially to flurbiprofen, in comparison with peripheral and glial cells. This is also supported by recent findings by several investigators reporting different degrees of selectivity in various cell systems (Table 1).

The Aβ-lowering effects seem to be restricted only to some COX unselective NSAIDs. In fact, COX-1 (sc-560) and COX-2-selective (sc-125 and celecoxib) compounds differentially affect Aβ40 and Aβ42 secretion. Celecoxib, sc-125 and sc-560 significantly increase Aβ42 secretion, but either do not change (sc-125 and sc-560) or decrease (celecoxib) Aβ40 release from cultures of N2a cells and rat primary cortical neurons (Gasparini *et al*. 2004). Similarly, Sagi *et al*. (2003b) reported that celecoxib and COX-2 selective derivatives of indomethacin and meclofenamic acid (Kalgutkar *et al*. 2000) increase the secretion of Aβ42 while decreasing Aβ38 levels in CHO and H4 neuroglioma cells overexpressing βAPP.

NSAIDs and cerebral β-amyloid load

Selected NSAIDs have been tested in animal models that reproduce various components of the genetic and pathological features of AD. Importantly, a significant Aβ-lowering activity has been shown in mice transgenic for mutant human βAPP following long-term treatment regimen (Table 2). Lim *et al*. (1999) first reported on the effects of ibuprofen on amyloid pathology in AD transgenic mice that express the Swedish mutant form of human βAPP, i.e. tg2576 mice. Chronic administration of ibuprofen in the diet significantly decreases Aβ immunoreactive plaques, total Aβ burden and inflammatory markers, e.g. interleukin-1β levels and astrocytosis, in tg2576 mice (Lim *et al*. 1999). These findings has been recently confirmed by others (Dedeoglu *et al*. 2003; Yan *et al*. 2003). Oral administration of ibuprofen for 4 months significantly reduces the levels of SDS-soluble Aβ42 and, to a lesser extent, Aβ40 and decreases the extent of microglia activation (i.e. reduction of CD11b and CD45 expression) in tg2576 mice (Yan *et al*. 2003). Furthermore, 3-month oral administration of ibuprofen decreases the Aβ42/40 ratio in the brain of βAPP mice crossbred with presenilin-1 (PS1) knock-in mice (APPxPS1), when the treatment starts at first appearance of plaques, i.e. at 3 months of age (Dedeoglu *et al*. 2003). Importantly, the maximum reduction in cerebral insoluble Aβ requires early and chronic ibuprofen treatment. The reduction of insoluble Aβ in tg2576 mouse brains is much greater when the treatment starts prior to plaque formation (i.e. at 10 months of age) and continues up to 16 months of age, compared with a 3-month treatment which starts after the beginning of plaques formation (i.e. at 14 months of age) (Lim *et al*. 2001). A significant *in vivo* Aβ-lowering effect was also observed with nitric oxide (NO)-releasing derivatives of flurbiprofen, i.e. HCT 1026 (Table 2, T. Van Groen, personal communication) and NCX 2216 (Jantzen *et al*. 2002). In particular, NCX 2216 reduces the cerebral amyloid load to a greater extent than ibuprofen when administered to tg2576xPS1$_{M146L}$ doubly transgenic mice for 5 months in the diet, starting at 7 months of age (Jantzen *et al*. 2002). Surprisingly, the effects of NCX 2216 on Aβ load are accompanied by increased activation of microglia in the peri-plaque area. It is worth noting that microglia activation is not observed in non-transgenic mice treated with NCX 2216, suggesting that activated microglia is somehow contributing to the clearance of amyloid plaques similar to the mechanisms of Aβ removal proposed for Aβ vaccination (Bard *et al*. 2000). Other NSAIDs, i.e. indomethacin and *R*-flurbiprofen, reduce amyloid pathology in tg2576 mice (Eriksen *et al*. 2003b; Praticò *et al*. 2003). In particular, indomethacin significantly reduces soluble Aβ40 and Aβ42 peptides in the cortex and hippocampus and decreases insoluble Aβ40 and Aβ42 in the hippocampus of tg2576 mice when given chronically (from 8 to 15 months of age; Praticò *et al*. 2003). The long-term treatment with *R*-flurbiprofen significantly

