MECHANISM OF ACTION OF STEROIDAL ANTI-INFLAMMATORY DRUGS IN ALZHEIMER’S DISEASE: ROLE OF RHOGTASES

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1. Summary of the project

Numerous epidemiological studies have established that treatment with non-steroidal anti-inflammatory drugs (NSAIDs) reduces the risk for Alzheimer’s disease, but the mechanism of action remains controversial. The general hypothesis of this project was that the Rho GTPase family that controls cytoskeleton dynamics is a molecular target of NSAIDs in brain, and that NSAIDs provide protection in Alzheimer’s disease via Rho-GTPases by reducing chronic inflammation and improving synaptic plasticity. The project presented as novel ideas that alterations of the actin cytoskeleton play a role in neurodegenerative diseases, and that NSAIDs can regulate synaptic plasticity, a role independent of their cyclooxygenase-mediated anti-inflammatory effects. The study was the coordinated effort of three groups to analyze the role Rho-GTPases in NSAID-mediated protection. Subproject 1 aimed at characterizing the interplay between NSAIDs and Rho-GTPases (Rho, Rac, Cdc42) in the inflammatory reactivity of astrocytes and microglia in cell and organotypic cultures Rho-GTPase activities were modulated either pharmacologically or molecularly, by infection with adenoviruses containing “double negative” or “constitutively active” forms of Rho GTPases. The key finding obtained in this subproject is that widely used NSAIDs like ibuprofen, and novel profen derivatives like R-flurbiprofen and CHF5074, altered cytoskeleton dynamics in astrocytes, and that the intracellular signalling involved RhoA, Cdc42, PAK and ERK. In Subproject 2 we examined whether the NSAID ibuprofen modulates LTP (a functional expression of synaptic plasticity) in hippocampal slices. The key finding is that ibuprofen restores the impairment of LTP induced by amyloid beta (A) in hippocampus by activating the cyclic GMP-dependent phosphorylation of AMPA receptors at synapses. Subproject 3 aimed at complementing the analysis of synaptic plasticity by looking at neuronal remodelling in culture, and by characterizing Rho GTPase expression in AD brains and in mice over-expressing the Aβ precursor protein (APP). We have found that RhoA is primarily expressed in axon terminals from glutamatergic neurons, and that this expression is severely altered in brains from animal models and in human brains, where RhoA appeared sequestered by neurofibrillary tangles. Overall, the project confirms the initial hypothesis that molecular targets exist for NSAIDs aside from cyclooxygenases, and extends such hypothesis to indicate different actions and underlying signalling pathways in astrocytes and neurons. In the former, NSAIDs may regulate cell polarity and
directed movement, whereas in neurons the drugs may control activity-dependent plasticity. Why is all this relevant? According to a combined analysis of all epidemiological data NSAIDs afford an average protection of 58%. If this figure is translated into patient numbers, NSAIDs confirm their standing as a preventive treatment worth considering, encompassing novel multifunctional and multicellular mechanisms as modus operandi.

2. Results

✓ Secretease-independent and Rho GTPase/PAK/ERK-dependent regulation of cytoskeleton dynamics in astrocytes by NSAIDs and derivatives

**Rationale:** Profens like ibuprofen, R-flurbiprofen and CHF5074 are being considered for the treatment of Alzheimer’s disease because epidemiological data indicate that non-steroidal anti-inflammatory drugs are protective against neurodegeneration. Rho GTPases are small G proteins, including RhoA, Cdc42, and Rac1, which control cytoskeleton dynamics. Because ibuprofen promotes axon growth via RhoA in neurons we examined whether profens modulate astrocyte plasticity via Rho GTPases by looking at morphological changes (i.e. stellation) and motion in a scratch-wound injury, in astrocyte primary cultures.

**Results:** We report that ibuprofen (100-500 µM), R-flurbiprofen (100-500 µM), and CHF5074 (10-30 µM) caused a concentration-dependent stellation of astrocytes in primary cultures, associated with the reorganization of GFAP and actin filaments (Fig. 1). The stellation was independent of COX2, or secretases as judged by the lack of effect of inhibitors of these enzymes. RhoA, PAK, and Cdc42, but not Rac1, accounted for the profen-mediated stellation, as concluded from the joint analyses of activities and reversal experiments with adenoviral or pharmacological manipulations. Ibuprofen accelerated migration in a scratch wound assay while R-flurbiprofen had no effect and CHF5074 caused deceleration. Cell polarity regulation by Cdc42 and ERK1/2 may underlie the paradoxical effects of profens on migration.
Conclusions:

- Ibuprofen and ibuprofen derivatives that are actively investigated in Alzheimer's disease therapeutics regulate cytoskeleton dynamics in astrocytes in a secretase-independent manner, COX, PPAR, and dependent on Rho GTPases, PAK, and ERK.

