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Correspondence and requests for materials should be addressed to K.R.F. (krfoster@rice.edu) or C.R.L.T. (christopher.thompson@man.ac.uk).

Migratory neural crest-like cells form body pigmentation in a urochordate embryo

William R. Jeffery, Allen G. Strickler & Yoshiyuki Yamamoto*

Department of Biology, University of Maryland, College Park, Maryland 20742, USA

* Present address: Evolutionary Anatomy Unit, Department of Anatomy & Developmental Biology, University College London, Gower Street, London WC1E 6BT, UK

The neural crest, a source of many different cell types in vertebrate embryos, has not been identified in other chordates[1–3]. Current opinion therefore holds that neural crest cells were a vertebrate innovation[4–7]. Here we describe a migratory cell population resembling neural crest cells in the ascidian urochordate *Ecteinascidia turbinata*. Labelling of embryos and larvae with the vital lipophilic dye DiI enabled us to detect cells that emerge from the neural tube, migrate into the body wall and siphon primordia, and subsequently differentiate as pigment cells. These cells express HNK-1 antigen and *Zic* gene markers of vertebrate neural crest cells. The results suggest that migratory cells with some of the features of neural crest cells are present in the urochordates. Thus, we propose a hypothesis for neural crest evolution beginning with the release of migratory cells from the CNS to produce body pigmentation in the common ancestor of the urochordates and vertebrates. These cells may have gained additional functions or were joined by other cell types to generate the variety of derivatives typical of the vertebrate neural crest.

Neural crest cells delaminate from the dorsal neural tube in an anterior to posterior sequence, migrate through stereotypical pathways, and differentiate into a variety of cell types in vertebrate embryos, including neuronal, glial, endocrine, skeletal and pigment cells[1,2]. Migratory neural crest cells have not been described in non-vertebrate chordates. In ascidian urochordates and amphioxus, however, cells at the border of the neural plate or within the neural tube are often proposed as evolutionary precursors of the neural crest[3,7–10].

The life cycle of the ascidians is usually separated into distinct larval (or embryonic) and adult developmental phases[11]. The tadpole larva consists of a trunk (or head) with a dorsal central nervous system (CNS) containing two melanized sensory organs (otolith and ocellus) and a tail with a notochord, spinal cord and muscle cells. During metamorphosis, the larval tail is destroyed and the head is extensively reorganized into a sessile filter-feeding adult. Previous searches for neural crest cells were restricted to the embryonic phase in ascidian species such as *Ciona intestinalis*, whose small larvae exhibit the conventional mode of development. These studies did not use cell-tracing methods, which originally defined the neural crest in vertebrates[1,2]. We have investigated the possibility of neural crest-like cells in urochordates using the colonial ascidian *Ecteinascidia turbinata* (Fig. 1a), which exhibits a giant tadpole that is suitable for cell-tracing approaches to detect migratory cells (Fig. 1b). In contrast to more commonly studied ascidians, the *Ecteinascidia* tadpole initiates adult development in its head during the embryonic phase, where adult features such as the pharynx, heart, siphons and stellate body pigment cells are formed precociously (Fig. 1c, d)[12,13].

Using the otolith and ocellus as landmarks, DiI was injected into the anterior neural tube at the early tailbud stage and the injected embryos were subsequently examined by fluorescence microscopy. There was no movement of DiI or labelled cells immediately after injection (Fig. 2a, b). By 4 h after injection, however, DiI-labelled cells were observed to migrate from the injection site towards the developing siphons (Fig. 2c, d). Migratory cells continued to be observed through the late tailbud stage (Fig. 2e, f). Some of these migrating cells had the stellate morphology typical of body pigment cells (Fig. 2f). At the mid-tailbud stage, DiI was injected into the anterior neural tube, the posterior neural tube, or the ventral head epidermis. As before, migrating cells emerged from the anterior neural tube and moved ventrolaterally into the body wall (Fig. 2g). Sectioning showed that cell migration occurred in two pathways: (1) through the dorsal mesoderm surrounding the neural tube, and (2) between the mesoderm and epidermis (Fig. 2j–l). In contrast, DiI-labelled cells did not migrate from the posterior neural tube (Fig. 2h) or the ventral epidermis (Fig. 2i), although local rearrangements of labelled cells occurred in these regions. The results show that migratory cells emerge from the neural tube during *E. turbinata* embryogenesis.

