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Axonal tract tracing for delineating interacting brain regions: implications for Alzheimer's disease-associated memory

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Abstract

We are studying the projections from the entorhinal cortex to the hippocampal formation in the mouse. The dentate gyrus is innervated by the lateral entorhinal cortex (lateral perforant path) and medial entorhinal cortex (medial perforant path). The entorhinal cortex also projects to hippocampal areas CA3 and CA1, and to the subiculum. In young transgenic Alzheimer's disease mouse models (before amyloid-β pathology), the connections are not different from normal mice. In Alzheimer's disease mice with pathology, two changes occur: first, dystrophic axon endings appear near amyloid-β plaques, and second, there are sparse aberrant axon terminations not in the appropriate area or lamina of the hippocampus. Furthermore, MRI–diffusion tensor imaging analysis indicates a decrease in the quality of the white matter tracts connecting the hippocampus to the brain; in other words, the fimbria/fornix and perforant path. Similar changes in white matter integrity have been found in Alzheimer's disease patients and could potentially be used as early indicators of disease onset.

Keywords

entorhinal cortex; hippocampus; limbic system; perforant path; tractography

The entorhinal cortex (EC) functions as the gateway to hippocampal formation because its output, through the perforant path, is the major cortical source of input to the hippocampus and, furthermore, together with the subiculum, it also provides the major output of the hippocampus [1]. Ramón y Cajal was the first to clearly describe fibers arising from the entorhinal area going to the fascia dentata and hippocampus proper (i.e., cornu Ammonis) [2]. More recently, modern tracing studies performed in many species have confirmed that

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the EC projects by way of the perforant path to the dentate gyrus and hippocampus [3–7]. It has been demonstrated by a large number of studies in several species that the superficial layers of the EC project to the hippocampus[4,5,7,8]. In the rat, the layer II cells of the EC have been shown to project primarily to the dentate gyrus [4,9–11]. The EC layer III cells have been shown to predominantly project to area CA1 of the hippocampus, and this projection to area CA1 is bilateral [4,7]. In early studies, it was concluded that a topography existed in the entorhinal–hippocampal projection [4,12]; in other words, the axons arising from the laterodorsal EC terminate in the septal (dorsal) and more ventral hippocampus, and medial bands of EC project to the temporal (ventral) hippocampus (Figure 1B). Similarly, we have shown that in the mouse, entorhinal–hippocampal projections are, for the most part, similarly organized to those in the rat; however, some differences between these two species have been shown to exist [7,13]. The current article describes the differences in projections from the EC to the hippocampal formation in the Alzheimer's disease (AD) mouse model compared with the C57BL/6 mouse and we show that after the development of pathology, abnormalities are present in these connections [14]. We have studied the changes in plasticity in these connections in transgenic (Tg) AD model mice at different ages [15,16].

Tracing studies in Tg AD model mice

Deeply anesthetized adult, male mice were injected either with an anterogradely transported tracer in the EC or with retrogradely transported tracers in the hippocampal formation [7]. Together, these data give the full spectrum of the connections between the EC and hippocampus. Furthermore, we have performed MRI–diffusion tensor imaging (DTI) measurements, by creating respiratory-gated MRI images of the mouse brain. They were obtained with a Bruker Biospec® (Bruker Biosciences Corporation, MA, USA) 9.4-T/21-cm horizontal-bore magnet spectrometer with a 7.2-cm resonator for radiowave transmitting and an active-decoupled 2.0-cm surface coil for signal receiving. T2-weighted magnetic resonance images were first acquired using spin echo sequence (rapid acquisition with relaxation enhancement) with acquisition parameters: repetition time/echo time: 4000/40ms; four slices of 0.75-mm thickness without gap; 128× 128pixels matrix; and field of view: 16 × 16mm. DTI data were collected using a standard spin echo sequence with similar imaging parameters to those used in T2. DTI parameter maps were calculated using the vendor's software to give fractional anisotropy (FA), mean diffusity, axial diffusivity and radial diffusivity. Our data indicate that in Tg AD model mice that have significant AD pathology; in other words, with large amounts of amyloid-β deposits, the FA of the white matter tract (fornix) leaving the hippocampus is reduced by 20%. We are currently investigating the changes in the perforant path in a Tg AD mouse model, but since this is a less organized fiber bundle, it is more difficult to obtain the data.

