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► **To cite this version:**

B. Delatour, V. Blanchard, L. Pradier, Charles Duyckaerts. Alzheimer pathology disorganizes cortico-cortical circuitry: direct evidence from a transgenic animal model. *Neurobiol Dis*, 2004, 16 (1), pp.41-7. 10.1016/j.nbd.2004.01.008 . hal-03236866

HAL Id: hal-03236866

<https://cnrs.hal.science/hal-03236866>

Submitted on 26 May 2021

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Alzheimer pathology disorganizes cortico-cortical circuitry:

Direct evidence from a transgenic animal model

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Abstract

It has been proposed that Alzheimer disease (AD) is associated with a “disconnection syndrome” due to the gradual loss of morphological and functional integrity of cortico-cortical pathways. This hypothesis derives from indirect neuropathological observations but, definitive evidence that AD primarily targets cortico-cortical networks is still lacking.

By means of neuroanatomical anterograde tracing methods we have investigated, in a murine transgenic model of AD, the impact of the amyloid burden on axonal terminals in different neural systems. Axonal tracings revealed, in accordance with the “disconnection syndrome” hypothesis, that cortico-cortical fibers are significantly disorganised. Terminal fields in local and distant cortical areas contained numerous swollen dystrophic neurites, often grouped in grape-like clusters, at the plaque periphery. In contrary to fibers of cortical origin, those originating from subcortical brain structures only showed limited signs of degeneration upon reaching their cortical targets. These observations suggest a selective disruption of cortico-cortical connections induced by AD brain pathology.

KEYWORDS: Alzheimer disease, connections, neurodegeneration, dystrophic neurites, amyloidosis, axonal tracing, cortex, transgenic mice, amyloid protein precursor, presenilin.

Introduction

Pathological hallmarks of Alzheimer disease (AD) are classified into two types: extracellular (deposition of A β peptide, which may become amyloid) and intracellular (neurofibrillary alterations associated with accumulation of hyperphosphorylated tau protein). Senile plaques are composite lesions involving a dense core of A β peptide surrounded by a crown of abnormal, sometimes tau-positive, neuritic processes. Studying the mechanisms of senile (neuritic) plaque formation may help understand how amyloidogenesis and neurofibrillary lesions are related. Degenerated fibers that innervate senile plaques are mainly axons; they, indeed, contain presynaptic vesicles (Terry *et al.*, 1964) and neurofilament epitopes (Schmidt *et al.*, 1991). A subset of these fibers are immunostained by ubiquitin and were originally classified as dystrophic because of their markedly altered morphology (Dickson 1997).

The impact of axon pathology in neurodegenerative diseases has been somewhat underestimated (Coleman and Perry 2002) despite it being obvious that degenerated neurites may seriously hamper brain connectivity and, hence, have functional consequences (Knowles *et al.*, 1999). The pathogenic importance of dystrophic neurites in AD has hence been repeatedly emphasized (Arai *et al.*, 1990; Masliah *et al.*, 1991; McKee *et al.*, 1991; Mirra *et al.*, 1991). Moreover, neuropathological data suggest that AD is associated with a cortical “disconnection syndrome” in which cortico-cortical pathways gradually lose their morphological and functional integrity (for reviews, see Arendt 2001; De Lacoste and White 1993).

If AD pathology involves connective alterations at the level of brain circuitries, are all neuronal pathways disrupted? If not, what is the topography of affected connections ?

To identify the anatomical origin of the processes associated with senile plaques, we carried out experimental studies in a transgenic mouse model based on the overexpression of human mutated genes (coding for the amyloid precursor protein - APP and for the presenilin 1

protein -PS1) found in familial AD. This model reproduces some of the key neuropathological features of the disease. Accelerated A β deposition in AD transgenic models is the major, but not unique histological lesion (Price *et al.*, 1998). In the vicinity of A β deposits, several neurodegenerative changes can be observed including dystrophic neurites (Masliah *et al.*, 2001; Masliah *et al.*, 1996; Sturchler-Pierrat *et al.*, 1997).

In the present study, experiments were carried out using an APPxPS1 model that develops A β deposits, in close association with neuritic alterations, at 2-3 months of age (Blanchard *et al.*, 2003; Wirths *et al.*, 2001). We applied *in vivo* neuronal tracing protocols to study brain connectivity in these genetically modified mice. To our knowledge, this latter approach has been taken in only one previous study in which the authors mapped the perforant pathway by injecting a tracer into the entorhinal cortex (Phinney *et al.*, 1999). They provided evidence of abnormal, ectopic terminals at the level of the hippocampus (dentate gyrus) and the thalamus. Our goal was to determine the extent to which cortico-cortical connections were altered in relation with amyloid pathology. For a comparative approach we investigated the morphology of other fiber tracts, namely subcortical-cortical connections.