DiI-labelling experiments were continued during later development. After DiI injection into the posterior neural tube at the late-tailbud stage, labelled cells migrated into the head but were excluded from the tail (Fig. 3a, b). At the same stage, migratory cells were still generated after DiI injection into the anterior neural tube, and many of these cells became associated with the developing siphons (see Fig. 2e, f; data not shown). These results suggest that migratory cells emerge in an anterior to posterior sequence during *Ecteinascidia* development. In contrast to vertebrate neural crest cells, however, the

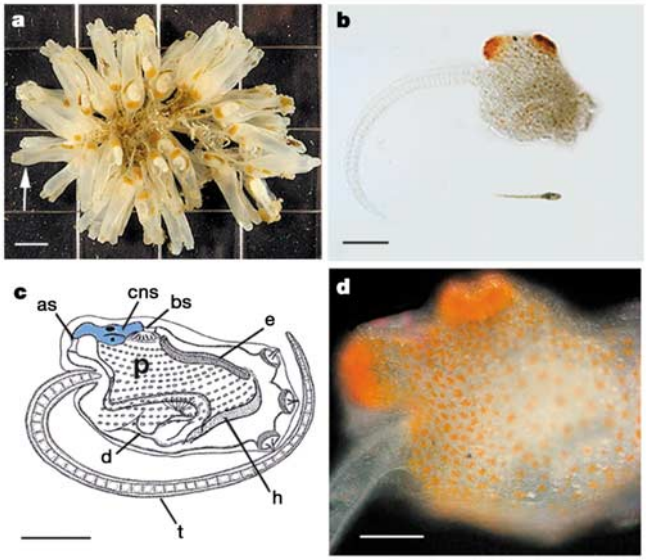

Figure 1 *Ecteinascidia turbinata*. **a**, A colony showing gravid zooids (arrow). **b**, The giant *Ecteinascidia* tadpole (above) compared to a small *Styela clava* tadpole (below). **c**, A diagram of the *Ecteinascidia* tadpole after ref. 12, showing the CNS (blue) with melanized sensory cells (black), branchial siphon (bs), atrial siphon (as), endostyle (e), heart (h), perforated pharynx (p), digestive tract (d), and tail (t). **d**, Orange pigment cells distributed throughout the body wall and concentrated in the siphon primordia of an *Ecteinascidia* tadpole. Scale bars: **a**, 1 cm; **b**–**d**, 800 μm.

Ecteinascidia cells migrated singly rather than in streams (Figs 2, 3), possibly owing to the absence of segmentation in the ascidian trunk. Also in contrast to vertebrate neural crest cells, the ascidian migratory cells appeared to emerge from all dorsal–ventral levels of the neural tube/CNS and for an extended time period after its formation from the neural plate. Injection of DiI into the CNS of mature tadpoles and examination of post-metamorphic adults by fluorescence microscopy detected labelled cells throughout the body wall and siphons (Fig. 3c–f). Most of the labelled cells were identified as pigment cells by their orange colour and stellate morphology (Figs 2f and 3g, h): in a single animal 28 of 36 DiI-labelled cells coincided exactly with a pigment cell. The remaining labelled cells were located near a pigment cell but their identity could not be determined with certainty. The results suggest that the migratory cells differentiate primarily into body pigment cells.

To determine whether the migratory cells express neural crest markers, embryos were stained with HNK-1 antibody or subjected to *in situ* hybridization with a *Zic* (*EtZic*) gene probe. HNK-1 antibody recognizes a surface glycolipid that is expressed in vertebrate neural crest cells during the migration phase but disappears shortly after terminal differentiation[14–17]. In *Ecteinascidia* larvae, HNK-1 expression was detected in dorsal body wall and siphon cells showing the typical morphology of pigment cells (Fig. 4a–d, h). *Zic* family genes encode C2H2 zinc finger transcription factors that are expressed in the neuroectoderm and migrating neural crest cells of vertebrate embryos and can lead to ectopic neural crest formation when misexpressed during *Xenopus* development[18–20]. As previously described in *Ciona* and amphioxus embryos[21,22], *Zic* was expressed throughout the *Ecteinascidia* neural tube at early developmental stages (data not shown). By the late-tailbud stage, however, *EtZic* expression was confined to dorsal body wall cells with typical pigment cell morphology (Fig. 4e, g). Cells expressing HNK-1 and *EtZic* were abundant in the developing siphons, which also accumulate migratory cells and have been suggested to be homologous to vertebrate placodes[23,24]. The results demonstrate the existence of migratory cells with several striking similarities to neural crest cells in a urochordate embryo. These cells emerge from the neural tube/CNS in an anterior to posterior sequence, migrate below the epidermis and through the mesoderm, express HNK-1 and *Zic* markers, differentiate into somatic pigment cells, and contribute to placode-like structures.