Table 1 Effects of NSAIDs on Aβ peptides secretion from cultured cells

Drug	Model	Aβ38	Aβ40	Aβ42	References
Compounds decreasing Aβ42					
Diclofenac	H4	n.d.	Unchanged	↓	Eriksen *et al.* 2003a
(±) Flurbiprofen	HEK293; H4	↑	Unchanged	↓	Sagi *et al.* 2003; Eriksen *et al.* 2003a
	N2a; primary rat cortical neurons	n.d.	↓	↓	Gasparini *et al.* 2004
	Primary guinea pig neurons	↓	↓	↓	Fici *et al.* 2003
R-Flurbiprofen	HEK293	n.d.	Unchanged	↓	Morihara *et al.* 2002; Sagi *et al.* 2003a
	SHSY-5Y	n.d.	↓	↓	Wrigley *et al.* 2003; Beher *et al.* 2004
	H4	↑	Unchanged	↓	Eriksen *et al.* 2003a
S-Flurbiprofen	HEK293; H4	↑	Unchanged	↓	Sagi *et al.* 2003a; Eriksen *et al.* 2003a
Fenoprofen	H4	n.d.	Unchanged	↓	Eriksen *et al.* 2003a
Ibuprofen	CHO; HEK293; H4	n.d.	Unchanged	↓	Weggen *et al.* 2001; Morihara *et al.* 2002; Eriksen et al. 2003a
	Primary guinea pig neurons; HEK293	↑	Unchanged	↓	Fici *et al.* 2003
Indomethacin	CHO; H4	n.d	Unchanged	↓	Weggen *et al.* 2001
Meclofenamic acid	H4	n.d.	Unchanged	↓	Eriksen *et al.* 2003a
	N2a	n.d.	Unchanged	Unchanged	Gasparini *et al.* 2004
Sulindac sulfide	CHO; Hs683; H4; primary mouse embryonic fibroblasts	↑	Unchanged	↓	Weggen *et al.* 2001; Eriksen *et al.* 2003a
	N2a; primary rat cortical neurons	n.d.	↓	↓	Takahashi *et al.* 2003; Gasparini *et al.* 2004
	SHSY-5Y	n.d.	↓	↓	Wrigley *et al.* 2003; Beher *et al.* 2004
	Primary guinea pig neurons; HEK293	↑	↓	↓	Fici *et al.* 2003
Compounds not affecting Aβ42					
Acetaminophen	N2a	n.d.	Unchanged	Unchanged	Gasparini *et al.* 2004
Aspirin	H4; N2a	n.d.	Unchanged	Unchanged	Weggen *et al.* 2001; Eriksen *et al.* 2003a; Gasparini *et al.* 2004
Dapsone	H4	n.d.	Unchanged	Unchanged	Eriksen *et al.* 2003a
Diflunisal	H4	n.d.	Unchanged	Unchanged	Eriksen *et al.* 2003a
Etodolac	H4	n.d.	Unchanged	Unchanged	Eriksen *et al.* 2003a
Fenbufen	H4	n.d.	Unchanged	Unchanged	Eriksen *et al.* 2003a
Ketoprofen	H4	n.d.	Unchanged	Unchanged	Eriksen *et al.* 2003a
Ketorolac	H4	n.d.	Unchanged	Unchanged	Eriksen *et al.* 2003a
Meloxicam	H4; N2a	n.d.	Unchanged	Unchanged	Eriksen *et al.* 2003a; Gasparini *et al.* 2004
Naproxen	CHO; H4; N2a	n.d.	Unchanged	Unchanged	Weggen *et al.* 2001; Eriksen *et al.* 2003a; Takahashi *et al.* 2003; Gasparini *et al.* 2004
Nabumetone	H4	n.d.	Unchanged	Unchanged	Eriksen *et al.* 2003a
Phenylbutazone	H4	n.d.	Unchanged	Unchanged	Eriksen *et al.* 2003a
Piroxicam	H4; N2a	n.d.	Unchanged	Unchanged	Eriksen *et al.* 2003a; Gasparini *et al.* 2004
Sulindac	CHO; N2a	Unchanged	Unchanged	Unchanged	Weggen *et al.* 2001; Gasparini *et al.* 2004
Sulindac sulfone	CHO; N2a	Unchanged	Unchanged	Unchanged	Weggen *et al.* 2001; Takahashi *et al.* 2003; Gasparini *et al.* 2004
Suprofen	H4	n.d.	Unchanged	Unchanged	Eriksen *et al.* 2003a
Compounds increasing Aβ42					
Celecoxib	N2a; Primary rat cortical neurons	n.d	↓	↑	Gasparini *et al.* 2004
	H4; CHO	↓	Unchanged	↑	Sagi *et al.* 2003b
Sc-125 (COX-2 selective)	N2a; Primary rat cortical neurons	n.d	Unchanged	↑	Gasparini *et al.* 2004
Sc-560 (COX-1 selective)	N2a; Primary rat cortical neurons	n.d.	Unchanged	↑	Gasparini *et al.* 2004

Table 2 Effects of long-term administration of selected NSAIDs on cerebral Aβ load, Aβ40 and Aβ42 levels in AD transgenic mice

Drug	Model	Dose, length of treatment	Aβ load	Aβ40 levels	Aβ42 levels	Aβ42/Aβ40 ratio	References
Celecoxib	tg2576x $PS1_{M146L}$	30 mg/kg/day, 5 months	Unchanged	n.d.	n.d.	n.d.	Jantzen *et al.* 2002
	βAPPxPS1	20 mg/kg/day, 3 months	n.d.	n.d.	n.d.	Unchanged	Dedeoglu *et al.* 2003
	tg2576	50 mg/kg/day, 4 months	n.d.	↑	↑		Sagi *et al.* 2003b
Ibuprofen	tg2576	~62.5 mg/kg/day, 6 months	↓	n.d.	n.d.	n.d.	Lim *et al.* 1999
		~62.5 mg/kg/day, 4 months	↓	↓[a]	↓[a]		Yan *et al.* 2003
	tg2576x $PS1_{M146L}$	~62.5 mg/kg/day, 5 months	↓	n.d.	n.d.	n.d	Jantzen *et al.* 2002
	βAPPxPS1	~62.5 mg/kg/day, 3 months	n.d.	n.d.	n.d.	↓	Dedeoglu *et al.* 2003
(±) Flurbiprofen	$βAPP_{swe}$x $PS1_{A246E}$	7 mg/kg/day, 6 months	Unchanged	n.d.	n.d.	n.d.	T. van Groen, personal communication
R-Flurbiprofen	tg2576	10 mg/kg/day, 8 months	n.d.	↓[d]	↓[d]	n.d.	Eriksen *et al.* 2003b
HCT 1026 (a NO-releasing flurbiprofen derivative)	$βAPP_{swe}$x $PS1_{A246E}$	30 mg/kg/day, 6 months	↓	n.d.	n.d.	n.d.	T. van Groen, personal communication
Indomethacin	tg2576	2.24 mg/kg/day, 8 months	↓[b]	n.d.	n.d.	n.d	Quinn *et al.* 2003
		10 mg/kg/day, 7 months	↓	↓[c]	↓[c]	n.d.	Praticò *et al.* 2003
NCX 2216 (a NO-releasing flurbiprofen derivative)	tg2576x $PS1_{M146L}$	~62.5 mg/kg/day, 5 months	↓	n.d.	n.d.	n.d	Jantzen *et al.* 2002
Nimesulide	tg2576	10 mg/kg/day, 7 months	Unchanged	Unchanged	Unchanged	n.d.	Praticò *et al.* 2003

