HHS Public Access

Author manuscript

Brain Res Bull. Author manuscript; available in PMC 2016 August 25.

Published in final edited form as:

Brain Res Bull. 2007 March 30; 71(6): 601–609. doi:10.1016/j.brainresbull.2006.12.002.

NUCLEUS REUNIENS OF THE MIDLINE THALAMUS: LINK BETWEEN THE MEDIAL PREFRONTAL CORTEX AND THE HIPPOCAMPUS

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Abstract

The medial prefrontal cortex and the hippocampus serve well recognized roles in memory processing. The hippocampus projects densely to, and exerts strong excitatory actions on, the medial prefrontal cortex. Interestingly, the medial prefrontal cortex, in rats and other species, has no direct return projections to the hippocampus, and few projections to parahippocampal structures including the entorhinal cortex. It is well established that the nucleus reuniens of the midline thalamus is the major source of thalamic afferents to the hippocampus. Since the medial prefrontal cortex also distributes to nucleus reuniens, we examined medial prefrontal connections with populations of nucleus reuniens neurons projecting to hippocampus. We used a combined anterograde and retrograde tracing procedure at the light and electron microscopic levels. Specifically, we made *Phaseolus vulgaris*-leuccoagglutinin (PHA-L) injections into the medial prefrontal cortex and Fluorogold injections into the hippocampus (CA1/subiculum) and examined termination patterns of anterogradely PHA-L labeled fibers on retrogradely FG labeled cells of nucleus reuniens. At the light microscopic level, we showed that fibers from the medial prefrontal cortex form multiple putative synaptic contacts with dendrites of hippocampally projecting neurons throughout the extent of nucleus reuniens. At ultrastructural level, we showed that medial prefrontal cortical fibers form asymmetric contacts predominantly with dendritic shafts of hippocampally projecting reuniens cells. These findings indicate that nucleus reuniens represents a critical link between the medial prefrontal cortex and the hippocampus. We discuss the possibility that nucleus reuniens gates the flow of information between the medial prefrontal cortex and hippocampus dependent upon attentive/arousal states of the organism.

Keywords

prelimbic cortex; working memory; mediodorsal nucleus; entorhinal cortex; rat

1. Introduction

The hippocampus and medial prefrontal cortex (mPFC) serve well recognized roles in memory processing [1,2,15–17,20–22,50,53]. The hippocampus distributes heavily to the medial prefrontal cortex [12,18,28–30,43,47] and exerts strong excitatory actions on the mPFC [18,31,33,34]. Despite the direct innervation/influence of the hippocampal formation (HF) on the mPFC, interestingly, there are no direct return projections from the mPFC to HF, and rather moderate mPFC projections to parahippocampal structures including the entorhinal cortex [6,9,27,34,37,39,40,45,49].

The nucleus reuniens (RE) of the ventral midline thalamus is the principal (or virtually sole) source of thalamic input to the hippocampus [8,24,42,54–57]. RE stimulation produces pronounced excitatory actions at CA1 of the hippocampus [7,14]. Bertram and Zhang [7] compared the effects of stimulation of RE with stimulation of the CA3 region of the hippocampus on population responses (field EPSPs and spikes) at CA1, and reported that RE actions on CA1 were equivalent to, and in some cases considerably greater than, those of CA3 on CA1. They concluded that the RE projection to the hippocampus "allows for the direct and powerful excitation of the CA1 region. This thalamohippocampal connection bypasses the trisynaptic/commissural pathway that has been thought to be the exclusive excitatory drive to CA1."

It has recently been shown [48,49] that the mPFC, particularly the infralimbic (IL) and prelimbic (PL) cortices, distribute prominently to RE. The combined demonstration, then, that mPFC projects to RE and RE in turn to HF, suggests that RE may represent an important relay between the mPFC and hippocampus. It remains to be determined whether mPFC fibers distributing to RE contact RE neurons projecting to the hippocampus. To assess this, we made anterograde tracer injections (PHA-L) in the ventral mPFC and retrograde injections (Fluorogold) in the CA1/subiculum of HF and examined, at the light and ultrastructural level, synaptic connections of mPFC fibers on RE neurons projecting to the hippocampus. We showed that mPFC fibers form asymmetric (excitatory) contacts predominantly on dendritic shafts of RE cells projecting to HF. RE thus appears to be a critical link between the mPFC and HF, completing an important loop between these structures (HF ▶ mPFC ▶ RE ▶ HF).

2. Materials and methods

2.1. Animals and surgical protocols

Twelve male Sprague-Dawley rats (Charles River, Wilmington, MA) weighing 275–350 grams were used. Experiments were approved by the Florida Atlantic University Institutional Animal Care and Use Committee and conform to all federal regulations and the National Institute of Health guidelines for the care and use of laboratory animals.

