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Annonacin, a Natural Complex I Inhibitor of the Mitochondrial Respiratory Chain, causes Tau Pathology in Cultured Neurons.

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B) Abstract……...……..

Escobar Khondiker M, Michel PP, Muriel MP, Champy P, Lannuzel A, Oertel WH, Hirsch EC, Ruberg M, Hoglinger GU. Annonacin, a natural complex I inhibitor, causes tau pathology in vitro. Fens abstr., vol.3, a200.10, 2006

C) Lebenslauf……

D) Acknowledgements……..

E) Akademische lehrer…….

F) Ehrenwörtliche erklärung……..

O. ABSTRACT.

Zusammenfassung in deutscher Sprache

Unter dem Begriff Tauopathien fasst man eine Gruppe chronisch progredienter neurodegenerativer Erkrankungen zusammen, die durch abnorme Akkumulation von hyperphosphoryliertem Tau Protein im Perykarion neuraler Zellen gekennzeichnet sind. Tau gehört zur Familie der axonalen Mikrotubuli-assoziierten Proteine. Die physiologische Funktion von Tau ist die Stabilisierung von Mikrotubuli und die Regulation axonaler Transportvorgänge. Eine neurodegenerative Tauopathie, die auf der karibischen Insel Guadeloupe endemisch ist, wurde epidemiologisch mit dem Konsum von *Annonaceae*-Pflanzen assoziiert. Diese enthalten Annonacin, den prototypischen Vertreter der Substanzklasse der Acetogenine, einer Gruppe von lipophilen Inhibitoren von Komplex I der Atmungskette. Vorausgehende experimentelle Arbeiten unserer Arbeitsgruppe konnten folgendes zeigen: 1) Chronische systemische Behandlung von Ratten mit Annonacin führt zu Nervenzellverlust im Gehirn in einem Verteilungsmuster, wie es bei den Patienten auf Guadeloupe in postmortem Untersuchungen gefunden wurden. 2) Annonacin führt durch Energie-Verarmung in Konzentrations-abhängiger Weise zu Zelltod in mesenzephalen Kulturen. Zur weiterführenden Überprüfung der Hypothese, dass Annonacin kausal an der Ätiologie der Erkrankung auf Guaedloupe beteiligt ist, untersuchten wir, ob Annonacin die Phosphorylierung und die intrazelluläre Verteilung des Tau Proteins beeinflusst.

Wir fanden, dass eine 48-stündige Behandlung von primären Nervenzellen aus dem Striatum embryonaler Ratten *in vitro* mit Annonacin zu einer Konzentrations-abhängigen Umverteilung von Tau aus dem Axon in das Perykarion führt. Das umverteilte Tau war an mehreren Epitopen phosphoryliert, wie durch Phospho-spezifische Antikörper nachgewiesen wurde. Der primäre molekulare Wirkmechanismus von Annonacin, die Inhibition von Komplex I, hat zwei primäre intrazelluläre Konsequenzen, nämlich erstens eine Zunahme von Sauerstoffradikalen und zweitens eine Abnahme der ATP-Konzentration. Obwohl Radikal-Fänger die Annonacin-induzierten Sauerstoffradikale neutralisieren konnten, verhinderten sie nicht die Redistribution von Tau. Dies wurde aber wohl verhindert durch eine Stimulation der

ATP-Produktion via anaerobe Glycolyse. Diese Beobachtungen legen nahe, dass die Annonacin-induzierte Umverteilung von phosphoryliertem Tau nicht aus oxydativem Stress, sondern aus Energie-Verarmung resultiert. Diese Interpretation wurde unterstützt durch die Beobachtung, dass andere zu Energie-Verarmung führende Neurotoxine (MPP^+, 3-NP) ebenfalls zu intrazellulärer Umverteilung von phosphoryliertem Tau führten. Eine Elektronen-mikroskopische Analyse zeigte, dass Annonacin auch zu einer Akkumulation von Mitochondrien im Perykarion von kultivierten Nervenzellen führt. Ca. 30% des phosphorylierten Tau im Zytoplasma erschien Elektronen-mikroskopisch an die äußere Membran von Mitochondrien gebunden. Video-Mikroskopie von lebenden Nervenzellen zeigte, dass Annonacin rasch zu einem umfassenden retrograden Transport von Mitochondrien aus den Neuriten in das Perykarion führte. Wir schlussfolgerten, dass die Annonacin-induzierte Umverteilung von Tau und Mitochondrien funktionell verknüpft sind, da Taxol, eine Substanz, die Microtubuli stabilisiert und Tau von Mikrotubuli verdrängt, sowohl den retrograden Transport von Mitochondrien als auch die Akkumulation von phosphoryliertem Tau im Perykarion vollständige blockierte. Schließlich fanden wir, dass Annonacin infolge der Tau-Umverteilung zu einer Tau-Verarmung in den Axonen und konsekutiv zu einer ultrastrukturell darstellbaren Destabilisierung von Mikrotubuli führte. Daher scheint Annonacin zu einer Akkumulation von phosphoryliertem Tau im neuronalen Perykarion durch retrograden axonalen Transport und zu konsekutiver axonaler Schädigung zu führen.

In ihrer Gesamtheit legen diese Untersuchungen nahe, dass Annonacin in der Lage ist, Veränderungen im Phosphorylierung-Zustand und in der intrazellularen Verteilung des Mikrotubuli-assoziierten Proteins Tau in einer Weise zu verändern, wie sie charakteristisch für humane Erkrankungen vom Typ der Tauopathien ist. Daher untermauern diese Ergebnisse weiter die Hypothese, dass regelmäßiger Konsum von *Annonaceae* Pflanzen in der Tat an der Ätiologie der Tauopathie auf Guadeloupe beteiligt sein könnte.

Summary in English language.

Tauopathy is the name given to a group of chronic progressive neurodegenerative disorders that share a common defining denominator, the accumulation of abnormally phosphorylated tau protein in the cytoplasm of neural cells. Tau belongs to the microtubule-associated protein family and is implicated in the stabilisation of microtubules and regulation of axonal transport. A particular tauopathy endemic to the Caribbean island of Guadeloupe has been associated with the consumption of *Annonaceae* plants that contain annonacin, the most abundant acetogenin, a family of potent lypophilic inhibitors of complex I of the mitochondrial respiratory chain. Previous experimental work has demonstrated the following: 1) Chronic systemic exposure of rats to annonacin induces neuronal cell loss in the brain in a similar pattern to what is observed in the brains of the Guadeloupean patients that have come to autopsy. 2) Annonacin causes concentration-dependent cell death in mesencephalic cultures by depleting cellular energy. To test the hypothesis that annonacin contributes to the aetiology of the human disease, we investigated, *in vitro*, whether if annonacin affects the phosphorylation state and the cellular distribution of the tau protein and the underlying mechanisms.

We found that in primary cultures of rat striatal neurons treated for 48 hours with annonacin, there was concentration-dependent redistribution of tau from the axons to the cell body. The redistributed tau was phosphorylated on several epitopes, as evidenced by phospho-specific antibodies. Complex I inhibition, the molecular mechanism of action of annonacin, has two major primary consequences, 1) an increase in oxidative stress and 2) a decrease in ATP levels. Although radical-scavengers (NAC, Trolox) neutralized the annonacin-induced radical oxygen species, they did not prevent the redistribution of tau. In contrast, stimulation of energy production via anaerobic glycolysis did, suggesting that the somatic redistribution of phosphorylated tau resulted from annonacin-induced energy depletion rather than from oxidative stress. This concept was strengthened by the observation that other energy-depleting neurotoxins (MPP^+, 3-NP) also induced somatic accumulation of phosphorylated tau. An electron-microscopic analysis demonstrated there was also a significant accumulation of mitochondria in the cytoplasm of annonacin-intoxicated neurons. About 30% of the

phopshorylated tau in the cytoplasm appeared ultrastructurally attached to the outer mitochondrial membrane. Videomicroscopy of living cells demonstrated that annonacin rapidly induced a comprehensive retrograde transport of mitochondria from the neurites to the neuronal soma. We concluded that the annonacin-induced somatic accumulation of tau and mitochondria were functionally linked, since we observed that taxol, a drug that stabilises microtubules and displaces tau, fully blocked the retrograde transport of mitochondria and somatic accumulation of phosphorylated tau. Finally, we found that annonacin led to a depletion of tau protein in axons, which in turn lead to the breakdown of microtubules, as evidenced ultrastructurally. Thus, annonacin appears to induce somatic accumulation of phosphorylated tau by retrograde axonal transport and to impair the axonal integrity.

Together, these data suggest that annonacin is capable of inducing changes in the phosphorylation state and intracellular distribution of the microtubule-associated protein tau in a way that has been observed in the human diseases. Therefore, these observations strengthen the hypothesis that regular consumption of A*nnonaceous* plants might be implicated in the aetiology of the Guadeloupean tauopathy.

I. INTRODUCTION.

In Europe and North America, idiopathic Parkinson's Disease is the main cause of parkinsonism while atypical parkinsonian syndromes represent only 20-30% (De Rijk *et al.,* 1997). Between 1995 and 2002, Caparros-Lefebvre, neurologist at the University Hospital Neurology Clinic (CHU) in Pointe-à-Pitre, Guadeloupe (a Caribbean island in the French West Indies), observed an abnormally high frequency (75%) of atypical forms of parkinsonism unresponsive to L-Dopa treatments, which are neuropathologically and biochemically defined as a tauopathy. Epidemiological surveys included in these studies (Caparros-Lefebvre *et al.,* 1999; Lannuzel *et al.,* Brain, in press) showed that atypical parkinsonian syndromes were predominant in patients who reported regular consumption of herbal tea and fruits of the *Annonaceae* family. In Guadeloupe, *Annona muricata* (also known as soursop, graviola, or corossol), is the most common plant belonging to the *Annonaceae* family and is known to contain neurotoxins: alkaloids and acetogenins, annonacin being the most abundant of the latter and a potent complex I inhibitor of the mitochondrial respiratory chain.

Since then, evidence that strengthens the hypothesis that consumption of these fruits is linked to the high prevalence of atypical parkinsonism in Guadeloupe increases every year. In 2003, Lannuzel and colleagues, showed that annonacin is toxic to mesencephalic neurons *in vitro* by impairment of energy metabolism. In 2004, Champy *et al.,* induced neurodegeneration of the *substantia nigra* and in the striatum *in vivo* with annonacin, Lannuzel *et al.,* (in press) defined the clinical phenotype of the atypical parkinsonism in the island. The aim of this thesis is to know if the neurotoxin annonacin contained in *Annona muricata* is capable of inducing, *in vitro,* a tauopathy like the one described in the patients in Guadeloupe.

A) TAU.

Tau is a protein belonging to the family of the microtubule associated proteins (MAP), described for the first time in 1975 by Weingarten and collaborators. In humans it is found mostly in axons (Binder *et al.,* 1985) where it interacts with tubulin, promotes their assembly and stabilises microtubules (Cleveland *et al.,* 1977). This major function of tau is controlled by two ways: by expression of different isoforms of tau and by the phosphorylation state of the protein.

1. TAU GENETICS.

Tau gene was localised to chromosome 17 by hybridization of a cDNA clone to flow-sorted and spot-blotted chromosomes (Neve *et al.,* 1986). By in situ hybridization it was mapped to the long arm of chromosome 17, 17q21 (Donlon *et al.,* 1987). The tau gene consists of 16 exons (Neve *et al.,* 1986). In the human central nervous system (CNS), 6 isoforms are generated by alternative splicing of the mRNA involving 11 of the 16 exons of tau (Himmler *et al.,* 1989; Kosik *et al.,* 1989; Goedert *et al.,* 1989). These six tau isoforms range from 352 to 441 amino acids in length depending on the splicing of exons 2, 3 and 10. Alternative splicing of exons 2 and 3 results in isoforms without, with one or two 29 amino acid inserts of unknown function in its N-terminal (called 0N, 1N, or 2N tau). Exon 10 encodes for a 31-amino repeat located in the C terminus which gives rise to 3 tau isoforms with 4 repeats each or 3 isoforms with 3 repeats each if exon 10 is spliced out (called 4R or 3R tau). In early developmental stages only the shortest isoform (exons 2, 3 and 10 spliced out) is found whereas all six isoforms are seen in adult brain (Kosik *et al.,* 1989; Goedert and Jakes, 1990).

The C-terminal repeat regions (R1-R4) that consist of 31–32 amino acids each are encoded by exons 9–12 (Gustke *et al.,* 1994; Trinczek *et al.,* 1995) and, together with some adjacent sequences, constitute the microtubule-binding domains (Lee *et al.,* 1989; Goode and Feinstein, 1994; Panda *et al.,* 1995; Hutton *et al.,* 1998). Goedert and Jakes (1990) described that, in normal adult human brain, 4R tau has more affinity for

the microtubules than 3R tau, that the ratio 3R: 4R tau isoforms is about one and that the 1N, 0N, and 2N tau isoforms comprise about 54%, 37%, and 9%, respectively, of total tau.

The six isoforms of tau are summarised in Fig. I. 1., a schematic representation taken from Buée *et al.*, 2000.

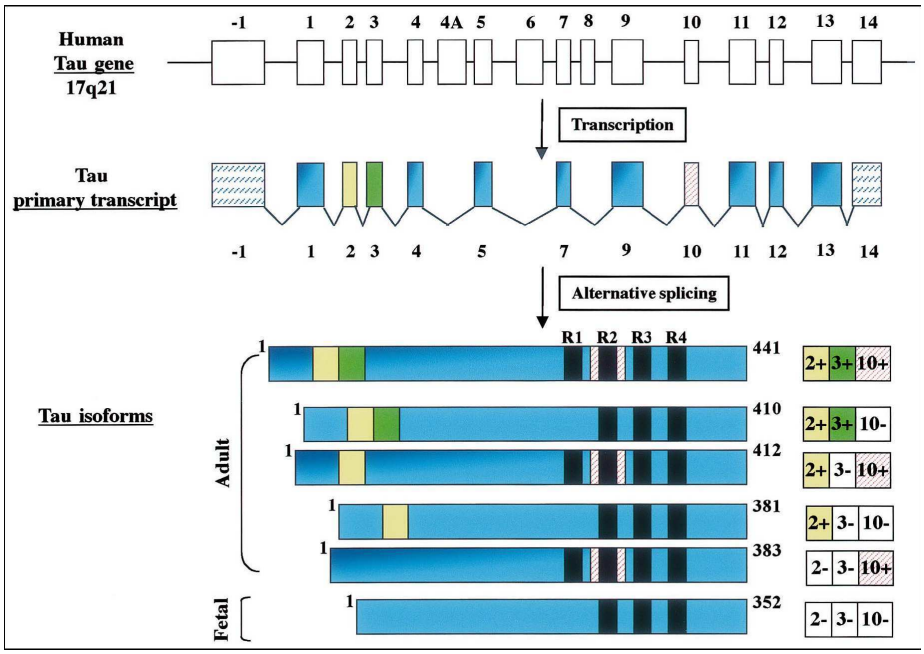


Fig. I. 1. Representation of the human tau gene, the human tau primary transcript and the six human CNS tau isoforms. The human tau gene is located over 100kb on the long arm of chromosome 17 at position 17q21. It contains 16 exons, the primary transcript contains 13 exons, since exons 4A, 6 and 8 are not transcribed in human (middle panel). Exons 1, 4, 5, 7, 9, 11, 12, 13 are constitutive, and exons 2, 3, and 10 are alternatively spliced, giving rise to six different mRNAs, translated in six different CNS tau isoforms (lower panel), which differ in absence or presence of one or two 29 amino acid inserts, encoded by exon 2 (yellow box) and 3 (green box) in the amino-terminal, in combination with either three (R1, R3 and R4) or four (R1–R4) repeat-regions (black boxes) in the carboxy-terminal. The fourth microtubule-binding domain is encoded by exon 10 (slashed box). The six isoforms consist of 441-amino acids (2+,3+,10+), 410-amino acids (2+,3+,10-), 412-amino acids (2+,3-,10+), 381-amino acids (2+,3-,10-), or 383-amino acids (2-,3-,10+). The shortest, 352-amino acids isoform (2-,3-,10-) is found only in the fetal brain, and thus referred to as fetal tau isoform **Taken from Buée *et al.*, 2000.**

2. POST-TRANSLATIONAL MODIFICATIONS.

Tau undergoes post-translational modifications like ubiquitination, polyamination, nitration, proteolysis, glycation (or non-enzymatic glycosylation) and phosphorylation.

Ubiquitination normally labels misfolded or damaged protein to undergo degradation via the ubiquitin-proteasome system. Tau contained in PHF (Paired Helical Filaments) has been described to be tagged for degradation by polyubiquitinylation (Bancher *et al.*, 1991; Iqbal and Grundke-Iqbal, 1991; Morishima and Ihara, 1994), or labelled by monoubiquitinylation (Morishima-Kawashima *et al.*, 1993), a modification which seems to modulate the location and activity of the protein rather than being a degradation target signal (Hicke, 2001).

Polyamination is the incorporation of polyamines to a protein such as tau. It has been reported by Tucholski *et al.*, (1999) that transglutaminase can incorporate these polyamines into tau. This post-translational modification does not affect tau's binding to microtubules, but renders the protein less susceptible to degradation by calpain (Murthy *et al.*, 1998; Tucholski *et al.*, 1999). In Alzheimer's disease (AD), tau has been shown to be **nitrated** (Horiguchi *et al.*, 2003).

Glycosylation is the addition of oligosaccharides to the side chain of polypeptides by various enzymes. Depending of the nature of the glycosidic bond, this can be an O-glycosylation [an O-linked N-acetylglucosamine (O-GluNAc) residue on Ser (Serine) or Thr (Threonine) in the proximity of a Pro (Proline) residue (Haltiwanger *et al.*, 1992)], or N-glycosylation [the sugar is linked to the amine group of Asp (Asparagine)]. Wang *et al.*, (1996) discovered the glycosylation of tau and described the necessity to deglycosylate and dephosphorylate PHF to restore the microtubule polymerisation activity of tau, suggesting that abnormal phosphorylation might promote aggregation of tau and inhibition of the assembly of microtubules, while glycosylation appears to be responsible for the maintenance of the PHF structure.

Phosphorylation. The addition of a phosphate group (PO_4) to a protein is done by kinases, whereas phosphatases are responsible for dephosphorylation. This modification of the tau protein will be dealt with in detail in the following paragraph.

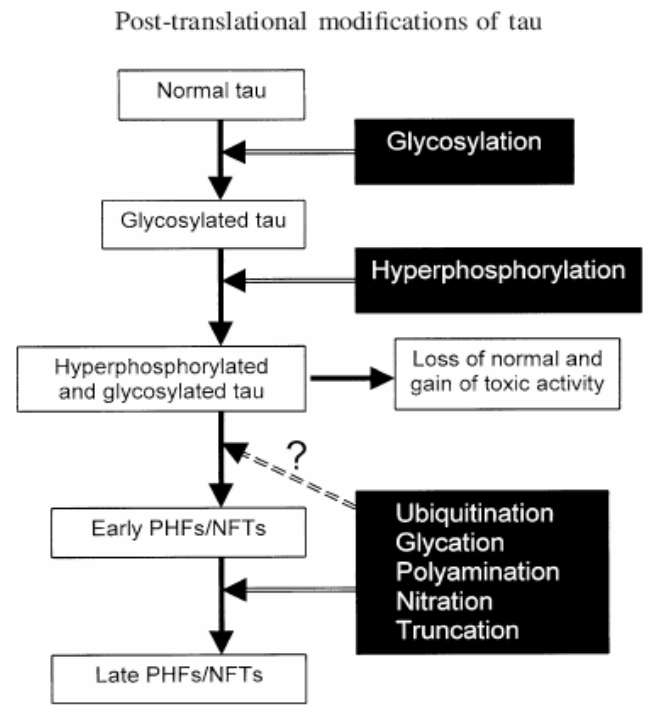


Fig I. 2. Post-translational modifications of tau. Some modifications of tau, such as glycosylation and phosphorylation, take place in the early stages of tau pathology. Hyperphosphorylated tau, on one hand, loses its normal activity to stimulate microtubule assembly and becomes toxic to the cell. On the other hand, hyperphosphorylation promotes tau's self-assembly into paired helical filaments (PHFs). PHF tau is further modified by ubiquitination, glycation, polyamination, nitration, and proteolytic truncation. (**Taken from Gong *et al.*, 2005**)

3. TAU PHOSPHORYLATION.

Tau is a phosphoprotein that normally contains 2–3 moles of phosphates per mole of tau (Ksiezak-Reding *et al.*, 1992; Köpke *et al.*, 1993; Kenessey and Yen, 1993). The phosphorylation level of tau isolated from autopsied AD brains are 3- to 4- folds higher than the normal human brains, this abnormal hyperphosphorylated tau loses its biological activity to bind to microtubules and stimulate their assembly (Lindwall and Cole, 1984; Iqbal *et al.*, 1986, 1994; Drechsel *et al.*, 1992; Bramblett *et al.*, 1993; Yoshida and Ihara, 1993; Biernat *et al.*, 1993; Alonso *et al.*, 1994, 1997; Wang *et al.*, 1995, 1996; Gong *et al.*, 2000).

Even if this process of hyperphosphorylation is not yet well understood, theoretically upregulation of tau kinases or downregulation of phosphatases could result in this phenomenon. Tau has at least 30 phosphorylation sites, some of which are found phosphorylated in PHF-tau, but not in normal brain, e.g. Thr212/Ser214, Thr231/Ser235 (Hoffmann *et al.,* 1997) and Ser422 (Bussiere *et al.,* 1999). *In vivo* studies have demonstrated that over a dozen protein kinases, including GSK-3β, cdk 5, and MAPK (Watanabe *et al.,* 1993), can phosphorylate tau (Table I. 1.) (for review, Billingsley *et al.,* 1997) at many residues, and mostly all phosphatases (except PP2C) can dephosphorylate tau (reviewed by Buée *et al.,* 2000; Lau *et al.,* 2002; Avila *et al.,* 2004). PP2A seems to be the major tau phosphatase in the brain (Goedert *et al.,* 1995; Sontag *et al.,* 1996, 1999; Gong *et al.,* 2000; Bennecid *et al.,* 2000; Kins *et al.,* 2001), but nothing is certified *in vivo*.