Overview of entorhinal hippocampal connections in mice

We have subdivided the EC into two main parts; in other words, the classical lateral entorhinal area (LEA) and the classical medial entorhinal area (MEA) (Figure 1) [17,18]. We use the division of the hippocampal formation into septal (i.e., dorsal) and temporal (i.e., ventral) poles of the hippocampus according to Blackstad (Figure 1) [19]. It should be noted that in the molecular layer of the dentate gyrus of the mouse, the two bands of the lateral and

medial perforant path are wider than the inner band of labeling (which occupies approximately 17% of the total stratum moleculare) [20]. The inner band of the molecular layer contains the terminals of the associational and commissural axons.

Laminar organization of projection of the LEA & MEA

LEA axons travel via the lateral perforant path; in the dentate gyrus, terminals are present only in the outer a third of the molecular layer (Figure 1) [7], while in CA3 and CA1, terminals are in the stratum lacunosum-moleculare, and in the subiculum, in the superficial part of the stratum moleculare (Figure 1) [7]. MEA axons enter the medial perforant path; in the dentate gyrus, terminals are present in the middle a third of the molecular layer (Figure 1) [7], and in CA3 and CA1, terminals are in the stratum lacunosum-moleculare (Figure 1) [7]. In general, the mouse shows a laminar organization that is similar to that displayed in the rat [9,21] and other species, including monkeys [22–24]; LEA is the origin of the lateral perforant path that terminates in the outer a third of the molecular layer of the dentate gyrus, and MEA is the origin of the medial perforant path that ends in the middle a third of the molecular layer of the dentate gyrus [7,25]. The Tg AD model mouse has no significant differences in the origin and distribution of axons originating in the LEA or in MEA, but they do show a small amount of aberrant axon terminals in inappropriate layers (Figure 2A–D) [25]. The number of incorrectly ending axonal terminals from the EC in the hippocampal formation is less then 1% of all axonal terminals, suggesting that they probably do not play a big role in memory dysfunction in these AD model animals.

Commissural connections

It is important to note that the entorhinal projections to the mouse hippocampus are bilateral to areas CA3 and CA1, but not to the dentate gyrus, where there is only a unilateral projection. This is different in rats where all projections are bilateral but, most likely, in humans, similar to monkeys [3,22], this projection is unilateral. In the mouse and rat, the hippocampus is strongly connected to the contralateral side of the brain; these connections arise from the the hilar cells and area CA3. However, the hippocampal commissural connections in monkeys (and most likely humans) are very limited; most intrinsic connections are unilateral [26,27]. In mice, both hippocampi are strongly interconnected [28,29], and the entorhinal cortices are connected.

Topographical organization

The projection of EC to the hippocampal formation is topographically organized, lateral parts of LEA project to the the dorsal, septal part of the hippocampal formation, whereas more medial parts of LEA terminate in the intermediate (septotemporal axis) part of the hippocampal formation. The most medial part of LEA terminates in the ventral, temporal part of the hippocampal formation (Figure 1B) [7]. MEA projections to the hippocampus display a similar septemporal distribution (Figure 1B) [7]. There are no significant differences present in the topographical organization between ‘normal’ and Tg AD model mice. Most species show a similar topographic organization of these connections [5,30].

Laminar organization in AD mice with plaques

Injections into LEA give rise to labeled axons in the lateral perforant path; in the dentate gyrus, labeled axons and terminals are primarily present in the outer a third of the molecular layer (Figure 3A) and in CA3 and CA,1 labeled axons and terminals are present in the stratum lacunosum-moleculare (Figure 3D) [25]. It should be noted that even with a significant amyloid-β load present in the molecular layer of the dentate gyrus (Figure 3B), the EC axonal endings are largely normal (Figure 3A). However, a few aberrant terminals can be observed in the inner molecular layer of the dentate gyrus (Figure 2A–D, arrows). Furthermore, at more advanced stages of pathology, the hippocampus shows shrinkage and the size of the dentate gyrus molecular layer is significantly decreased [31], probably indicating decreased synaptic innervation from the EC. Similarly, AD patients show shrinkage of the hippocampus [32–34].