Preliminary reports on these findings have been published in abstract form (Delatour *et al.*, 2002).

Materials and Methods

Transgenic mice

A double thy1 APP751 SL (Swedish mutation KM670/671NL, London mutation V717I introduced in the human APP751 sequence) x HMG PS1 M146 L transgenic mouse line was generated and established in accordance with standard procedures using CBA/C57Bl6 hybrids as described elsewhere (Blanchard *et al.*, 2003; Wirths *et al.*, 2001). Ten aged (between 14 and 24 months) male (n=4) and female (n=6) APPxPS1 transgenic mice were used for the present study.

Neuroanatomical tracings and immunohistochemistry

Biotinylated dextran amine –BDA (Brandt and Apkarian 1992; Reiner *et al.*, 1993; Veenman *et al.*, 1992) was used as an anterograde tracer to visualize axonal tracts and normal or dystrophic terminal boutons. BDA is also transported retrogradely raising the possibility that collateral axons from retrogradely labeled neurons could contaminate the preparation.

However, to minimize the impact of reverse transport, fine micropipettes were used (Reiner *et al.*, 1993).

Injections of BDA (Molecular Probes, Eugene, USA, 10% in 0.01 M phosphate buffer - PB) were placed in the cortex (posterior cingulate cortex, n= 1 mouse injected ; visual cortex, n= 2 and hippocampus, n= 1) or subcortical nuclei (septum, n= 3 and thalamus, n= 3). BDA injections in cortical and subcortical areas were equally distributed in male and female transgenic mice. Results from subsequent tracing studies did not show any noticeable effect of gender. A pilot study was previously performed in six non-transgenic mice to determine parameters for the injections in selected anatomic targets (stereotaxic coordinates, volume of tracer to be injected). No neuritic dystrophy was found in those control animals.

Mice were anesthetized with a mixture of 4:3 ketamine-xylazine. BDA was stereotaxically injected using a glass micropipette (inner diameter: 15 μ m), using coordinates adapted from the Franklin and Paxinos atlas (Franklin and Paxinos 1997). Tracer was iontophoresed by applying a positive pulsed DC current (6 μ A; 7 s on / 7 s off) for 10-15 min. After a survival time of 7-10 days, animals were sacrificed with an overdose of sodium pentobarbital and were perfused transcardially with a fixative solution containing 4% paraformaldehyde in 125 mM PB. After removal, the brains were postfixed for one hour in the same fixative used for perfusion. Brains were stored overnight in a solution of 20% glycerin and 2% dimethylsulfoxide in 125 mM PB for cryoprotection. Brains were subsequently sectioned (frontal 40 μ m-thick sections) on a freezing microtome, collected and rinsed with PB (125mM). Sections were then washed in a 0.05 M triton X-100 saline phosphate buffer (PBS) solution and incubated, at room temperature, with an avidin-biotin-peroxidase complex (ABC Vectastain standard kit, Vector Laboratories, Burlingame, USA, 1:400) for 90 min. The following reaction made use of nickel-enhanced diaminobenzidine, which precipitated as a gray/dark product. In a second step, labeling of amyloid plaques was done by standard Congo red staining (30 min in a 80% ethanol solution saturated with congo red and sodium chloride ; adapted from Puchtler *et al.*, 1962) or by A β immunohistochemistry. For the latter method, we used polyclonal antibodies directed against A β 1-40 or A β 1-42 (FCA-3340 and FCA-3542, gift from F. Checler, dilution: 1/500) that were revealed using peroxidase-anti-peroxidase (PAP) reaction rather than avidin-biotin-peroxidase method. This method was chosen to avoid cross-reactions with the biotin residue of the tracer. The chromogen used to visualize A β was the diaminobenzidine which precipitates as a brown product.

Synaptophysin immunostaining was carried out to determine the occurrence and morphology of axon terminals at the periphery of the plaques. A primary polyclonal anti-synaptophysin antibody (Dako, Glostrup, Denmark, 1:200) was used and detected with the PAP method.

Degeneration of traced terminals was finally assessed using double (BDA/ubiquitin) fluorescent immunolabelings that were analyzed by means of a confocal laser-scanning microscope (TCS SP1, Leica, Rueil Malmaison, France). BDA was revealed using a streptavidin-cyanine 2 complex (Jackson ImmunoResearch, West Grove, USA, dilution: 1:200). Ubiquitin was detected with a polyclonal antibody (Dako, 1:500) and subsequently a goat anti-rabbit secondary antibody conjugated cyanine 3 (Jackson ImmunoResearch, dilution: 1:1000).

