LAMINAR AND MODULAR ORGANIZATION OF PREFRONTAL PROJECTIONS TO MULTIPLE THALAMIC NUCLEI

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Abstract—The prefrontal cortex projects to many thalamic nuclei, in pathways associated with cognition, emotion, and action. We investigated how multiple projection systems to the thalamus are organized in prefrontal cortex after injection of distinct retrograde tracers in the principal mediodorsal (MD), the limbic anterior medial (AM), and the motor-related ventral anterior/ventral lateral (VA/VL) thalamic nuclei in rhesus monkeys. Neurons projecting to these nuclei were organized in interdigitated modules extending vertically within layers VI and V. Projection neurons were also organized in layers. The majority of projection neurons to MD or AM originated in layer VI (~80%), but a significant proportion (~20%) originated in layer V. In contrast, prefrontal neurons projecting to VA/VL were equally distributed in layers V and VI. Neurons directed to VA/VL occupied mostly the upper part of layer V, while neurons directed to MD or AM occupied mostly the deep part of layer V. The highest proportions of projection neurons in layer V to each nucleus were found in dorsal and medial prefrontal areas. The laminar organization of prefrontal cortico-thalamic projections differs from sensory systems, where projections originate predominantly or entirely from layer VI. Previous studies indicate that layer V cortico-thalamic neurons innervate through some large terminals thalamic neurons that project widely to superficial cortical layers. The large population of prefrontal projection neurons in layer V may drive thalamic neurons, triggering synchronization by recruiting several cortical areas through widespread thalamo-cortical projections to layer I. These pathways may underlie the synthesis of cognition, emotion and action. © 2009 IBRO. Published by Elsevier Ltd. All rights reserved.

Key words: *Macaca mulatta*, mediodorsal thalamic nucleus, anterior thalamic nuclei, layer V pyramidal neurons, cortico-thalamic pathway.

The prefrontal cortex in macaque monkeys has lateral, orbitofrontal and medial sectors that have complementary roles in behavior. The lateral prefrontal sector has a key role in working memory, and the orbitofrontal and medial sectors are associated with distinct aspects of emotional processes [reviewed in; Goldman-Rakic, 1988; Fuster, 1989; Petrides, 1994; Barbas, 2000]. The entire prefrontal cortex is connected with several thalamic nuclei including the mediodorsal, the anterior medial, the ventral anterior, the medial pulvinar, midline and intralaminar nuclei [e.g.; Kievit and Kuypers, 1977; Goldman-Rakic and Porrino, 1985; Preuss and Goldman-Rakic, 1987b; Barbas et al., 1991; Morecraft et al., 1992; Dermon and Barbas, 1994; Bachevalier et al., 1997; Cavada et al., 2000; Xiao and Barbas, 2002a,b, 2004; Zikopoulos and Barbas, 2007]. However, the organization of multiple projections from prefrontal areas to several thalamic nuclei is not well understood. We sought to address this issue by investigating prefrontal cortical projections to its principal thalamic mediodorsal (MD) nucleus, the limbic anterior medial (AM) nucleus, and the motor related ventral anterior (VA) nucleus in the same animals. These nuclei are robustly connected with prefrontal cortices, and have some related and complementary functions [reviewed in; Jones, 2007]. The MD and AM receive projections from the amygdala, which is associated with emotional processing. The AM nucleus receives robust projections from the hippocampal formation associated with memory, and the VA and MD have a key role in motor and cognitive functions through connections with the basal ganglia and prefrontal cortex (Alexander et al., 1986; Armstrong, 1990; Groenewegen et al., 1990; Mitchell et al., 1999; Middleton and Strick, 2000; Graybiel, 2000; Eichenbaum, 2000; Haber and McFarland, 2001; Xiao and Barbas, 2002b, 2004).