Laminar origin

The entorhinal projection to the dentate gyrus predominantly originates from neurons in layer II (Figure 2) [4,7] and this projection is confined to the ipsilateral dentate gyrus, whereas the entorhinal projection to CA3, CA1 and the subiculum is bilateral, and it predominantly originates from neurons in layer III. No significant changes are present in Tg AD model mice (Figures 2 & 3). Importantly, it has been shown that the neurons in the superficial layers of the EC degenerate early in AD patients [35–37], thereby probably leading to decreased innervation of the hippocampus [38,39].

MRI–DTI measurements in mice & AD patients

To study the changes in connections in AD model mice compared with normal; in other words, C57BL mice, we performed preliminary MRI–DTI studies. The data indicate that small changes occur in hippocampal-related tracts in the Tg AD model mice that have developed significant pathology(Figure 4). The most easily identifiable changes occur in the fimbria/fornix; in the Tg AD model mice, there is a decrease in tract integrity (FA values increased by approximately 15%), as indicated by increased diffusion anisotropy, similar changes are observed in AD patients [40,41]. These changes in the integrity of myelin (white matter) correlate with observations of the white matter quality in the hippocampus of AD model mice by immunohistochemical methods [42]. In humans, it has been demonstrated that fornix FA both cross-sectionally correlated with and longitudinally predicted memory decline and progression to AD. Manually drawn regions of interest within the fornix show promise comparable with hippocampal volume as a predictive biomarker of progression [40]. Similarly, it has been demonstrated that the perforant path undergoes synaptic changes in the course of aging and dementia. Yassa *et al*. report direct evidence of age-related perforant path degradation in humans *in vivo* using ultra-high-resolution microstructural DTI [43]. They did not find evidence of white matter loss in a control pathway, the alveus, suggesting that these findings are not evidence for a global decline in white matter integrity. The extent of perforant path degradation correlated with performance on a word-list learning task sensitive to hippocampal deficits. They also show evidence for gray matter diffusion signals consistent with pyramidal dendrite orientation in the

hippocampus and cerebral cortex [43]. Perforant path degradation is a unique biomarker that can be used in combination with traditional structural and functional neuroimaging methods to enhance detection of AD in its earliest stages.

Plasticity in connections

In general, the magnitude and significance of sprouting, both normal and aberrant, in AD have been underestimated [44,45]; most studies have been focused on degeneration in brain connections, especially on the entorhinal to hippocampal connections [38,46].

Only a few studies have analyzed the effects of EC lesions on hippocampal plasticity in the mouse [31,47]. Our studies have shown that, surprisingly, even in Tg AD model animals with a high level of amyloid-β pathology, the response to an EC lesion is not significantly different from control age-matched mice [31]. We and others have shown that partial EC lesions lead to reinnervation of the denervated entorhinal–hippocampal pathway, both in control and Tg AD model mice. This, taken together with the use of Tg mice in studies on the involvement of the perforant path in AD [48] indicates that detailed anatomical information on the EC and its hippocampal connections in each species is important. Thus far, there is only one other study on the changes in the entorhinal–hippocampal connection in AD model mice [25]. Similar to our findings, their findings suggest that cerebral amyloid deposition has neurotropic effects and is the main cause of aberrant sprouting in the AD brain [49]. Furthermore, dystrophic axon terminals were found surrounding plaques; it should be noted that synaptic pathology is a major neurobiological substrate for cognitive dysfunction in AD [50].

Synaptic dysfunction is one of the first hallmarks of neurodegenerative disease. Spine pathology has been observed in association with many brain disorders, such as AD, Parkinson’s disease, prion diseases, schizophrenia, mental retardation and epilepsy. However, it is currently unclear how these phenotypes causally relate to disease progression. For example, in the vicinity of a cerebral infarct in mice, dendrites become exceptionally plastic, characterized by a long-lasting increase in the rate of spine turnover. These structural changes might provide a substrate for the long-term functional changes in the representational cortical maps that are observed after stroke models. Similarly, in mouse models of AD, the vicinity of amyloid plaques is characterized by highly dysmorphic neurites and spine turnover, causing a net loss of spines [51]. This phenotype could be caused by amyloid-β oligomers, which have been shown to block long-term potentiation and directly induce long-term depression, spine loss and memory loss [52].