Results

Axon dystrophic terminals

All injection sites were located in plaque-enriched (cortical and subcortical) areas. In each case a high density of abnormally enlarged neurites containing the anterograde tracer was observed, close to the injection site, surrounding amyloid plaques (Figure 1-A). These observations indicate that local circuitries, involving short axonal projections, were affected in both cortical and subcortical structures. In regard to long distance connections, only a subset of traced projections showed morphologically altered terminals around amyloid deposits (see below).

In both cortical and subcortical brain areas, swollen distended neurites were often observed to coalesce in grape-like clusters around the plaques. These were made of a dense core of Congo red positive material strongly immunoreactive to A β antibodies. Abnormal neurites were not found in diffuse deposits of A β peptide i.e. those >30 μ m in diameter, Congo red negative and with indistinct contours. Double immunofluorescence indicated that most of BDA-traced puffy terminals showed ubiquitin labeling (see Figure 2-A,B,C), suggesting that they had undergone some degenerative processes.

In rare cases (n=2), BDA injections induced retrograde labeling in neurons located in adjacent brain regions (e.g. in the parietal cortex following injection in the posterior cingulate cortex).

In these cases neuronal cell bodies and their dendritic tree were filled, with a Golgi-like appearance (see Figure 1-B). The morphology of dendritic spines did not appear to be modified in the vicinity of the A β deposits. On the contrary, synaptophysin immunostaining revealed a large number of synaptophysin-positive, distorted, enlarged axon terminals surrounding A β deposits (Figure 1-C).

Cortical projections

BDA was injected at various cortical loci (eg Figure 2-D). Plaque-associated dystrophic neurites were noted close to the injection site (Figure 2-F) in all areas.

Projections from the associative visual cortex gave rise, at some distance, to pathological endings in the retrosplenial cortex (Figure 2-H). When the tracer was injected in the posterior cingulate cortex, terminals were found in various cortical targets that were either in the near vicinity of the injection site (eg presubiculum) or at a greater distance (eg dorsomedial prefrontal cortex). In all cases we found evidence of numerous pathological terminal fields around A β deposits. One mouse received an injection in the dorsal CA1 field of the hippocampus; direct projections, ending with abnormally dilated boutons, were observed in the plaque-enriched subicular complex.

To summarize, whatever target cortical area was considered, numerous BDA-positive dystrophic neurites were noted at the plaque periphery. Not only short cortico-cortical axons (such as CA1-subicular connections) but also long distance fiber association pathways (such as cingulo-frontal fibers that map the whole caudo-rostral extent of the brain to reach their targets) showed marked morphological alterations in relation to amyloid deposits.

Hence, neuroanatomical tracing revealed that cortico-cortical fibers were significantly disorganised in the context of amyloid pathology, both at the level of intrinsic and extrinsic circuitries.

Subcortical projections

In other animals, the tracer was iontophoresed in different subcortical locations (eg Figure 2-E). As mentioned above, we noticed that intrinsic connections, at the level of the injection site, were affected around plaques. Hence, when thalamic and septal nuclei were injected, a large number of dystrophic neurites were observed directly adjacent to the injection sites in

these structures (Figure 2-G). Subcortical circuitries involving connections between distant structures were also disrupted. For example, after injections in the septal area, projections in the accumbens nucleus were associated with distorted enlarged terminals observed at the plaques' periphery.

While subcortical-subcortical connections were found to be morphologically altered in relation with A β deposits, we found no evidence that a similar process occurred for projections terminating in cortical areas. In all examined cases only a very small number of dystrophic neurites were noticed at the level of cortical axonal terminal fields. For instance, despite very dense projections from the mediodorsal thalamic nucleus to the orbital and dorsomedial prefrontal cortices, no clear evidence of pathological boutons were seen close to the amyloid deposits in these cortical regions (Figure 2-I). Furthermore, only rare dystrophic terminals were noted at the level of thalamo-insular or septo-hippocampal connections (Figure 3).

To summarize, as opposed to cortico-cortical connections, fibers originating in subcortical brain structures, did not show obvious signs of neurodegeneration when reaching their cortical targets. However, fibers remaining in subcortical networks presented pathological endings associated with neighbouring A β plaques.

Discussion

By means of tracing experiments we provided direct confirmation of plaque-associated axonal dystrophy in APPxPS1 mice. Traced axons were abnormal both at the morphological (enlarged boutons) and immunohistochemical (labeling with antibodies raised against ubiquitin) levels (see also Masliah *et al.*, 1996).

Terminal endings in dystrophic neurites surrounding plaques were evidenced by their immunoreactivity for synaptophysin. These observations, pointing to the presence of synaptic vesicles in dystrophic neurites, replicate previous observations obtained in the same transgenic line (Blanchard *et al.*, 2003) as well as in other mouse models (Phinney *et al.*, 1999) and in AD brains (Masliah *et al.*, 1991). Electron microscopy has also provided strong evidence that synaptic elements are present in dystrophic neurites in both humans and genetically modified mice (Phinney *et al.*, 1999; Terry *et al.*, 1964).