Under sodium pentobarbital anesthesia (50 mg/kg, ip), each rat received an injection of *Phaseolus vulgaris*-leucoagglutinin (PHA-L) in the medial prefrontal cortex and an injection of Fluorogold (FG) in the hippocampus. Powdered lectin from *Phaseolus vulgaris*-leucoagglutinin (Vector Labs, Burlingame, CA) was reconstituted to 5% in 0.05 M sodium

phosphate buffer, pH 7.4. The PHA-L solution was iontophoretically deposited in the brains of anesthetized rats by means of a glass micropipette with an outside tip diameter of 40–60 μm. The stereotaxic coordinates were: AP, + 2.6 to 2.8 mm to bregma, L, 0.5 mm and V, 4.5 to 5.2 mm. Positive direct current pulses (5–10 μA) were applied through a Grass stimulator (Model 88) coupled with a high voltage stimulator (Frederick Haer Co., Brunswick, ME) at 2 seconds "on"/2 seconds "off" intervals for 40–50 minutes.

Fluorogold (Fluorochrome, LLC, Denver, CO) was dissolved in a 0.1M sodium acetate buffer (pH 5.0) to yield a 5% concentration. FG was either iontophoretically deposited in the hippocampus or pressure injected using a 1 μl Hamilton syringe. FG was iontophoretically injected through a glass micropipette with an outside tip diameter of 40–50 μm for 5–10 min using the same protocol described above for PHA-L. The pressure injections (0.03 μl) were made slowly over 10–15 min and the syringe was left in place lasted for an additional 10 min to prevent the tracer from spreading up the injection path. The stereotaxic coordinates were: AP, − 5.6 − 5.9 mm to bregma, L, 5.4 to + 5.8 mm, and V, 4.6 to 6.2 mm.

After a survival period of 7–10 days, rats were deeply anesthetized and perfused transcardially with 50 ml of heparinized saline, followed by a fixative containing 4% paraformaldehyde, 0.1% glutaraldehyde, and 15% picric acid in 0.1M phosphate buffer (PB) (pH 7.4). The brains were removed and postfixed overnight in 4% paraformaldehyde and 15% picric acid in 0.1M PB. The brains were then washed with 0.1M PB subsequently sectioned (50 μm) with a vibratome and collected in 0.1 M PB for light and electron microscopic analysis.

2.2. Light Microscopic Procedures

Two procedures were used for the dual visualization of PHA-L labeled fibers and FG retrogradely labeled neurons. The first procedure involved an intensification of FG retrogradely labeled neurons and the second procedure an intensification of PHA-L anterogradely labeled fibers. All sections were initially treated with 1% sodium borohydride in 0.1 M PB for 30 min to remove excessive aldehydes. They were then washed 3 times for 5 minutes each (3 × 5 minutes) in PB and incubated for 60 minutes in 0.5% bovine serum albumin in 0.1M Tris-buffered saline (TBS, pH 7.6) at room temperature (RT) to minimize nonspecific labeling. Following this, sections were incubated for 20 hours at RT in diluent [0.1% bovine serum albumin (BSA) in TBS containing 0.25% Triton X-100] and biotinylated goat anti-PHA-L (Vector Labs, Burlingame, CA) at a dilution of 1:500.

For the first procedure sections were washed (0.1M PB) and placed for 1 hour in 1:400 concentration of biotinylated rabbit anti-goat immunoglobulin (IgG) and diluent. Following another PB wash, sections were incubated for 1 hour in 1:100 concentration of peroxidase-avidin complex (ABC) from the Vector Elite kit and diluent. The peroxidase reaction product was visualized by incubation in a solution containing 0.022% 3,3' diaminobenzidine (DAB) and 0.003% H_2O_2 in TBS for 6 minutes. Sections were then re-incubated in 0.5% BSA in 0.1M TBS for 15 minutes, and incubated for 48 hrs at RT in 1:200 concentration of rabbit anti-FG (Fluorochrome, LLC, Denver, CO) and diluent. Sections were then washed (0.1M PB) and placed for 2 hours in a 1:100 concentration of a secondary antiserum, donkey anti-rabbit immunoglobulin (Jackson ImmunoResearch Laboratories, West Grove, PA) and

diluent. Following another PB wash, sections were incubated for 2 hours in a 1:200 concentration of rabbit peroxidase-anti-peroxidase (Jackson ImmunoResearch Laboratories, West Grove, PA) and diluent. Following 0.1M PB washes, sections were re-incubated in the same concentrations in donkey anti-rabbit immunoglobulin and rabbit peroxidase-anti-peroxidase for 2 hours each -- double bridge procedure. After final washes (0.1M PB), the reaction product was visualized by an incubation in a solution containing 0.015% 3,3' diaminobenzidine, 0.015% cobalt acetate and 0.003% H_2O_2 in TBS for 6 minutes.