AD & plasticity

The mechanisms that are involved in structural adaptive plasticity, allowing for the constant readjustment of connectivity providing the basis for ‘higher brain function’, are not very well understood. The integrative theory of neuro-plasticity suggests that no distinctions should be made between developmental, adaptive or restorative plasticity. It is thus reasonable to propose that the mechanism of reactive synaptic plasticity in the adult brain is identical to that involved in the natural turnover of synapses. The impact of experience on the CNS likely requires a lifelong high turnover of synapses that might, therefore, involve

the same molecules as ‘reactive synaptogenesis’, as occurs, for example, after a lesion of the EC. Some of the proposed mechanisms are:

- Neurotrophic factors such as NGF, BDNF, IGF-1, FGF or TGF-β;
- Growth-associated proteins, such as GAP-43;
- Neural cell-adhesion molecules, such as NCAM, and several synaptic proteins, such as synaptophysin and SNAP 25;
- Cellular lipids and lipid carrier proteins, such as ApoE;
- Changes in the expression and subcellular distrubution of microtubule-associated proteins and other cytoskeletal proteins [53,54].

In organs other than the brain, cell activation seems to increase ‘wear and tear’, for example, by increased free-radical formation, and thus cause an increased rate of aging. However, activation of nerve cells within the physiological range seems to lead to maintenance of neurons during aging and AD, possibly by preferentially stimulating the action of protective mechanisms, such as DNA repair. This ‘use it or lose it’ principle might explain why certain neurons degenerate in aging or AD, while others do not, and why recovery of various neuronal systems during aging has been obtained by restoration of the missing stimulus [55]. Consequently, neuronal activation may provide a means of prolonging neuronal function for the full length of our natural lifespan. There is a regional pattern of amyloid deposits and tangle pathology in AD brains that changes with disease duration [56]. Tangle density in AD shows systematic regional differences that can be observed both during aging and in AD [57]. The intensity of dendritic remodelling that can be observed during aging, as well as in AD, is regionally different and decreased in the following order: transentorhinal region > limbic areas (entorhinal region and hippocampus) > nonprimary association areas > primary sensory association areas > primary sensory and motor cortex [56]. These regional differences of neuronal plasticity follow the same pattern as the regional vulnerability to tangle formation in AD [57,58]. Furthermore, brain areas affected by AD pathology are primarily those structures that are involved in the regulation of ‘higher brain functions’ [59]. The functions these areas subserve, such as learning, memory, perception, self-awareness and consciousness, require a life-long adjusting of synaptic contacts that allows for the acquistion of new information, a process based on a particularly high degree of structural plasticity. Thus, there seems to be a relation between the development of AD pathology and disturbed neuronal plasticity [49,55].

AD & pathology spread

Many age-associated neurodegenerative diseases have, as the main pathology, the aggregation of specific proteins within the nervous system. For instance, in AD, the insidious pathogenic process begins many years before the symptoms emerge and the lesions that characterize the disease; in other words, senile plaques and neurofibrillary tangles are present throughout the brain. However, the pathology does not show up at random: a sequence of pathological events occurs [56,57]. There is clear evidence that both the amyloid-β and tau proteins, which aggregate to form senile plaques and neurofibrillary tangles, respectively, spread through the brain [48,60] following anatomical pathways.

Recent data also indicate that the spread of these lesions from one site to another is mediated by the cellular uptake, transport and release of endogenous seeds formed by the cognate proteins; in other words, amyloid-β and tau [61,62].

AD & connections

It has been demonstrated that patients with AD experience a brain network breakdown [63], reflecting disconnection at both the structural and functional system levels [14,53,64]. Resting-state functional MRI studies demonstrated that the regional coherence of the functional MRI signal is significantly altered in patients with AD and amnestic mild cognitive impairment [65]. MRI–DTI has made it possible to track fiber bundle projections across the brain, revealing a substantially abnormal interplay of 'critical' white matter tracts in these conditions [66]. Regional cortical atrophy and cognitive function of AD patients have been shown to correlate with the structural changes of white matter. Synchronized structural changes of cingulum bundle and fornix, both of which are limbic tracts, were revealed. Widespread yet distinctive structural changes were demonstrated in limbic, commissural, association and projection tract groups between control and AD subjects [67,68]. In order to improve our understanding of the pathobiology of these findings, studies in Tg AD model mice are required [69].