In the present study, short and long cortico-cortical axons were disrupted at the plaque periphery. Intrinsic circuitries within cortical or subcortical areas underwent a similar neurodegenerative process. However, striking differences were seen between cortical and subcortical afferent fibers to cortical plaques. For instance, while cingulate cortical projections degenerated in the frontal cortex, thalamic fibers terminating in the same area showed no obvious sign of axonal pathology despite heavy amyloid burden. Interestingly, human neuropathological data also indicate that there is no evidence of a thalamic innervation of cortical senile plaques (Duyckaerts *et al.*, 1986). Extending previous observations (Phinney *et al.*, 1999), our results support the hypothesis that neurites of cortical senile plaques are mainly axons from adjacent or distal cortical areas. No clear evidence was found that these neurites could have a subcortical origin. We recently immunolabeled catecholaminergic fibers (using tyrosine hydroxylase immunohistochemistry) in the cortex of

APPxPS1 mice to evaluate morphological changes in cortical axon terminals arising from cell bodies located in subcortical nuclei. Once again we failed to demonstrate high number of dystrophic neurites associated with cortical plaques (unpublished observations). Supporting these observations, Cervera and collaborators (1990) also showed the lack of tyrosine hydroxylase positive senile plaques in the neocortex of AD patients.

This selective involvement of cortico-cortical fibers in plaque-associated neuritic degeneration can be paralleled with neuroimaging data obtained in human subjects. MRI studies have demonstrated, in AD patients, disruptions of connections linking cortical areas, as revealed by callosal atrophy (Hampel *et al.*, 1998; Teipel *et al.*, 2002). Similarly, functional brain imaging studies (using PET-based functional connectivity mapping or EEG coherence recordings) indicate alterations of cortico-cortical connections (e.g. between prefrontal and medial temporal lobe regions) in AD patients (Grady *et al.*, 2001; Leuchter *et al.*, 1992). Dementia severity has been correlated with the degree of callosal atrophy (Hampel *et al.*, 1998; Teipel *et al.*, 2002), suggesting that hodological alterations during the course of AD might lead to a disconnection syndrome with clinical (disrupted cognitive functioning) consequences.

Our observations in the APPxPS1 transgenic model, suggest that vulnerability of neuronal connections is selective. While some fiber systems are affected (e.g. cortico-cortical and subcortico-subcortical connections) others are largely preserved (e.g. subcortico-cortical connections). Several factors could explain this differential vulnerability. The amyloid cascade hypothesis would predict that axonal dystrophy develops within neuronal processes in response to A β aggregation. In accordance with this hypothesis, dystrophic, degenerated (ubiquitine positive) neurites are found at 2-3 months of age in the transgenic line used here (Blanchard *et al.*, 2003) when A β deposition starts. They develop in parallel with A β

deposition. A β peptide could act as a trophic substance to trigger pathological sprouting of afferent fibers (Geddes *et al.*, 1986; Masliah *et al.*, 1991) or conversely might have direct neurotoxic effects (Koh *et al.*, 1990; Neve and Robakis 1998; Yankner *et al.*, 1989). While numerous data indicate a detrimental effect of A β , as well as other plaque-associated peptides on neuronal cells and processes (Forloni *et al.*, 2000; Marcon *et al.*, 1999; Yang *et al.*, 2000), it remains to be determined how A β could alter selectively distinct axon populations.

Differential vulnerability could depend on neurochemical properties of neural connections. Glutamatergic pathways, that are mainly involved in cortico-cortical projections, have been repeatedly reported to be altered (at both pre- and postsynaptic levels) in AD brains (Greenamyre and Young 1989; Lee *et al.*, 2002; Myhrer 1993). Neuropeptidergic systems have also been shown to be differentially affected, in relation to their excitatory/inhibitory properties, in the brain of transgenic mice (Diez *et al.*, 2000). According to another hypothesis, selective vulnerability of neural pathways is related to the content of neurofilaments and calcium-binding proteins in the sensitive neurons (Hof *et al.*, 1990; Morrison *et al.*, 1998) or to the degree of axon myelination (Braak *et al.*, 2000).

Disruption of cortico-cortical fibers might also rely on the specific topographical distribution of A β deposits: Armstrong and collaborators (2001) recently demonstrated that senile plaques in AD brains are evenly spaced in clusters paralleling the organisation of feedforward and feedback cortico-cortical pathways. This regular, non-random distribution of A β deposits could therefore determine degeneration of selected neural pathways.