All vertebrate body pigment cells, including melanophores, iridophores, and xanthophores (which are most similar to *Ecteinascidia* orange pigment cells), are derived from the neural crest[25]. Although most DiI-labelled cells appear to differentiate into pigment cells, the possibility that some of these cells may have additional fates cannot be excluded. The migratory pigment cells we have identified are restricted to the developing head of *Ecteinascidia* larvae, suggesting that they may be special features of the adult rather than the larval development phase. This interpretation is consistent with evidence for smaller-scale migration of adult neuronal precursors from the CNS during ascidian meta-

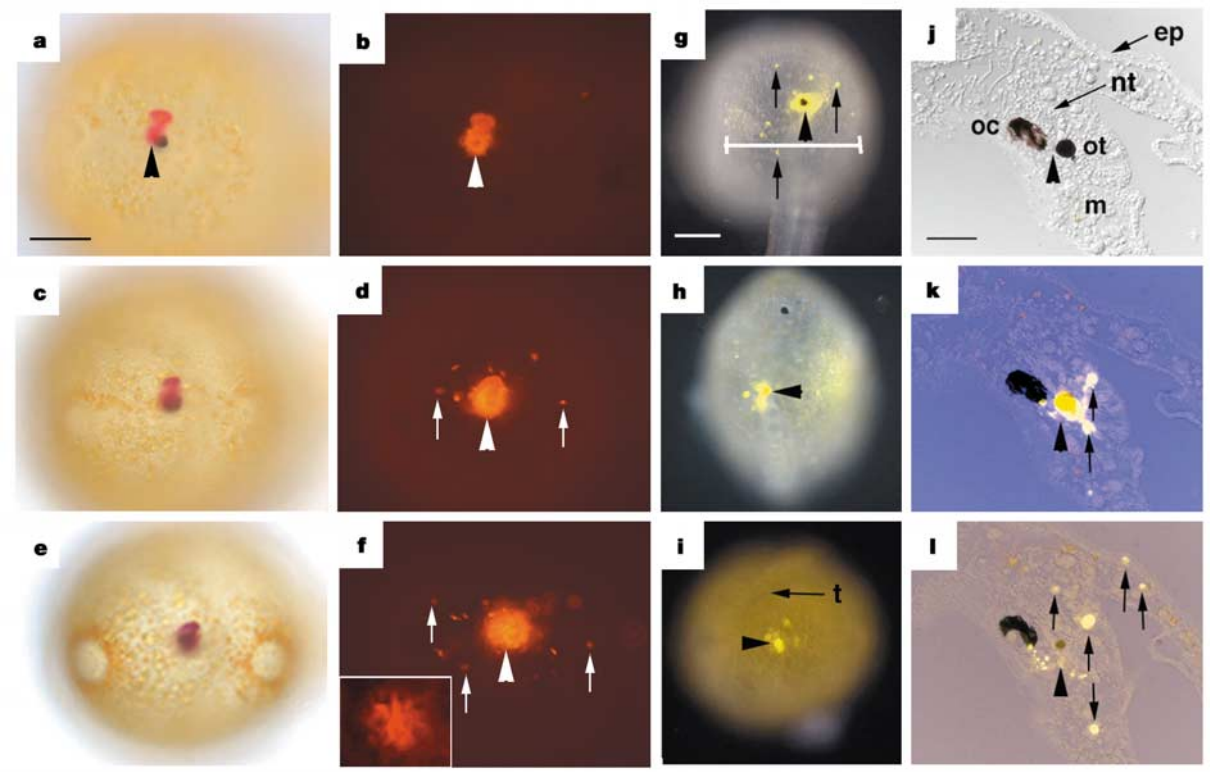

Figure 2 DiI tracing of migratory cells in embryos. **a**–**f**, Bright-field (**a**, **c**, **e**) and fluorescence (**b**, **d**, **f**) images of the same embryo injected in the anterior neural tube near the otolith/ocellus (black dot) at the early-tailbud stage. Inset in **f**, High magnification of stellate migrating cell. Images shown at 10 min (**a**, **b**), 4 h (**c**, **d**), and 14 h (**e**, **f**) after injection. **g**–**i**, Fluorescence images of DiI-labelled cells after injection into the anterior neural tube near the otolith/ocellus (**g**), the posterior neural tube region near the junction of the trunk and tail (**h**), and the ventral epidermis (**i**) at the mid-tailbud stage. The white bracketed horizontal line in **g** indicates the plane of section in **j**–**l**. **j**–**l**, Bright-field (**j**) and fluorescence (**k**, **l**) images of a mid-tailbud embryo cross-sectioned serially with respect to the