Conclusion

Patients with AD demonstrate a brain network breakdown, reflecting disconnection at both the structural and functional system level. Our studies and many other studies are helping to establish the exciting potential of tract tracing as a neuropathological measure and as a biomarker of disease progression in AD.

Future perspective

Together, the data presented indicate that deposition of cerebral amyloid-β leads to synaptic pathology, which causes the disruption of neuronal connectivity which, in turn, significantly contributes to AD dementia. Imaging provides a powerful quantitative measure of changes in structural connectivity measures derived from MRI–DTI methods. These methods offer additional markers of neuropathology arising from the secondary changes in axonal caliber and myelination that accompany decreased neuronal activity and neurodegeneration. DTI can especially be used for more finely mapping neurodegenerative changes in AD in patients and defining neuro-pathological changes in white matter. The differences between AD and control subjects, and those between mild cognitive impairment and control subjects indicate a progressive pattern of white matter disruption from limbic and commissural tracts to other tracts [67]. Together, the high correlation between FA, mean diffusity, and radial diffusivity measurements from limbic tracts and cortical atrophy suggests that the disruption of the limbic tracts is caused by neuronal damage [67], as is also shown by our anatomical studies [25].

Visualizing neuropathology can be enhanced by using more specific DTI measures and interpreting them relative to knowledge of local white matter anatomy in the healthy brain [68].

The available data suggest that AD risk is associated with a decline in white matter integrity in a subset of tracts. Specifically, AD risk has been associated with white matter integrity declines in tracts that connect gray matter structures associated with memory function [59]. These tracts include parahippocampal white matter, including the cingulum and the splenium of the corpus callosum. Some studies have indicated that AD risk declines are characterized by increases of radial diffusivity, raising the possibility that a myelin-related pathology may contribute to AD onset [67]. Together, these findings justify future research aimed at a more complete understanding of the neurobiological basis of DTI-based declines in AD. With continued refinement of imaging methods, DTI holds promise as a method to aid in the identification of presymptomatic AD [41,68,70–72].

Together, our and many other studies are helping to establish the exciting potential of tract tracing as a neuropathological measure and a biomarker of disease progression. The viability of these white matter tract integrity metrics as potential neuroimaging biomarkers of the earliest stages of AD and disease progression is unquestionable, but basic studies in Tg AD model mice are still urgently needed to validate these changes.

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EXECUTIVE SUMMARY

- Lateral entorhinal area projects via the lateral perforant path to the dentate gyrus in the outer third of the molecular layer.
- The Medial entorhinal area gives rise to labeled axons in the medial perforant path ending in the middle third of the molecular layer of the dentate gyrus.
- The projection of the entorhinal cortex to the hippocampal formation is topographically organized. Neurons in lateral parts of the medial and lateral entorhinal cortex project to the dorsal, septal part of the hippocampal formation, medial parts of the entorhinal cortex project to the temporal, ventral hippocampal formation.
- It is likely that cerebral amyloid deposition early on has neurotrophic effects and is the main cause of aberrant sprouting in the Alzheimer's disease (AD) brain. Furthermore, dystrophic axon terminals were found surrounding plaques; it should be noted that synaptic pathology is the major neurobiological substrate for cognitive dysfunction in AD.
- Small changes occur in hippocampal-related tracts in transgenic AD mouse models that have developed significant pathology.
- The earliest and most easily identifiable changes occur in the fimbria/fornix, as indicated by increased diffusion anisotropy.
- These changes are also observed in AD patients.
- Patients with AD demonstrate a brain network breakdown, reflecting disconnection at both the structural and functional system level.
- Together, our studies and many other studies are helping to establish the exciting potential of tract tracing as a neuropathological measure and biomarker of disease progression in AD.