[a]SDS-soluble peptides; [b]in the hippocampus, but not in the cortex; [c]soluble peptides in cortex and hippocampus and insoluble ^{Aβ40} and ^{Aβ42} in the hippocampus; [d]using formic acid extraction. βAPPxPS1: βAPP mice × PS1 knock-in mice. n.d., not determined.

reduces the cerebral levels of both Aβ40 and Aβ42 peptides and improves the cognitive performance of tg2576 mice in the Morris water maze (Eriksen *et al*. 2003b).

Celecoxib and nimesulide, which are COX-2-selective NSAIDs, have also been tested in transgenic mouse models of AD (Jantzen *et al*. 2002; Dedeoglu *et al*. 2003; Praticò *et al*. 2003; Sagi *et al*. 2003b). Neither celecoxib nor nimesulide affects Aβ levels in tg2576 (Praticò *et al*. 2003), APPxPS1 (Dedeoglu *et al*. 2003) or amyloid plaques in tg2576x$PS1_{M146L}$ mice (Jantzen *et al*. 2002). Conversely, Sagi *et al*. (2003b) have recently reported that long-term administration of celecoxib (50 mg/kg/day for 4 months, starting at 7 months of age) *increases* the levels of Aβ40 and Aβ42 in tg2576 mice (Sagi *et al*. 2003b). Thus, based on present *in vitro* and *in vivo* evidence, COX-2-selective drugs do not seem to possess an activity that is relevant to the treatment of amyloid pathology. This is also supported by findings in triple transgenic mice expressing $βAPP_{swe}$, $PS1_{A246E}$ and neuronal human COX-2 (hCOX-2). The expression of hCOX-2 has only a mild and very late effect on Aβ load, indicating a marginal role for COX-2 in the development of amyloid pathology in this model (Xiang *et al*. 2002a,b).

While the effects on Aβ load following long-term treatment of transgenic mice are clear and consistent for a few NSAIDs, there is controversy as to whether the same drugs reduce Aβ peptides levels in the brain following acute or short-term treatment (Weggen *et al*. 2001; Eriksen *et al*. 2003a; Lanz *et al*. 2003; Van der Awera and Gasparini, unpublished data). It has been reported that some NSAIDs, including ibuprofen, flurbiprofen, indomethacin and sulindac, reduce the levels of Aβ42 in the brain of tg2576 mice following 3-day oral treatment (Weggen *et al*. 2001; Eriksen *et al*. 2003a). Other NSAIDs either do not alter, e.g. naproxen and aspirin, or increase, e.g. celecoxib, cerebral Aβ42 levels when given orally for 3 days (Eriksen *et al*.

2003a; Sagi *et al*. 2003b). However, other recent studies show that short-term (i.e. for 3–5 days) oral administration of flurbiprofen, sulindac or ibuprofen do not change the levels of either Aβ40 or Aβ42 in the cortex, hippocampus and CSF of AD transgenic mice (Table 3; Lanz *et al*. 2003; Van der Awera and Gasparini, unpublished data). It is still unclear whether differences in tissue extraction procedures or Aβ measurements could explain the discrepancies between different studies (Weggen *et al*. 2001; Eriksen *et al*. 2003a). This is a key issue that has important implications in the drug discovery process. In fact, short-term treatment protocols would significantly speed up proof-of-the-concept trials on new compounds; they would provide hints of the mechanisms of action of test drugs and support the use of biomarkers in clinical studies. For example, compounds acting as γ-secretase inhibitors lowers Aβ cerebral levels within hours from their administration to young tg2576 mice (Lanz *et al*. 2004), while other Aβ-lowering mechanisms would require long-term drug administration.

Potential mechanisms of NSAIDs effects on Aβ metabolism

The effects on Aβ metabolism seem unrelated to the COX inhibitory activity of NSAIDs. It has been shown that sulindac sulfide maintains the Aβ42-lowering effect in COX-1 and COX-2-deficient fibroblasts (Weggen *et al*. 2001). Conversely, naproxen, a non-selective COX inhibitor, does not alter Aβ metabolism at concentrations that produce a 90% inhibition of COX activity (Cryer and Feldman 1998; Weggen *et al*. 2001). In addition, the two COX-selective compounds sc-560 (COX-1 selective) and sc-125 (COX-2 selective) achieve the same effects on Aβ metabolism independently of their selectivity for any COX isoform (Gasparini *et al*. 2004). Lately, a splice variant of COX-1, i.e. COX-3, has been identified (Chandrasekaran *et al*. 2002). COX-3 is expressed in most CNS areas and its expression seems to be modestly reduced in AD brain (Cui *et al*. 2004). However, COX-3 is not expressed in neurons derived from rat brain (Kis *et al*. 2003), ruling out an involvement of COX-3 in the effects of NSAIDs on Aβ in cultures of rat cortical neurons (Gasparini *et al*. 2004). This is also supported by the lack of effects on Aβ by acetaminophen (Gasparini *et al*. 2004), which has been hypothesized to selectively inhibit COX-3 (Chandrasekaran *et al*. 2002).