For the second procedure, sections were washed (0.1M PB) and placed for 1 hour in 1:400 concentration of biotinylated horse anti-goat immunoglobulin (IgG) and diluent. Following another PB wash, sections were incubated for 1 hour in 1:100 concentration of peroxidase-avidin complex (ABC) from the Vector Elite kit and diluent. After a final PB wash, the peroxidase reaction product was visualized by incubation in a solution containing 0.015% cobalt acetate, 0.022% 3,3' diaminobenzidine (DAB) and 0.003% H_2O_2 in TBS for 5 minutes. Sections were then re-incubated in 0.5% BSA in 0.1M TBS for 15 minutes, and then incubated for 48 hrs at RT in 1:200 concentration of rabbit anti-FG (Fluorochrome, LLC, Denver, CO) and diluent. Sections were then washed (0.1M PB) and placed for 2 hours in a 1:400 concentration of a secondary antiserum, biotinylated donkey anti-rabbit immunoglobulin (Jackson ImmunoResearch Laboratories, West Grove, PA) and diluent. Following another PB wash, sections were incubated for 1 hour in 1:100 concentration of peroxidase-avidin complex (ABC) from the Vector Elite kit and diluent. Following a final PB wash, the peroxidase reaction product reaction was visualized by an incubation in a solution containing 0.015% 3,3' diaminobenzidine and 0.003% H_2O_2 in TBS for 5 minutes. All sections were re-rinsed in PBS (3 × 1 minutes) and mounted onto chrome-alum gelatin-coated slides. An adjacent series of sections from each rat was stained with cresyl violet for anatomical reference. Sections were examined using light and darkfield optics.

The first procedure gave rise to brown appearing PHA-L labeled fibers on black FG labeled cells and the second procedure to black PHA-L labeled fibers on brown FG-labeled cells. The lightfield photomicrographs of injection sites, labeled fibers and cells were taken with a Nikon DXM1200 camera mounted on a Nikon Eclipse E600 microscope. The captured images were then assembled into photomontages using Image-Pro Plus software (Media Cybernetics, Inc., Silver Spring, MD). Single photomicrographs (non-montages) were taken with ACT-1 software (Nikon, USA). Digital images were enhanced (brightness and contrast) using Adobe PhotoShop 9.0 (Mountain View, CA).

2.3. Electron Microscopic Procedures

To allow for antibody penetration, the sections were incubated in a cryoprotective solution (20% sucrose in 0.1M PB) until sections sank and freeze-thawed in liquid nitrogen. The tissue was then blocked with a diluent solution (1% BSA, 0.1 % glycine, 0.1 % L-lysine, and 4% normal goat serum in 0.1M PB) for 30 minutes to reduce non-specific labeling and then incubated for 48 hours in a cocktail of primary antibodies against both the PHA-L and FG as described above for light microscopic procedures. Following copious washes in PB, sections were incubated in ABC at a concentration of 1:50 in 0.1 M PB for 2 hours. Following further washes, sections were immersed for four minutes in a nickel-intensified DAB/

glucose oxidase solution (15 mg DAB, 12 mg ammonium chloride, 0.12 mg glucose oxidase, 600 ul of 0.05 M nickel ammonium sulphate, and 600 ul of 10 % B-D-glucose in 40 ml 0.1M PB) to visualize the PHA-L reaction product. FG was visualized using silver intensification of a gold secondary antibody. Following a DAB reaction, the tissue was washed and blocked again in preparation for incubation in 1.2 nm gold conjugated anti-rabbit (Nanoprobes, Yaphank, NY) for 30 minutes in diluent (same as above with 1% cold water fish gelatin and 0.05% Tween-20 added). The tissue was incubated for two hours in the secondary antibody and washed with PB. The sections were then treated with 1% glutaraldehyde in 0.1 M PB for ten minutes to fix the gold particles. Following initial washes of PB and secondary washes of double-distilled water (DDW), sections were placed for two minutes in a HQ Silver Kit (Nanoprobes, Yaphank, NY) to intensify the gold reaction. The tissue was washed with DDW followed by PB. Sections were then osmicated (1% osmium tetroxide in 0.1 M PB) for 10 minutes, followed by copious washes of initially PB and then DDW. The tissue was then dehydrated in ethanol, 50% to 70%. Sections were immersed in 70% ethanol with 1% uranyl acetate for one hour, washed with 70% ethanol, and further dehydrated using 95% and100% ethanol followed by propylene oxide. The tissue was then exposed to a 1:1 mixture of propylene oxide and a Durcupan mixture (ACM Fluka, Bucks, Switzerland) overnight. Sections were immersed in the Durcupan mixture for four hours and mounted between liquid release agent-coated slides and coverslips and baked at 60°C for 48 hours to allow for polymerization. Selected areas were photo-documented for putative synapses and trimmed for sectioning. Ultrathin sections (80–85 nm) were collected serially on single-slot Formvar-coated grids and contrasted with lead citrate. The ultrastructure was examined with a Tecnai 12 transmission electron microscope equipped with an AMT Advantage 4.00 HR/HR-B CCD camera system (Advanced Microscopy Techniques, Danvers, MA).

3. Results

Using a combined anterograde-retrograde tracing procedure, we describe the distribution of PHA-L labeled fibers from the mPFC onto populations of cells of nucleus reuniens that project to the hippocampus. In accord with previous work [48,49], an injection of PHA-L into the ventral mPFC (Fig. 2a), essentially confined to the infralimbic and prelimbic cortices, gave rise to pronounced labeling of RE. Labeled fibers were not, however, restricted to RE but were also present in other nuclei of the midline thalamus including the paraventricular, paratenial, mediodorsal, intermediodorsal, interanteromedial, central medial and rhomboid nuclei of the thalamus. Figure 1a depicts extra-RE labeling antero-dorsally in the thalamus within the paratenial nucleus and less so in the medially adjacent paraventricular nucleus.