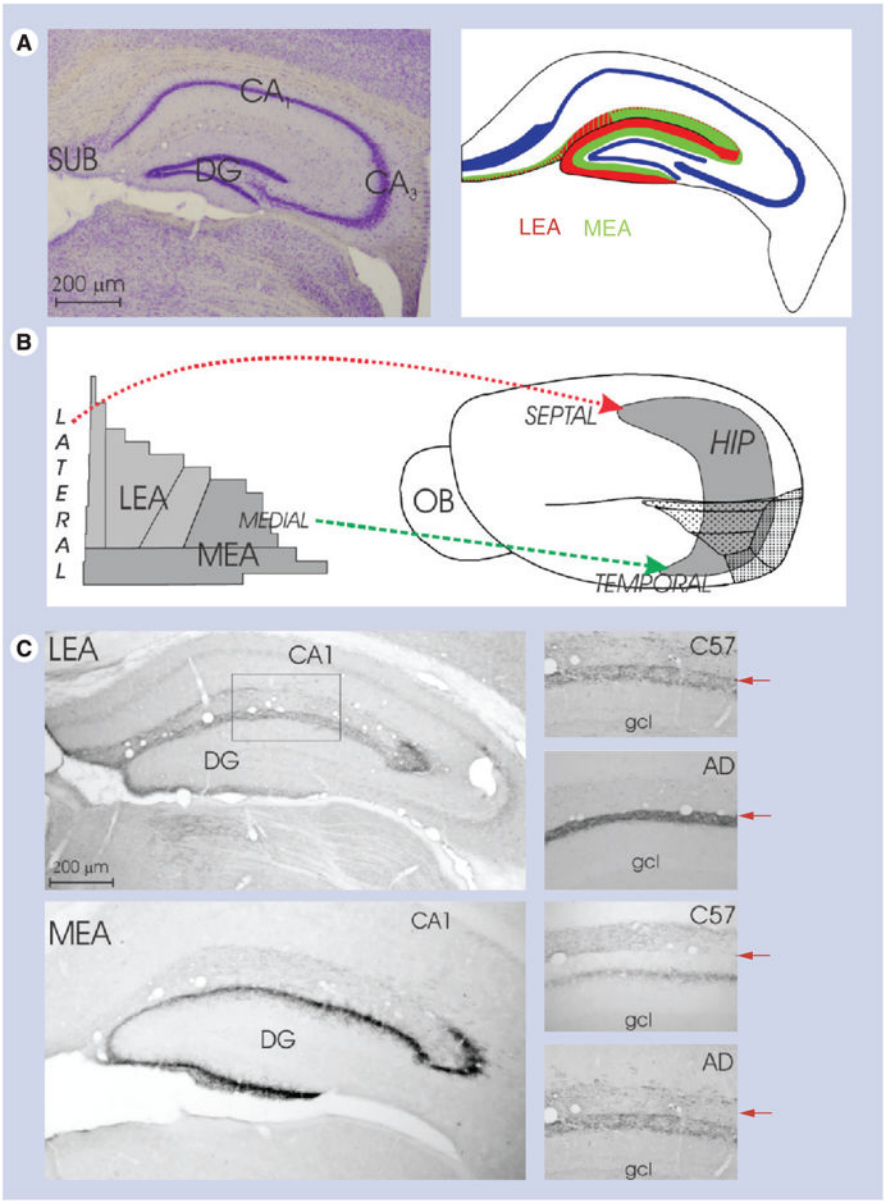

Figure 1. Overview of entorhinal–hippocampal connections

(A) Low-power photomicrograph of a coronal section of the septal hippocampal formation and 2D reconstruction map of the HIP to demonstrate the labeling pattern of the entorhinal cortex axons in the HIP; **(B)** 2D reconstruction maps of the entorhinal cortex (left) and mouse brain (right) to demonstrate the septotemporal distribution of the entorhinal cortex axons in the HIP; **(C)** left, two low-power photomicrographs of coronal sections through the septal hippocampal formation to demonstrate the labeling patttern of the entorhinal cortex axons in the HIP. Right, four higher-power images of the molecular layer of the DG showing the terminals of the LEA and MEA. Top image: C57BL mouse; bottom image: young (2 month) transgenic AD mouse model. The arrows indicate hippocampal fissure.

AD: Alzheimer's disease; DG: Dentate gyrus; gcl: Granule cell layer; HIP: Hippocampus; LEA: Lateral entorhinal cortex; MEA: Medial entorhinal cortex; OB: Olfactory bulb; SUB: Subiculum.