It is well known that some of the effects of non-selective NSAIDs on cellular functions are mediated by COX-independent mechanisms. For example, it has been proposed that they may act through the activation of peroxisome proliferator-activated receptor γ (PPARγ) (Tegeder *et al*. 2001). However, it seems unlikely that this mechanism is involved in the effects of NSAIDs on Aβ in basal conditions. Naproxen and sulindac sulfide are potent PPARγ agonists (Jaradat *et al*. 2001; Wick *et al*. 2002), while flurbiprofen has little or no activating activity on PPARγ (Pang *et al*. 2003; Bernardo *et al*. in preparation). Indeed, it has been recently reported that PPARγ and other non-COX mechanisms (i.e. nuclear factor-κB, lipooxygenase and inhibitor of κB kinase) are not involved in the effects of sulindac sulfide on basal Aβ metabolism (Sagi *et al*. 2003a). However, PPARγ could mediate the effects of NSAIDs on cytokine-stimulated Aβ metabolism (Sastre *et al*. 2003). It has been reported that ibuprofen inhibits the secretion of Aβ40 and Aβ42 from SK-N-SH cells stimulated by TNFα and INFγ (Blasko *et al*. 2001). Similarly, ibuprofen and indomethacin inhibit TNFα/IFNγ-induced secretion of Aβ and sAPPα in N2a cells. These effects of NSAIDs are mimicked by the PPARγ agonist pioglitazone and are suppressed by PPARγ antagonists (Sastre *et al*. 2003), suggesting the involvement of PPARγ in modulating Aβ metabolism under inflammatory conditions.

Recently, it has been proposed that the Aβ-lowering effects of NSAIDs could be mediated by inhibition of the Ras signalling pathway (Zhou *et al*. 2003). Sulindac sulfide, a prototypic Aβ-lowering NSAID, also inhibits Ras and Ras-like GTP-binding proteins (Herrmann *et al*. 1998; Gala *et al*. 2002). Zhou *et al*. (2003) demonstrated that the small GTP-binding protein Rho and its effector, Rho-associated kinase (Rock), regulate the amount of Aβ42 produced by SHSY-5Y cells. In particular, activation of Rho by geranylgeranyl pyrophosphate increases Aβ42 secretion. In contrast,

Table 3 Effects of short-term administration of NSAIDs on cerebral Aβ load and Aβ peptides levels in AD transgenic mice

Drug	Model	Dose, length of treatment	Aβ40 levels	Aβ42 levels	References
Flurbiprofen	tg2576	50 mg/kg/day, 3 days	Unchanged	↓	Eriksen *et al*. 2003a
		25 mg/kg/day, 3 days	Unchanged	Unchanged	Lanz *et al*. 2003
	βAPP-Lo[a]	50 mg/kg/day, 5 days	Unchanged	Unchanged	Van der Awera and Gasparini, unpublished data
Ibuprofen	tg2576	50 mg/kg/day, 3 days	Unchanged	↓	Weggen *et al*. 2001; Eriksen *et al*. 2003a
		50 mg/kg/day, 3 days	Unchanged	Unchanged	Lanz *et al*. 2003
Sulindac	tg2576	50 mg/kg/day, 3 days	Unchanged	↓	Eriksen *et al*. 2003a
			Unchanged	Unchanged	Lanz *et al*. 2003

Aβ peptides were extracted in guanidinium buffer (Lanz *et al*. 2003), formic acid (Weggen *et al*. 2001; Eriksen *et al*. 2003a) or TBS (Van der Awera and Gasparini). [a]βAPP-Lo: transgenic mice expressing the London mutant βAPP in neurons (Moechars *et al*. 1999).

inhibition of Rho pathway by using Rho dominant negative constructs or the Rock inhibitor Y27632 decreases Aβ42 production. Importantly, Aβ42-lowering NSAIDs, including sulindac sulfide, ibuprofen and indomethacin, also inhibit Rho, while other NSAIDs, i.e. naproxen, meloxicam, piroxicam and sc-560 influence neither Rho nor Aβ in SHSY-5Y cells. This suggests that the Rho-Rock pathway regulates βAPP processing and may mediate the effects of a subset of NSAIDs on Aβ metabolism.