Although RE was a main, but not the sole, target of labeled fibers from mPFC, labeled cells were virtually restricted to RE following retrograde FG injections in HF. As depicted for three levels of RE (Fig. 1b–d), labeled neurons were present rostrocaudally throughout RE following a HF injection (Fig. 2b), most densely concentrated ipsilaterally (left side) within rostral, ventral and lateral subdivisions of RE [38,44]. Very few labeled neurons were observed within the thalamus outside of RE. Accordingly, RE was the only nucleus of the thalamus showing a convergence of anterogradely labeled fibers (from mPFC) and

retrogradely labeled neurons (from HF). As further shown in Figure 1, there was a significant overlap of PHA-L labeled fibers (brown) and FG labeled cells (black), at rostral (Fig. 1b), intermediate (Fig. 1c) and caudal (Fig. 1d) levels of RE, most pronounced rostrally – the site of the heaviest concentration of retrogradely labeled neurons in RE.

Figure 2c shows a dense plexus of labeled fibers interspersed among, and in close proximity to, labeled cells in RE. As depicted at higher levels of higher magnification (Fig. 2d–f) labeled terminals overlay and form putative synaptic contacts with proximal dendrites (arrows in Fig. 2d–f) of retrogradely labeled RE neurons.

At the ultrastructural level, PHA-L labeled terminals formed asymmetric connections with both FG-labeled (Fig. 3a–d) and unlabeled (Fig. 4a) dendrites of RE neurons. The consecutive series of ultrathin sections of Figure 3 show PHA-L labeled terminals (F) making asymmetric contacts with a labeled proximal dendrite (D) of a retrogradely labeled RE neuron -- identified by the presence of several silver intensified gold particles (arrows). As further shown (Fig. 3), an unlabeled terminal (A) asymmetrically connects with this same dendritic element. Although unknown, it is possible that the unlabeled axon also originates from the mPFC; that is, PHA-L injections in mPFC would likely only labeled a small subset of prefrontal cells projecting to RE.

In a similar manner, as shown in Figure 4, PHA-L labeled (F) and unlabeled (A) terminals were found to form (predominantly) asymmetric connections with unlabeled dendrites (D) of RE cells (arrows in Fig. 4a). These contacts (labeled or unlabeled fibers with unlabeled dendrites) does not negate the possibility that the unlabeled dendrites belong to cells that also project to the hippocampus. Specifically, FG injections in HF (or those restricted to the ventral CA1/subiculum) would undoubtedly only retrogradely fill a small percentage of RE cells projecting to HF. Figure 4b shows a labeled terminal (F) in close proximity, but without synaptic contact, on a labeled cell body (C) in RE. No labeled terminals were found to make synaptic connections with cell bodies. Figure 4c depicts a unlabeled (A) symmetric contact on a labeled cell body (C), indicating local or extra-RE inhibitory connections with hippocampally projecting RE neurons.

4. Discussion

At the light microscopic level, we demonstrated a pronounced overlap in nucleus reuniens of PHA-L labeled fibers from the medial prefrontal cortex and retrogradely-labeled cells from the hippocampus, as well as multiple putative contacts of labeled terminals onto dendrites of RE cells projecting to the hippocampus. At the ultrastructural level, we showed that mPFC fibers form asymmetric contacts predominantly with dendritic shafts of hippocampally projecting RE cells. These findings suggest that mPFC exerts excitatory actions on populations of RE cells that project to the hippocampus, and as such RE may serve as an important relay between the ventral mPFC and the hippocampus.

Although the inputs and outputs of RE have been described in separate studies [see, 51] the present report is the first to examine RE afferents/efferents in a single study, and by so doing

demonstrated a strong convergence within RE of fibers from the mPFC onto RE hippocampally projecting cells (see Fig. 1).

Medial prefrontal fibers were shown to form asymmetric connections with proximal dendrites of RE neurons projecting to HF. Unlabeled fibers also made asymmetric connections with labeled dendrites, and in some instances the same (labeled) dendrites were contacted by labeled and unlabeled mPFC fibers (Fig. 3). Since even large PHA-L injections in mPFC would undoubtedly only label a small percentage of mPFC cells, it is very possible that many of the unlabeled profiles on labeled dendrites also represent fibers originating from mPFC. In a similar manner, labeled fibers forming connections with unlabeled dendrites could represent contacts on hippocampally projecting RE neurons since retrograde injections were restricted to a small region of the terminal RE field in HF [8,13,38,54,57].

RE fibers innervating the hippocampus appear to mainly utilize glutamate. Specifically, RE is densely populated by glutamatergic cells [26] and RE neurons were shown to be retrogradely labeled following [^{3}H]D-aspartate injections in HF [8]. Halasy et al. [23] recently described a tight band of VGLUT2 terminals in stratum lacunosum-moleculare of CA1 that were thought to originate from RE. Taken together, this suggests that mPFC can indirectly exert excitatory actions on the hippocampus, mediated through an excitatory, glutamatergic, relay within RE. Finally, RE selectively targets CA1 and the subiculum but avoids CA3 and the dentate gyrus [8,13,38,54,57, present results), and the CA1/subiculum is the source of origin of efferents to the mPFC [12,18,28–30,43,47]. This indicates that projections between structures are directly routed through CA1 (and subiculum): CA1 → mPFC → RE → CA1.