Residue	Site	PHFs Tau	CaMkII	Casein kinase I	Casein kinase II	Cdc2	Cdk2	Cdk5	DYRK	MAPK (Erk1/2)	GSK3 α	GSK3 β	MARK	PKA	PKB/AKT	PKC	PKN	pk35/41	Phosphorylase kinase	SAPK1g/JNK	SAPK2a/P38α	SAPK2b/P38β	SAPK3/P38δ	SAPK4	Tau tubulin kinase	PHFs Kinase
S	416		X											X												
S	413	X									X															
S	412	X																								X
S	409	X	X											X				X								
S	404	X		X	X	X	X	X		X	X	X											X	X		
T	403											X														
S	400	X										X														
S	396	X		X	X	X	X	X		X	X	X								X	X	X	X	X		
T	377															X										
T	361																									X
S	356		X								X		X	X					X	X	X	X	X	X		
S	352																X		X							
S	324										X		X	X		X		X								
S	320												X	X		X										
S	305												X	X		X			X							
S	293												X	X		X		X								
S	285		X																X							
S	262	X	X								X	X	X	X				X	X							
S	258															X	X									
T	245													X							X	X	X	X		
S	237																		X							
S	235	X				X		X		X	X	X								X	X	X	X	X		
T	231	X				X		X		X	X	X								X			X	X		
T	217	X								X		X								X	X	X	X	X		
S	214	X						X				X		X	X											
T	212	X				X		X	X	X	X	X		X						X	X	X	X	X		
S	210	X																							X	
S	208	X										X													X	
T	205	X				X	X	X				X		X						X	X	X	X	X		
S	202	X				X	X	X		X	X	X		X						X	X	X	X	X		
S	199	X								X	X	X														
S	198	X										X		X												
S	195					X		X				X		X												
S	185																				X	X	X			
S	184										X	X														
T	181	X								X		X								X	X	X	X	X		
T	175									X		X								X	X	X	X			
T	153							X		X		X								X	X	X	X	X		
T	69									X		X														
T	50					X				X		X														
S	46									X		X									X	X	X			
T	39				X																					

Table I. 1. Kinases involved in the phosphorylation of tau and the phosphorylation sites. T=threonine, S=serine, their position is labelled with numbers and at the bottom the kinases that phosphorylate tau in one or more sites (sites labelled with an X). Taken from http://www.alzheimer-adna.com/Images/TablePhosphoTau.gif

A panel of antibodies is available, which recognise phosphorylated sites of tau, some of which are pathological, since they do not occur in a healty brain, e.g. AT 100, PHF-27 (Hoffmann *et al.*, 1997) and AP 422 (Bussiere *et al.*, 1999) (Fig. I. 3.).

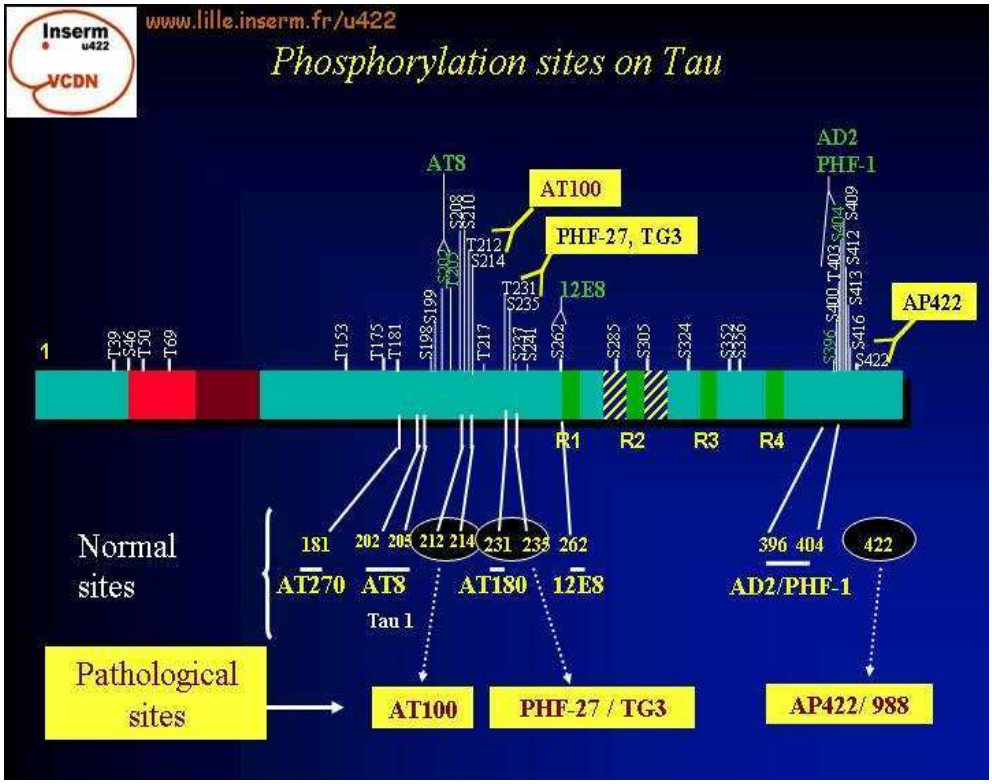


Fig. I. 3. Antibodies that recognise different phosphorylation sites of tau. Phosphorylation sites on tau are written in white with the type of amino acid susceptible to such modification. The antibodies that recognise pathological sites of phosphorylation are in a yellow box and antibodies which recognise normal phosphorylation sites are written in green. Exons 2 and 3 are highlighted in red. The microtubule binding domains are highlighted in green. The slashed boxes delineate exon 10. Taken from http://www.alzheimer-adna.com/Images/TauSitePhos.gif

4. TAU STRUCTURE.

In the vicinity of the N-terminal region of the tau protein, there is a highly acidic region followed by a basic proline-rich area, referred to as the projection domain since it projects from the microtubule surface and interacts with other cytoskeletal elements, membranes (Hirokawa *et al.*, 1988; Brandt *et al.*, 1995) and cytosolic organelles such as mitochondria (Rendon *et al.*, 1990). These interactions determine the diameter of the axon (Chen *et al.*, 1992). The proline-rich sequence is also involved in signal transduction pathways when interacting with SH3 of the src-family non-receptor tyrosine kinases like fyn (Lee *et al.*,1998).

Near the C-terminus, encoded by exons 9-12, are the microtubule binding domains (R1-R4). These are highly conserved regions of 18 amino acids separated by inter-repeat regions of less conserved 13-14 amino acids (Lee *et a.*, 1989).

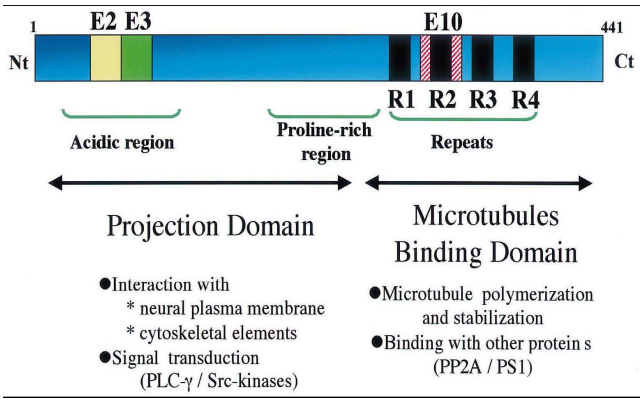


Fig. I. 4. Taken from Buée *et al.*, 2000. Schematic representation of the functional domains of the longest tau isoform (2+,3+,10+). The projection domain, includes the acidic and the proline-rich regions and interacts with cytoskeletal elements, determining the space between microtubules in axons and with proteins, such as PLC-g and Src-kinases. It is therefore involved in signal transduction pathways. The C-terminal is the microtubules binding domain and regulates the rate of microtubules polymerization. It is also involved in the binding with functional proteins as protein phosphatase 2A (PP2A) or presenilin 1 (PS1).

5. TAU FUNCTIONS.

Tau is a microtubule-associated protein. It has a major role in the polymerisation of tubulin, stabilisation of microtubules (Fellous *et al.,* 1977) and regulation of microtubule dynamics (Trinczek *et al.,* 1995), the constant assembly and dis-assembly of the microtubules depending on the GTP-GDP exchange. During polymerisation of tubulin molecules to microtubules, both α- and β-tubulin subunits are bound to a molecule of GTP. The GTP bound to α-tubulin is stable, but the GTP bound to β-tubulin may be hydrolysed to GDP shortly after assembly. This GDP-tubulin is prone to depolymerise and begin a rapid shrinkage of the whole microtubule. This process of microtubule assembly and dis-assembly is essential to maintain the cells function and viability, and therefore needs to be tightly regulated (Gonçalves *et al.,* 2001). This regulation is achieved by the binding of tau to the microtubules (Cleveland *et al.,* 1977; Lee *et al.,* 1989; Butner *et al.,* 1991; Drechsel *et al.,* 1992; Brandt *et al.,* 1993; Goode *et al.,* 1994; 1997; Billingsley *et al.,*1997; Panda *et al.,* 1999;2003), hence the activity of the tau protein is also strongly controlled by two major mechanisms. The first is the alternative splicing which leads to the expression of the two classes of tau proteins (3R and 4R). It has been shown that 4R tau is more efficient than 3R in stimulating tubulin polymerisation (Goedert *et al.,*1990; Butner *et al.,* 1991). Secondly, the microtubule-binding activity of tau is regulated by phosphorylation via kinases and phosphatases on its approximately two dozens of phosphorylation sites (reviewed by Buée *et al.,* 2000).

As a consequence of its interaction with the microtubules tau is essential to establish the polarity of neuronal cells, to promote axonal outgrowth during development, to maintain axonal morphology and is involved in axonal transport in mature cells (Cleveland *et al.,* 1977; Caceres and Kosik 1990; Hanemaaijer *et al.,* 1991; Brandt and Lee 1993; Esmaeli-Azad *et al.,* 1994; Harada *et al.,* 1994; Paglini *et al,.* 2000; Dawson *et al.,* 2001). In addition, other physiological functions of tau have been proposed, such as a role in development (Lee *et al.,*1998) or binding to RNA or DNA (Kampers *et al.,*1996; Hua and He 2003). Alterations in the functions of tau can lead to neurodegeneration (reviewed by Brandt *et al.,* 2005).

B) TAUOPATHIES.

In 1907, Alois Alzheimer described senile plaques and neurofibrillary tangles in the neocortex and hippocampus of a middle-aged woman with memory and cognitive function loss (Alzheimer, 1907), this was the first published description of Alzheimer's Disease. It took almost 80 years to identify the principal components of the senile plaques and of the neurofibrillary tangles: β-amyloid (Glenner and Wong, 1984) and tau protein (Brion *et al.*, 1985).

The role of the microtubule associated protein tau in the onset and progression of AD is still not clear, but the study over the past decades of sporadic and familial neurodegenerative diseases, other than AD, has led to the realisation that the tau protein is involved in a large group of diseases. They present a large diversity with regard to clinical manifestations, brain dysfunction, pattern of neurodegeneration and aetiology. Still, all of these diseases are chronic and progressive neurodegenerative disorders that have abundant, filamentous tau pathology and brain degeneration (Lee *et al.*, 2001). These disorders were named "tauopathies" and are summarised in Table I. 2.

TABLE I. 2. Diseases with tau-pathology. **Taken from Lee *et al.*, 2001**.

Alzheimer's disease*
Amyotrophic lateral sclerosis/parkinsonism–dementia complex*
Argyrophilic grain dementia*
Corticobasal degeneration*
Creutzfeldt-Jakob disease
Dementia pugilistica*
Diffuse neurofibrillary tangles with calcification*
Down's syndrome
Frontotemporal dementia with parkinsonism linked to chromosome 17*
Gerstmann-Sträussler-Scheinker disease
Hallervorden-Spatz disease
Myotonic dystrophy
Niemann-Pick disease, type C
Non-Guamanian motor neuron disease with neurofibrillary tangles
Pick's disease*
Postencephalitic parkinsonism
Prion protein cerebral amyloid angiopathy
Progressive subcortical gliosis*
Progressive supranuclear palsy*
Subacute sclerosing panencephalitis
Tangle only dementia*

* Diseases in which tau-positive neurofibrillary pathology are the most predominant neuropathologic feature.

In all tauopathies, the cytoplasmic, filamentous protein aggregates are found, which consit mainly of hyperphosphorylated and abnormally phosphorylated tau protein (Grundke-Iqbal *et al.*, 1986; Goedert *et al.*, 1989, 1992; Hauw *et al.*, 1994). This hyperphosphorylation is believed to be an early event in the pathway that leads from soluble to insoluble and filamentous tau protein (Braak *et al.*, 1994). Several cell culture and animal models, provide converging evidence that aberrant tau phosphorylation, even in the absence of large tau aggregates, can cause a neurodegenerative phenotype similar to that seen in human tauopathies (Table I. 3.).

Model	Experimental set-up	Main conclusion	References
I. Cell culture models			
Neural cell line (PC12)	transfection of "pseudo-hyperphosphorylated tau"	• induces cytotoxicity associated with an induction of apoptotic mechanisms in the absence of detectable tau aggregates	[166]
Human model neurons	infection of "pseudo-hyperphosphorylated tau"	• induces cytotoxicity associated with an induction of apoptotic mechanisms	[166]
Non-neuronal cell line (COS7)	co-transfection of tau, GSK-3β and ubiquitin	• co-expression of tau and GSK-3β resulted in accumulation of soluble phosphorylated tau, which is cytotoxic • co-expression along with CHIP rescues this type of cell death	[53]
Human cortical neuronal cell line (HCN2A)	delivery of pathological hyperphosphorylated human tau	• pathological hyper-phosphorylated tau induced cell death associated with apoptosis • Hsp27 prevents tau induced cell death	[188]
II. Animal models			
D. melanogaster	overexpression of human tau constructs	• neurodegeneration due to abnormal tau phosphorylation in the absence of NFTs • enhanced neurotoxicity with FTDP-17 mutant tau	[171,173]
C. elegans	overexpression of human tau constructs	• neuronal dysfunction as evident from Unc phenotype and more severe in FTDP-17 mutation • accumulation of soluble phosphorylated mutant tau correlates with the Unc phenotype before aggregation of tau	[174]

Table I. 3. Summary representation of experimental evidence for a role of direct cytotoxicity of altered tau protein in tauopathies. Taken from Brand *et al.*, (2005). Ref 53= Shimura *et al.*, 2004a; Ref 166= Fath *et al.*, 2002; Ref 171= Wittmann *et al.*, 2001; Ref 173= Jackson *et al.*, 2002 ; Ref 174=Kraemer *et al.*, 2003 ; Ref 188= Shimura *et al.*, 2004b

1. NEUROPATHOLOGICAL DESCRIPTION.

Neuropathologically, all tauopathies are characterised by prominent intracellular accumulations of the microtubule associated protein tau (Avila, 2000; Caceres and Kosik, 1990). In different diseases tau accumulations are found in different cell types and therefore receive different names: in astrocytes they are named tuffs; in oligodendrocytes, coiled bodies; in neurons, Pick's bodies, tangles or pre-tangles and in axons, neuropil threads (Lee *et al.*, 2001). Depending on the structure adopted by tau, the accumulations can be called Paired Helical Filaments (PHF) as in AD or Straight Filaments (SF) as in Progressive Supranuclear Palsy (PSP) as shown in Fig. I. 5.

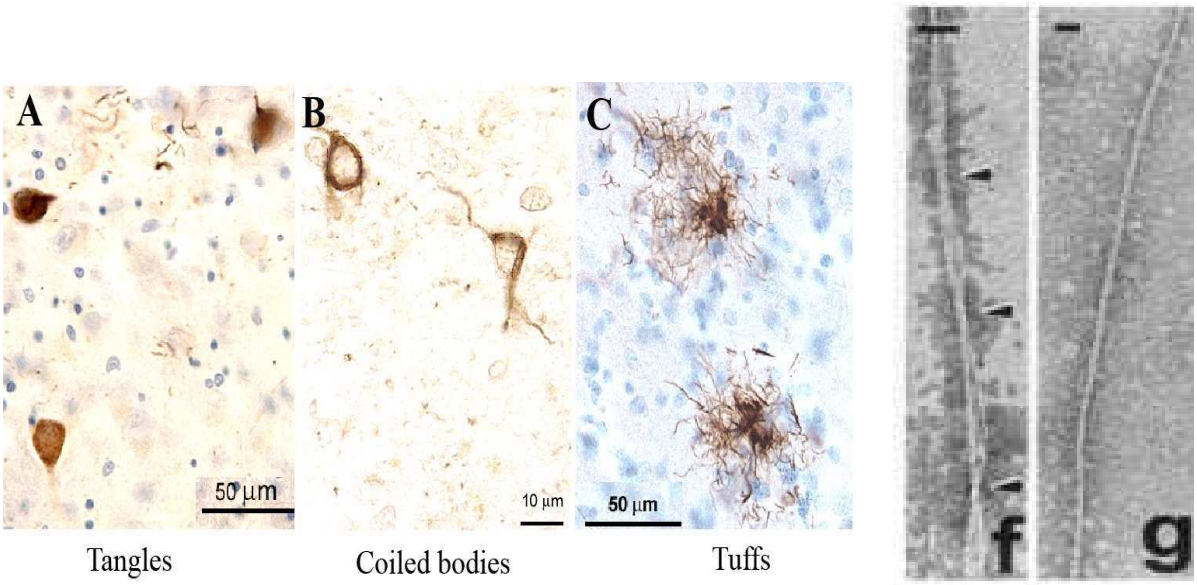


Fig. I. 5. Neuropathology of tau. Intracellular accumulation of tau in neurons (tangles) (A), in oligodendrocites (coiled bodies) (B) and in astrocytes (tuffs) (C). The formation of tangles of PHF and SF from *in vitro* hyperphosphorylated tau. **Taken from Alonso *et al.*, 2001**. tau, 0.5 mg/ml, was incubated with rat brain extract in the presence of ATP to induce hyperphosphorylation of tau that induced its self-polymerization into tau protofilaments forming PHF (*f*) or '15-nm straight filament, (*g*). Bars represent 40 nm.

2. BIOCHEMISTRY OF TAUOPATHIES.

There are two groups of isoforms of tau, 3R and 4R, which are expressed in a normal human brain in a ratio of 1:1. 4R tau binds to microtubules approximately 3 fold stronger than the 3R (Goode *et al.,* 2000) and is more effective in assembling the microtubules (Lee *et al.,*1989; Goedert and Jakes 1990). 3R is known to sequester soluble tubulin, necessary for self-assembly of tubulin to microtubules, and is more prone to self-aggregation (Utton *et al.,* 2001; Stanford *et al.,* 2003). This conclusion is supported by experimental work, when the ratio 3R:4R is disrupted in favour of an overexpression of the 3R tau in transgenic mice, which leads to the formation of hyperphosphorylated aggregates of the tau protein (Ishihara *et al.,* 1999). In tauopathies like AD, the amount of 3R and 4R tau accumulated is more or less the same, but in others there is a predominant isoform of tau that accumulates; in Pick's Disease for example, 3R tau is the major component of the PHF and in PSP, it is the 4R. (Reviewed by Buée *et al.,* 2000).

All tauopathies are defined neuropatholologically by the deposits of the tau protein found. A disease-specific alteration of tau is represented in its electrophoretic profile. Fig. I.6., shows tau of 55kDa which results from the phosphorylation of the fetal isoform (2-, 3-, 10-), tau of 64kDa resulting from the phosphorylation of tau patients with variants with one cassette exon (2+,3-,10- or 2-, 3-, 10+) and tau of 69kDa resulting from the phosphorylation of tau variants with two cassette exons (2+, 3+, 10- or 2+, 3-, 10+). Phosphorylation of the longest tau isoform (2+, 3+, 10+) induces the formation of the additional hyperphosphorylated tau 74kDa variant. There are three different patterns of tau on Western blots when human brain tissue is separated electrophoretically. The "3 bands" consists of tau 69, 64 and 55 kDa, classical for AD, FTDP-17 and ALS/PDC (Amyotrophic Lateral Sclerosis/Parkinson Dementia Complex). The "2 bands": tau of 69 and 64 kDa is common for PSP, CBD (CorticoBasal Degeneration) and some forms of FTDP-17, all related to the aggregation of 4R hyperphosphorylated tau. Pick's Disease has also 2 bands, the two shorter isoforms of tau, which relate to the aggregation of hyperphosphorylated 3R tau in what is called Pick's bodies.

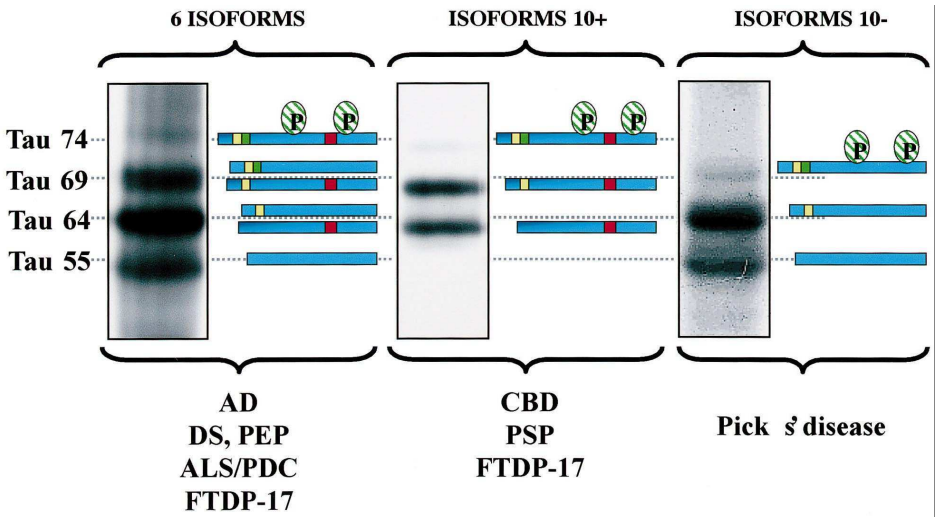


Fig. I. 6. Taken from Buée *et al.*, 2000. Typical electrophoretic profiles of pathological tau proteins using the phosphorylation-dependent monoclonal antibody AD2, with schematic representation of isoform composition (right of each frame). The six tau isoforms are involved in the formation of the typical AD-triplet with the minor tau 74 variant. This pattern is also described in Down syndrome (DS), post-encephalitic parkinsonism (PEP), ALS/PDC (Amyotrophic lateral schlerosis/Parkinson dementia complex and some families with FTDP-17 (Frontotemporal dementia with parkinsonism linked to chromosome 17) (left panel). The typical PSP/CBD (Progressive supranuclear palsy/Corticobasal degeneration) doublet tau 64, 69 is related to the aggregation of hyperphosphorylated tau isoforms with exon 10 (4R). The FTDP-17 families with mutations in exon 10 or intron 10 exhibit the same profile (middle panel). Hyperphosphorylated tau proteins without exon 10 (3R) aggregated in Pick's disease are detected as a tau 55, 64 doublet (right panel). Color codes: yellow box: insert encoded by exon 2, green, by exon 3, red by exon 10.