Other potential mechanisms can be hypothesized to explain the change in Aβ levels by some NSAIDs. First, NSAIDs could achieve their effects on Aβ by modulating the vesicular trafficking or the degradation of Aβ peptides. These mechanisms are not involved in the effects of sulindac sulfide on Aβ secretion (Weggen *et al*. 2001), but this has to be explored for other NSAIDs. Second, Aβ-lowering NSAIDs could reduce β- or γ-secretase activities. Yet, Aβ-lowering COX-unselective NSAIDs change neither the levels of βAPP C-terminal fragments nor Notch S3-site cleavage in cultured cells and in membrane-based γ-secretase activity assays, suggesting that such NSAIDs do not alter β- or γ-secretase overall activities (Weggen *et al*. 2001, 2003b; Gasparini *et al*. 2004). However, different results have been obtained by Takahashi *et al*. (2003). It has been demonstrated that sulindac sulfide inhibits γ-secretase by non-competitive mechanisms in detergent solubilized membranes from HeLa cells, leading to a preferential, but not exclusive, reduction of Aβ42 formation. Indeed, sulindac sulfide also inhibits Aβ40 generation and, at very high concentrations (250–500 μM), Notch cleavage. Interestingly, NSAIDs could interfere with Aβ metabolism via inhibition of β-secretase expression in cytokine-stimulated cells (Sastre *et al*. 2003). It has been shown that ibuprofen and indomethacin inhibit TNFα/IFNγ-induced secretion of Aβ and sAPPα in N2a cells and these effects are mediated by inhibition of cytokine-induced β-secretase expression and activity (Sastre *et al*. 2003). Lastly, Aβ-lowering NSAIDs could reduce Aβ secretion by shifting γ-secretase activity towards the production of short Aβ peptides; they could stimulate the activity of an exopeptidase that converts full-length Aβ (e.g. Aβ42) peptides in shorter Aβ species (e.g. Aβ40 or 38) (Weggen *et al*. 2001, 2003a) or they could act on a presenilin-independent secretase activity (Wilson *et al*. 2002). However, further investigation is warranted in order to ascertain these hypotheses in neuronal cultures.

Other COX-independent mechanisms may be involved. Reid *et al*. (2003) recently reported that some NSAIDs, including flurbiprofen, ibuprofen and indomethacin, interact with and inhibit a member of the ATP-binding cassette transporter family, namely the multidrug resistance protein-4 (MRP4). MRP4 is widely expressed in several tissues, including neuronal tissue and it transports several physiological substrates, e.g. cyclic nucleotides, steroid conjugates, folate and prostaglandins (Reid *et al*. 2003; Warner and Mitchell 2003). Some MRP4 substrates, i.e. cAMP or unconjugated estradiol, are known to regulate βAPP/Aβ metabolism (Xu *et al*. 1996; Marambaud *et al*. 1998; Xu *et al*. 1998). Thus, NSAIDs may also indirectly affect Aβ metabolism by altering MRP4 transport.

Overall, studies in *in vitro* and *in vivo* models relevant to AD pathology consistently indicate that a selected group of NSAIDs, including ibuprofen, flurbiprofen, indomethacin and sulindac, significantly inhibit amyloid formation and deposition by mechanisms still poorly understood.

Classical anti-inflammatory mechanisms of NSAIDs: could they help in AD?

COX and AD: the relevance of COX inhibition

The seminal observations by Vane (1971) first pointed out that NSAIDs suppress inflammation primarily through their ability to inhibit the COX enzyme. COX is the key regulatory enzyme of the eicosanoid biosynthetic pathway, which produces a variety of pro-inflammatory mediators. The inhibition of COX by NSAIDs would limit the production of pro-inflammatory eicosanoids at the site of injury. Different variants of COX have been described: COX-1, which is constitutively expressed in nearly all tissues and mainly has 'housekeeping' functions; COX-2, which is induced at injury sites by inflammatory stimuli and play a key role during inflammation; and the recently identified COX-3, whose functions are still unknown. Based on this general functional distinction, an effort was made to design drugs which selectively inhibit COX-2 and thereby are devoid of the adverse effects associated with the blockade of both COX-1 and COX-2, e.g. gastrointestinal lesions.

A recent attempt to apply this strategy to treat AD did not yield positive results. Patients with mild to moderate AD taking the COX-2-selective inhibitor rofecoxib did not show any cognitive improvement after 12-month treatment (Aisen *et al*. 2003; Reines *et al*. 2004). It is worth noting that epidemiological data concerning the benefits of NSAIDs in AD mainly refer to COX-unselective NSAIDs, while data on COX-2 selective compounds are still missing because of their more recent clinical use. Furthermore, biological data indicate that both COX-1 and COX-2 may contribute to inflammatory processes, particularly in the CNS and AD (Dubois *et al*. 1998; Graham and Hickey 2003; Schwab and Schluesener 2003). Increasing evidence indicates that COX-2 may also exert anti-inflammatory effects during the resolution of inflammation and has physiological functions in the CNS, e.g. in synaptic remodelling and function (Bazan 2001). Either up-regulation or down-regulation of COX-2 expression has been reported in AD brain (Lukiw and Bazan 1997; Ho *et al*. 1999, 2001; Kitamura *et al*. 1999; Yermakova and O'Banion 2001). A reduction of the COX-2 positive neurons has been observed in AD (Yermakova and

O'Banion 2001). This is consistent with the observation that the CSF levels of prostaglandin E_2 (PGE_2), which is the major product of COX-2 enzymatic activity, decrease in AD patients when the severity of dementia increases (M. Combrick and L. Minghetti, manuscript in preparation). However, elevated levels of PGE_2 have also been observed in AD hippocampus (Montine *et al*. 1999). COX-1 expression is not altered in CA3 hippocampal neurons, but more microglial cells express COX-1 in AD tissue than in controls (Yermakova *et al*. 1999). Only a few data are available on COX-2 expression in AD transgenic models. Neuronal expression of COX-2 is similar in tg2576xPS1$_{M146L}$ and wild-type mice, while its expression in glia is increased only in a few astrocytes surrounding some, but not all, Aβ deposits in tg2576xPS1$_{M146L}$ mice (Matsuoka *et al*. 2001). Thus, COX-2 seems to play only a minor role in amyloid pathology in this AD model.