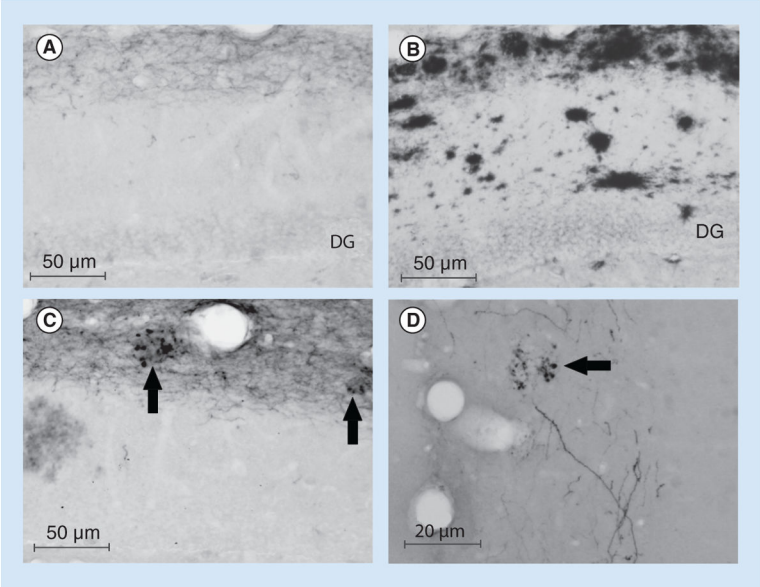

Figure 2. Four high-power photomicrographs of coronal sections through the dorsal hippocampal formation to demonstrate the labeling pattern in the hippocampus following injections of biotinylated dextran amine into the entorhinal cortex in transgenic Alzheimer's disease mouse models that have Alzheimer's disease pathology

(A) Demonstrates labeling in the dentate gyrus following an injection into the lateral entorhinal cortex, while **(B)** shows the adjacent section stained for amyloid-β (WO-2 antibody). **(C & D)** Demonstrate labeling in the hippocampus following injections into the lateral entorhinal area and medial entorhinal area, respectively. The presence of plaques and dystrophic axon endings is highlighted by the arrows.

DG: Dentate gyrus.