Most of the phosphorylation sites on tau are located within the microtubule repeat domain and flanking sequences and affects structural properties of the protein (Hagestedt *et al.*, 1989). Phosphorylation on these sites influences its interaction with microtubules (Lindwall *et al.*, 1984; Trinczek *et al.*, 1995). Therefore, many groups have been interested in the kinases and phosphatases involved in the phosphorylation state of tau. Patrick *et al.*, (1999) reported upregulation of cdk5 in AD brains, but Yoo and Lulec, (2001) and Taniguchi *et al.*, (2001), were not capable of reproducing this result. On the other hand, PP2A in AD brains has been found reduced in both expression and activity (Gong *et al.*, 1993, 1995; Vogelsberg-Ragaglia *et al.*, 2001; Loring *et al.*, 2001; Sontag *et al*, 2004), hence, downregulation of phosphatases could contribute to tau hyperphosphorylation (Matsuo *et al.*, 1994). Despite these results, attempts to produce tangles or PHFs in animal model merely by alteration of the activity

of individual phosphatases and/or kinases has not yet been successful, hence a combination of sites are likely to be involved. Thus, hyperphosphorylation of tau appears to be the result of a deregulation of phosphorylation and/or dephosphorylation events, and may have critical consequences. For example, hyperphosphorylated tau isolated from patients with AD is unable to bind to microtubules and to promote microtubule assembly, both are restored after enzymatic dephosphorylation (Lu and Wong 1993; Alonso *et al.*, 1994; Iqbal *et al.*, 1994). These lines of evidence link hyperphosphorylation to tauopathies. However, it is still not clear how this hyperphosphorylation is involved in the neuronal degeneration.

Fig. I. 7. shows a summary of the processing of tau, from its transcription via posttranslational processing to its pathological aggregation and the disease-specific electrophoretical fingerprint:

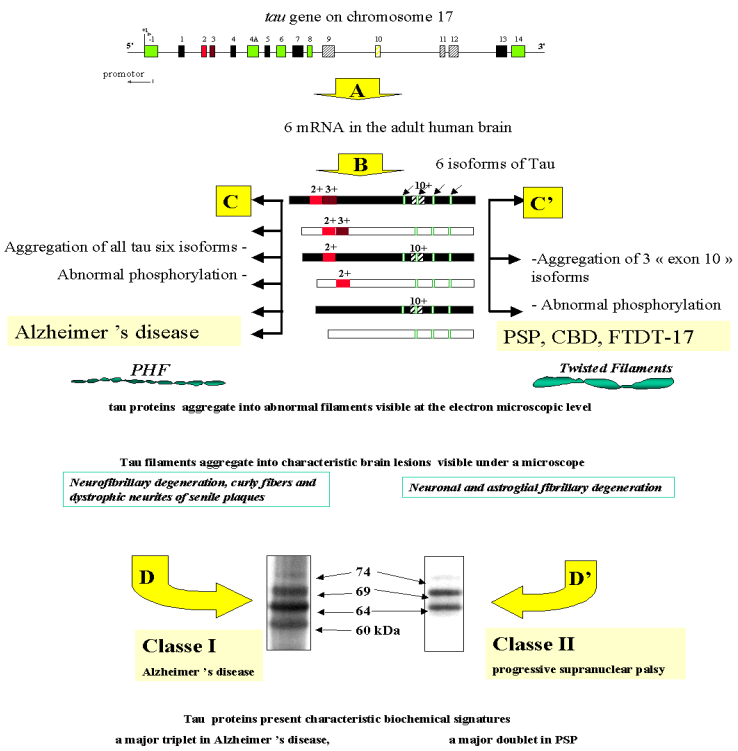


Fig. I. 7. Summary of tau's genetics and its relation with pathology. Tau gene is represented at the top. Red is exon 2, dark red is exon 3 and yellow exon 10, in green exons that are not transcribed. C and C´ show the 6 isoforms of tau and their relation with pathology; the characteristic lesions seen at electron microscopic level and their characteristic electrophoretic print are shown in D and D´. Taken from http://www.alzheimer-adna.com/Psp/signaturePSP.html

3. MAJOR TAUOPATHIES.

a. Alzheimer's Disease (AD).

AD is the most common and best-studied tauopathy. It affects approximately 10% of the population over 65 years of age (Rocca *et al.*, 1991). It is a progressive neurodegenerative disorder that leads to dementia (loss of intellectual functions, such as thinking, remembering, and reasoning), and behavioural disorders. Neuropathologically, it results in widespread atrophy in the temporal and parietal lobes and is characterised by the presence of NFT composed of PHFs and straight filaments (SFs), both being aggregates of tau (Kidd, 1963), and senile plaques (β- amyloid deposits), the two necessary hallmarks to define a neurological disorder as AD according to classical criteria (McKhann *et al.*, 1984, Hyman and Trojanowski, 1997). The tau aggregates are composed of the hyperphosphorylated state of all 6 isoforms, and therefore are biochemically characterised by the appearance of three major electrophoretic bands of the tau protein at weights 55, 64 and 69 kDa (Delacoute *et al.*, 1990; Lee *et al.*, 1991; Goedert *et al.*, 1994).

b. Progressive Supranuclear Palsy (PSP).

PSP has an estimated prevalence of 5-6 per 100 000 people (Schrag *et al.*, 1999), the average onset of the disease is 50-70 years (Santacruz *et al.*, 1998) and has a mean duration of approximately 8 years. It favours neither race nor gender, although there is a tendency for males to be more affected (Golbe *et al.*, 1988).

PSP, also known as the Steele, Richardson, and Olszewski disorder, presents clinically as parkinsonim, characterized by bradykinesia (slow movements) and prominent postural instability (Steele *et al.*, 1964). It also presents dysarthria (slurred speech) and dysphagia (swallowing problems) and, in the later stages, subcortico-frontal dementia. Supranuclear gaze palsy is the characteristic clinical sign of PSP. Tremor is usually absent. The symptoms are associated with marked frontal lobe glucose hypometabolism (Brooks *et al.*, 1994), but the aetiology remains unknown. There is not

yet an effective treatment. Particularly, the dopaminergic therapy used in Parkinson's disease, is of little or absent benefit in PSP.

Neuropathologically, PSP is characterized by atrophy of the basal ganglia, subthalamus, and brainstem, with corresponding neuronal loss and gliosis. Within these brain regions, there is a high density of fibrillary tau pathology, including neuropil threads, and NFTs that are typically round or globose (Pollock *et al.*, 1986; Hauw *et al.*, 1994; Litvan *et al.*, 1996a). Glial fibrillary tangles in both astrocytes and oligodendrocytes are abundant (Hauw *et al.*, 1990; Yamada *et al.*, 1992; Komori, 1999). Ultrastructural analysis of these neurofibrillary lesions revealed 15- to 18-nm straight filaments, with a long periodicity (Tellez-Nagel and Wisniewski 1973; Roy *et al.*, 1974). The biochemical identification of insoluble, hyperphosphorylated tau at 68 and 64 kDa (Flament *et al.*, 1991; Vermersch *et al.*, 1994), corresponds to the 4R tau (Spillantini *et al.*, 1997; Sergeant *et al.*, 1999). Furthermore, in PSP, the relative abundance of tau mRNA containing E10 has been reported to be increased in the brainstem but not in the cortex, which is consistent with the distribution of the neurofibrillary pathology (Chambers *et al.*, 1999).

c. Frontotemporal Dementia with Parkinsonism Linked to Chromosome 17 (FTDP-17).

The first such disorder was linked to chromosome 17 when Wilhelmsen *et al.*, (1994) described a familial disease they called "disinhibition-dementia-parkinsonism-amyotrophy complex" and demonstrated genetic linkage of this disease to chromosome 17q21-22. Subsequently, a number of related neurodegenerative disorders were linked to the same region on chromosome 17 (Wijker *et al.*, 1996, Bird *et al.*, 1997, Foster *et al.*, 1997, Heutink *et al.*, 1997, Murrell *et al.*, 1997, Lendon *et al.*, 1998).

This group of syndromes consists of autosomal-dominantly inherited neurodegenerative diseases with diverse, but overlapping, clinical and

neuropathological features (Foster et al 1997). Clinically, the disease commonly begins with behavioural, cognitive or motor manifestations and is characterized primarily by a progressive frontotemporal Dementia (FTD) and parkinsonism (Foster *et al.,* 1997). The neuropathology of FTDP-17 is characterized by marked neuronal loss, frontotemporal atrophy, gliosis, extensive neuronal and glial fibrillary pathology composed of hyperphosphorylated tau protein (reviewed in Spillantini *et al.,* 1998, Crowther and Goedert, 2000, Hong *et al.,* 1998), but without evidence of β-amyloid deposits or other disease-specific brain lesions (Murrell *et al.,* 1999, Lippa *et al.,* 2000, Rizzini *et al.,* 2000, Spillantini *et al.,* 2000). In some cases the biochemical pattern is similar to AD, with 69, 64 and 55 kDa forms of tau, while in other cases the pattern is like CBD and PSP, where only the 69 and 64 kDa tau is found. The latter phenotype is due to intronic mutations that result in the forced expression of exon 10 (Grover *et al.,* 1999; Hutton *et al.,* 1998; Spillantini *et al.,* 1998; Varani *et al.,* 1999). Some factors involved in the alternative splicing of tau have been already identified (D'Souza and Schellenberg, 2002). More than 25 mutations in the tau gene have been identified in exons 1, 9, 10, 11, 12, and 13 as a cause of FTDP-17. These mutations may affect mRNA splicing or the protein levels of tau and occur preferentially in the microtubule binding region.

d. Corticobasal Degeneration (CBD).

It is an adult-onset progressive neurodegenerative disorder characterised by cortical disturbances like aphasia and apraxia, moderate dementia and markedly asymmetric motor disturbances, such as rigidity, limb dystonia and tremor. Neuropathological examination indicates depigmentation of the substantia nigra (Rebeiz *et al.,* 1967, 1968) and frontoparietal atrophy, neuronal loss with spongiosis and gliosis. Tau pathology is found in neurons as intracytoplasmic aggregates (Iwatsubo *et al.,* 1994; Mori and Ihara1991) and in glial cells as characteristic astrocytic plaques. A striking feature of CBD is the extensive accumulation of tau-immunoreactive neuropil threads throughout gray and white matter (Feany and Dickson 1995; Feany *et al.,* 1996). The tau filaments in CBD include both PHF-like filaments and straight filaments

(Ksiezak-Reding et al 1994, Komori 1999). Electrophoretically, hyperphosphorylated tau is only found in the 69 and 64 kDa forms.

e. Pick's Disease (PiD).

PiD is a rare form of neurodegenerative disorder characterised by a distinct dementing process producing disturbances in language (Constantinidis *et al*, 1974), as well as depression and behavioural changes associated with frontal lobe atrophy. This disorder is characterized by the presence of cytoplasmic tau inclusions in neurons of the frontal lobe, known as Pick bodies (Brion *et al.*, 1991). The granular cells of the dentate gyrus are also affected. Biochemical analysis using Western blot with phosphorylation-dependent tau, reveals that in all cases of PiD a major 55 and 64 kDa tau doublet (Buee-Scherrer *et al.*, 1996; Delacourte *et al.*, 1996), indicating the absence of exon 10 expression.

There are other tauopathies like postencephalic parkinsonism, Down's Syndrome, myotonic dystrophy, prion disorders with NFT, Niemann-Pick Type C disease or the Parkinsonism-Dementia-Complex of Guam that will not be described here, but are reviewed in detail by Buée *et al.*, (2000), Ingram and Spillantini, (2002) and Avila *et al.*, 2004.

4. PATHOPHYSIOLOGY OF TAUOPATHIES.

The role of tau in neurodegeneration is still a subject of ongoing debate. Two major hypotheses have been proposed: (1) a gain of toxicity of the tau protein, where intracellular accumulation of tau is the cause of cell death, as it has been demonstrated for FTDP-17, where a mutation in tau is sufficient to cause the protein aggregation in the cytoplasm and the second cell death (Goedert *et al.,* 1998); (2) a loss of function of tau, where the protein loses its biological function of stabilising the microtubules and cell death results from an altered microtubule dynamic and its consequences (Feinstein and Wilson, 2005).

5. AETIOLOGY OF TAUOPATHIES.

There are 3 possible aetiologies described to date.

A **genetic origin** was first described for FTDP-17, which refers to a group of autosomal dominant inheritance with mutations in the tau-gene (reviewed by Brandt *et al.,* 2005). In 1998, tau mutations in introns and exons were identified and associated with FTDP-17 (Hutton *et al.,* 1998; Poorkaj *et al.,* 1998; Goedert *et al.,* 1998), establishing the dysfunction of tau due to mutations as a possible cause of neurodegeneration. This is the only tauopathy with a proven genetic cause, where mutations in the tau protein are sufficient to trigger the disease.

Tau **polymorphisms** may be a risk factor for PSP and CBD (de Yebenes *et al.,* 1995; Rojo *et al,* 2000). Conrad and colleagues, (1997), were the first to demonstrate an allelic association of a dinucleotide polymorphism in intron 9 of the tau gene with PSP, showing that variations in the tau gene could be a genetic predisposition in sporadic PSP. This observation was confirmed for PSP and CBD by others (Di Maria *et al.,* 2000; Houlden *et al.,* 2001). The allelic association to PSP and CBD was later extended to a series of polymorphisms, defining the PSP-associated haplotype H1, distinct from

the haplotype H2, which is not PSP-associated (Baker *et al.*, 1999; Ezquerra *et al.*, 1999; da Silva, 2003).

Another hypothesis links distinct tauopathies to **environmental triggers**. Natural toxins contained in food products are hypothesised to cause several tauopathies. In the island of Guam for example, PDC has been linked to the consumption of *Cycas* rumpii, a false sago palm, which contains β-D-glucosides, which may cause the disease by glutamate neurotoxicity (Khabazian *et al.*, 2002; Wilson *et al.*, 2002). Atypical parkinsonism associated with the consumption of plants from the *Annonaceae* family have been described in the island of New Caledonia (Angibaud *et al.*, 2004) and in the Caribbean island of Guadeloupe (Caparros-Lefebvre *et al.*, 1999). The latter disease is the main interest of this thesis.

C) ATYPICAL PARKINSONISM IN GUADELOUPE.

In Europe and North America, idiopathic Parkinson's Disease is the major form of parkinsonism, about 20% are atypical parkinsonism (De Rijk *et al.*, 1997) and less than 4% of patients have Progressive Supranuclear Palsy (PSP) (Golbe *et al.*,1988). Parkinsonism is defined as a combination of symptoms, which include bradykinesia and at least one of the following: tremor, rigidity or postural instability (Calne *et al.*, 1992). Parkinson's Disease has a unilateral onset and is levodopa responsive (Gibb and Lees, 1988). The major clinical symptoms result from a deficit in striatal dopamine due to the loss of dopaminergic neurons in the *substantia nigra pars compacta* (SNpc) (Agid, 1991). PSP differs from Parkinson's Disease because it is levodopa-unresponsive and presents a unique characteristic to this disorder, a supranuclear gaze palsy, which is essential for clinical diagnosis of PSP (Litvan *et al.*, 1996b). Patients that do not fulfil classical criteria for PSP, by presentation of hallucinations or delusions, severe autonomic dysfunction, cortical sensory deficit or alien limb phenomena (Litvan *et al.*, 1996a), are referred to as atypical parkinsonism.

In Guadeloupe, a Caribbean island in the French West Indies, Caparros-Lefebvre and Elbaz (1999) found a high prevalence (33%) of PSP and atypical parkinsonism unresponsive to levodopa (33%). Three PSP-like patients that came to autopsy had neuropathological features that resemble those of PSP: high accumulations in the perikarion of abnormally phosphorylated tau (Caparros-Lefebvre *et al.*, 2002). In two clinical studies (Caparros-Lefebvre and Elbaz, 1999; Lannuzel *et al.*, in press), a relationship between the consumption of herbal tea and fruits from the *Annonaceae* family (custard apple or pawpaw family) has been described. In the island of Guadeloupe, the most common *Annonaceae* plant is *Annona muricata*, which is known to contain potent neurotoxins which inhibit complex I of the mitochondrial respiratory chain, so called annonacous acetogenins. Annonacin is the most abundant of these acetogenins produced exclusively by *Annonaceae* and has become a candidate neurotoxin that might be involved in the aetiology of the disease (Degli Esposti, 1998; Höglinger *et al*, 2005).

1. Gd-PSP CLINICAL PHENOTYPE.

Lannuzel *et al.*, (in press), based on a cohort of 160 parkinsonian patients, defined in detail the clinical phenotype of the atypical parkinsonism in Guadeloupe. From 160 patients, 104 (63%) had atypical forms of parkinsonism. Fifty-one (50%) of these had supranuclar ocular motor dysfunction and are referred to as Gd-PSP (Guadeloupe PSP-like syndrome). In this group, 12 of these patients fulfilled the NINDS-SPSP diagnostic criteria for possible or probable PSP (Litvan *et al.*, 1996a), and the majority (n=39; 76%) present at least one of the mandatory exclusion criterion for PSP (hallucinations and /or delusions, severe autonomic dysfunction or both). The remaining 50 atypical parkinsonian patients had a homogeneous clinical syndrome, which differs from the Gd-PSP because of the absence of oculomotor problems and will be referred to as Gd-PDC (Guadeloupe parkinsonism dementia complex). Apart from this feature, Gd-PSP and Gd-PDC are indistinguishable.

Tremor was the most frequent initial symptom in Gd-PSP and Gd-PDC patients followed by the other classic parkinsonian symptoms: rigidity and bradykinesia, which were in many cases symmetrical and both Gd-PSP and Gd-PDC patients were little or non-responsive to levodopa. In classical PSP, postural instability and falls are frequently initial symptoms (Litvan *et al.,* 1996b), while in Gd-PSP and Gd-PDC they appeared later (2,5 and 3,4 years after disease onset respectively). Many patients with Gd-PSP and Gd-PDC presented exclusion criteria for classical PSP like cognitive or psychiatric dysfunctions (memory impairment, personality changes or hallucinations) and unexplained autonomic dysfunction (like arthostatic hypotension and urinary incontinence). Almost all had fronto-subcortical dementia. The only statistically significant difference found between Gd-PSP and Gd-PDC was the existence or absence of oculomotor dysfunction respectively.

2. Gd-PSP NEUROPATHOLOGY, BIOCHEMISTRY AND GENETIC ANALYSIS.

Three patients with a PSP-like clinical manifestation from Guadeloupe came to autopsy. Neuropathological examinations (Caparros-Lefebvre *et al.,* 2002) showed accumulations of tau protein, predominantly in the midbrain, which resembles that of PSP (Litvan *et al.,* 1996b) as shown in Fig. I. 8. Immunohistochemistry revealed tau positive processes (threads) and cell bodies (pretangles, i.e., diffuse immunostaining of the cell body) or true neurofibrilly tangles which were rare and astocytic tuffs, considered as highly suggestive of PSP (Hauw *et al.,* 1990; Komori *et al.,* 1998), were virtually absent in case 1.

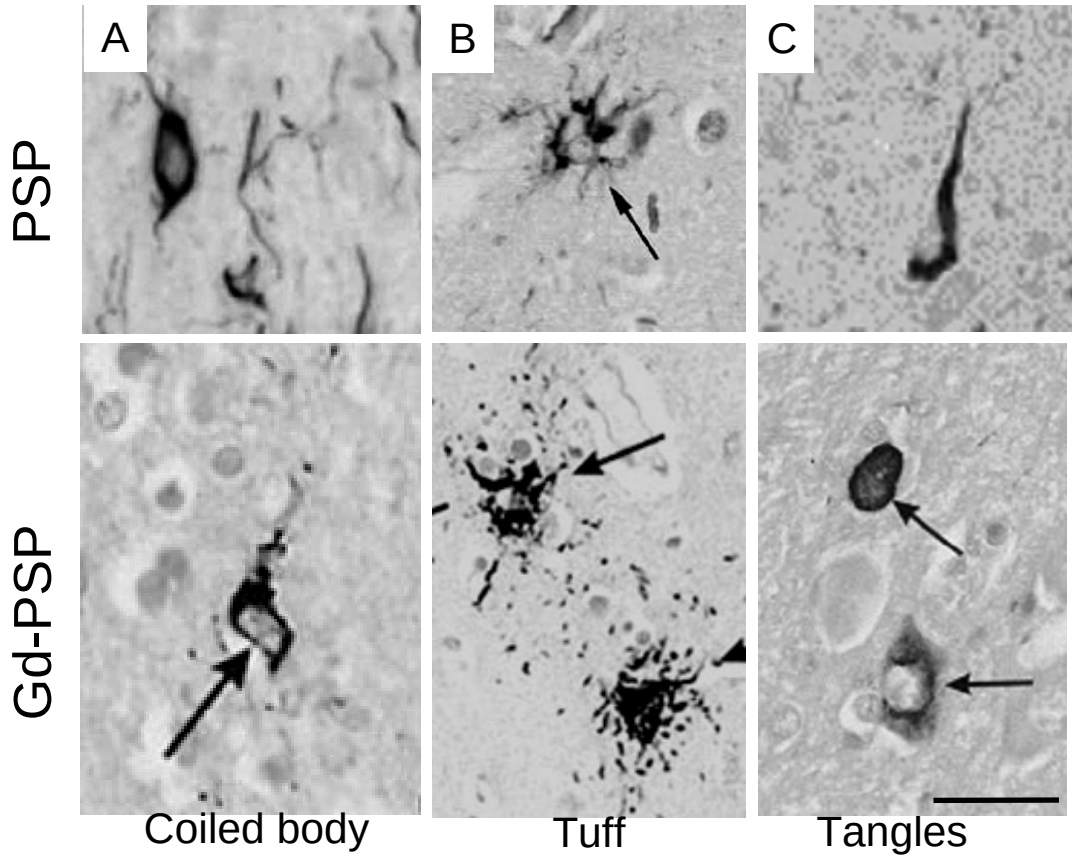


Fig. I. 8. Comparison of histopathology between Guadealoupean PSP (Gd-PSP) (case 3) (lower row) and PSP (upper row) in the striatum. A) Coiled bodies (Tau accumulation in astrocytes). B) Astrocytic tuffs (tau accumulated in astrocytes). C) Tangles (tau inmunoreactivity). Polyclonal anti-tau from Dako was used. Scale bar ~ 50µm.**Taken from Caparros-Lefebvre *et al.*, 2002.**

Biochemical studies of brain tissue homogenates detected the classical doublet at 64 and 69 kDa of tau (Caparros-Lefebvre *et al.*, 2002), which is characteristic for tauopathies like PSP, which accumulate hyperphosphorylated tau isoforms containing the exon 10 sequence (Mailliot *et al.*, 1998; Sergeant *et al.*, 1999).