Finally, a combination of the different factors that we have considered might explain why degenerating neurites are principally axons, and why only some axons degenerate. Both plaque properties (local factors differentially expressed in amyloid deposits depending on cerebral topography) and morphological characteristics of axon populations (related to their anatomical origin) could interact and promote neurodegenerative effects.

Further research effort is required to untangle this multicausal process and to find out how dystrophic neurites (as well as other neurofibrillary alterations) are related to A β aggregation .

Interestingly, it has been shown that A β injection in the hippocampus of transgenic mice overexpressing P301L mutant human tau retrogradely promotes tangle formation in neurons of brain regions that project to the locus of injection (Gotz *et al.*, 2001). This suggests that neural connections could be a substrate of interaction between A β deposits and tau-positive neurofibrillary lesions.

Both A β deposition and neuritic alterations compromise the integrity of brain circuitry. A treatment targeting abnormal A β metabolism and deposition should also prevent the lesions of the connective network in order to have clinical impact.

Figure legends

Figure 1

A: Innervated plaques, at a short distance from the injection site, in the visual cortex of an APPxPS1 mouse. The amyloid deposits (stars) were immunostained with an anti-A β antibody. BDA-positive enlarged terminals (black) were observed at the periphery of plaques (see arrows).

B: Retrogradely labeled pyramidal cells in the parietal cortex (black). Note that the dendritic trees were not altered in the vicinity of A β deposits (indicated by stars).

C: Synaptophysin immunoreactivity in the cortex of an APPxPS1 mouse. Abnormally enlarged synaptophysin-positive terminals (arrow heads) were constantly observed around A β deposits (stars).

Scale bars: 50 μ m

Figure 2

A-C: Colocalisation of BDA (A) and ubiquitin (B) in the same puffy axon terminals is illustrated in the merged photograph (C). In some dilated neurites, the tracer BDA was present and colocalized with ubiquitin (arrows), while non-traced dystrophic terminals contained only ubiquitin (arrow heads). Neurites were in contact with a deposit of A β peptide (star).

Confocal microscopy: BDA was visualized using a streptavidin-CY2 complex (red channel) and ubiquitin by a conjugated CY3 complex (green channel).

D: Example of cortical injection site (visual cortex)

E: Example of subcortical injection site (mediodorsal thalamic nucleus)

F: Intrinsic cortico-cortical connections. The tracer was injected in the visual cortex; short-distance axonal projections (black) ending in the same cortical area showed marked morphological alterations (arrows) around amyloid deposits (brown).

G: Intrinsic subcortico-subcortical connections. BDA was iontophoresed in the thalamus and local projections (black) showed signs of dystrophy (arrows) at the periphery of thalamic amyloid plaques (stained with Congo red).

H: Extrinsic cortico-cortical connections. The tracer was injected in the visual cortex and terminal fields were observed in the retrosplenial cortex. Note the presence of numerous BDA positive enlarged terminals (black; see arrows) around A β deposits (brown).

I: Subcortico-cortical connections. Thalamo-prefrontal projections (black) were traced after BDA injection in the mediodorsal nucleus of the thalamus. No obvious signs of axonal dystrophy were observed in contact with prefrontal A β plaques (brown). Inset in the right upper corner shows at higher magnification the area delimited by dotted outlines (compare with figure 1-A).

Scale bars: 10 μ m (A-C) and 50 μ m (D-I).

Figure 3

Illustration of septo-hippocampal fibers. The tracer was injected in septal nuclei and terminal fields were observed in the hippocampus. Note the absence of dystrophic terminals at the plaque (star) periphery.

Scale bar: 25 μ m

Acknowledgements

This work was supported by the Aventis Pharma® Alzheimer network and by the Fondation pour la Recherche Médicale.

We thank Jean-Jacques Hauw for stimulating discussions and continuous encouragements.

We are grateful to Frédéric Checler for the gracious gift of the FCA-3340 and FCA-3542 antibodies.

The help of H. El Hachimi for histological work is greatly acknowledged. Our appreciation goes to J. Horwood for her indispensable aid in the redaction of the manuscript.