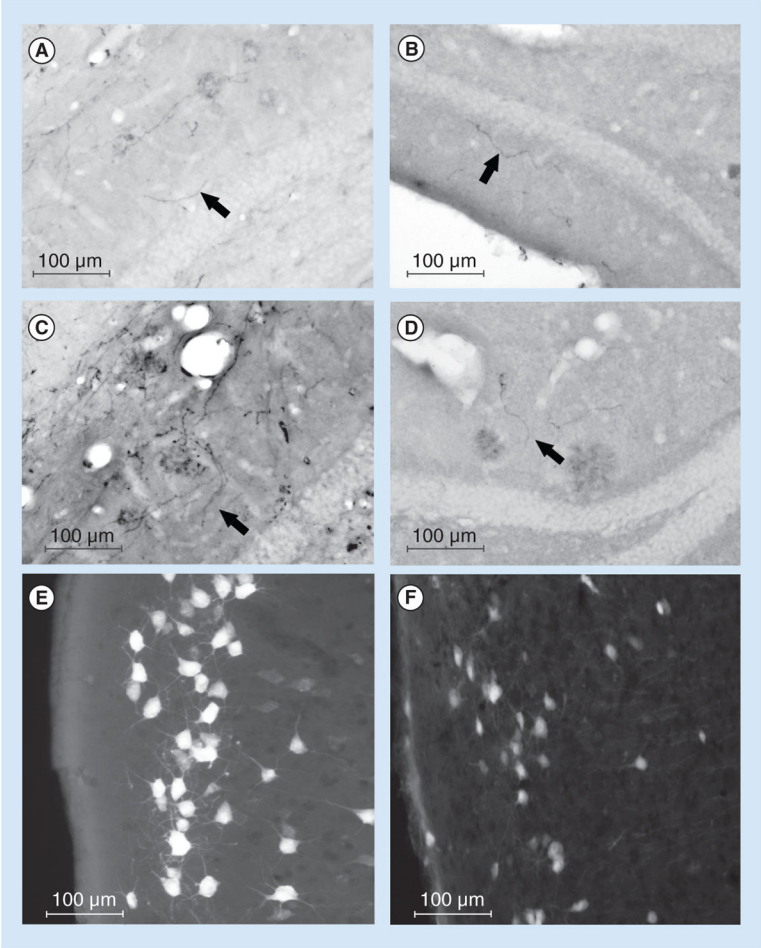

Figure 3. Changes in terminal fields with Alzheimer's disease

(A–D) Four high-power photomicrographs to demonstrate improper localization of axons in the dentate gyrus in a transgenic Alzheimer's disease mouse model with pathology. Arrows indicate incorrect location of axon endings. **(E & F)** High-power photomicrographs to demonstrate the pattern of labeled neurons in the entorhinal cortex following injections of retrogradely transported tracers into the dorsal hippocampus. **(E)** Demonstrates FluoroGold™-labeled (Fluorochrome, LLC, CO, USA) neurons in layer II of the entorhinal cortex following an injection into the dentate gyrus; **(F)** demonstrates labeled neurons in a transgenic Alzheimer's disease mouse model with amyloid-β pathology.

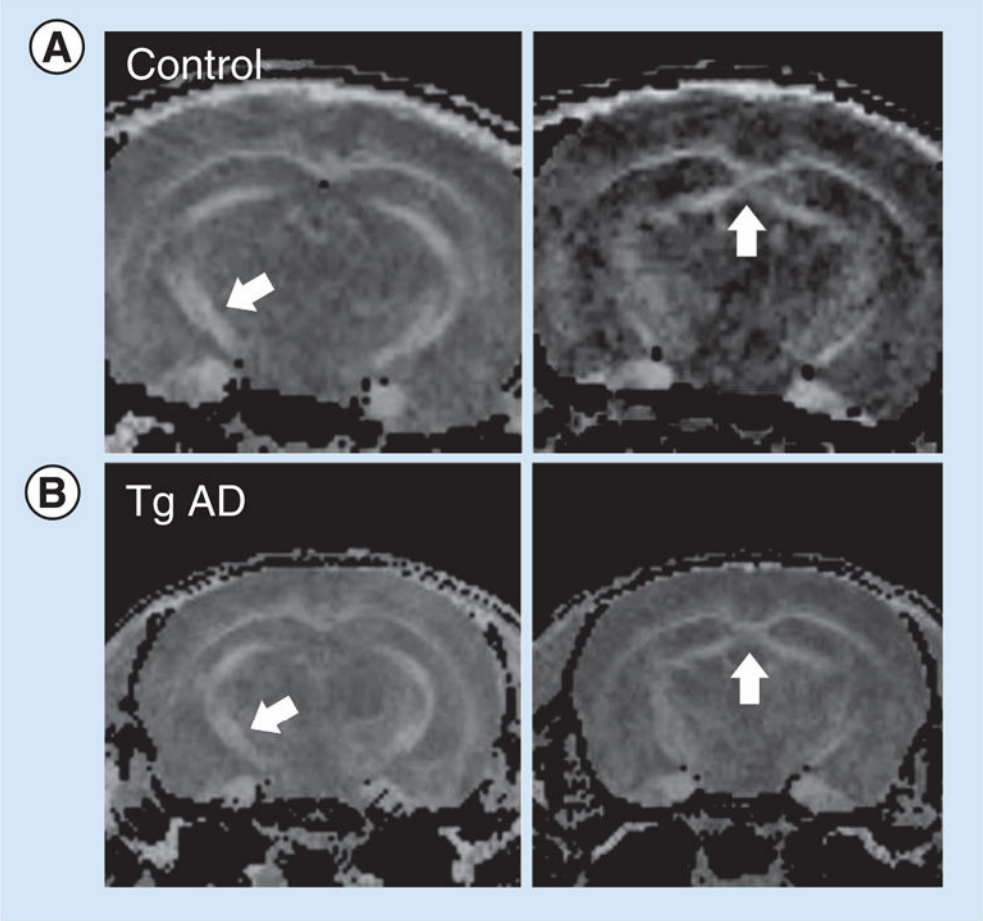

Figure 4. Four MRI–diffusion tensor imaging images of the mouse brain

(A) Shows the normal mouse and **(B)** shows the Tg AD mouse model at 6 months of age. Arrows indicate areas with changes in fractional anisotropy between normal and Tg AD model mice; in other words, the fornix and perforant path. The fractional anisotropy has been reduced by 20% in the AD mice.

AD: Alzheimer's disease; Tg: Transgenic.