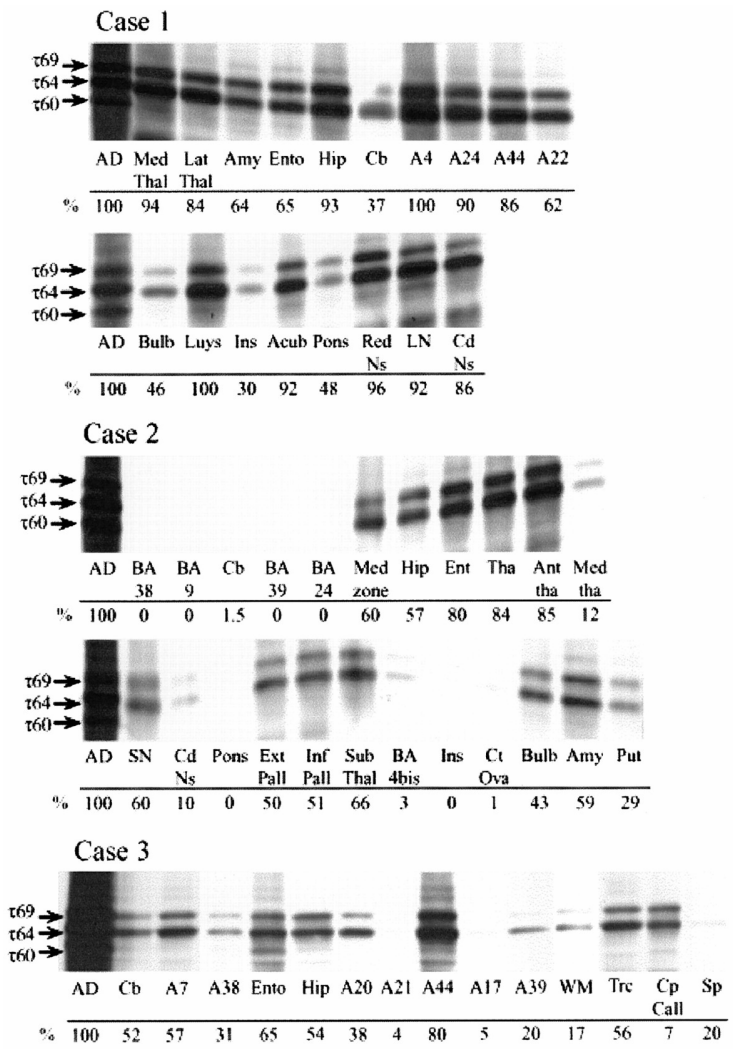


Fig. I. 9. Biochemical mapping of pathological tau of three Guadeloupean cases. 18, 24 and 15 brain regions of Case 1, Case 2 and Case 3 respectively were analysed for their pathological tau content by Western blotting. Antibody AD2 detected a doublet of pathological tau at 64 and 69 kDa in the three cases, whereas the typical triplet of pathological tau is observed in the Alzheimer brain tissue samples (AD lanes). AD = Alzheimer's disease; Accu = nucleus accumbens septi; Amy = amygdala; Ant tha = anterior thalamus; BA = Brodmann area; Bulb = bulbe; Cb = cerebellum; Cd Ns = caudate nucleus; Cp Call = corpus callosum; Ct Ova = centre ovale; Ento = entorhinal cortex; Ext Pall = external pallidum; Hip = hippocampus; Inf Pall = internal pallidum; Ins = insula; LN = locus niger; Lat Thal = ventral lateral nucleus; Med zone = medial zone; Med tha = median thalamus; Put = putamen; Red Ns = red nucleus; SN = substantia nigra; Sub Thal = subthalamic nucleus (nucleus of Luys); Tha = thalamus; Trc = brainstem; WM = white matter. **Taken from Caparros-Lefebvre *et al.*, 2002.**

In FTDP-17 mutations have been described (Hutton *et al.,* 1998; Poorkaj *et al.,* 1998; Goedert *et al.,* 1998), and tau haplotype tau polymorphisms confer a genetic risk for PSP (Baker *et al.,* 1999) and CBD (Corticobasal Degeneration) (Di Maria *et al.,* 2000; Houlder *et al.,* 2001) but not for other tauopathies like PiD (Pick's Disease) (Russ *et al.,* 2001). Although none of the patients had a family history of parkinsonism or dementia, a genetic study of these 3 autopsied patients from Guadeloupe was carried out and no mutation of the tau gene was observed. They were all homozygous for the H1 haplotype (Caparros-Lefebvre *et al.,* 2002).

3. EPIDEMIOLOGICAL STUDY ON GUADELOUPE.

Out of the 31 PSP-like patients (now called Gd-PSP) and the 30 with atypical parkinsonism (renamed Gd-PDC) examined by Caparros-Lefebvre and Elbaz (1999), all reported regular consumption of either pawpaw fruit or herbal teas. These groups consumed significantly more fruits and drank more herbal tea than controls (fruit: odd ratio 20·7; herbal tea: 6·48). Caparros-Lefebvre and Elbaz (1999) therefore postulated a link with the consumption of herbal tea and fruits from the *Annonaceae* family (specially *Annona muricata* which is the most common in the region), which contain high concentrations of neurotoxins like benzyltetrahydroisoquinoline alkaloids (Leboeuf *et al.,* 1980) and annonaceous acetogenins (Li *et al.,*1990; Cavé *et al.,*1997).

More evidence has been recently collected (Lannuzel *et al.,* Brain, in press) from a cohort of 160 parkinsonian patients. The consumption of fruit and infusion of leaves of *Annona muricata* was evaluated in Gd-PSP and 31% Gd-PDC patients and converted into mg of annonacin (major acetogenin in *Annona muricata*), as determined by mass spectroscopy (Champy *et al.,* 2005). One fruit and one cup of infusion are estimated to contain 15mg and 0,14mg of annonacin, respectively. Thereof, the amount of annonacin ingested during the patient's life was estimated and is summarised in Table I. 4. The percentage of high consumption (≥10 fruits or cups per day for one year) of annonaceous products in Gd-PSP and Gd-PDC did not differ significantly and the

estimated ingestion of annonacin was significantly increased compared to control subjects in both groups.

	N	% High consumption of annonaceous products	Estimated amounts of annonacin ingested in grams (mean±S.E.M.)	P
Controls	75	33	33±6	
Gd-PSP	21	76	148±40	***
Gd-PDC	23	74	156±48	***

Table I. 4. Comparison of high consumption of annonaceous products (in percentage) and estimation of ingestion of annonacin between controls and atypical parkinsonisms of Guadeloupe. Gd-PSP and Gd-PDC patients are higher consumers of *annonaceae* products than controls. High consumption refers to ≥ 10 fruits or cups of annonaceous products per day in one year. The estimation of the amount of annonacin ingested during the patients' life is expressed as gram (mean±S.E.M), knowing that each fruit contains 15mg of annonacin and each cup 0,14mg. ***Significantly different than control values ($p<0.001$, one-way ANOVA followed by Student-Newman-Keuls test)

D) AN ENVIRONMENTAL TOXIN HYPOTHESIS.

The absence of Mendelian inheritance and of mutations in the tau-gene, the ethnic heterogeneity of the affected population (Caparros-Lefebvre *et al.*, 1999, 2002; 2005, Lannuzel *et al.*, Brain, in press), and the high prevalence of sporadic cases, argue against a genetic cause of Guadeloupean atypical parkinsonism. The geographical clustering of this disease on the islands of Guadeloupe and its association with the regular consumption of *Annona muricata* (Caparros-Lefebvre *et al.*, 1999; Lannuzel *et al.*, in press) are arguments in favour of a natural toxin-related aetiology of this PSP-like disease.

Other associations between the consumption of *Annonaceae* and atypical parkinsonism have been reported in other geographically isolated regions such as New Caledonia (Angibaud *et al.*, 2004). These associations in independent populations with genetically distinct backgrounds also argue in favour of the hypothesis that toxic compounds contained in fruits from the *Annonaceae* family, which are very deeply-rooted in the daily life of certain populations, may be responsible for the observed neurodegenerative syndrome.

Intriguing evidence linking these two effects (consumption of *Annonaceae* products and Guadeloupean disease) was described by Caparros-Lefebvre (1999) when five patients under the age of 65 showed remarkable improvement when they stopped consuming anonnaceous products.

E) EXPERIMENTAL WORK SUPPORTING THE TOXIN-HYPOTHESIS IN GUADELOUPE.

Isoquinolin-alkaloids and acetogenins are the two major groups of neurotoxins contained in the *Annonaceae* plants.

The alkaloids are found in many plants, they are hydrophilic and energy poisons, but their molecular mechanism of action has not been clearly elucidated (Kotake *et al.*, 2004). To investigate if they cause neuronal degeneration, and therefore play a role in the Guadeloupean disease, Lannuzel and collaborators (2002), studied their toxicity and possible mechanism of action on dopaminergic neurons *in vitro*. In this study alkaloids killed non-selectively (i.e., dopaminergic and GABAergic neurons). The analysis of the mechanisms of action showed that glucose protected against annonacin by glycolitic stimulation (Lannuzel *et al.*,2002), suggesting that alkaloids might contribute to the toxicity of the plants *in vivo* by energy depletion.

The acetogenins are a group of over 400 different molecules, which constitute a unique and structurally homogenous class of polyketides (fatty acid derivatives). They are highly lypophilic because of their fatty-acid-derived chain (Degli Esposti *et al.*, 1998), which allows them to penetrate into cells by passive diffusion, they are stable to light, pH, temperature, are specific to the *Annonaceae* plants (Cavé *et al.*,1997; Alali *et*

al., 1999) and are potent and specific inhibitors of mitochondrial complex I (Tormo *et al.,* 1999; Motoyama *et al.,* 2002).

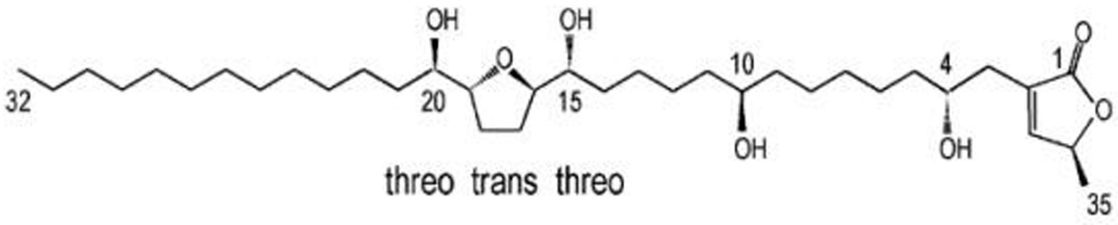


Fig I. 10. Molecular structure of annonacin. Annonacin has an aliphatic tail, which confers lipophilicity allowing it to cross the blood-brain-barrier and cell membranes, and an aromatic end.

The toxicity and mechanisms of neurotoxicity of annonacin (Fig. I. 10), the most abundant acetogenin in *Annona muricata* (Laprévote *et al.,* 1993; Gleye, 1998), was studied *in vitro* and compared to other complex I inhibitors such as MPP^+, known to kill dopaminergic neurons (Langston *et al.,* 1999), and rotenone (Höglinger *et al.,* 2003). Annonacin penetrates by passive diffusion and affects equally dopaminergic and non-dopaminergic neurons (Lannuzel *et al.,* 2002). It inhibits the complex I activity of submitochondrial particles with an IC_{50} of about 30 nM (Tormo *et al.,* 1999) and kills neurons by ATP depletion, thereby being about 50-times more toxic to dopaminergic neurons and 2000-times more toxic to non-dopaminergic neurons than MPP^+ (Lannuzel *et al.,* 2003).

From this moment onwards, more attention was paid to the acetogenin annonacin mainly because of two reasons: 1) acetogenins were toxic at nanomolar concentrations, whereas for alkaloids to be toxic, micromolar concentrations were necessary, 2) acetogenins are known mitochondrial toxins, like other parkinsonian-inducing compounds such as MPP^+ (Langston *et al.,* 1999) or rotenone (Höglinger *et al.,* 2004).

To test the hypothesis that annonacin is capable of inducing neurodegeneration in a living system, it was administrated intravenously to rats for 28 days (Champy *et al.,* 2004). It was shown that annonacin enters the rat brain parenchyma, decreases brain

ATP levels and induces pronounced and widespread neurodegeneration in basal ganglia and brainstem nuclei, whereas hippocampus, cerebellum, and cerebral cortex are only moderately affected. This mimics the distribution of brain lesions seen in patients with atypical Guadeloupean parkinsonism (Caparros-Levebvre *et al.*, 2002).

Champy *et al.*, in 2005, quantified the concentration of annonacin in different extracts of fruit and leaves of *Annona muricata* and estimated that an adult who consumes one fruit or can of nectar a day during a whole year, would have consumed a cumulative dose/kg (106 mg/kg) equivalent to the dose administrated to the rats (3,8 mg/kg/day) (Champy *et al.*, 2004).

In Table I. 5., the concentrations of annonacin in different preparations are shown.

Preparation	Extract concentration	Estimated dose/unit	Estimated dose/ year (mg/kg)
Pulp[a]	525 mg/kg[b]	15 mg/fruit[c]	78
Nectar (25% pulp)	109 ± 30 mg/L[d]	36 mg/can (330 ml)	188
	100 ± 27 mg/L[e]	—	—
Leaves			
MeOH extract	318 ± 88 mg/kg[d]	—	—
	299 ± 133 mg/kg[e]	—	—
Infusion	56 ± 22 mg/kg[d]	0.14 ± 0.05 mg/250 ml	0.73
Decoction	56 ± 14 mg/kg[d]	0.14 ± 0.03 mg/250 ml	0.73

Values are expressed as the mean ± SD of three preparations.
*Cumulative dose for an average adult (70 kg) consuming 1 fruit, 1 can of nectar, or 1 cup of infusion per day.
[a]One kg of lyophilized pulp = approximately 30 fruits.
[b]Value obtained by quantitative extraction of the molecule.
[c]Average fruit, approximately 800g.
[d]Values obtained by MALDI MS with ^{18}O solamin as internal standard.
[e]Values obtained by RP HPLC.

Table I. 5. Concentrations of annonacin in different *Annona muricata* preparations. The average content of annonacin in one fruit is estimated to be about 15mg, in a can of commercial nectar 36mg and in a cup of infusion 140µg. **Taken from Champy *et al.*, 2005**.

F) AIM OF THIS THESIS.

The epidemiological and experimental data described above are strongly suggestive of a relationship between the consumption of *Annona muricata,* rich in the acetogenin annonacin, and the widespread neurodegeneration in the brain which resembles that of the tauopathy PSP. The aim of my thesis is to demonstrate that the acetogenin annonacin, a lipophilic and potent complex I inhibitor, is capable of inducing a tau pathology consistent with that described in the patients on the island of Guadeloupe. To reproduce a tauopathy in presence of annonacin and to study the underlying mechanisms, an *in vitro* model is most adequate.

In Parkinson's disease and in atypical parkinsonism like PSP, one of the most affected regions is the *substantia nigra* , specifically the dopaminergic neurotransmitter system (Agid, 1991; Ruberg *et al.,* 1995). We decided upon an *in vitro* model of primary cultures of rat striatum instead, because the striatum was a major site of pathology in the three Guadeloupean patients that came to autopsy (Caparros-Lefebvre *et al.,* 2002) and also in annonacin treated rats (Champy *et al.,* 2004).

II. RESULTS.

A) CELL DEATH AND TAU REDISTRIBUTION IN ANNONACIN TREATED STRIATAL CULTURES.

1. DOES ANNONACIN INDUCE CELL DEATH IN STRIATAL NEURONS IN CULTURE?

The striatum was dissected from the brains of rat embryos at embryonic day E16,5 as described (see methods chapter). The cells were dissociated and cultured for 3 days in presence of the antimitotic agent AraC to eliminate proliferating astrocytes and treated with annonacin at DIV 5/6 for 48h.

First, we determined the concentration of annonacin at which the inhibition of complex I became toxic after 48h. This time point was chosen so that the cell death process could occur slowly enough to allow us to study the events that lead to it. We treated the cells with an increasing range of concentration of annonacin: 0, 25, 50, 75, and 100nM and quantified cell survival after 48h with the Live/Dead kit (see materials) which distinguishes living cells labelled with calcein (green) from dead cells labelled with ethidium homodimer (red) which intercalates in their DNA. The dose response curve for cell survival in annonacin treated cultures after 48h of treatment is shown in Fig. II. 1A. Cell survival is presented as the percentage of control levels, approximately 7 x 10^5 cells / 16 mm wells. Fifty percent of cell survival was obtained at a concentration of 50nM and almost all cells were dead at double the concentration after 48h.

Hence, annonacin is capable of inducing cell death in striatal neurons of primary cultures, as it does in the striatum of living animals (Champy *et al.*, 2004), indicating that we can use this *in vitro* model to study whether annonacin induces tau pathology.

2. IS TAU ACCUMULATED IN THE PERIKARYON OF ANNONACIN-TREATED CULTURES?

In human tauopathies, like Alzheimer's Disease (AD) or Progressive Supranuclear Palsy (PSP), the tau protein is found in the cytoplasm of the cells accumulated or aggregated in neurofibrillary tangles (NFT) (Reviewed by Lee *et al.*, 2001).

Our interest is to know if annonacin is capable of inducing such a tauopathy, i.e., the accumulation or aggregation of tau in the soma of striatal cultures. For this purpose, we performed an immunocytochemical analysis of the tau protein in striatal cultures treated with the same range of increasing concentrations of annonacin that were used to evaluate cell death. Tau was detected with the phosphorylation-dependent AD2 tau antibody, used to diagnose AD (Reig *et al.*, 1995) CBD and PSP (Buee-Scherrer *et al.*, 1996), which labels an epitope on tau phosphorylated at serine residues 396 and 404.

In striatal cells, annonacin induced an increase in the number of neurons with AD2-positive cell bodies in a dose-dependent way as shown in Fig. II. 1B. Representative images of AD2- labelled striatal neurons treated with annonacin 25, 50, 75 and 100 nM are shown in Fig. II. 1C. Tau immunoreactivity was found mainly in the neurites in control conditions, but in annonacin-treated cultures, the neuritic network became less dense and the tau labelling was found mostly in the perikaryon. An identical distribution of tau was obtained under the same experimental paradigm when tau was immunolabelled with other phosphorylation-dependent antibodies against other serine or threonine residues: AT8: serine 202 / threonine 205; AT270: threonine 181 and AT100: threonine 212 / serine 214, which recognises pathologically phosphorylated tau, see Fig. II. 1D.

These findings suggest that annonacin induces tau relocalisation prior to the cell death of striatal neurons in a dose dependent way.

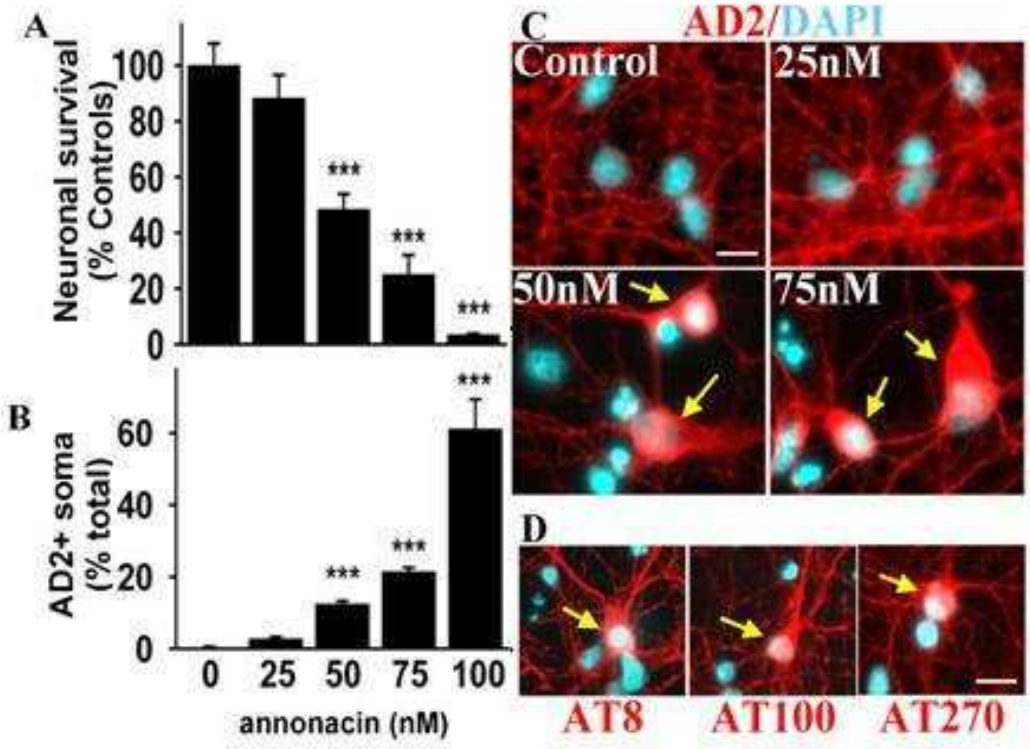


Fig. II. 1. Annonacin treatment induces cell death and redistribution of hyperphosphorylated tau in striatal cultures. Treatment of striatal neurons in culture for 48h with increasing concentrations of annonacin caused a dose dependent decrease in cell survival (A) and a redistribution of tau labelled with the AD2 antibody to the cell body (B). Cell survival (mean ± s.e.m. of 4 independent experiments) is presented as a percentage of the control value (658944 cells / 16 mm well). ***Significantly different than control values (p<0,001, one-way ANOVA followed by post hoc Dunnett test). C) Representative microscopic fields of striatal cultures treated with increasing doses of annonacin for 48h labelled with the anti-tau antibody AD2 (red). D) AT8, AT100 and AT270 (red). Antibodies raised against other phosphorylation sites on tau also evidence tau redistribution like AD2. DAPI (blue) labels nuclei. Scale bar 20µm (C and D).