anterior–posterior axis showing the injection site between the otolith (ot) and ocellus (oc) and labelled cells migrating from the neural tube (nt) into the dorsal mesoderm (m) and below the epidermis (ep) (arrows). Migratory cells were observed leaving the injection site in every case ($n = 14$) in which DiI was injected into the anterior neural tube. Views are dorsal in **a**–**h** and ventral in **i**; **a**–**f**, anterior on the right; **g**, **h**, anterior on top. Arrowheads, DiI injection sites. Arrows, DiI-labelled migrating cells. Scale bars: **a**, **g**, 200 μm; **j**, 30 μm; magnification is the same in **a**–**f**, **g**–**i** and **j**–**l**.

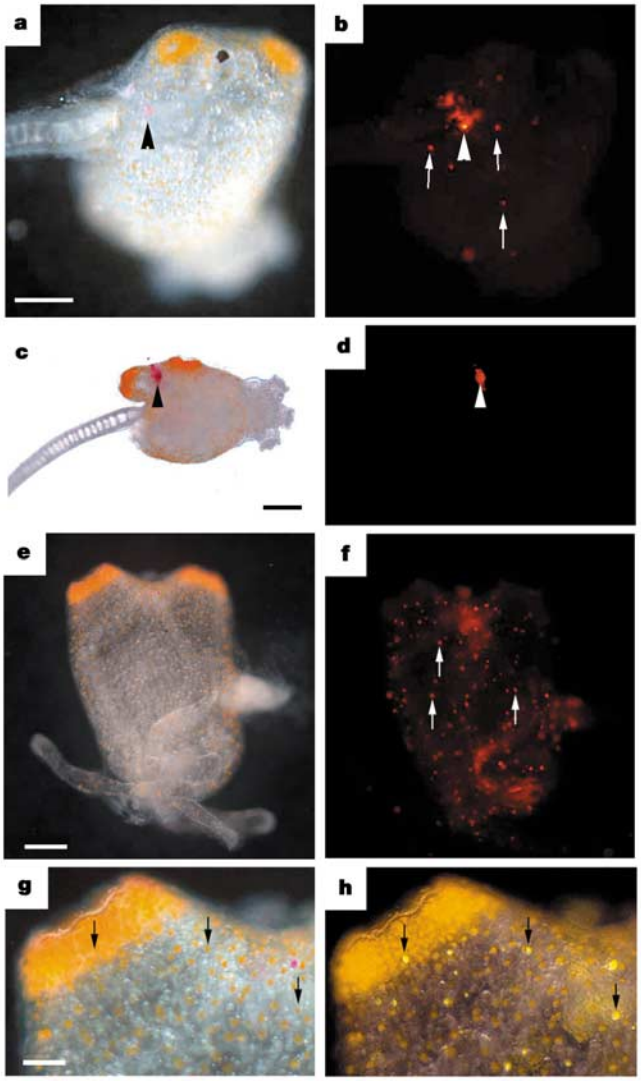

Figure 3 DiI tracing and fate of migratory cells in developing tadpoles and post-metamorphic adults. **a**, **b**, Bright-field (**a**) and fluorescence (**b**) images of the same embryo injected with DiI in the posterior neural region at the late-tailbud stage. **c**–**f**, Bright-field (**c**, **e**) and fluorescence (**d**, **f**) image of a DiI-injected mature tadpole (**c**, **d**) showing labelled cells 12 h later after metamorphosis (**e**, **f**). **g**, **h**, Fate of DiI-labelled cells in a post-metamorphic adult. **g**, Bright-field image showing individual orange pigment cells in the body wall. **h**, Fluorescence image of the same animal as in **g** showing DiI-labelled cells (in yellow). All views are lateral. Arrows indicate some of the orange pigment cells that also show DiI labelling. Scale bars: **a**, 250 μm; **c**, 200 μm; **e**, 100 μm; **g**, 20 μm; magnification is the same in **a** and **b**, **c** and **d**, **e** and **f**, and **g** and **h**.