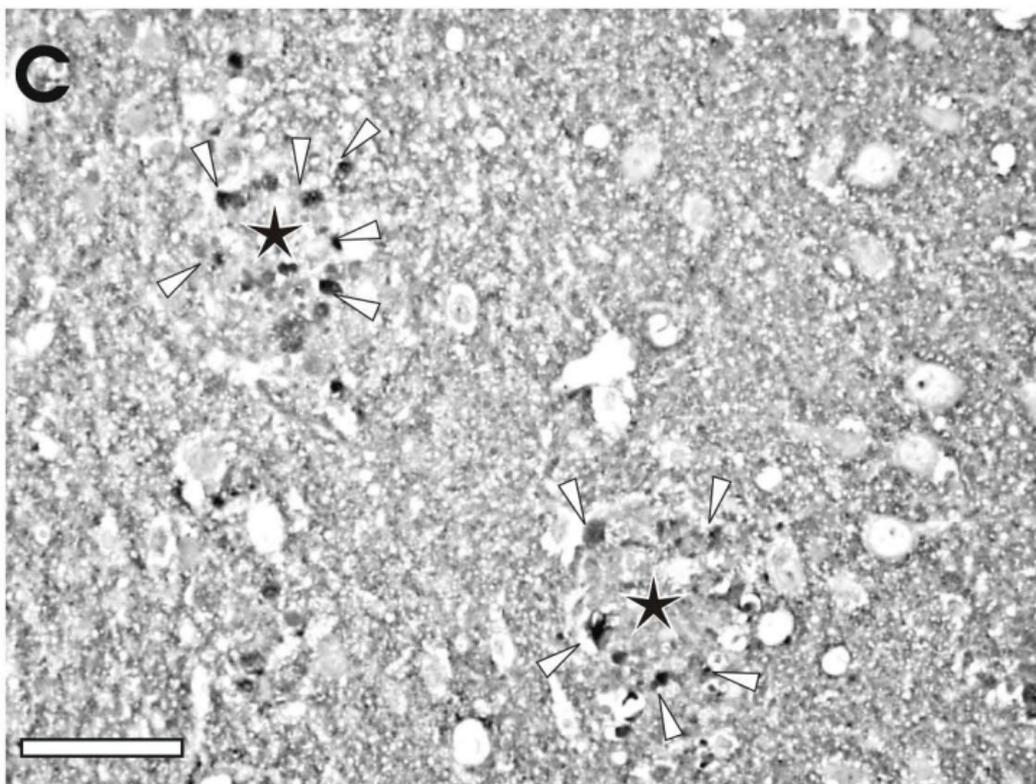
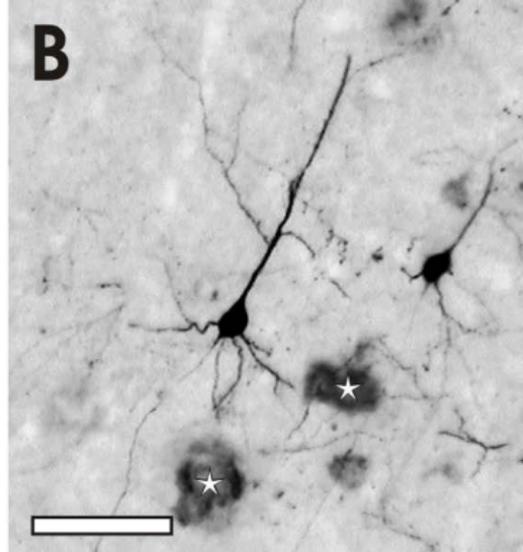
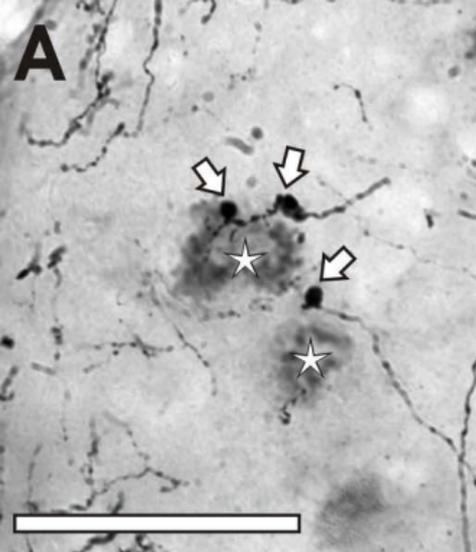


Figure1 Delatour et al (NBDI 123)