4.1. Functional considerations

As discussed, in the absence of direct projections from the mPFC to the hippocampus, the nucleus reuniens appears to represent an important link from the mPFC to HF involved in memory processing. The mnemonic function commonly associated with the prefrontal cortex, and one extensively examined, is working memory – or the temporary storage and utilization of information over short intervals [1,2,20,22].

As well documented for monkeys [22], the mPFC of rats, or specifically the prelimbic cortex of mPFC [51], has been shown to participate in tasks requiring the maintenance of information over time – or working memory. The reversible or irreversible inactivation of the ventral PFC (or PL) produces marked deficits in delayed response tasks involving short or long delays [19,51]. Further, cells of the ventral mPFC in rats show sustained activity during the delay period of delay tasks [3,4,36].

Whereas information is retained for the short term in the PFC, it is widely acknowledged the long term storage of information requires the hippocampus and associated structures of the medial temporal lobe [15–17,41,58]. Regarding the dual (and interactive) roles of the PFC and HF in memory processing, Buckner and colleagues [10,11] recently put forth the intriguing hypothesis that the prefrontal cortex uses information in the short term (working memory) to deal with impending task demands and its long term storage may be simply a by product of its use in meeting immediate behavioral requirements. The conversion of short

(working memory) to long term stores might then involve a transfer of information from mPFC to the hippocampus/parahippocampus. In the absence of direct mPFC inputs to the HF, indirect projections from PFC to HF via RE may represent an important route for the transfer of information from PFC to the medial temporal lobe involved in memory processing.

Although speculative, it is possible that information relayed through RE (in contrast to direct cortical-hippocampal projections) may be subject to modulating influences acting on RE that affect that transfer. For instance, Van der Werf et al. [46] have proposed that the midline thalamus, including RE, regulates processes such as arousal, attention and awareness. In support of this, the midline thalamus receive pronounced input from 'arousal related' sites of the brainstem [32,35,52] as well as from 'wake-promoting' orexin neurons of the lateral hypothalamus [5,25]. It is possible that by modulating levels of arousal/attention, RE serves to gate the flow of information between the mPFC and HF thereby controlling the type of prefrontal information that gains access to the hippocampus for its long term storage.

Acknowledgments

This research was supported by NIMH grants MH35883 and MH63519 to RPV, and NIH and NIMH grants NS 042644 and MH 060858 to CL.

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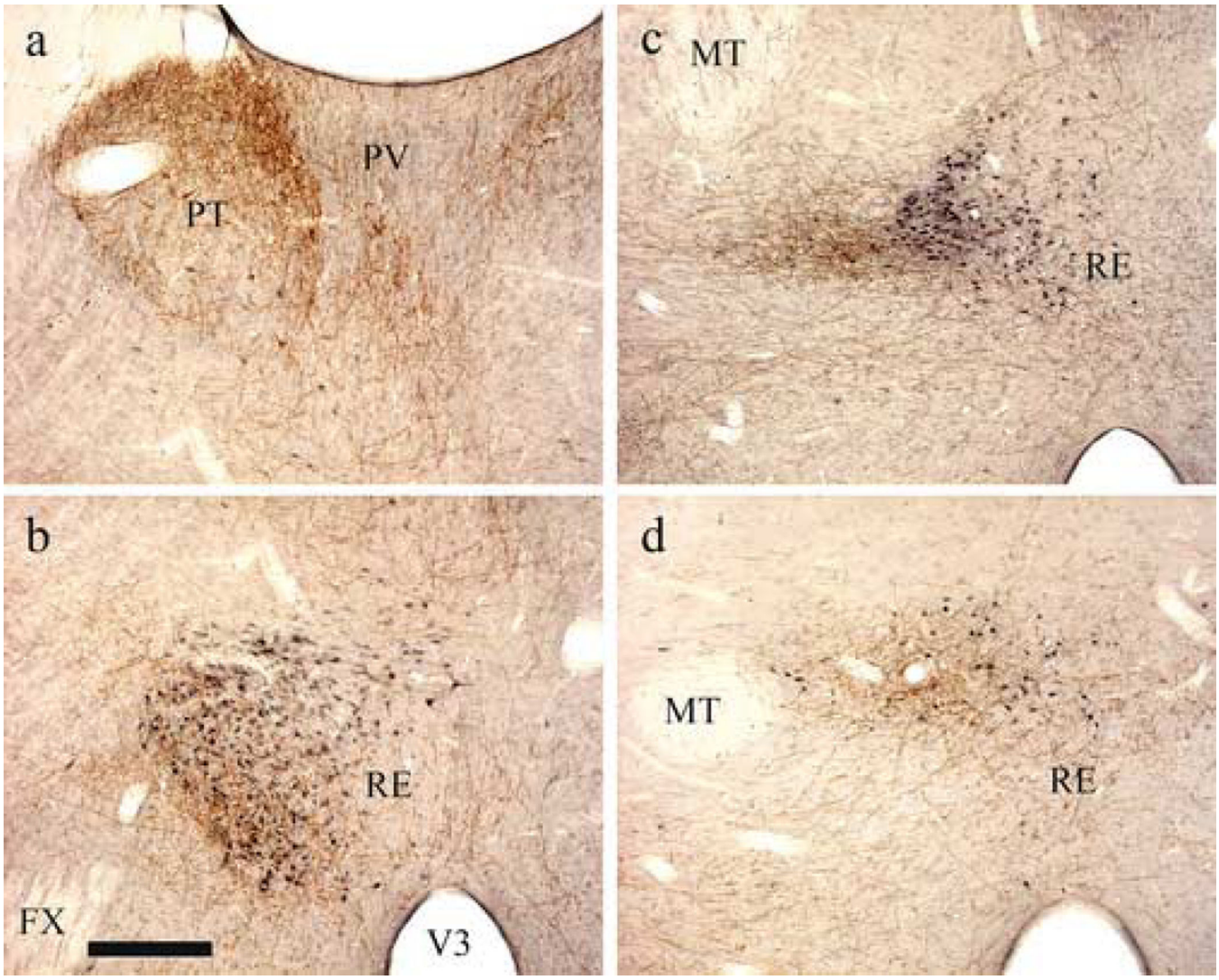