3. DOES ANNONACIN INDUCE THE ACCUMULATION OF OTHER PROTEINS ASSOCIATED WITH NEURODEGENERATIVE DISEASES?

To investigate if the effect of annonacin is specific to the tau protein, annonacin-treated striatal cultures were immunolabelled with antibodies raised against proteins related to other human neurodegenerative diseases, such as α-synuclein, a major component of Lewy bodies in PD (Spillantini *et al.*, 1997) and the peptide β-amyloid, constitutive of the senile plaques seen in AD patients (Glenner and Wong, 1984; Masters *et al.*, 1985).

Although the antibodies recognized the proteins on western blots of rat brain extracts and identified Lewy bodies and amyloid plaques in the brains of patients with Parkinson's and Alzheimer's disease, respectively (not shown), no accumulation of α-synuclein or β-amyloid were detected in the cell bodies of annonacin treated striatal neurons under our experimental conditions.

This indicates that cytopathological effects of annonacin are specific to the tau protein among those that form the histopathological hallmarks of other neurodegenerative diseases.

4. ANNONACIN DOES NOT INDUCE AGGREGATION OF TAU.

Aggregation of the tau protein has been described for other human neurodegenerative diseases, such as the NFTs of patients with AD (reviewed by Goedert, 1993; Trojanowski *et al.,* 1993) or PSP (Chambers *et al.,* 1999) and insoluble aggregates of tau have also been found in the brains of patients with atypical parkinsonism in Guadeloupe (Caparros-Lefebvre *et al.,* 2002). These aggregates can be recognised with Thioflavin S, a marker for amyloid-like protein aggregates with a secondary β-sheet structure (Bancher *et al.,* 1989; Braak *et al.,* 1994). To know whether annonacin induced the formation of tau aggregates, we labelled annonacin treated cultures with the amyloid-binding dye Thioflavin S.

No Thoflavin S positive aggregates were observed (data not shown), suggesting that tau in annonacin-treated cultures, did not adapt a β-sheet secondary structure. To confirm this observation and determine ultrastructurally how tau is redistributed in the neurons of annonacin-treated cultures, we used immuno-electron microscopy analysis studying the distribution of tau labelled with AD2 in control cultures and cultures treated for 48h with 50nM of annonacin. Fig. II. 2. shows a representative neuron from each condition. In control cultures, tau labelling is abundant in the neurites and absent in the soma, whereas in the annonacin treated cultures, tau had almost disappeared from the neurites and was increased in the cytoplasm, where it remained dispersed and did not form aggregates.

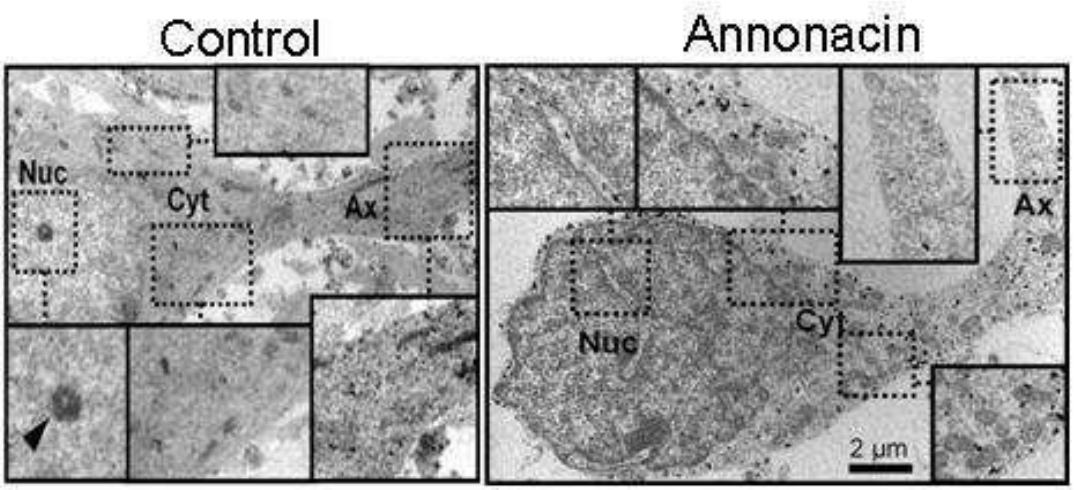


Fig. II. 2. **Tau does not form aggregates in annonacin treated cultures.** Tau was immunolabelled with the AD2 antibody and observed by electron microscopy in control cultures and cultures treated for 48h with 50nM of annonacin. No evidence of aggregation was seen, but rather an accumulation of the protein in the cytoplasm of the cell. No tau was found in the nuclei. Scale bar 2µm.

This series of experiments in a striatal cell culture model from rat E16,5 embryos, shows that annonacin-induced neuronal death is associated with the accumulation of hyperphosphorylated tau in the soma of striatal neurons in the form of pre-tangles (diffuse distribution in the cytoplasm). This mimics the pathology described on the island of Guadeloupe (Caparros-Lefebvre *et al.*,2002), where the striatum contains one of the neuronal populations that degenerate in the brains of the patients which was reproduced in rats after chronic intoxication with the complex I inhibitor (Champy *et al.*, 2004).

This cell culture model was used in further experiments to approach the question of how annonacin can induce such a redistribution of the tau protein.

B) INHIBITION OF COMPLEX I AS THE ULTIMATE CAUSE OF THE REDISTRIBUTION OF TAU.

Annonacin is an inhibitor of the mitochondrial respiratory chain at the level of complex I. Inhibition of complex I has two direct consequences, namely 1) an increase of free radical production, especially superoxides ($^{\cdot}O_2^{-}$) ions that are converted to hydrogen peroxide (H_2O_2) by the superoxide dismutase and transformed in the Fenton reaction into highly reactive hydroxyl radicals ($^{\cdot}OH$) (Fridovich, 1998), and 2) an ATP depletion. Both, oxidative damage and an energy deficit in the cell, could be the cause of the redistribution of tau in the presence of annonacin as a consequence of complex I inhibition. Therefore we have examined both possibilities.

1. ARE THE REACTIVE OXYGEN SPECIES, PRODUCED BY INHIBITION OF COMPLEX I RESPONSIBLE FOR THE ANNONACIN-INDUCED CHANGE IN THE DISTRIBUTION OF TAU?

It has been proposed that reactive oxygen species (ROS) may contribute to the ageing process and play an important role in mitochondrial pathology (Harman 1981; Berlett and Stadtman 1997). As mitochondria are the main consumers of molecular oxygen and the respiratory chain has been shown to generate large amounts of ROS in the presence of specific inhibitors (Cadenas and Boveris 1980; Turrens *et al.,* 1985), we wanted to know whether mitochondrial free radical production is implicated in the redistribution of tau.

Firstly, to determine if annonacin induced free radical production in striatal neurons, ROS levels were measured and visualised with the fluorescent dye DHR-123

(Dihydrorhodamine-123), a positively charged rhodamine derivative, which enters mitochondria and fluoresces when it is oxidised by free radicals. Striatal cultures where treated with 100nM of annonacin for 6h at DIV 5 or 6 and then incubated with DHR-123 for 30 min before quantitative analysis of individual neurons.

Annonacin induced an increase in DHR-123 fluorescence in striatal cultures as illustrated in Fig. II. 3A (Fig. II 3B). Annonacin increased by about 40% the intracellular production of ROS after 6h of intoxication with annonacin compared to controls. To reveal if the produced free radicals could be scavenged, two structurally different anti-oxidant agents, trolox 10 μM (a vitamin E derivative) and N-acetylcysteine 5 mM (NAC), were added approximately 1 h before the addition of annonacin. The pre-treatment with both ROS scavengers, reduced the ROS induced by annonacin to control levels (Fig. II. 3A,B).

To determine if ROS are responsible for annonacin-induced cell death and tau redistribution, striatal cultures were treated with 50nM of annonacin for 48h in the presence and the absence of these anti-oxidants and then analysed for cell survival and tau redistribution with the anti-tau antibody AD2. Neither of the scavengers were capable of preventing annonacin-induced cell death (Fig. II. 3C) or tau redistribution (Fig. II. 3D).

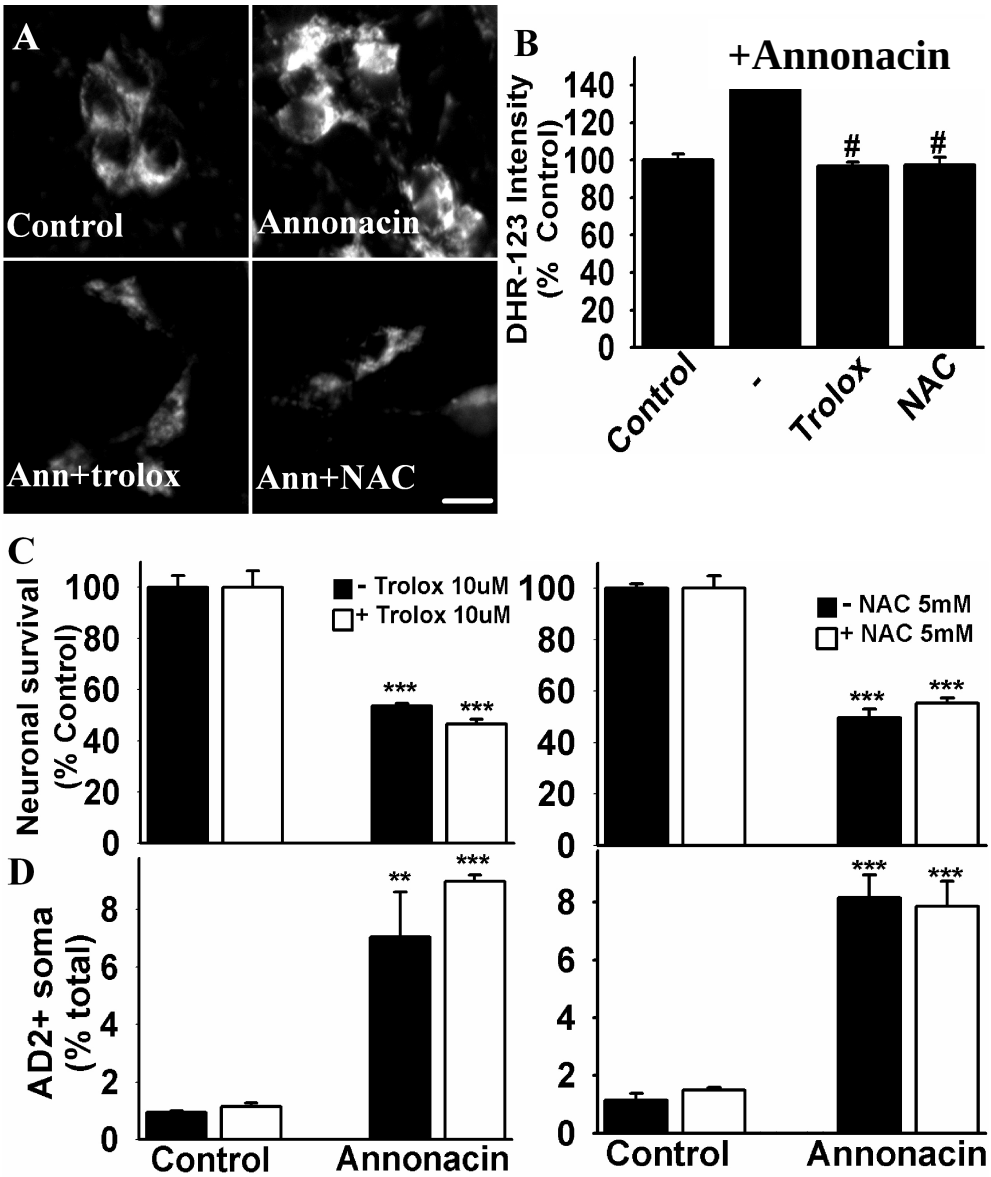


Fig. II. 3. Free radical production is not responsible for the cell death and tau redistribution caused by annonacin. A) Representative fields of DHR-123 labelling in control cultures and treated cultures with annonacin in the presence and the absence of two ROS scavengers (5mM NAC and 10μM trolox) for 6h. Scale bar, 20μm. B) Quantification of the intensity of DHR-123 in the presence and in the absence of ROS scavengers. The percentage is shown as the mean ±s.e.m. in respect to control levels. *Significantly different than control values ($p<0.05$, one-way ANOVA followed by Dunnett's test). # Significantly different to their respective control, but not significantly different to control values ($p<0.05$, one-way ANOVA followed by Dunnett's test). Neuronal survival (C) and $AD2^+$ neuronal cell bodies (D) in control and annonacin treated cultures after 48h in presence (open bars) or absence (black bars) of the anti-oxidant trolox (left) or NAC (right). *** Significantly different than control values ($p<0.001$, one-way ANOVA followed by Dunnett's test). ** Significantly different than control values ($p<0.01$, one-way ANOVA followed by Dunnett's test).

These results show that mitochondrial free radical production is increased by annonacin and that ROS levels can be decreased with anti-oxidants, but they do not play a role in either the cell death or in the redistribution of the tau protein caused by inhibition of complex I by annonacin in striatal neurons.

2. IS ATP DEPLETION RESPONSIBLE FOR THE EFFECTS OF ANNONACIN?

The inhibition of complex I implies the break down of the proton motive force ($\Delta\psi_H$) which is used by complex V to phosphorylate ADP to ATP, an energy source readily usable by cells. However, the cell has a second and less efficient anaerobic system for producing energy in the form of ATP, namely glycolysis, which transforms glucose into ATP in the cytoplasm, and is independent of the mitochondria. A previous study in mesencephalic cultures (Lannuzel *et al.*, 2003), showed that high extracellular glucose levels prevent the cell death induced by annonacin by activating glycolysis. We sought to protect our striatal cultures from tau re-location by the same means. Therefore, we increased the concentration of glucose in the medium from 250 µM, which is close to the extracellular levels in the brain (Fellows *et al.*, 1992; Netchiporouk *et al.*, 2001), to 50 mM throughout the exposure of the neurons to the toxin. As shown in Fig. II. 4A, the stimulation of glycolysis by high concentrations of glucose (50 mM) was sufficient to prevent cells death of striatal neurons and also the accumulation of tau in the soma (Fig. II. 4B). The effect of 50 mM of glucose on AD2 labelled striatal cultures treated with 50nM of annonacin is illustrated in Fig. II. 4C. These observations suggest that energy depletion is responsible for both of the effects annonacin.

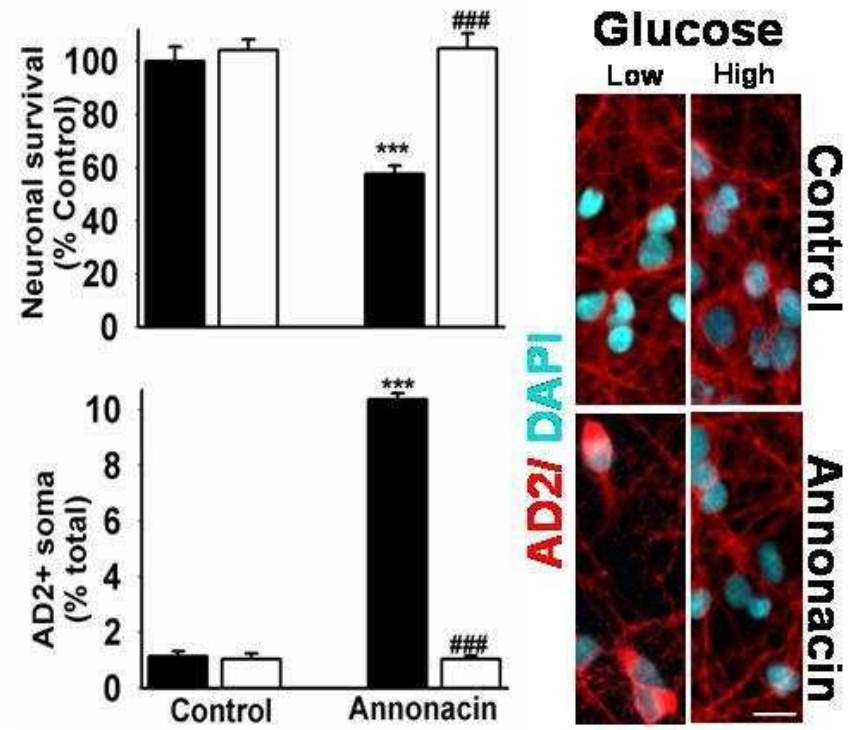


Fig.II. 4. Glucose prevents from annonacin-induced cell death and tau redistribution. Neuronal survival (A) and $AD2^+$ cell bodies (B) in control and annonacin treated cultures in presence of 250 µM (open bars) or 50mM (black bars) of glucose. *** Significantly different to control levels ($p < 0.001$ one-way ANOVA followed by Dunnett's test). ### Significantly different to their respective control but not significantly different to control levels ($p < 0.001$ one-way ANOVA followed by Dunnett's test). C) Representative fields of control cultures (top) and annonacin treated cultures (bottom) in presence of low (250µM) (left) or high (50mM) (right) amounts of glucose. Tau is labelled in red with the AD2 antibody and nuclei are visualised with DAPI staining. Scale bar 20µm.

3. DO OTHER ENERGY DEPLETING NEUROTOXINS INDUCE THE SAME EFFECTS AS ANNONACIN?

We tested the hypothesis of energy depletion as the ultimate cause of cell death and tau redistribution with other neurotoxins capable of depleting cellular ATP, such as MPP^+ (1-methyl-4-phenylperidinium), another complex I inhibitor, and 3-NP (3-nitroproprionic acid), an irreversible inhibitor of the complex II the respiratory chain.

Complexes I and II both provide reducing equivalents to the respiratory chain for oxidative phosphorylation.

MPP^+ is the active metabolite of MPTP (1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine), a meperidine analogue that induced parkinsonian syndromes in drug addicts (Langston *et al.,* 1983a, b). Since then, it has become the drug most commonly used to model PD in animals (reviewed by Przedborski and Vila, 2003). It is a complex I inhibitor that differs from annonacin in its molecular structure and binding site on complex I (Degli Esposti, 1998).

3-NP, an irreversible inhibitor of complex II (the second metabolic entry to the mitochondrial respiratory chain) is known to produce a very selective striatal degeneration and ATP depletion and is therefore used commonly to model Huntington's Disease (HD) (Borlongan, *et al.,* 1997).

After 6h of intoxication with 25 μM MPP^+ or 300 μM 3-NP, when the cells had not yet begun to show signs of degeneration, ATP levels were measured by chemoluminescence and were found to be significantly depleted (Fig. II. 5C). After 48h of treatment with increasing concentrations of MPP^+ (0, 10, 25 and 50μM) or 3-NP (0, 100, 300 and 1000 μM), there was a concentration dependent cell death (Fig. II. 5A) and a redistribution of tau to the cytoplasm of the cultured neurons as seen with annonacin (Fig. II. 5B). The concentration at which MPP^+ killed 50% of the striatal neurons was of 25μM, that is 500 times greater than the effective concentration of annonacin (50 nM). 3-NP was 50% effective with regard to cell death at 300μM, a concentration 6000 times greater, indicating that annonacin is a much more potent neurotoxin. Figure 5D illustrates the effect of both respiratory chain inhibitors at a 50% efficiency concentration on striatal neurons.