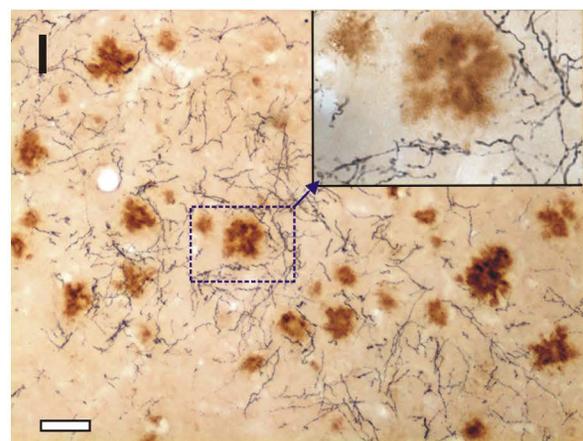
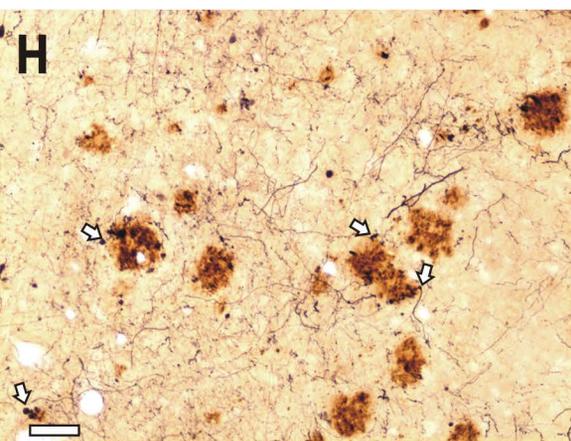
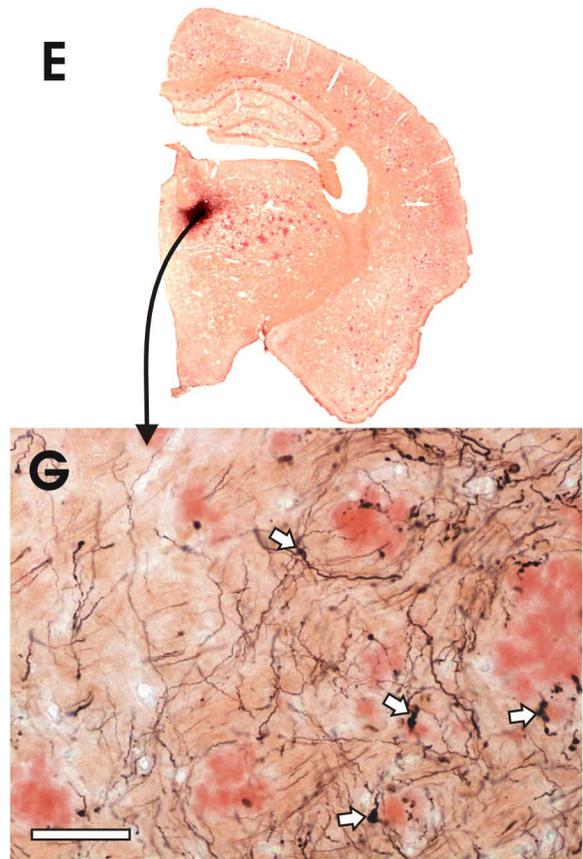
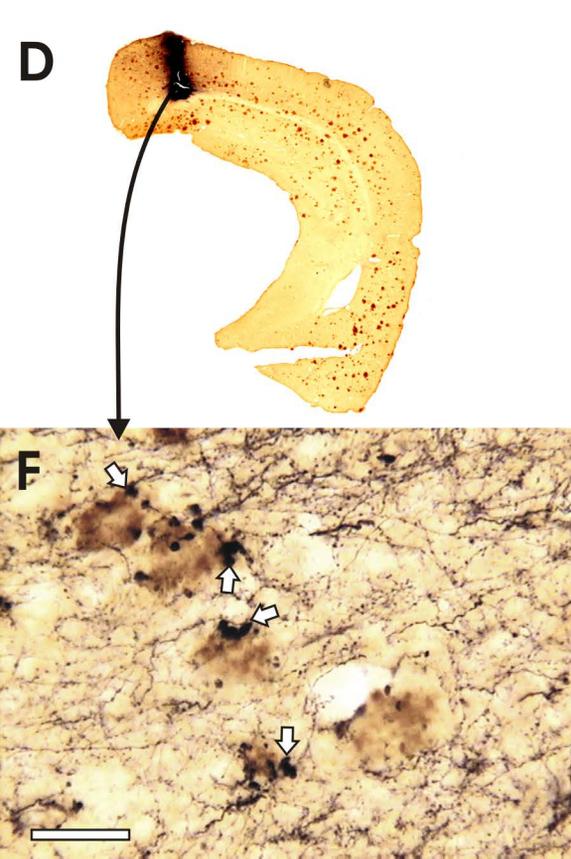
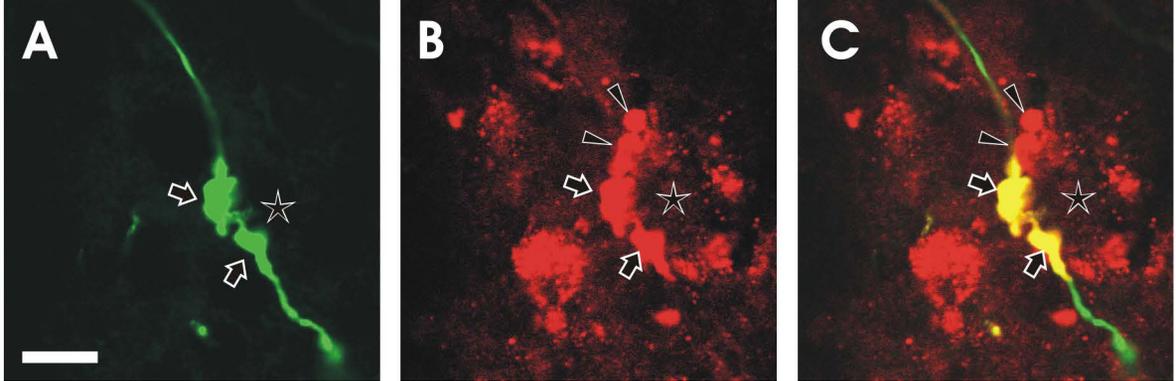