Previous studies have shown that the majority of projection neurons to the thalamic MD or AM nuclei originate from layer VI (Giguere and Goldman-Rakic, 1988; Yeterian and Pandya, 1994; Xiao and Barbas, 2002b). Layer VI gives rise to the large majority of all cortico-thalamic projections [reviewed in; Steriade et al., 1997; Jones, 2007]. However, a significant proportion of prefrontal neurons projecting to the thalamus originates in layer V, constituting about 20% of neurons projecting to AM or MD, and making up nearly half of projection neurons directed to the VA (Xiao and Barbas, 2002b, 2004; Zikopoulos and Barbas, 2007). Cortical pathways emanating from layers VI and V

are thought to innervate distinct classes of thalamo-cortical neurons [reviewed in; Guillery, 1995; Rouiller and Welker, 2000]. In the reverse pathway, thalamic neurons innervated by cortical layer VI project focally to the middle cortical layers. In contrast, thalamic neurons innervated by cortical layer V project widely to the superficial cortical layers, and likely participate in high-order association processes [reviewed in; Jones, 1998a; Rouiller and Welker, 2000; Jones, 2003]. Reciprocal pathways linking the cortex with the thalamus are thought to have distinct functions and are affected in a variety of neurologic and psychiatric diseases (Scheibel, 1997; Aggleton and Brown, 1999; Llinas et al., 1999; Van Der Werf et al., 2003; Llinas and Steriade, 2006).

Here we investigated the organization of prefrontal projection neurons directed to its principal MD nucleus, the limbic AM nucleus, and the motor related VA nucleus. We provide evidence that multiple prefrontal projection systems to distinct thalamic nuclei have a dual organization into modules and layers. These prefrontal pathways may underlie the functional specialization of thalamo-cortical interactions in recruiting cortical and other thalamic structures associated with cognition, emotion and action.

EXPERIMENTAL PROCEDURES

Surgical procedures

Experiments were conducted on five young adult rhesus monkeys (*Macaca mulatta*) of both sexes under sterile procedure, according to the National Institutes of Health (NIH) Guide for the Care and Use of Laboratory Animals (DHEW publication no. [NIH] 80-22, revised 1996, Office of Science and Health Reports, DRR/NIH, Bethesda, MD, USA). The protocol on the ethical use of animals was approved by the Institutional Animal Care and Use Committee at Boston University, Harvard Medical School and New England Primate Research Center. Procedures involving animals were designed to reduce the number of animals needed and minimize animal suffering.

To inject tracers in the VA, AM and MD nuclei it was necessary to first obtain a map of the thalamus using magnetic resonance imaging (MRI). We used the interaural line as a reference by filling hollow ear bars of the stereotaxic apparatus with Betadine salve (containing polymyxin-B sulfate, bacitracin zinc and pramoxine HCl, Purdue Frederick Company, Norwalk, CT, USA), which is visible in MRI. Brain scans were obtained from monkeys anesthetized with a mixture of ketamine hydrochloride (10 mg/kg, intramuscularly (i.m.)) followed by sodium pentobarbital, administered intravenously (i.v.) through a femoral catheter (to effect). The stereotaxic coordinates for the VA, AM and MD were calculated in three dimensions using the interaural line as reference.

One week after MRI surgery was performed to inject neural tracers. Monkeys were anesthetized first with ketamine hydrochloride (10 mg/kg, i.m.), intubated, and then anesthetized with gas anesthetic (isoflurane) until a surgical level of anesthesia was achieved. Overall physiological condition was monitored, including heart rate and temperature. A small hole was made above the injection site for penetration of the injection needle. Small amounts of fluorescent retrograde tracers were delivered to the thalamic nuclei (Table 1). The types and amount of tracers injected were as follows: Diamidino Yellow (which fluoresces yellow and labels only the nucleus; Sigma, St. Louis, MO, USA; 3% solution, volume of 0.3–1.6 μl); Fast Blue (fluoresces blue and labels the soma and proximal dendrites; Sigma; 1% solution, volume of 0.8–2 μl); Fluoroemerald (fluoresces green and labels the soma and proximal dendrites; Molecular Probes, Carlsbad, CA, USA; 10% solu-

Table 1. Injection sites, cases and tracer types in thalamic VA, MD and AM nuclei

Injection sites	Cases				
	Case BD (male)	Case BE (male)	Case BG (female)	Case BB (female)	Case AZ (male)
VA or VA/VL	FE (L)	FR (L)	FR (R)		
MD	FB (L)	DY (L)	FB (R)	FE (L)	FR (L)
AM	FR (L)			DY (L)	DY (L)

Abbreviations: FB, Fast Blue; FE, Fluoroemerald; FR, Fluororuby; DY, Diamidino Yellow; L, left side; R, right side.

tion, volume of 3–4 μl); and Fluororuby (fluoresces red and labels the soma and proximal dendrites; dextran tetramethylrhodamine, Molecular Probes; 10% solution, volume of 3–4 μl).