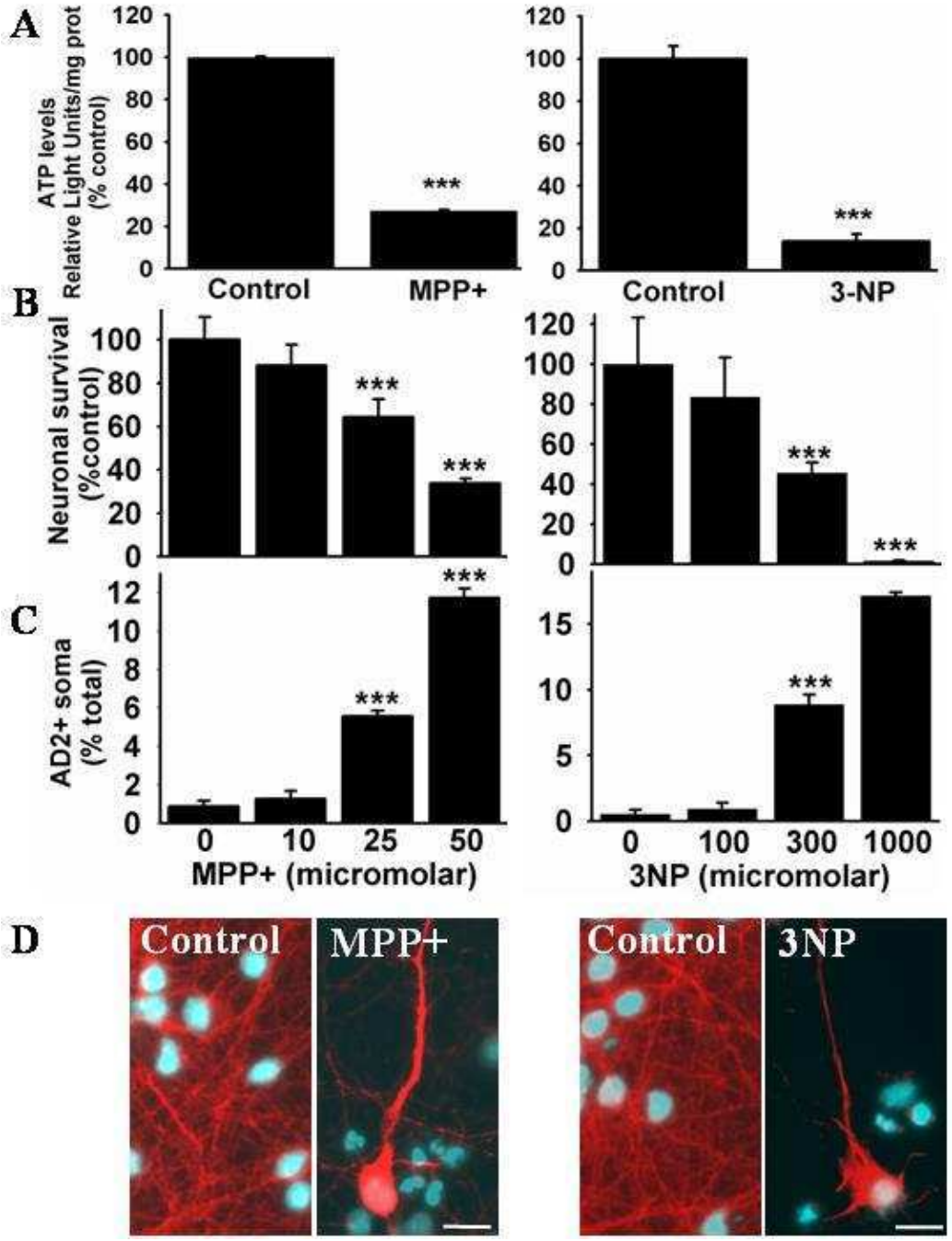


Fig. II. 5. The complex I inhibitor MPP$^+$ and the complex II inhibitor 3-NP also induce a dose-dependent neuronal death and redistribution of tau in primary cultures of rat striatum. A) ATP levels were measured by chemical luminescence in extracts of control cultures and cultures treated for 6h with the toxin. The measurements were normalised with the amount of protein in each extract. Neuronal survival (B) and AD2$^+$ cell bodies (C) are presented as the percent of control values. The mean ± s.e.m. of 4 different experiments is shown. ***Significantly different than control values ($p<0,001$, one-way ANOVA followed by post hoc Dunnett test). D) Representative fields of control and intoxicated cultures after 48h. Tau is labelled with the AD2 antibody (red) Nuclei are labelled with DAPI (blue) for nuclei. Scale bar 20μm.

These results show that inhibition of the respiratory chain, at the level of complex I with annonacin or with other inhibitors that induce a decrease in ATP levels induces both the displacement of tau from neurites to cell bodies and cell death. They also suggest that the aetiology of neurodegenerative disorders where tau is disregulated, may implicate a deficiency in energy metabolism.

The next question to be answered was, how tau is re-located in the soma of striatal neurons in presence of annonacin.

C) POSSIBLE MECHANISMS FOR THE REDISTRIBUTION OF TAU.

Tau is physiologically located mainly in the axons (Binder *et al.*, 1985), but under the effects of annonacin it is accumulated in the perikaryon of striatal neurons. This can be due to two main reasons. The first is a passive process related to the "dying back" mechanism where the axon of an unhealthy neuron degenerates progressively from the distal part and therefore tau may be "pushed" back into the soma. The second is an active process which involves the retrograde axonal transport. We have tried to distinguish between these two processes.

1. IS TAU IN THE SOMA BECAUSE THE NEURITES RETRACT?

Neuronal death in cell cultures is often preceded by neurite retraction as a self-destruct programme against an external insult (reviewed by Raff *et al.*, 2002). We therefore examined whether the redistribution of $AD2^+$ tau to the cell bodies of annonacin treated striatal neurons was secondary to or preceded neurite loss by evaluating the integrity of the neurites. For this, control and annonacin treated cultures were co-immunolabeled with the dendritic marker MAP-2 and the axonal marker beta-III-tubulin and then observed their behaviour with respect to that of tau labelled with the AD2 antibody. If the neuritic labelling disappeared together with the AD2, it would mean that neuritic retraction is responsible. If on the other hand, AD2 disappears first, this would be a sign that it occurs before neurite retraction.

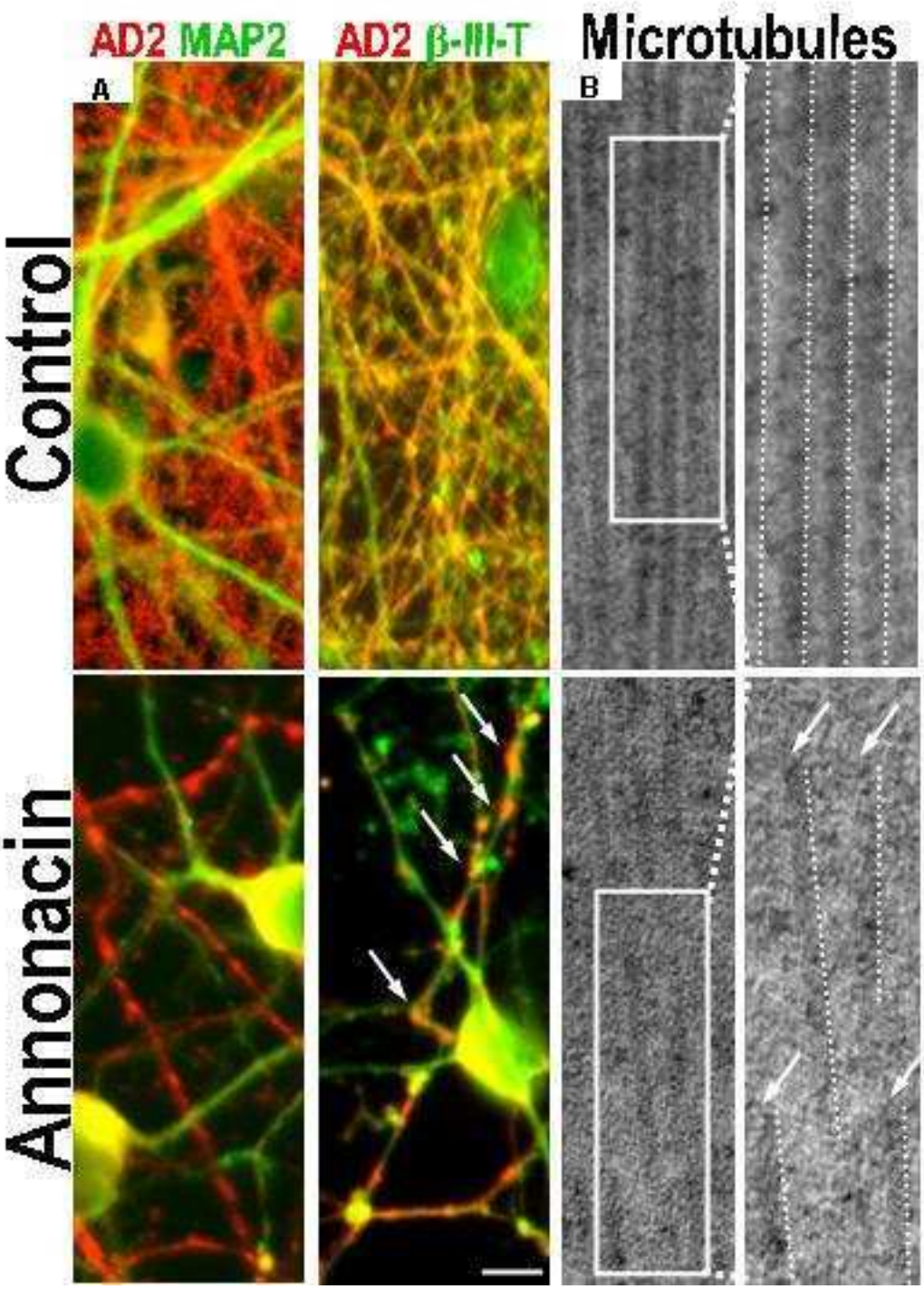
In control cultures, there was no co-localisation between MAP-2 and β-III tubulin confirming that they label in a mutually exclusive way dendrites or axons, respectively. In control cells, AD2 immunoreactivity colocalized with beta-III-tubulin but not with MAP-2 and was not present in cell bodies (Fig. II. 6A), indicating that tau is physiologically restricted to axons. In annonacin-treated cultures, the neuritic network was less dense than in control cultures and, in the remaining neurons, AD2 immunoreactivity acquired a discontinuous punctuate distribution in the neurites,

whereas beta-III-tubulin labelling remained continuous. These observations indicate that neuritic loss followed rather than preceded changes in the cellular tau localization in annonacin-treated cells.

2. DOES ANNONACIN ALTER THE MICROTUBULE NETWORK?

The best-known function of tau is the maintenance of microtubule stability and the polymerisation of tubulin which is necessary for cellular transport. We have shown that, after annonacin-treatment, tau is displaced from its physiological location and is hyperphosphorylated, a process that induces tau to loose its biological activity (Gong et al., 2005). To address the question of what happens to the microtubules after tau is misplaced, we examined them by electron microscopy (Fig. II. 6B). Microtubule profiles in neurites were profoundly altered in annonacin treated cells (50 nM, 48 h). In control cells, long segments of parallel microtubules were visible, whereas in annonacin treated cultures, the segments were much shorter and disorganised. The difference in length of the segments visualised in 8 neurites in each condition, was statistically significant (410±40 nm in control cells, 164±14 nm in annonacin-treated cells, $p < 0.001$).

Fig. II. 6. The redistribution of tau from the neurites to the cell bodies of annonacin-treated neurons precedes neurite retraction. A) Dendrites labelled with an antibody against MAP-2 (left, green) and axons with an antibody against β-III-tubulin (right, green). Colabelling with the anti-tau antibody AD2 (red) shows that tau co-localises with β-III-tubulin but not with MAP-2. In annonacin treated cultures (bottom), both MAP-2 positive and β-III-tubulin positive neurites were less dense and AD2 acquired a punctate structure (arrows), whereas both MAP-2 and β-III-tubulin labelling remained continuous. Scale bar 20µm. B) Electron microscopic photographs of control (top) and annonacin-treated (bottom) neurites (50 nM, 48 h). The controls have a long, continuous and parallel microtubular network while annonacin induces their breakdown rendering microtubules significantly shorter (410±40 nm in control cells, 164±14 nm in annonacin-treated cells, $p < 0.001$) and disorganised.



These experiments suggest that the effect of annonacin on tau destabilises the microtubule network before neurites retract. This raises a new question as to how cellular transport is affected.

D) MECHANISM OF MITOCHONDRIAL TRANSPORT IN PRESENCE OF ANNONACIN.

There is evidence that 1) there is an interaction between mitochondria and tau (Jung *et al.*, 1993) 2) that tau allows binding between microtubules and mitochondria (Sergeant *et al.*, 2005),and 3) that tau participates in mitochondrial transport (Stamer *et al.*, 2002). Therefore we asked if annonacin affects mitochondrial transport in the same way that it affects the distribution of tau.

1. ARE MITOCHONDRIA REDISTRIBUTED LIKE TAU IN ANNONACIN-TREATED CULTURES?

To observe the intracellular distribution of mitochondria, we labelled them by immunofluoresce with an antibody against cytochrome C, a protein associated with the inner membrane of the mitochondrion, in control and annonacin treated cultures.

In control conditions, we find mitochondria distributed throughout the entire cell with an equal intensity of labelling between the perikaryon and the neurites (Fig. II. 7), suggesting a homogeneous distribution of the organelle throughout the neuronal cytoplasm. In annonacin-treated cultures (50 nM, 48 h), however, they became concentrated in the cell body and were less abundant in the neurites (Fig. II.7), showing that mitochondria, like tau, are redistributed from neurites to the cell bodies of annonacin treated neurons.

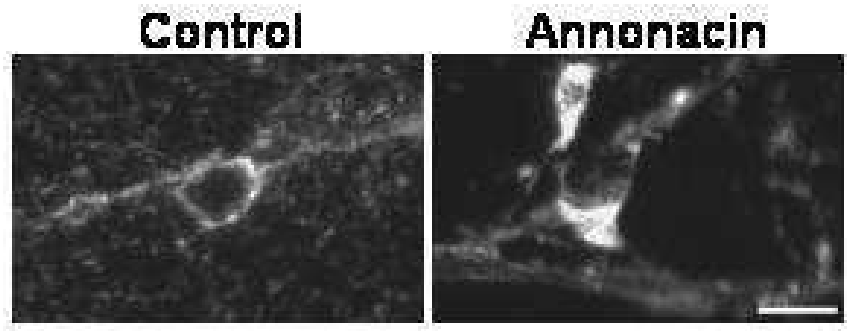


Fig. II. 7. Mitochondria are redistributed to the cell bodies of annonacin-treated neurons. Mitochondria were labelled with an antibody against Cytochrome C (CytC). In control cultures, CytC is distributed throughout the cell, but in annonacin treated cultures, CytC labelling increases in the cell bodies and decreases in neurites. Scale bar 20μm.

To verify if, in our experimental paradigm, there was an association between tau and mitochondria, we looked at the ultrastructural level in control and annonacin-treated neurons (50 nM, 48 h) immunolabelled with the anti-tau antibody AD2 (Fig. II.8). Electron microscopy confirmed the distribution seen with the antibody against Cyt C and allowed the quantification of this process (Table II.1). Analysis of twenty cells per condition showed that annonacin provoked the swelling of the cytoplasm, shown by an increase in the surface of the proximal segment and a significant increase in the number of mitochondria retrieved in the cell bodies. The percentage of mitochondria immunolabelled with the anti-tau antibody AD2, however, was not altered by annonacin treatment, suggesting that tau and mitochondria are associated and that their cytoplasmic localization is affected equally by annonacin (Table II.1). Figure 8 shows electron micrographs of representative control and annonacin treated cells.

These findings suggest that annonacin has the same effect on tau and on mitochondria, and that they are associated.

	Axon Hill Area (μm^2)	Number of mitochondria	AD2-labelled mitochondria	% AD2-labelled mitochondria/free mitochondria
Control	27,20±3,10	18,55±1,73	4,55±0,43	27.1±2.8
Annonacin (50nM)	39,20±2,92	28,45±3,12	6,80±0,47	26,59±1,89
P <	0,01	0,01	0,001	0,729

Table II.1. **Mitochondria and tau are associated in striatal neurons.** Annonacin (50 nM, 48 h) induces a significant swelling of the axon hill of the striatal cultures, a significant increase in the amount of mitochondria in the axon hill, but the percentage of AD2-labelled mitochondria does not vary from control to annonacin treated cultures. Values are expressed as the mean ± s.e.m. of 20 cells analysed per condition. P, analysis by t-test.

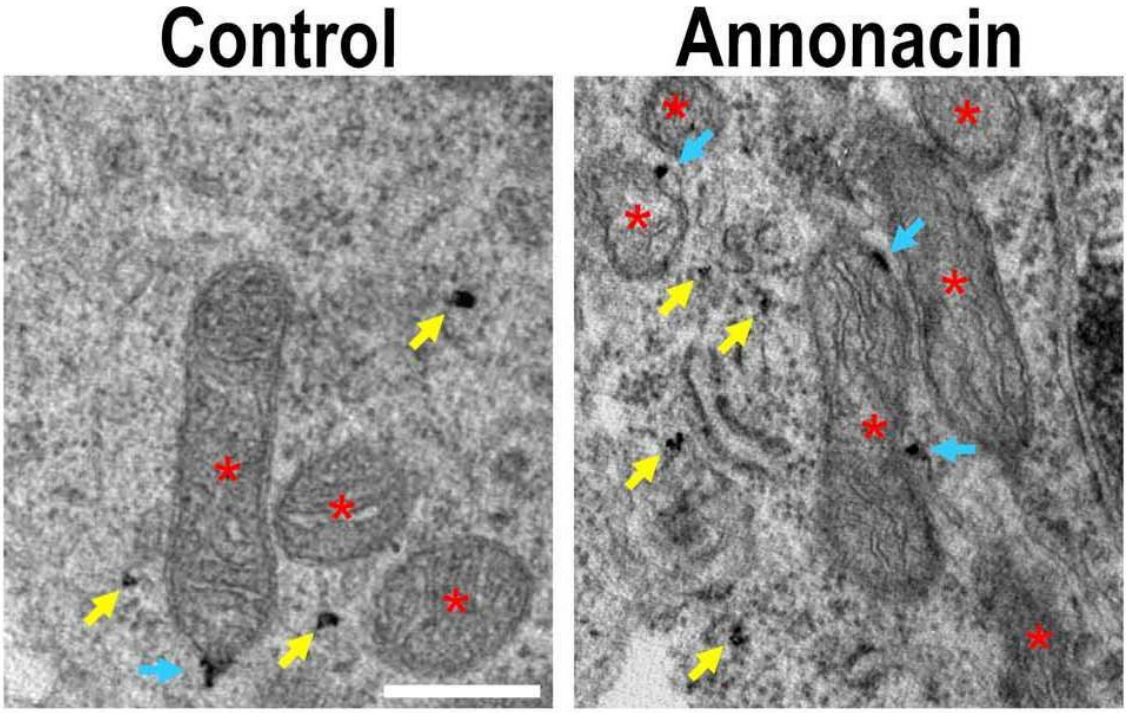


Fig. II. 8. **Association between tau and mitochondria.** In the axon hill of cultured neurons, we analysed mitochondria (red asterisks) and AD2-labelled tau (arrows) that was either free in the cytoplasm (yellow arrows) or attached to the outer membrane of mitochondria (turquoise arrows). In annonacin treated cultures, there were more mitochondria and more AD2+ tau, but the relation AD2-labelled mitochondria to free mitochondria was not affected. Scale bar 500 nm.

2. ARE MITOCHONDRIA RETROGRADELY TRANSPORTED IN THE PRESENCE OF ANNONACIN?

We studied axonal transport because it has been shown that hyperphosphorylation of tau causes inhibition of axonal transport in diseases like AD (Smith *et al.*, 2002; Takeda *et al.*, 2000; Mandelkow *et al.*, 2003; 2004; Liu *et al.*, 2005). We used mitochondria as a marker for transport because their transport depends on microtubules (Grafstein and Forman, 1980; Hollenbeck 1996), because they are distributed like tau in annonacin-treated neurons.

To determine whether the Annonacin-induced redistribution of mitochondria was due to a disruption of cellular transport, we used time-lapse video microscopy to follow mitochondria labelled with an auto-fluorescent green marker (Mitotracker) that is taken up by the mitochondria of living cells. Striatal neurons were plated at low density (9×10^5 cells/16mm well) to permit visualisation of individual neurites and images where taken at DIV5 in 5 different planes on the z axis every 10 min for 6 hours starting from the addition of annonacin, which was used at a high concentration (50nM) to accelerate its effects. We also used bright field microscopy to verify the morphology of the neurons before and after the treatment.

Fig. II. 9A shows bright field images of a control and an annonacin treated neuron at the beginning (t0) and at the end of the treatment (t360). After 6h control cells maintain their original morphology, whereas annonacin treated neurons have lost their neurites. Fig. II. 9B shows the position of mitochondria at different time points. Mitochondria in control cultures move in all directions, anterogradely towards the nerve terminal and retrogradely towards the soma. However, in annonacin-treated cultures, the mitochondria in the neurites are rapidly after addition the 50 nM annonacin transported into the perikaryon and accumulate there, before signs of degeneration appear at 180 min of treatment.

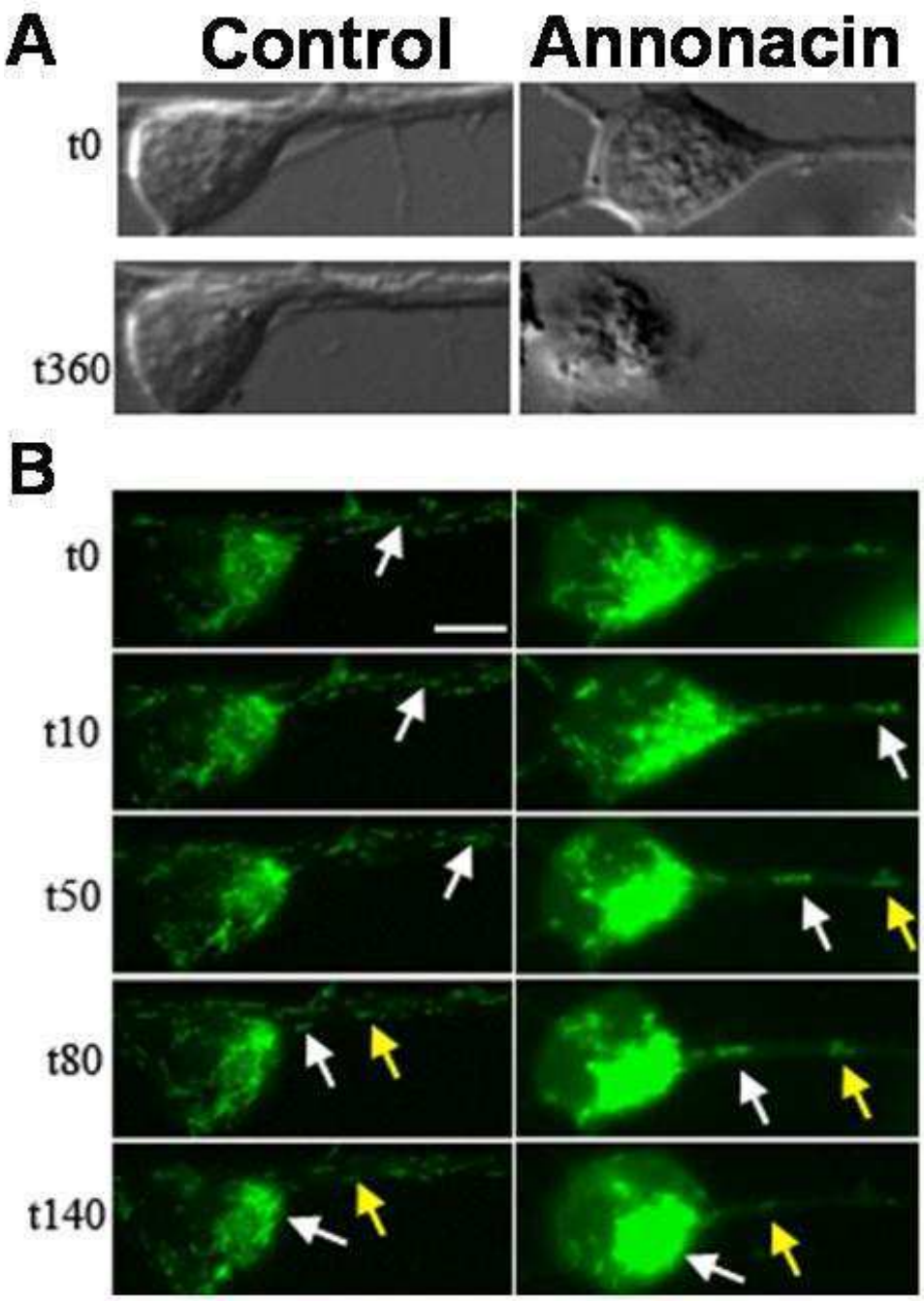


Fig. II. 9. Annonacin induces a rapid clustering of mitochondria in the perikarion. A) Bright field images of representative neurons in a control and an annonacin-treated culture of striatal neurons, a concentration of 50nM was used to induce the neurodegenerative process. B) Time lapse photos were taken every 10 minutes over a 6h period. Images of control and annonacin-treated cultures were taken at the beginning of annonacin treatment (t0) and 10, 50, 80 and 140 min later. Arrows show both retrograde and anterograde movement of mitotracker-labelled mitochondria in a control neuron and exclusively retrograde movement of mitochondria in an annonacin-treated neuron. Scale bare 5µm.