Figure 1.

(a–d) Series of low magnification rostrocaudally aligned photomicrographs through the thalamus showing: (1) the distribution of PHA-L labeled fibers (brown) in the paratenial (PT), paraventricular (PV) and reuniens (RE) nuclei of the midline thalamus produced by a PHA-L injection in the medial prefrontal cortex (mPFC, see Fig. 2a), and (2) retrogradely-labeled cells (black) in RE produced by a Fluorogold injection in the hippocampus (Fig. 2b). Note the significant overlap of PHA-L labeled fibers and retrogradely labeled cells at three rostrocaudal levels of RE, but no overlap in PT and PV due to the absence of retrogradely labeled cells in PT/PV. Scale bar, 300 μm. FX, fornix, MT, mammillothalamic tract, PT, paratenial nucleus of thalamus; PV, paraventricular nucleus of thalamus; RE, nucleus reuniens of thalamus, V3, third ventricle.

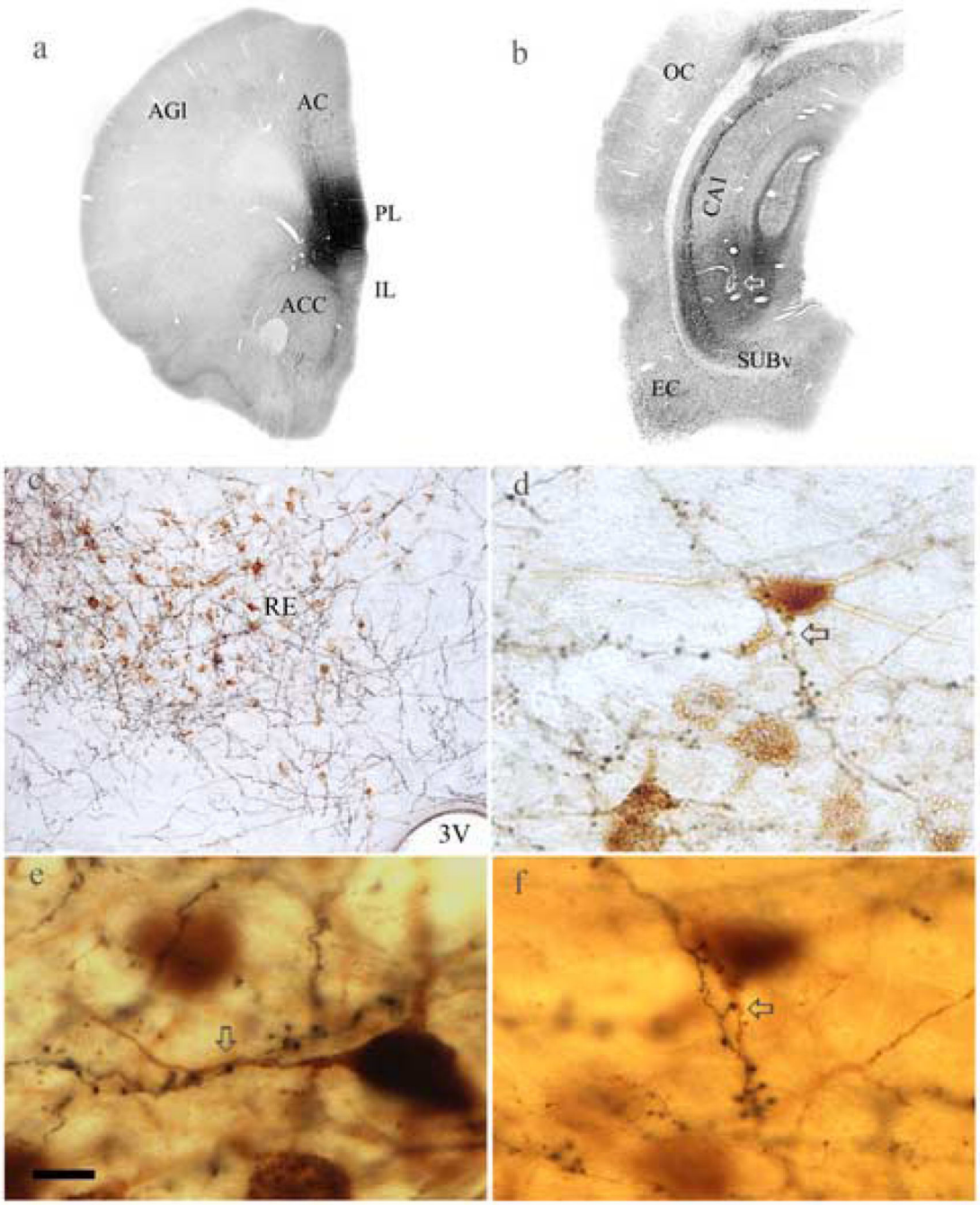

Figure 2.