- While all profens induced stellation, different actions were observed in migration: while ibuprofen (and other standard NSAIDs such as naproxen or indomethacin) accelerated migration, the novel profen CHF5074 disturbed polarity concomitantly to reduced ERK activation.

- A schematic representation of possible targets of profens in stellation and migration is shown in Fig. 2 (A and B). In summary, for stellation we posit that profens induce cytoskeleton disarrangement by concerted actions on RhoA, Cdc42 and PAK, all of which converge into inhibiting MLCK. A key idea in migration is that differences among profens may result from the extent of ERK1/2 activation acting on compartmentalized Rho GTPase signalling governing polarity and motion.
Since migration is a hallmark of astrocyte response during inflammation we propose that in addition to (or instead of) lowering Aβ42 via secretases, some NSAIDs may prevent Alzheimer’s disease by modulating astrocyte reactivity through Rho GTPase/PAK/ERK-dependent signalling. Pending in vivo evaluation, the data cast doubts on the novel derivative CHF5074, already in phase 2, which appears to impair migratory abilities of astrocytes.

*Ibuprofen restores the impairment in long term potentiation caused by amyloid beta by targeting cyclic GMP-dependent pathways.*

**Rationale:** Aβ42 rapidly impairs LTP and cognitive function in rats. The aims of this study were to assess whether ibuprofen prevents and/or rescues amyloid-induced LTP impairments in hippocampal slices and to analyze the role of the NO/cyclic GMP-protein kinase G pathways, which previous studies from Dr Felipo’s group implicated in LTP, as well as AMPA receptor phosphorylations as a novel mechanism associated to LTP.

**Results:** Amyloid impairs tetanus-induced activation of guanylate cyclase and cyclic GMP increase, preventing protein kinase G activation, phosphorylation of GluR1 in Ser845 and AMPA receptors translocation to synaptic membranes, which is responsible for LTP impairment by amyloid. Ibuprofen prevents the LTP impairment by Aβ (Fig. 3) by restoring guanylate cyclase activation, the increase in cyclic GMP and, subsequently, activation of protein kinase G, phosphorylation of GluR1 in Ser845 and synaptic expression of AMPA receptors. The cyclic GMP analogue 8BrcGMP mimicked the effect of ibuprofen, and restored LTP in the presence of amyloid. Measurements of RhoA activation by assessing translocation to membrane revealed late changes, indicating that RhoA
implication, if any, occurred at the maintenance rather than the onset of the restorative effects.

**Conclusions:**

- AMPA phosphorylation is a downstream target of NO/cyclic GMP-dependent pathways stimulating LTP.
- Altered phosphorylation of AMPA receptors underlies the damage in LTP caused by amyloid beta consistent with general impairment of cyclic GMP pathways.
- Ibuprofen reverses the effect by targeting cyclic GMP-dependent signalling. The exact mechanism of action appears to be very upstream since exposure to the NSAID restores amyloid-produced changes at all measured steps, from protein kinase G activation to AMPA phosphorylation.

![Fig. 3. Ibuprofen restores the decrease in LTP caused by amyloid beta](image-url)

**✓ Altered RhoA distribution in Alzheimer’s disease**

**Rationale:** Rho GTPases control cytoskeleton dynamics thereby modulating synaptic plasticity. Because Alzheimer’s disease is characterized by synaptic dysfunction, we sought to determine whether the expression, activity, or localization of the GTPases RhoA, Rac1 and Cdc42, as well as p21-PAK, a downstream target of Rac1/Cdc42, were altered in 18-month-old AbetaPP Tg2576 mice (Swedish mutation) or in brains from patients with Alzheimer’s disease. For comparison, the analysis was extended to brains with Pick’s disease, a neurodegenerative disorder characterized by hyper-phosphorylated tau accumulation. Technical approaches were single immunohistochemistry, double-labelling immunofluorescence and confocal microscopy, electron microscopy and...
western blot analysis of cytosolic and membrane fractions to assess protein translocation as an indication of changes in GTPase activity.