Overall, based on data available so far, it would appear that the inhibition of both COX-1 and COX-2 would be desirable to achieve beneficial effects in CNS inflammation. Further investigation is still required to better understand whether COX-1 and COX-2 contribute to AD neurodegenerative and inflammatory processes and how their inhibition by NSAIDs would affect AD.

NSAIDs effects on the cellular component of neuroinflammation

Evidence from animal studies indicates that the effects of NSAIDs in AD might also be mediated by the suppression of the cellular inflammatory reaction (i.e. microglia and astrocyte activation) associated with Aβ plaques. In models of inflammation consequent to Aβ deposition or infusion, NSAIDs reduce the glial reaction. For example, indomethacin significantly reduces microglia activation surrounding the intraparenchymal Aβ deposit developing after Aβ infusion in the lateral ventricle of rats (Netland *et al*. 1998). Flurbiprofen and its NO-releasing derivatives (HCT 1026 and NCX 2216) decrease the activation of microglia and astrocytes induced by Aβ42 injection in the nucleus basalis magnocellularis (NBM) of rats (Prosperi *et al*. 2004).

NSAIDs also modulate the activation of microglia in other rat models of neuroinflammation. Rofecoxib attenuates the activation of microglia and prevents the loss of cholinergic neurons induced by the injection of the excitotoxin quisqualic acid in the rat NBM after 7-day treatment (Scali *et al*. 2003). In the same excitotoxic model, nimesulide and the NO-releasing derivative of flurbiprofen HCT 1026 significantly inhibit the microglial reaction and reduce the levels of inflammatory molecules, e.g. IL-1β and PGE_2 (Scali *et al*. 2000; Prosperi *et al*. 2001). Both HCT 1026 and NCX 2216 were also investigated on neuroinflammation induced by chronic infusion of lipopolysaccharide (LPS) into the 4th ventricle of rats, a model that reproduces many of the behavioural, neurochemical and neuropathological changes associated with AD (Hauss-Wegrzyniak *et al*. 1998, 2000, 2002). The chronic LPS infusion in rat brain produces an extensive inflammation that is particularly evident in the hippocampus, subiculum and enthorinal and piriform cortices. HCT 1026 and NCX 2216 significantly attenuate the brain inflammation as indicated by the decreased density and reactive state of microglia. Importantly, anti-inflammatory treatment attenuates LPS-induced microglia activation in young and adult rats, but not in old animals (Hauss-Wegrzyniak *et al*. 1999a). These findings suggest that, once the activation of microglia is fully established, treatment with anti-inflammatory drugs would be ineffective. This hypothesis is also supported by recent evidence obtained in the same animal model with a compound combining anti-inflammatory and antioxidant properties (Wenk *et al*. 2004), i.e. the anti-inflammatory activity is no longer present when drug administration starts 14 days after initiation of LPS infusion.

PPARγ: a molecular switch for microglia activation?

COX-independent mechanisms, particularly the activation of the nuclear receptor PPARγ, could mediate the effects of NSAIDs on microglia activation. PPARγ is expressed in cells of macrophagic lineage and its activation has anti-inflammatory effects, resulting from suppression of the production of pro-inflammatory mediators (Landreth and Heneka 2001). PPARγ is up-regulated in activated macrophages and negatively regulates the expression of inflammatory genes, including inducible NO synthase, gelatinase B and scavenger receptor A (Ricote *et al*. 1998). Moreover, PPARγ ligands inhibit the production of pro-inflammatory cytokines, i.e. IL-1β, TNFα and IL-6, in human monocytes (Jiang *et al*. 1998) and arrest the differentiation of monocytes into activated macrophages (Combs *et al*. 2000). It has been shown that some NSAIDs, including indomethacin and ibuprofen, bind and activate PPARγ in adipocytes (Lehmann *et al*. 1997), microglia and THP-1 monocytes (Combs *et al*. 2000). In particular, indomethacin and ibuprofen inhibit the Aβ-stimulated secretion of pro-inflammatory and neurotoxic products by microglia and monocytes via PPARγ, thus preventing microglial- and monocyte-mediated neurotoxicity *in vitro* (Klegeris *et al*. 1999; Combs *et al*. 2000). IL-6 and TNFα gene expression is also inhibited in THP-1 monocytic cells by indomethacin, ibuprofen and other PPARγ ligands (Combs *et al*. 2000).

As previously discussed, PPARγ is probably not involved in the effects of NSAIDs on Aβ metabolism at basal conditions. However, there is evidence that this nuclear receptor mediates the inhibitory effects of indomethacin, ibuprofen and other PPARγ agonists on Aβ secretion in neuroblastoma cells challenged by pro-inflammatory cytokines (Sastre *et al*. 2003). Still, recent findings *in vivo* do not support this hypothesis. In fact, long-term treatment with pioglitazone, which is a PPARγ agonist, does not alter Aβ

load in tg2576 mice. A poor bioavailability of pioglitazone in the brain has been claimed to explain the lack of effects on Aβ load (Yan *et al*. 2003). Further investigation is required to fully understand the role of PPARγ in neuroinflammation and amyloid clearance.