(a) Low magnification photomicrograph of a PHA-L injection in the medial prefrontal cortex, virtually confined to the prelimbic cortex. (b) Low magnification photomicrograph of a FG injection in the ventral hippocampus, with the core of the injection (arrow) centered in stratum lacunosum-moleculare of ventral CA1. (c) Low magnification photomicrograph showing pronounced overlap in RE (dorsolateral to the third ventricle) of PHA-L labeled fibers (black) and FG labeled cells (brown). (d) High magnification photomicrograph showing putative synaptic contacts of a PHA-L labeled fiber (arrow) on a dendrite of a FG

labeled cell. (e,f) High magnification photomicrographs showing putative synaptic contacts (arrows) of PHA-L labeled fibers on proximal dendrites FG labeled cells (f, higher magnification of d). Scale bars, a,b, 1200 μm; for c, 60 μm; for d, 25 μm; for e,f, 15 μm. AC, anterior cingulate cortex; ACC, nucleus accumbens; AGl, lateral agranular (frontal) cortex; CA1, CA1 field of Ammon's horn of hippocampal formation; EC, entorhinal cortex; IL, infralimbic cortex, OC, occipital cortex; PL, prelimbic cortex; SUBv, ventral subiculum of hippocampal formation.

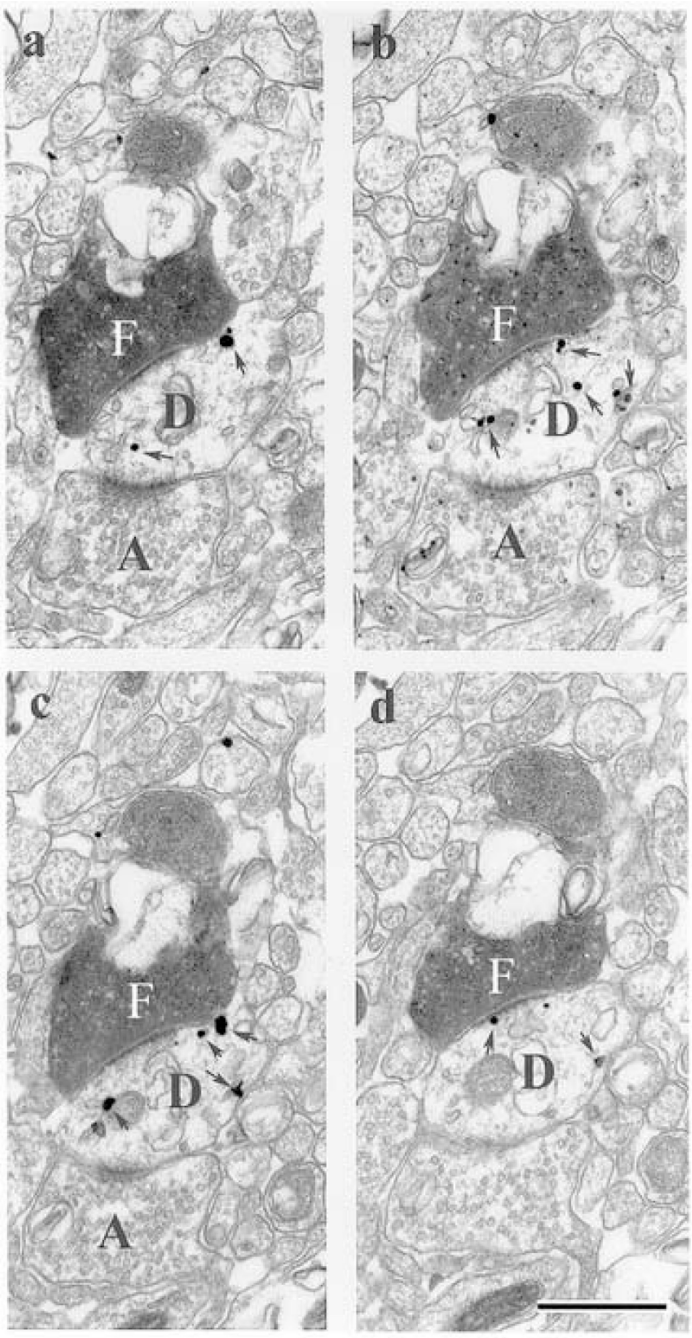

Figure 3.