3. IS THE RETROGRADE TRANSPORT OF MITOCHONDRIA IN ANNONACIN-TREATED NEURONS DEPENDENT ON MICROTUBULES?

Taxol (paclitaxel), is a pharmacological agent commonly used in the treatment of cancer because it blocks microtubule dynamics by stabilizing GDP-bound tubulin and by binding to the N-terminal of β-tubulin in the microtubule (Rao *et al.*, 1994, 1995; Nogales *et al.*,1995). Thereby, taxol stabilizes the microtubule network and displaces tau from the microtubules (Samsonov et al., 2004). We have used it to determine whether these effects of taxol alter the toxicity of annonacin.

We first determined the concentration of taxol that was subtoxic to neurons in our culture system (5nM, data not shown). Control and annonacin-treated striatal cultures were followed by videomicroscopy, in the absence or the presence of 5nM of taxol for 6h. Figure II-10 shows the movement of mitochondria in the neurites of neurons that are representative of each experimental condition. In taxol treated cultures, with and without annonacin, the movement of mitochondria (labelled with different colour arrows) was totally arrested, suggesting that displacing tau from the microtubules with taxol prevents both retrograde and anterograde transport of mitochondria in striatal neurons. Next we addressed the question if taxol can also prevent the cell death induced by annonacin?

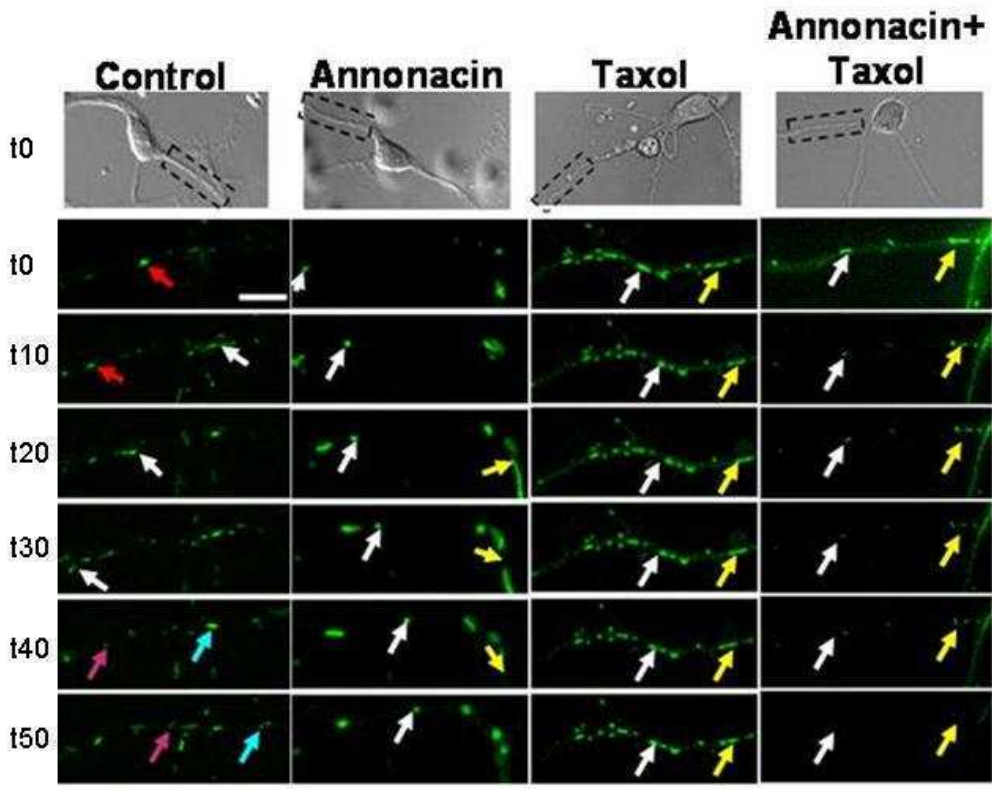


Fig. II. 10. Treatment of striatal cultures with subtoxic concentrations of taxol protects from annonacin induced mitochondrial redistribution. A) DIC (Diferential Interference Contrast) images of cultured neurons taken at t=0 min in all treatments (control conditions; 50 nM Annonacin; 5nM Taxol; 50 nM Annonacin plus 5nM Taxol). B) Distribution of mitochondria labelled with the fluorescent dye mitotracker (green), shown at different time points (0, 10, 20, 30, 40 and 50 min) after the addition of respective substances. In control cultures the movement is bidirectional. In annonacin-treated cultures, mitochondria only move towards the cell body. In taxol treated cultures in presence and absence of annonacin, mitochondria present no movement. Individual mitochondria are labelled with differently coloured arrows. Scale bare 5μm. Photos where taken every 10min for 6h.

4. CAN TAXOL PREVENT ANNONACIN-INDUCED NEURONAL CELL DEATH?

In many tauopathies, like AD, it is thought that tau accumulation in cell soma is the ultimate cause of cell death (Arriagada *et al.* 1992). Consistent with this hypothesis, a number of studies *in vitro* (Fath et al, 2002; Shimura *et al.*, 2004), in animal models (Wittmann *et al.*, 2001; Jackson *et al.*, 2002; Kraemer *et al.*, 2003; Andorfer *et al.*, 2003) and in patients with certain tau mutations (Bird *et al.*, 1999) show that the increase in hyperphosphorylated soluble tau in the cell body is deleterious. Therefore, we wanted to determine, whether if in our experimental model, preventing hyperphosphorylated tau from being re-localised from the axon to the perikaryon in annonacin-treated cultures, would promote neuronal survival.

To do so, cultures were treated with taxol (5 nM) 1h before annonacin (50 nM) at DIV5 and analysed for cell survival and tau redistribution after 48h of treatment.

As shown in figure II. 11A taxol induced a hypertrophy of neurites (arrows), compared to neurites in taxol-free cultures. In annonacin-treated cultures, the addition of taxol prevented the redistribution of phosphorylated tau to the cell bodies of the neurons (Fig. II. 11A). Annonacin-induced cell death, however, was not prevented by taxol (Fig. II. 11B).

These findings suggest that neither the effect of annonacin on tau, nor the destabilisation of the microtubule network is responsible for cell death in annonacin treated neurons. The redistribution of tau, which results from microtubule destabilisation, is therefore an epiphenomenon of the energy depletion caused by complex I inhibition and is not directly instrumental in neuronal death.

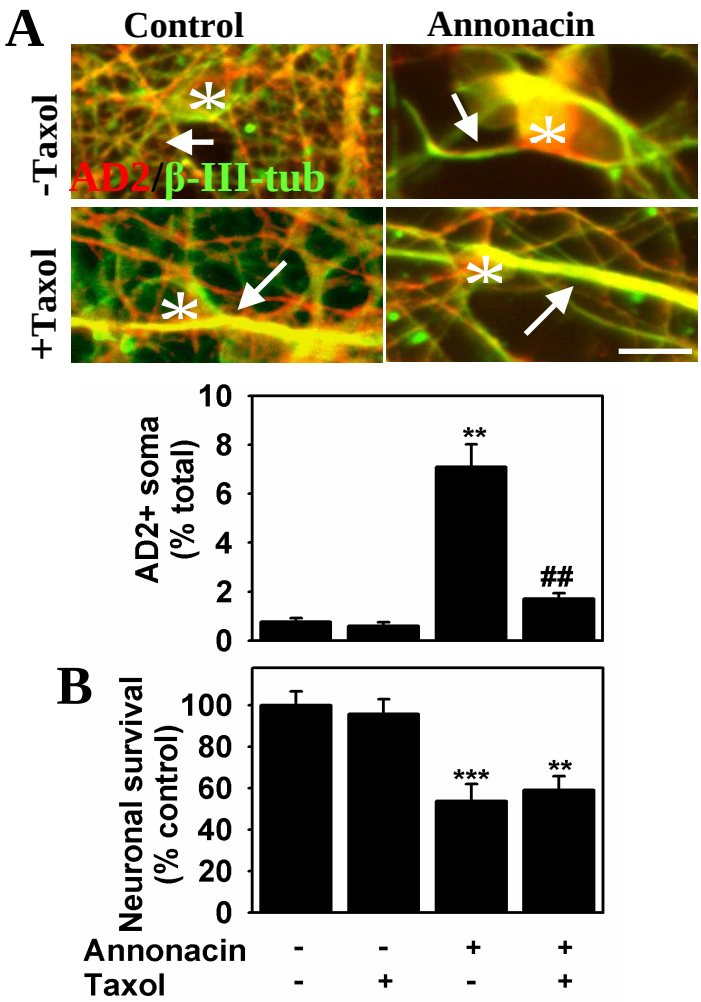


Fig. II.11. The redistribution of tau is not responsible for annonacin-induced neuronal death. A) The microtubule network in axons labelled with an antibody against β-III-tubulin (green) and tau visualised with the AD2 antibody (red) were observed in control and annonacin (50 nM, 24 h) treated cultures in the absence and the presences of taxol (5 nM). Taxol alone induced a hypetrophy of axons (arrows) and it also prevented the redistribution of tau to cell bodies of annonacin-treated cultures. Scale bar 20 µm. B) The quantification of neuronal survival showed that preventing the redistribution of tau to the perikaryon of annonacin-treated neurons with taxol did not prevent neuronal death. Values shown are the mean ± s.e.m. of three independent experiments, analysed by ANOVA, followed by bonferroni's test (** $p<0.01$, *** $p<0.001$ vs. controls; ## $p<0.01$ vs. Annonacin alone.

III. DISCUSSION.

A) ANNONACIN CAUSES A TAU-PATHOLOGY BY DEPLETING CELLULAR ENERGY.

In this study, we have shown that low nanomolar concentrations of annonacin cause neuronal cell death and the redistribution of highly phosphorylated tau protein from the axon to the cell body of striatal neurons *in vitro* as happens in the brains of the three patients with a PSP-like disease from Guadeloupe that came to autopsy. The results presented in this thesis support the hypothesis that exposure to the environmental toxin annonacin might be implicated in the aetiology of the atypical parkinsonism in Guadeloupe.

Under physiological conditions, the primary function of the axonal microtubule-associated protein tau is to promote polymerisation of tubulin monomers into microtubules and regulate transport along the microtubule network (Mandelkow and Mandelkow, 1998). These functions are controlled by phosphorylation at its over a dozen phosphorylation sites (Buée *et al.*, 2000). Accumulation of phospho-tau in the cell body, however, is the defining neuropathological hallmark of a group of heterogeneous neurodegenerative disorders termed tauopathies, exemplified by PSP, frontotemporal dementia with parkinsonism linked to chromosome 17 (FTDP-17), the parkinsonism–dementia complex of Guam and atypical parkinsonism in Guadeloupe (Braak *et al.*, 1994; Delacourte and Buee, 2000; Goedert *et al.*, 2000; Caparros-Lefebvre *et al.*, 2002; Lee *et al.*, 2005).

In tauopathies, somatic phospho-tau may be present in an un-aggregated or in an aggregated amyloid-like form (Bancher *et al.*, 1989; Braak *et al.*, 1994; Komori, 1999). In our experimental paradigm, neither electron microscopy nor thioflavin S staining, which recognises amyloid-like β-sheet secondary structure, provided evidence of tau aggregates in annonacin-treated neurons. The fact that tau was un-aggregated in cell bodies of our striatal neurons in culture may have many explanations: (1) rodents only have the 4-repeat tau, which is more stable and therefore less prone to aggregate than 3-repeat tau (Goedert *et al.*, 1994; Götz *et al.*, 1995); (2) our conditions might not be chronic enough to induce the structural modifications of tau needed for it to form

aggregates; (3) to form pathological PHF (Paired Helical Filaments) tau may need to undergo further posttranslational modifications such as further hyperphosphorylation, glycation or nitration(Buée *et al.,* 2000). However, in our conditions, annonacin induced a pre-tangle form of un-aggregated somatic tau accumulation, as it has been reported in the brains of the three patients with atypical Guadeloupean parkinsonism that have been autopsied (Caparros-Lefebvre *et al.,* 2002).

The redistribution of phospho-tau induced by annonacin is mediated by complex I inhibition. The major cellular consequences of complex I inhibition are increased ROS production and reduced ATP production (Höglinger *et al.,* 2003). Stabilisation of cellular ATP levels by anaerobic glycolysis stimulated by an excess of glucose prevented the annonacin-induced tau-pathology. This and the inability to prevent annonacin-induced somatic redistribution of phospho-tau with antioxidants (trolox or NAC), suggests that this phenomenon is a consequence of cellular energy depletion. This conclusion is supported by the observation that somatic redistribution of phospho-tau also occurred after inhibition of the respiratory chain with other compounds that inhibit complex I (MPP^+) or complex II (3-NP), all of which result in reduced ATP levels. Further support for this interpretation comes from the observation that cultured neurons transfected with the NADH-quinone-oxidoreductase (NDI1) of Saccharomyces cerevisiae, which can restore NADH oxidation in complex I-deficient mammalian cells (Seo *et al.,* 2000; 2002) were significantly protected against annonacin-induced ATP depletion, neuronal death and redistribution of phospho-tau (Khondiker *et al.,* submitted).

Consistent with these observations, accumulation of the tau protein with MPP^+ was observed in other culture models (Song and Ehrich, 1998) and upregulation and phosphorylation of tau with MPTP, the precursor of MPP^+, have previously been detected *in vivo* in the substantia nigra of mice (Miller and Sheetz 2004; Smith *et al.,* 2003). Furthermore, in rats with profoundly reduced cerebral ATP levels after treatment with the complex I inhibitor rotenone, accumulations of phospho-tau were seen in neural cells, some of which were stained by thioflavine S and Gallyas silver impregnation and were fibrillary in nature as shown by electron microscopy (Höglinger *et al.,* 2005). The concept that ATP depletion can lead to the somatic accumulation of phospho-tau is particularly intriguing, since deficits in cellular energy metabolism have

been described in sporadic human tauopathies (Foster *et al.*, 1988; Brooks *et al.*, 1994; Martinelli *et al.*, 2000; Swerdlow *et al.*, 2000; Albers *et al.*, 2001).

B) POSSIBLE MECHANISMS OF ACTION OF ANNONACIN.

Annonacin causes cell death and redistribution of phospho-tau. The question arises by which mechanisms ATP depleting neurotoxins, such as annonacin, lead to somatic accumulation of phospho-tau. Our work demonstrated that annonacin caused an immediate backward movement of mitochondria from neurites to accumulate in the soma. This observation is consistent with previous work showing that other drugs inducing damage to mitochondria, such as depolarisation of the mitochondrial membrane potential or inhibition of ATP production, lead to an increase in retrograde transport of mitochondria (Miller and Sheetz, 2004), to perinuclear clustering (De Vos *et al.*, 1998; Desagher and Martinou, 2000) and to their elimination by autophagy (Lemasters *et al.*, 1998; Xue *et al.*, 2001).

Microtubules serve as tracks for the movement of mitochondria (Morris and Hollenbeck, 1995). Microtubule-associated proteins such as tau stabilise the microtubule tracks and tau-phosphorylation regulates the transport of mitochondria along microtubules (Mandelkow *et al.*, 2004; Tatebayashi *et al.*, 2004). Several pieces of evidence point to an implication of tau in the backward transport of damaged mitochondria. 1) Tau plays a role in the interaction between microtubules and mitochondria (Jung *et al.*, 1993). 2) Overexpression of tau is sufficient to induce a retrograde transport of mitochondria from the neurites to cluster near the nucleus (Ebneth *et al.*, 1998; Trinczek *et al.*, 1999; Stamer *et al.*, 2002). 3) A high percentage of the mitochondria clustered in the soma in annonacin-treated cultures appeared to have phospho-tau attached to their outer membranes. 4) Treatment with taxol, a drug stabilising microtubules, but displacing tau from microtubules (Samsonov *et al.*, 2004), blocked both retrograde transport of mitochondria and somatic accumulation of phospho-tau. These arguments suggest a model in which annonacin-induced damage to

mitochondria leads to their retrograde, tau-dependent transport to the soma for repair or elimination. The resulting depletion of both tau and mitochondria in neurites are destabilisation of microtubules and further neuritic ATP depletion, subsequently causing impairment of axonal transport.

Consistent with our work, deleterious effects on microtubule stability have also been reported with other complex I inhibitors such as rotenone (Ren *et al.*, 2005) and MPP^+ (Cappelletti *et al.*, 1999). Destabilisation of the microtubule network and alteration of the microtubule dynamics are considered as consequences of the decreased ability of hyperphosphorylated tau to bind to the microtubules and has been reviewed by (Brandt *et al.*, 2005). These data suggests a link between complex I inhibition, tau redistribution and microtubule destabilisation, but further work is required to validate this model.

Cellular tau pathology has great significance for the dysfunction or degeneration of neurons, since the number of neurons containing abnormal accumulations of phospho-tau in the cell body has been closely correlated with behavioural deficits in Alzheimer's disease (Arriagada *et al.*, 1992) and in a transgenic mouse model expressing human tau with the P301L mutation that causes FTDP-17 (Santacruz *et al.*, 2005). It is already clear from rare genetic diseases, that mutations in the tau gene causing dysfunction of the tau protein is in itself sufficient to cause neurodegeneration (Goedert *et al.*, 2000, Lee *et al.*, 2005). Intriguingly, in a transgenic mouse model of a tauopathy, based on overexpression of the shortest human brain tau isoform, the microtubule-stabilising drug taxol has been shown to reverse fast axonal transport deficits by functionally substituting for tau, which is sequestered into inclusions (Zhang *et al.*, 2005). In the annonacin-induced model of a sporadic tauopathy, taxol prevented the redistribution of phospho-tau and mitochondria, but not the cell death induced by annonacin, suggesting that, in this rather acute model, the redistribution of phospho-tau to the cell body is not involved in either neuroprotective or neurodegenerative pathways. It may rather be considered to be a stigma characteristic of neuronal death induced by energy failure.

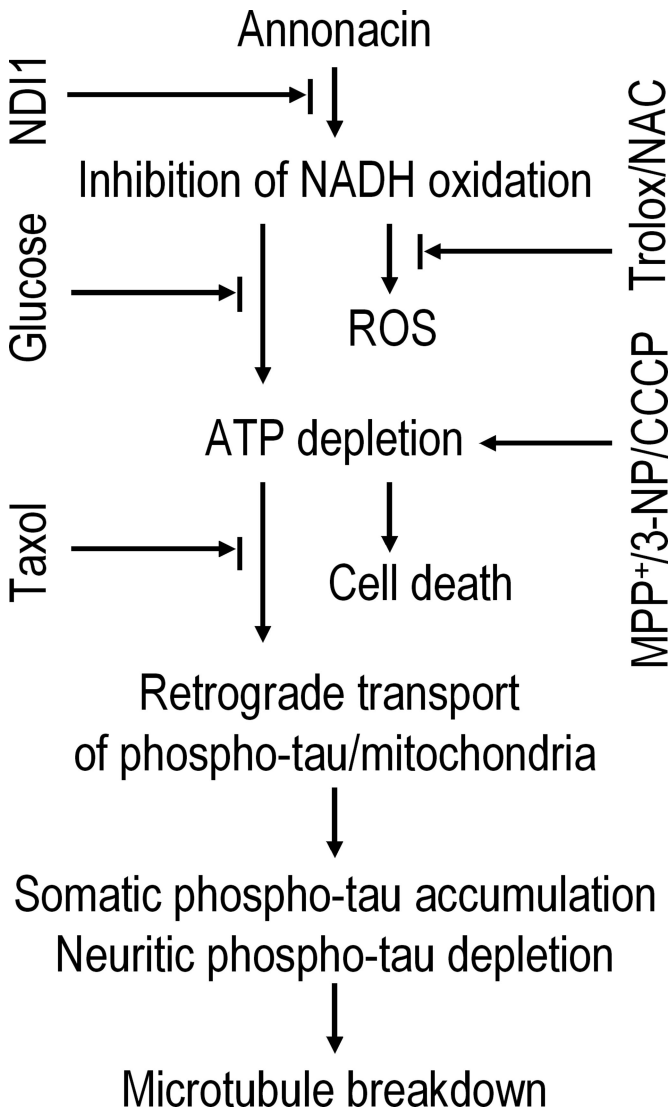


Figure III. 1. The sequence of pathological events induced by annonacin in primary cultures of striatal neurons. Annonacin inhibited the NADH-quinone-oxidoreductase activity of mitochondrial complex I leading to increased ROS and decreased ATP production. The latter appears to be responsible for the retrograde transport of phospho-tau from neurites to the cell soma and, consequently, microtubule breakdown, since the antioxidants trolox and NAC had no effect on cell death or the distribution of phospho-tau, whereas increasing ATP levels by the expression of the yeast NADH oxidase NDI1 or the stimulation of anaerobic glycolysis with high glucose concentrations prevented both cell death and the redistribution of phospho-tau. ATP depletion induced by the respiratory chain inhibitors MPP^+ and 3-NP or by the mitochondrial uncoupler CCCP mimicked annonacin-induced cell death and redistribution of phospho-tau. Taxol prevented the redistribution of phospho-tau and mitochondria, but not the cell death induced by annonacin, suggesting that, in this experimental model, the redistribution of phospho-tau to the cell body is not involved in either neuroprotective or neurodegenerative pathways. It may rather be considered to be a stigma characteristic of neuronal death induced by energy failure.