**Observations:** Immunohistochemical analyses with subcellular markers revealed a distinct localization of each Rho GTPase. RhoA was detected in glutamatergic axon terminals and the shafts of dendrites of pyramidal neurons. Rac 1 was mostly present in astrocytes, while Cdc42 appeared localized in synapses highly concentrated on neuronal bodies in cortex and hippocampus. The association of RhoA with synapses and dendritic microtubules was confirmed by electron microscopy. In A PP mice, RhoA expression decreased in synapses and increased in dystrophic neurites, suggesting altered subcellular targeting of RhoA. In postmortem brains with Alzheimer’s disease, RhoA immunostaining decreased in the neuropil and markedly increased in neurons, co-localizing with hyperphosphorylated tau inclusions, as though RhoA were sequestered by neurofibrillary tangles (**Fig. 4**). Additionally, total RhoA protein was lower in the brain hippocampus from Alzheimer brains, reflecting loss of the membrane bound, presumably active, GTPase. RhoA colocalized with hyperphosphorylated tau in Pick’s disease, again suggesting that altered subcellular targeting of RhoA is related to neurodegeneration. No major immunohistochemical changes were observed for Rac1, Cdc42, or p21-PAK.

**Conclusions:**
- The study provides new information about the distinct cellular and intracellular distribution of Rho, Rac1, and Cdc42. Despite the overwhelming evidence that Rho GTPases regulate synaptic plasticity in vitro, no studies have addressed cellular localization in vivo. Presence of RhoA in postsynaptic elements is in accordance with the prevailing view...
that RhoA promotes spine stability. By contrast, presence of RhoA in presynaptic excitatory terminals supports the novel notion that RhoA may regulate glutamate release in the mammal brain. Also novel is the localization of RhoA in dendrites, associated with microtubules, as confirmed by electron microscopy. Overall, it appears that RhoA-dependent signalling is a negative regulator of microtubule polymerization, consistent with the well documented capacity of RhoA to abort axon growth. Finally, RhoA was found in nuclear bodies and mitochondrion membranes by electron microscopy, suggesting cytoskeleton-independent roles of the small GTPase in brain. By contrast, Rac1 or Cdc42 did not colocalize with markers of axon terminals. Rac1 was ubiquitously spread in a fine grained pattern in astrocytes as though performing a general role in these cells, perhaps related to control of oxidative stress. Cdc42 location, finally, suggested control of inhibitory synapses.

- The combined evidence from mouse and human brains prompts the conclusion that RhoA shifts from synapses to microtubules thereby supporting the notion that altered subcellular targeting of RhoA is associated to Alzheimer pathology.

3. Relevance and possible clinical applicability of the final results

- A very worrisome fact in Alzheimer’s disease is that all clinical trials (up to 20) with disease-modifying drugs have failed in the past years. The view is emerging that the reason for this failure is that drugs were administered too late, when brain damage is well advanced and perhaps irreversible. Thus, a consensus is forming that Alzheimer’s disease progresses in a clinically silent manner for many years (some authors indicate as many as 20 years) before dementia is manifest. This forces the view that preventive treatments should be applied to people on the path to developing Alzheimer’s disease.

- According to epidemiological data and a recent primary prevention trial (ADAPT) NSAIDs are highly protective in Alzheimer’s disease. Indeed, they are the only treatment that has shown any preventive benefit. Attention should thus be paid to NSAIDs with two courses of action: 1) more primary prevention trials should be carried out to provide support to the
conclusions of epidemiological data; and 2) the mechanism of action of NSAID needs to be unveiled, since cumulative evidence indicates that the canonical target of NSAID, cyclooxygenase, is not involved.

✓ The project funded by the Marató-TV3 Foundation has advanced knowledge concerning the use of NSAIDs for Alzheimer’s disease therapeutics by revealing novel molecular and functional targets. Overall, the results indicate that NSAID may modulate astrocyte migration and synaptic plasticity, and that signalling involves Rho GTPases and/or cyclic GMP-dependent pathways.

✓ Of note, some of the aforementioned concepts and results have been confirmed by other laboratories, or by parallel research by the Marató-TV3 groups

  o Evidence that NSAIDs may promote axon regeneration via RhoA (Fu et al., J Neuroscience 27:4154-4164, 2008) is consistent with our observations that RhoA is physically associated with microtubules and, moreover, that association to neurofibrillary tangles is a hallmark of Alzheimer’s disease.

  o Evidence that ibuprofen reverses LTP (Kotilinek et al., Brain 131, 651-664, 2008) is thoroughly consistent with our observations, which extend the mechanism to define the implication of cyclic GMP.


  o Dr. Felipo’s group has shown the therapeutic benefits of ibuprofen in hepatic encephalopathy (Cauli et al., Hepatology. 200746:514-9, 2007).
4. Publications


Monfort P, Felipo V. *Amyloid-β impairs, and ibuprofen restores, the cGMP pathway, synaptic expression of AMPA receptors and long-term potentiation in the hippocampus.* J Alzheimers Dis. 22:795-809, 2010. IF 3,83 5-year IF 4.81