Figure2 Delatour et al (NBDI 123)

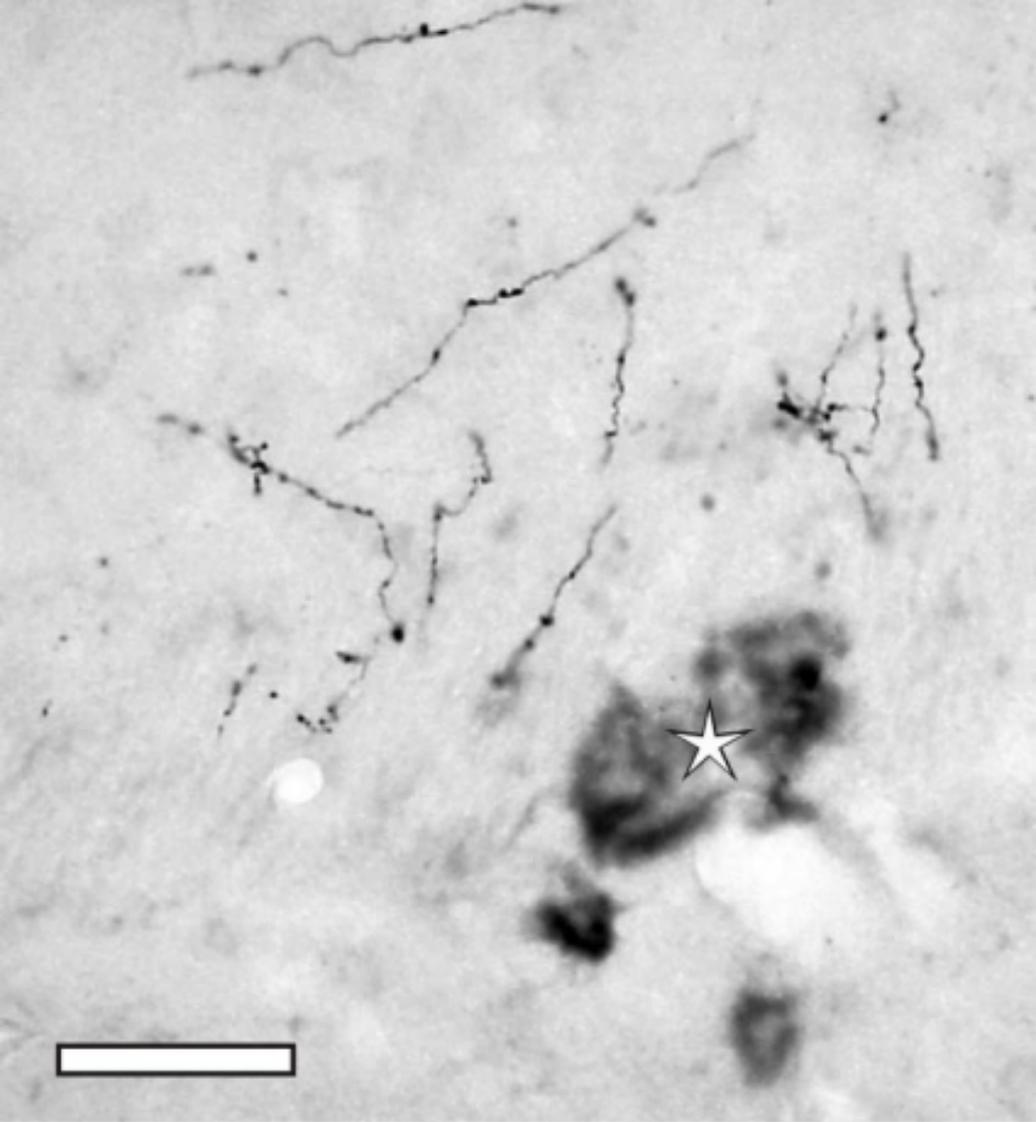


Figure3 Delatour et al (NBDI 123)

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