C) ADVANTAGES AND LIMITS OF OUR MODEL.

Our model of annonacin-treated primary cultures of rat striatum is very interesting, because to date there has been no model, neither *in vivo* nor *in vitro*, for PSP. Therefore, it represents a novel and valid tool to learn about the pathophysiology of pure tauopathies, like PSP. The major advantages of this novel model are, 1) that it relies on brain tissue of wild-type rodents and a natural neurotoxin, 2) that the model easily allows mechanistic studies of pathophysiological processes 3) that it can be easily used to screen pharmacological agent and 4) that results in this in vitro system can be obtained relatively quickly. As all models, there are also some limits to our model, like the absence of the normal cellular environment in an integrated in vivo system. Unfortunately we cannot keep the cells alive *in vitro* for long periods of time, therefore no chronic treatment can be studied. Therefore, the further development of the annonacin-based in vivo model (Champy et al., 2004) is urgently warranted.

D) IMPORTANCE OF OUR FINDINGS.

There is evidence suggesting impairment of mitochondrial energy metabolism in PSP (Albers and Beal, 2002): 1) Reduced cerebral glucose and ATP metabolism have been shown in functional imaging studies in PSP patients (Forster *et al.*, 1988; Brooks *et al.*, 1994; Martinelli *et al.*, 2000). 2) Cybrid cells harboring mitochondrial genes from PSP patients have decreased ATP levels and complex I activity (Swerdlow *et al.*, 2000; Albers *et al.*, 2001; Chirichigno *et al.*, 2002). These clinical observations suggest that mitochondrial dysfunction might also play a role in the aetiology of PSP. In summary, these data suggest that mitochondrial dysfunction, particularly complex I inhibition, may contribute crucially to the aetiology of PSP. This concept is strengthened by the experimental work presented in this thesis. This body of evidence provides the first pathophysiological concept of PSP and offers the first therapeutic target, namely mitochondrial energy production, that might help to reduce the burden PSP imposes on

patients, caregivers, and society. Moreover, both the *in vivo* and *in vitro* models of atypical parkinsonism of Guadeloupe demonstrate the possible implication of annonaceous acetogenins in the aetiology of the disease and offers the possibility of eradicating the disease by preventive health care measures. In the rat it is known that annonacin penetrates, because of its lypophilic chain, into the brain parenchyma where it is accumulated, inhibits mitochondrial complex I and decreases ATP levels (Champy *et al.,* 2004), but nothing has been shown in the brains of patients. No study has been made about the bioavailability of annonacin or any other known acetogenin, nor about how they are metabolized (or even if they are) in the organism. The only strong piece of evidence we have is that the intake of *Annonaceae* products which contain a cocktail of acetogenins (principally annonacin), alkaloids and maybe other neurotoxic substances, has been related to parkinsonian symptoms characteristic from the island of Guadeloupe. This has been validated experimentally with annonacin which is capable of inducing widespread degeneration in an organism (Champy *et al.,* 2004), in mesencephalic cultures (Lannuzel *et al.,* 2003) and tau pathology *in vitro* as has been described in the neuropathological examinations of three patients that came to autopsy (Caparros-Lefebvre *et al.,* 2002). But this does not constitute a conclusive proof that the regular consumption of annonaceous food products causes the disease, although the recovery of the few patients that had arrested the intake of these fruits is encouraging (Caparros-Lefebvre *et al.,* 2002) In terms of public health, the consumption of these products should probably be discouraged. The gradual disappearance of the disease would constitute the best proof of the toxic origin of the disease, and obviate the need for therapeutic strategies. This thesis not only helps to understand a bit more the disease on the island of Guadeloupe and maybe that in New Caledonia (Angibaud *et al.,* 2004) thought to be related with the consumption of *Annonaceae* products, but it also contributes to the understanding of possible mechanisms by which a large group of molecules similar to the annonacin (functionally or structurally), can affect the normal functioning of the cell and induce harmful effects. This is important to better understand the aetiology of many neurodegenerative processes where mitochondrial dysfunction and energy depletion are involved.

E) FUTURE APPLICATIONS OF THIS MODEL.

Our model of annonacin-treated primary cultures of rat striatum has successfully mimicked the hallmarks of a sporadic disease in the geographically isolated Caribbean island of Guadeloupe. Therefore it can be used to screen other potential natural toxins in our environment, as candidates for a putative role in sporadic tauopathies. A large number of compounds with similar molecular properties as annonacin, lypophilic complex I inhibitors of natural origin, are found in edibles and in bacteria also in moderate climate zones. . Kinetic studies suggest that these inhibitors can be grouped into two (Friedrich *et al.,* 1994), or even three (Degli Esposti *et al.,* 1994) classes, represented by piericidin A (class I/Atype), rotenone (class II/B-type), and capsaicin (C-type), respectively. Such substances may be screened in the model presented in this thesis for putative implication in the etiology of sporadic tauopathies.

Lannuzel *et al.,* (2003), demonstrated that cell death was arrested when annonacin was withdrawn from the culture medium at an early time point, and a few younger patients that eliminated *Annona muricata* products from their diet experienced a decrease in their parkinsonian symtoms (Caparros-Lefebvre *et al.,* 1999). Therefore, it could be interesting to study *in vitro* the process by which dying cells recover, study the restoration of the ATP levels, the localisation and the phosphorylation state of the tau protein and the movement of the mitochondria after annonacin withdrawal to better understand how annonacin induces cell death in cultured neurons and how a recovery from such an insult may be enforced therapeutically.

The model used in this thesis was a pure neuronal system, and lacked the glial cells (astrocytes, microglia) that constitute the normal environment of the neurons. The next step towards a detailed understanding of the annonacin-effects would therefore be to study the influence of annonacin on striatal neurons in their physiological cellular environment, to determine whether the presence of the glia, known to have protective as well as deleterious effects (Iwata-Ichikawa *et al.,* 1999; Mogi *et al.,* 1996; reviewed by Hirsch, 2000) protect from or exacerbate annonacin toxicity.

This model for sporadic tauopathies can also be used to screen potential drugs that might interfere with the mechanism of action of energy depleting neurotoxins (e.g.

Coenzyme Q10), or that affect microtubule dynamics. Other drugs of therapeutic interest are those that act on the phosphorylation state of the tau protein.

Finally, this model of an environmentally-caused tauopathy provides for the first time the possibility to study the molecular basis of the interaction of genetic predisposition and environmental triggers in the pathophysiology of sporadic tauopathies. As described in detail in the introduction of this thesis, there seem to be a variety of genetic predispositions for sporadic tauopathies, such as the tau haplotype H1. In recent years, although we have considerably increased our knowledge about the mechanisms of neuronal cell death due to particular genetic backgrounds, systematic studies on the role of gene-toxin interaction are a minority. Therefore, we have now the unique possibility to determine whether there is a pathogenetic interaction between the exposure to complex I inhibitors and the expression of FDTP-17 tau mutations or of the H1 tau haplotype or the 4R tau variant in cell culture systems.

F) CONCLUSION.

The present work shows that very low concentrations of annonacin, a natural complex I inhibitor of the respiratory chain, three and four orders of magnitude lower than MPP^+ and 3-nitroproprionic acid, respectively, can induce a tau pathology *in vitro*, due to cellular energy depletion, similar to what is seen in the postmortem brains of patients with atypical parkinsonism in Guadeloupe and in the brains of rats infused with annonacin. Thus, the present study suggests that environmental factors leading to cerebral energy depletion might also be an important aetiological factors in other sporadic tauopathies and therefore presents them as possible therapeutic targets to eradicate this disease, the atypical parkinsonism found in New Caledonia (which is associated with consumption of *Annonaceae* products as well) and possibly other neurodegenerative diseases with similar aetiologies.

IV) MATERIALS AND METHODS.

A) CELL CULTURE.

All the experiments have been done using an *in vitro* model of primary cultures of embryonic rat striatum treated with an antimitotic agent to prevent glial proliferation and thus to obtain an almost pure neuronal system.

1. PREPARATION FOR THE DISSECTION.

Since neurons do not attach directly to plastic, the day before the dissection, the 24 well plates (Costar), where the cells were going to be cultured, were coated overnight with 1 mg/ml polyethyleneimine (PEI) (Sigma), a cationic polyelectrolyte with an average molecular weight of 10,000 g/mole and one protonizable amine group (NH) per monomer unit, to favour attachment. The day after, the PEI was washed trice with PBS before adding the culture medium.

2. DISSECTION OF THE RAT STRIATUM.

Animals were treated in accordance with the Guide for the Care and Use of Laboratory Animals (National Institutes of Health, Bethesda, MD), the European Communities Council Directive 86/609/EEC, and the guidelines of the institutional ethics committee. All efforts were made to minimize the number of animals used and their suffering.

To make pure primary cultures of rat striatum, embryos from pregnant Wistar rats (Janvier Breading Centre) were used. The mother was anaesthetised with 40mg/km of pentobarbital (Essex), decapitated and the embryos were removed at embryonic day 16,5. The striatum was dissected as described (Hartikka and Hefti, 1988). Briefly, the brain was removed from the embryo, then the cortexes of both hemispheres were removed, leaving the nucleus basalis, the striatum and the septum visible. The septum and nucleus basalis of Meynert were then removed.

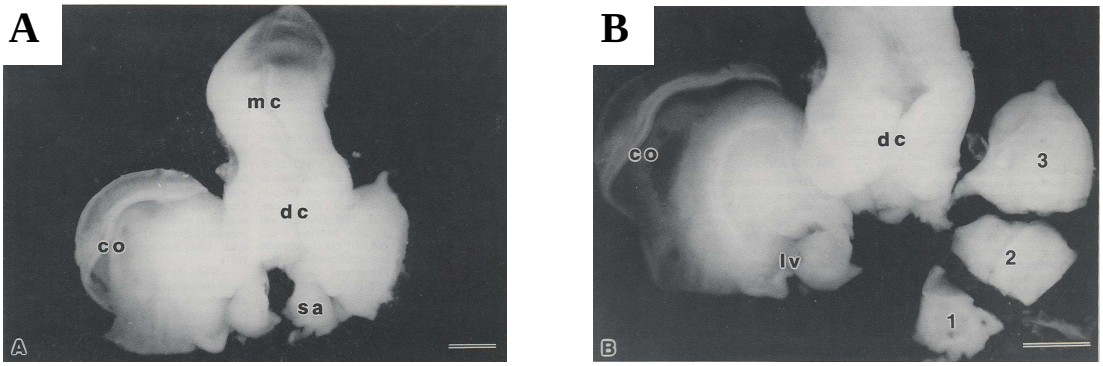


Figure I. Taken from Hartikka and Hefti, 1988. Dissection of striatal region from fetal rat of embryonic age E17. A) The brain was carefully pushed out of the skull and put on its ventral surface under a stereomicroscope. The cortical hemispheres were slightly pulled away from the brain's midline. This procedure makes visible the striatal area, which appears denser than the cortical tissue adjacent to it B) Disection. Using a small scalpel, the cortex was teared away, leaving visible the septum (1), striatum (2) and nucleus basalis (3). A vertical cut was made at the borderline separating the striatal area from the diencephalons and making 2 transversal cuts the striatum is separated of the septal and the nucleus basalis structures. Scale bar, 1 mm. Abbreviations: co, cortex; dc, diencephalon; mc, mesencephalon; sa, septal area; st, striatum; ve, ventricle.

A suspension of striatal cells was then prepared by mechanical trituration without proteolytic enzymes in L15 medium because it gives a better survival rate after plating than culture medium. Cell suspension was centrifuged at 1000rpm for 6min and plated at a density of $1{,}6x10^6$ cells/well in a 24 well culture plate. To count the cells, 10µl of the cell suspension was mixed with the same volume of trypan blue (Sigma), which excludes living cells, and were counted with a modified Neubauer counting chamber.

3. MATURATION OF CULTURES.

From DIV (Day In Vitro) 0-3 the cells were maintained in 500 µl of N5 (Kawamoto and Barrett, 1986) medium supplemented with 5% horse serum (HS) and 2.5% fetal calf serum (FCS), to favour cell attachment. To achieve the purest neuronal culture, 2µM of cytosine arabinoside (1-β-D-arabinofuranosylcytosine, AraC) (Sigma) were added during the following 24h of the dissection, to prevent the proliferation of non-neuronal cells. In the absence of astrocytes which normally take up glutamate, 2µM of the NMDA receptor antagonist dizocilpine maleate (MK-801) (Sigma) was also added to avoid glutamate-induced neurotoxicity. N5 medium was used because it reproduces the most closely the composition of the cerebral spinal fluid (CSF) and because the lack of non-neuronal cells is not due to toxic effects of the medium, since the addition of whole serum to this medium results in the rapid proliferation of fibroblasts and glia (Kaufman and Barrett, 1983).

After the first three days *in vitro*, the concentration of FCS was brought down to 0.5% for the next two days. At DIV5, when the cells were treated in the experimental paradigm, the concentrations of HS and FCS were lowered to 1 and 0.1%, respectively to limit possible interference by serum components.

The process of maturation took place under controlled conditions of temperature, humidity and CO_2 in an incubator at 37°C with a 5% CO_2 atmosphere.

B) TREATMENTS.

Striatal cultures underwent various treatments with different pharmacological and natural agents were prepared as stock solutions, stocked at -20ºC in aliquots which were never refrozen. Serial dilutions in N5 medium were made prior to their use.

All treatments began at day *in vitro* 5-6 and lasted for 48h without changing the medium or addition of new treatment.

1. ANNONACIN

Annonacin, the main toxin of interest of my thesis, was extracted as described (Champy *et al.*, 2005), it is extracted from the leaves and roots of *Annona muricata*. The lyophilised powder of this lypophilic agent was dissolved in DMSO (dimethylsulfoxide), stocked at 1mM at –20ºC in aliquots that were not refrozen. Serial dilutions were prepared in culture medium, so that the final concentration of DMSO never exceeded 0,01% of the final volume.

2. OTHER TREATMENTS

All the other pharmacological agents used were water soluble. We used, two antioxidants, N-acetylcysteine (NAC) (5mM) and trolox (10µM), which scavenge reactive oxygen species (ROS), two other respiratory chain inhibitors, 3-NP (3-nitropropionic acid) (0, 100, 300, 1000 µM), a complex II inhibitor, MPP^+ (0, 10, 25, 50µM), another complex I inhibitor with a different molecular structure, which was prepared freshly for every experiment due to its rapid oxidation and taxol (paclitaxel) (5mM), a pharmacological agent, that stabilises microtubules.

C) CELL VIABILITY.

After treating the striatal cells, cell viability was assed with the live-dead kit (Molecular Probes). Living cells incorporate calcein-acetometylester (excitation 495 nm, emission 515 nm) that fluoresces in green after cleavage of the ester by non-specific esterases. Dead cells were detected with ethidium homodimer (excitation 495 nm, emission 635 nm) which will intercalate into the nucleic acid of a dead cell because of their permeable membrane and be observed in red.

D) IMMUNOCYTOCHEMISTRY.

All treatments lasted for 48h from DIV 5. At DIV 7, cultures were washed once with warm PBS and then fixed with 4% paraformaldehide (PFA) (Sigma) for 12 min and then washed three times with PBS.

When immunocytochemistry was needed, cultures where incubated overnight at 4° with the primary antibodies diluted in PBS 0,1M, Thimerosal 0,02%, an anti-fungical (Sigma) 5% NGS (Norma Goat Serum), to avoid non-specific labelling (Vector) and 0,2% Triton x100, a detergent to break membranes and allow the entrance of the antibody (Sigma). Secondary antibodies, diluted in the same solution, were incubated with the cells for 2h at room temperature.

Immunolabelled cells were visualized under an epifluorescence microscope (Nikon TE-300) equipped with a ORCA-ER digital camera (Hamamatsu, C4742-95) and a computer-based image analysis system (Simple-PCI, Cimaging Systems, 6.1).

The following primary antibodies were used to study the phosphorylation state and distribution of the tau protein: monoclonal antibodies against hyperphosphorylated tau AD2 (1:1000) (Bio-Rad), AT8 (1:20), AT180 (1:1000), AT270 (1:300), AT100 (1:100) (all from Innogenetics); monoclonal antibody against tau Tau5 (1:1000) (Chemicon), polyclonal antibody against hyperphosphorylated tau pS^{262} (1:1000) (Biosource). Other primary antibodies were used to try and identify proteins which are pathological hallmarks of other diseases, such as a polyclonal anti-α-synuclein antibody (Chemicon) (1:500) (as in Parkinson's Disease) and polyclonal anti-β-amyloid (Santa Cruz) (1:100) (Alzheimer's Disease). To differentiate between the processes, polyclonal antibody against the dendritic marker microtubule associated protein 2 (MAP2, 1:500) (Sigma) and axonal marker beta-III-tubulin (1:300) (Sigma), were used. Monoclonal Cytochrome C (1:1000) (BD PharMingen) detected mitochondria.

After washing with PBS, the cells were incubated with the appropriate secondary antibody: Cyanide 3-conjugated goat anti-mouse IgG (1:500) (Jackson Immunoresearch) and Alexa 488 goat anti-rabbit (1:500) (Molecular Probes) for 2h at room temperature. Nuclei were visualized with the fluorescent DNA stain Hoechst 33342 (Sigma).

E) ELECTRON MICROSCOPY.

After 48h of treatment starting at DIV 5-6, cells were fixed with a mixture of 2% PFA (Sigma) and 0.2% glutaraldehyde in PBS for 60 min at room temperature, to preserve the morphology of the cells without altering the epitopes targeted by the antibodies, and then incubated with the primary antibody (AD2, 1:1000) for 24h at 4°C, rinsed in PBS, then incubated in 0.5% BSA (in PBS supplemented with 0.1% fish gelatine (Aurion) for 1h at room temperature, followed by incubation for 24h at room temperature with a secondary IgG conjugated to ultrasmall gold particles (0.8nm, Aurion) diluted 1:50 in PBS-BSA-fish gelatine (Aurion). The reaction was stopped by washes in sodium acetate 2% (Fischer) and intensified by silver enhancement for 35min at room temperature in the dark. The cells were then immersed in gold chloride (0.05%) (Riedel haën) for 10min at 4°C and in sodium thiosulfate (0.3%) (Fischer) twice for 10min at 4°C and ten rinsed in PBS. Immunogold reacted cultured cells were post-fixed in osmium tetroxide (Fisher) (1% in PBS for 20min at 4°C), rinsed, dehydrated and embedded in Epon. Ultrathin sections were cut, contrasted with uranyl acetate (Fisher) and lead citrate and observed with a JEOL 1200 EX electron microscope at 80kV.

F) THIOFLAVIN S STAINING.

To visualize the presence of amyloid-like structures, fixed cultures were incubated for 8 min in 0,05% thioflavine S (Sigma), washed in PBS and differentiated in 70% ethanol for 5 min, as described (Höglinger *et al.*, 2005).

G) DETECTION AND QUANTIFICATION OF REACTIVE OXYGEN SPECIES.

DHR-123, dihydrorhodamine-123 (Molecular Probes), is a positively charged rhodamine derivative that accumulates in the mitochondria and is the most common substance used to measure free radicals. To detect ROS in the cultures, we used it as described previously (Darios *et al.*, 2003). On DIV 5, the cells were treated for 6 hours with or without the antioxidants with and without annonacin. DHR-123 was then added to the cultures for 30 min and then replaced by the original treatment. The cutures were observed under a fluorescent microscope and 10 random fields were photographed and the intensity of the labelling per cell was quantified by computer assisted image analysis (Simple-PCI Cimaging System, 6.1). Results are expressed as the mean pixel intensity per cell.

H) QUANTIFICATION OF ATP LEVELS.

To estimate the intracellular ATP levels, the Vialight HS kit (Cambrex) was used according to the manufacturer's protocol. Briefly, the cells were treated for 6h, then washed twice with cold PBS and immediately afterwards, scrapped off the wells in 100µl of distilled water. If these samples were not processed immediately, they were stored at –80ºC. ATP levels were measured in 1–5µl of the cell lysates, in a tube luminometer and the results expressed as RLU (Relative Light Unites) normalised with respect to the concentration of protein in the extract.

I) LIVE IMAGING.

Coverslips of 11mm in diameter were placed in 24 well plates, coated with PEI overnight washed three times with PBS before adding the medium. Neurons were plated at low density ($1x10^6$ cells/well): to see the neurites individually and identify the cell they belong to.

On DIV5, the coverslips were removed from the plate, and placed upside down in 1ml of N5LS (Low Serum content) in an adequate chamber for the microscope Leica DMI 6000 Fluorescence Microscope. Temperature was kept at 37ºC with a temperature controlled chamber, and the CO_2 levels were stabilised by adding HEPES (Sigma) 50mM in the medium and then analysed.

Taxol (placlitaxel) (Sigma), a pharmacological agent which stabilises microtubules dynamics was added to the cultures one hour before intoxication with annonacin at a subtoxic concentration (5nM) to allow it to interact with the microtubules.

Mitochondria in living cells were followed using Green-Fluorescent MitoTracker Green FM (Molecular Probes), excitation of 490nm and an emission at 516nm. It labels live mitochondria by accumulating in the mitochondrial matrix where it binds covalently to the free thiol group of serine residues of mitochondrial proteins (Presley *et al.*, 2003). The medium was exchanged for 100µl of mitotracker at 200nM, left for 30 min at 37ºC, re-exchanged for the treatment and immediately analysed using a Leica DMI 6000 Fluorescence Microscope with the Leica LAS software package during 6h. Photos were taken every 10 minutes during 6 hours and in five different planes on the z axis, to be able to observe the whole cell.

J) DATA AQUISITION AND ANALYSIS.

All data result from the analysis of at least 3 wells per experimental condition in at least 3 independent experiments. Data are expressed as the mean ± S.E.M. of at least three different experiments. Means were compared by a one-way ANOVA followed by either Bonferroni's or Dunnett's correction. The t-test was used when appropriate. All data were treated on SigmaStat 2.03.

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