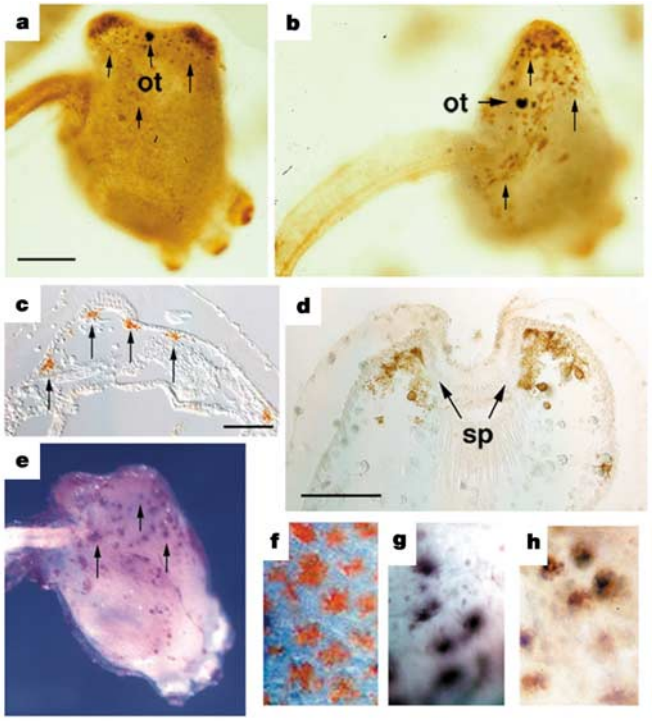

Figure 4 Neural crest markers. Expression of HNK-1 antigen (**a**–**d**, **h**) and the *EtZic* gene (**e**, **g**) in developing larvae. **a**, **b**, Lateral (**a**) and dorsal (**b**) views showing HNK-1 expressing cells (arrows). **c**, **d**, Sections through the dorsal body wall (**c**) and oral siphon primordium (sp) (**d**) showing HNK-1-stained cells (arrows). **e**, Lateral view of *in situ* hybridized larva showing *EtZic*-expressing cells (arrows) in the dorsal body wall. **f**–**h**, Comparison of orange pigment cells (**f**), *EtZic*-expressing cells (**g**), and HNK-1-stained cells (**h**) in the larval body wall. Scale bars: **a**, 250 μm; **c**, **d**, 30 μm; magnification is the same in **a**, **b** and **e**; **f**–**h** are × 10 the magnification of **a**.

morphosis[26–28] and the presence of calcitonin-producing cells, a vertebrate neural crest derivative[1,2], in the adult pharnyx[29].

We conclude that migratory neural crest-like cells are present in the ascidian *Ecteinascidia*. Although these cells could have evolved independently within the urochordate line or even within *Ecteinascidia*, HNK-1 and *Zic* expression suggests that they are likely to share a common origin with vertebrate neural crest cells. In support of a common evolutionary heritage of ascidian migratory cells and vertebrate neural crest cells, one of us (W.R.J.; manuscript in preparation) has recently discovered similar HNK-1 positive cells in four other ascidian species from evolutionarily diverse families. We therefore propose the following scenario for neural crest evolution in the chordates.

First, the neural tube/CNS attained the capacity to generate migratory pigment cells, possibly as a means to protect a sessile ascidian-like chordate ancestor from the harmful effects of sunlight in shallow marine habitats. These migratory pigment-generating cells may have been lost secondarily in amphioxus, which live buried in marine sediment and lack body pigmentation. Alternatively, it is conceivable that urochordates are the true sister group of vertebrates and that the migratory cells evolved in their common ancestor after the divergence of amphioxus from the chordate lineage. Later in chordate evolution, probably at or near the base of the vertebrate radiation, the primitive migratory cells gained additional functions and/or were joined by other cell types to generate the multiple derivatives characteristic of the neural crest. □

Methods

Animals

Ecteinascidia turbinata was obtained from Gulf Specimens, Inc. or collected near the Bermuda Biological Station, St Georges West, Bermuda. Tailbud-stage embryos and larvae were dissected from viviparous adults and cultured in Millipore-filtered sea water (MFSW) at 19 °C. The chorion of pre-hatching stage embryos was removed by dissection with sharpened tungsten needles.