(a–d) Series of consecutive electron micrographs within nucleus reuniens showing asymmetric contacts of a single PHA-L labeled (F) fiber (from the mPFC) on a labeled dendritic shaft, identified by the presence of numerous silver intensified gold deposits (arrows in D), of a RE cell retrogradely from the hippocampus. Note also the presence of asymmetric contacts of an unlabeled fiber (A) on the same labeled dendrite (D). Scale bar, 1 um.

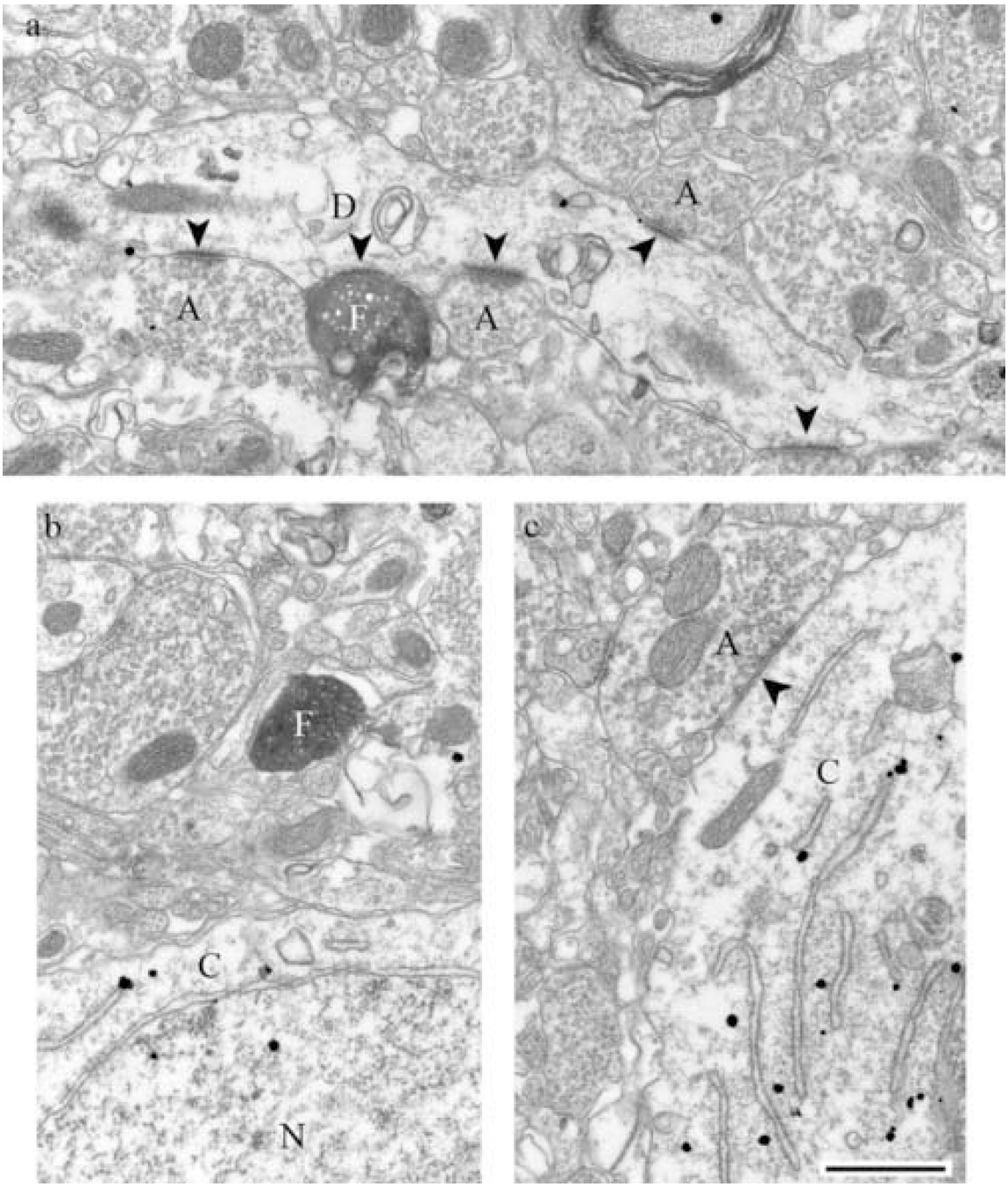

Figure 4.

(a) An electron micrograph showing a PHA-L labeled fiber (F, arrow head) from the mPFC forming an asymmetric contact with an unlabeled dendrite within RE and several (four) unlabeled asymmetric contacts (A, arrow heads) with the same dendritic process. (b) A PHA-L labeled fiber (F) from the mPFC in close proximity but without synaptic contact on a labeled RE cell body (C) and nucleus (N). (c) An unlabeled symmetric contact (A, arrow head) with a labeled RE cell body (C). Scale bar, 1 μm.