Neuroprotection and neurogenesis

Other mechanisms may contribute to the potential beneficial effects of NSAIDs in AD. For example, neuroprotective effects of NSAIDs have been suggested. It has been reported that nimesulide significantly reduces hippocampal neuronal damage after transient global ischemia in gerbils, providing long-lasting neuroprotection (Candelario-Jalil *et al*. 2002). Furthermore, an NO-releasing derivative of flurbiprofen, i.e. HCT 1026, attenuates the toxicity of inflammation upon cholinergic cells and reduces caspases 3, 8 and 9 activity in the caudate/putamen of LPS-infused rats (Wenk *et al*. 2000). Aspirin and sodium salicylate protect rat primary neuronal cultures and hippocampal slices against glutamate-induced neurotoxicity through blockade of the transcription factor nuclear factor-κB (NF-κB; Grilli *et al*. 1996). In addition, other NSAIDs, including aspirin, mefenamic acid, indomethacin and ketoprofen, protect neuronal cultures from toxicity induced by high concentrations of NO, possibly through direct scavenging of NO radicals (Asanuma *et al*. 2001).

Recent evidence indicates that inflammation is detrimental for hippocampal neurogenesis in adult brain. Inflammation induced by either LPS injection or irradiation strongly impairs basal hippocampal neurogenesis in rats (Ekdahl *et al*. 2003; Monje *et al*. 2003). Intriguingly, hippocampal neurogenesis could be restored by blocking inflammation with indomethacin (Monje *et al*. 2003) or with minocycline (Ekdahl *et al*. 2003), which is a derivative of tetracycline with anti-inflammatory properties. This indicates another potential mechanism by which NSAIDs could exert beneficial effects in neuroinflammatory diseases.

Clinical relevance of non-COX mediated effects of NSAIDs: can they be achieved at therapeutic dosage?

The COX-independent effects of NSAIDs, such as those on Aβ metabolism, are achieved at high drug concentrations *in vitro*. For example, flurbiprofen lowers Aβ at concentrations of 50 μM and above in cultured cells, whereas it inhibits COX isoforms with an IC_{50} ranging from nM to low μM (Engelhardt *et al*. 1996; Cryer and Feldman 1998). It should be pointed out that the relationship between *in vitro* inhibition of COX activity, reduced prostanoid formation and changes in prostanoid-dependent cell function *in vivo* is not necessarily linear (Patrono *et al*. 2001). For instance, significant inhibition of tromboxane synthesis in human platelets *in vivo* is obtained only by doses of aspirin which completely inhibit tromboxane production *ex vivo* (Reilly and FitzGerald 1987). This suggests that the IC_{50} for *in vitro* COX inhibition is a poor predictor of COX-dependent clinical activities by NSAIDs. It remains to be investigated whether and how COX-independent effects of NSAIDs correlate with *in vitro* COX-inhibiting concentrations.

It is worth noting that the concentrations effective on Aβ are similar to those achieved in plasma in humans after a standard therapeutic dose, i.e. C_{max} is about 60 μM after a single oral dose of flurbiprofen at 100 mg (Cryer and Feldman 1998; Nicox, internal data). In transgenic mice, ibuprofen (∼50 mg/kg/day, orally; Eriksen *et al*. 2003a) or the flurbiprofen derivative HCT 1026 (∼30 mg/kg/day, orally; Van Groen, personal communication) lower Aβ levels or deposition with a plasma exposure at steady state of about 40 μM ibuprofen (Eriksen *et al*. 2003a) and flurbiprofen (Van Groen, personal communication), respectively. Thus, studies available show that plasma exposure for certain NSAIDs is comparable between animal models of AD and humans.

A critical point to be considered is whether NSAIDs cross the blood–brain barrier and which drug level is achieved in the brain. The anti-pyretic effects of NSAIDs and the central side effects of certain compounds, e.g. indomethacin, suggest that NSAIDs get into the brain (Bannwarth *et al*. 1989). There are, however, only a few and scattered data available on the CNS levels of these drugs, possibly because they were originally developed for peripheral inflammatory diseases. In this regard, NSAIDs greatly differ for their degree of lipophilicity and, consequently, for their potential penetration into the brain with indomethacin, ibuprofen and flurbiprofen showing the highest degree of lipophilicity (Matoga *et al*. 1999; Pehrourcq *et al*. 2004). In general, brain penetration of NSAIDs is low: the levels in the CSF correspond to 1–2% of the plasma levels achieved by therapeutic doses of NSAIDs in humans and laboratory animals, for example from 0.9 to 1.5 μM for flurbiprofen and enantiomers of ibuprofen (Bannwarth *et al*. 1995; Matoga *et al*. 1999). The kinetics of brain penetration of different NSAIDs could also influence their effectiveness in CNS chronic affections. Peak levels in the CSF occur between 1 and 3 h for flurbiprofen in the rat (Matoga *et al*. 1999; Wallace *et al*. 2004) and at 3 h for ibuprofen in humans (Bannwarth *et al*. 1995) and their levels rapidly decline thereafter. Interestingly, one NO-releasing derivative of flurbiprofen, NCX 2216, shows a long-lasting suppression of prostaglandin production in the brain and CSF (Wallace *et al*. 2004). This effect may contribute to its potent inhibition of β-amyloid deposition observed in transgenic mice (Jantzen *et al*. 2002).