DiI cell tracing

Dechorionated embryos and hatched larvae were immobilized for microinjection in 0.2% agar/MFSW. The vital lipophilic dye 1, 1′-dictadecyl-3, 3, 3′-tetramethylindocarbocyanine perchlorate (DiI) (C-7000, Cell tracker, Molecular Probes) was dissolved to a final concentration of 37.5 μg ml^{-1} in 92% *N*, *N*-dimethylformamide/8% 2 × distilled water by briefly heating to 50 °C, and glass micropipettes were back-filled with the DiI solution. Before DiI injection a small puncture was made with a tungsten needle in the larval tunic above the preferred DiI injection site. The DiI-containing micropipette was then inserted into the puncture and a bolus of DiI was pressure-injected. After microinjection, the embryos and larvae were washed several times in MFSW, and viewed with bright-field and

fluorescence optics. The DiI-injected embryos were fixed in 4% paraformaldehyde, embedded in polyester wax, sectioned at 10 μm, and sections were viewed unstained with bright-field and fluorescence optics.

HNK-1 staining

Larvae were fixed in 4% paraformaldedyde in PBS for 30 min at room temperature and then in 100% methanol for 5 min at −20 °C. The fixed larvae were washed in PBS containing 1% Triton X-100, and stained with a 1:20 dilution of HNK-1 antibody (Biopharmagen) for 24 h at 4 °C using the Vectastain ABC Peroxidase kit (Vector Laboratories). Antibody incubations and blocking were carried out in 2% Superblock and controls used non-immune mouse serum.

EtZic isolation and *in situ* hybridization

The oligodexoynucleotide primers AARGCMAAATACAARCTRATCAACC (forward) and ACYTTCATGTGCTTSCKRAGRGAGC (reverse) were used to isolate part of an *Ecteinascidia Zic* gene by polymerase chain reaction with reverse transcription (RT–PCR) from total larval RNA. BLAST searches and tree building suggested that *EtZic* is most closely related to *CsZicr2* (ref. 21), *AmphiZic* (ref. 22), and the vertebrate *Zic2* (refs 18–20) genes. *In situ* hybridization with a digoxygenin-labelled probe was carried out as described previously[30].

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Correspondence and requests for materials should be addressed to W.R.J. (jeffery@umd.edu). The EtZic DNA sequence has been deposited in GenBank under accession number AY176563.

Role for a cortical input to hippocampal area CA1 in the consolidation of a long-term memory

Miguel Remondes & Erin M. Schuman

Caltech/HHMI, Division of Biology, 114-96, Pasadena, California 91125, USA

A dialogue between the hippocampus and the neocortex is thought to underlie the formation, consolidation and retrieval of episodic memories[1–4], although the nature of this cortico-hippocampal communication is poorly understood. Using selective electrolytic lesions in rats, here we examined the role of the direct entorhinal projection (temporoammonic, TA) to the hippocampal area CA1 in short-term (24 hours) and long-term (four weeks) spatial memory in the Morris water maze. When short-term memory was examined, both sham- and TA-lesioned animals showed a significant preference for the target quadrant. When re-tested four weeks later, sham-lesioned animals exhibited long-term memory; in contrast, the TA-lesioned animals no longer showed target quadrant preference. Many long-lasting memories require a process called consolidation, which involves the exchange of information between the cortex and hippocampus[3,5,6]. The disruption of long-term memory by the TA lesion could reflect a requirement for TA input during either the acquisition or consolidation of long-term memory. To distinguish between these possibilities, we trained animals, verified their spatial memory 24 hours later, and then subjected trained animals to TA lesions. TA-lesioned animals still exhibited a deficit in long-term memory, indicating a disruption of consolidation. Animals in which the TA lesion was delayed by three weeks, however, showed a significant preference for the target quadrant, indicating that the memory had already been adequately consolidated at the time of the delayed lesion. These results indicate that, after learning, ongoing cortical input conveyed by the TA path is required to consolidate long-term spatial memory.

To assess the role of the TA pathway in the acquisition and retention of spatial memory in the rat, we identified stereotaxic coordinates to target the TA axons for electrolytic ablation. We used histology, electrophysiology, and retrograde tracing techniques to verify the extent and specificity of the TA lesions. We included in our behavioural analysis data from animals with relatively restricted lesions in the stratum lacunosum moleculare, the region where TA axons terminate in area CA1 (Fig. 1a,b and Supplementary Figs 1 and 2); we excluded animals in which the lesion extended significantly into the perforant path input to the dentate gyrus. We performed electrophysiological recordings on a subset of slices, and assessed synaptic transmission in the perforant path–dentate gyrus, TA–CA1 and CA1–subiculum pathways with extracellular stimulation and recordings (Fig. 1c). Brain slices from sham-lesioned animals showed normal synaptic transmission at all three sets of synapses (Fig. 1c). Slices from TA-lesioned animals exhibited