Accumulation of NSAIDs in specific areas of the brain or in subcellular compartments should also be considered as it may significantly impact on their activity. In particular, it has been suggested that acidic NSAIDs (e.g. diclofenac,

ibuprofen) reach fairly high and sustained levels in inflamed tissues (Brune and Neubert 2001). However, whether or how this applies to inflammation in the brain is still unknown.

Overall, the positive results emerging from cell-based assays and transgenic animals for certain NSAIDs provide conceptual support to the data from epidemiology, but still need to be translated into the effective brain concentration in patients. This is a critical point to be clarified as it will influence both the dose-window and the length of drug exposure in clinical trials designed to evaluate whether the anti-inflammatory strategy with selected NSAIDs will bring significant benefits in AD.

Clinical perspectives

NSAIDs use seems to be associated to reduced risk of developing AD and PD, slower progression and decreased severity of dementia (In't Veld *et al*. 2002; Chen *et al*. 2003; Etminan *et al*. 2003). However, the benefits of NSAIDs in AD are still a matter of debate. Post-mortem evaluation of the effects of NSAIDs administration on AD pathology leads to controversial results. The degree of amyloid plaques and NFT pathology is similar in AD brains from NSAIDs users versus non-users, though NSAIDs use significantly reduced the number of peri-plaque activated microglial cells in one study (Mackenzie and Munoz 1998) but not in another (Halliday *et al*. 2000). In addition, recent randomized, placebo-controlled 1-year clinical trials failed to detect any effect on cognition impairment by naproxen or rofecoxib administration in mild to moderate AD patients (Aisen *et al*. 2003; Reines *et al*. 2004). This failure could be due to a number of factors. These studies were performed in patients with established dementia. It may be that people with mild to moderate AD are at a stage of neuronal damage that is too advanced for clinical benefit from anti-inflammatory therapy. Initiation of NSAIDs therapy before any dementia symptoms, or at the stage of mild cognitive impairment and treatment for a period of years, may be required to achieve appreciable benefits. Epidemiological observations provide strong support for a reduction in risk of AD (as opposed to slowing of the progression of clinically apparent disease) with long-term NSAIDs use. Indeed, the Baltimore Longitudinal Study of Aging (Stewart *et al*. 1997) and the Rotterdam Study (In't Veld *et al*. 2001) suggest that NSAID treatment for at least 2 years may be necessary for beneficial effects.

A large primary prevention trial to assess the benefits of naproxen and celecoxib in the elderly has been initiated (ADAPT, Prevention of Alzheimer's and cognitive decline; http://www.2stopad.org). In this trial, several thousand cognitively normal elderly individuals will be randomly assigned to receive naproxen, celecoxib or placebo for a period of 7 years and the primary outcome measure of the trial will be the diagnosis of AD. The design of the ADAPT study has raised criticisms, mainly because of the two test drugs which have been selected. In fact, the pharmacological characteristics of the drugs (i.e. activity on Aβ metabolism) and their blood–brain penetration properties might hamper the success of this study.

Recent studies in experimental models (Weggen *et al*. 2001; Jantzen *et al*. 2002; Morihara *et al*. 2002; Eriksen *et al*. 2003a; Praticò *et al*. 2003; Gasparini *et al*. 2004) suggest that both naproxen and COX-2-selective inhibitors might not be able to reduce Aβ burden in AD brain. Conversely, there is robust experimental evidence that certain NSAIDs have Aβ-lowering activity which could be a useful pharmacological feature in AD therapy. Lately, flurbiprofen has been proposed as a candidate drug for the treatment of AD. To avoid the gastrointestinal side effects which limit its chronic use, two different strategies have been identified. One is the use of the *R* enantiomer of flurbiprofen which maintains the Aβ-lowering properties of the racemate, but does not cause gastric damage due to the lack of COX-inhibitory activity (Morihara *et al*. 2002). The other strategy is based on the use of NO-releasing derivatives of flurbiprofen (i.e. HCT 1026 and NCX 2216) which have been shown to reduce brain inflammation (Hauss-Wegrzyniak *et al*. 1999a,b; Wenk *et al*. 2000; Prosperi *et al*. 2001; Rosi *et al*. 2003; Prosperi *et al*. 2004) and Aβ burden (Jantzen *et al*. 2002; Van Groen, personal communication) without producing gastrointestinal damage (Bertrand *et al*. 1998; Wallace *et al*. 2004). Clinical investigations will clarify whether these strategies will lead to innovative and effective treatment for AD.

Concluding remarks

Current treatments for AD have only moderate symptomatic effects on the disease, but do not modify the progression of dementia. The inflammatory process represents an interesting target for potential disease-modifying drugs that may influence several important steps of AD pathogenesis, including glial activation and Aβ cerebral load. Among the mechanisms of action of NSAIDs, it is still unknown which one would be the most effective to ameliorate and possibly cure AD, or whether the combination of anti-inflammatory and anti-amyloidogenic properties is required to reach a satisfactory therapeutic effect. Ongoing clinical trials will possibly provide clues on the effectiveness of certain NSAIDs in the prevention or treatment of AD. However, while awaiting the results from these studies, the increased insight on the potential mechanisms of action of NSAIDs has already open new avenues in the drug discovery process. For example, the identification of compounds which lower the amyloid burden without affecting other important physiological pathways (e.g. Notch cleavage) might represent an unequalled start in the discovery of new chemical entities for the treatment of neurodegenerative disorders. Although this is a long-term and challenging process, it might ultimately lead to the identification of disease-modifying drugs for the treatment of AD and other neurodegenerative disorders.

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