After injection of tracers the wound was closed in anatomic layers and the skin sutured. At the completion of surgical procedures the animals were monitored until recovery from anesthesia, and they were given antibiotics and analgesic (Buprenex, i.m.) every 12 h, or as needed.

Perfusion and tissue processing

Animals were given an overdose of anesthetic (sodium pentobarbital, >50 mg/kg, i.v. to effect) and perfused through the heart with a fixative (4% paraformaldehyde in 0.1 M sodium phosphate buffer, PB, pH 7.4) 18 days after injection of tracers. The brain was then removed from the skull, photographed and transferred through a graded series of sucrose solutions for cryoprotection (10%, 15%, 20%, 25% and 30% in 0.1 M PB with 0.05% azide). After cryoprotection the brain was frozen in −75 °C isopentane for 2 h, and cut on a freezing microtome coronally at 50 μm in 10 matched series. Adjacent sections in each series were thus separated by 500 μm. Two matched series of sections were mounted, dried under darkness, and stored at 4 °C. One series was used to map labeled neurons in prefrontal cortices and the other was coverslipped with Krystalon (EM Science) for long-term storage and photography.

Data analysis

Mapping labeled neurons in areas and layers. After injection of retrograde tracers in MD, VA/ventral lateral nucleus (VL) and AM, we mapped all labeled neurons in prefrontal cortices in one series of sections, using unbiased, uniform random sampling (one in 10 sections), and exhaustive sampling within the entire prefrontal cortex. Section outlines and the location of labeled neurons in coronal sections through the ipsilateral prefrontal cortex were viewed with a microscope under fluorescence illumination (Nikon, Optiphot). Maps of projection neurons were plotted on paper using a digital plotter (Hewlett Packard, 7475 A), which is electronically coupled to the stage of the microscope and to a PC computer (all cases, except case BG). Movement of the stage was recorded through linear potentiometers (Vernitech, Axsys, San Diego, CA, USA) mounted on the X and Y-axes of the microscope stage. Analog signals were converted to digital signals via an analog-to-digital converter (Data Translation, Marlboro, MA, USA). Software designed in our laboratory ensured that each neuron was counted only once, as described previously (Barbas and De Olmos, 1990). In case BG, tissue sections were viewed with a microscope under fluorescence illumination (Olympus Optical BX60, Thornwood, NY, USA) and plotted using a commercial system (Neurolucida, Microbrightfield, Williston, VT, USA). After plotting, sections were counterstained with Thionin and returned to the microscope to delineate layers, and count labeled neurons by layer in individual prefrontal areas. The neuron profile counts estimated by this exhaustive plotting were used to calculate den-

sity and laminar proportions of prefrontal projection neurons within and between different prefrontal areas.

Measurement of modules of labeled neurons through layers VI and V. We also estimated the width of columnar modules of neurons projecting to each of the thalamic nuclei throughout the prefrontal cortex, using exhaustive sampling in one series (one in 10 sections) in every case. For each injection site, the modules consisted of clusters of projection neurons that extended vertically within layers VI and V. We operationally defined modules as clusters of neurons separated by a distance of at least 150 μm, as illustrated in Fig. 1D. Adjacent modules of labeled neurons directed to two or three thalamic nuclei overlapped especially at the fringes, as shown in Fig. 1.

Three-dimensional reconstruction of labeling. We compared the topography and distribution of retrogradely labeled neurons across prefrontal areas and cases, by reconstructing in three dimensions a reference rhesus monkey brain using the free, open source software Reconstruct (Fiala, 2005). We then imported the traced outlines of prefrontal areas, containing three-dimensional information about the quantitative topography of labeling in Reconstruct, aligned and registered them with corresponding levels of the reference brain, and generated three-dimensional models as described (Medalla and Barbas, 2006; Zikopoulos and Barbas, 2006, 2007; Barbas and Zikopoulos, 2007; Fiala et al., 2007). As a result, all markers and traces were stereotaxically registered and superimposed on the three-dimensional model.

Photography. Photographs of labeled neurons in prefrontal cortices were captured with a CCD camera using a software system (Neurolucida, Microbrightfield). Images were transferred into Adobe Photoshop (Adobe Systems Inc., San Jose, CA, USA) for arrangement and adjusting of contrast, but were not retouched. Images of sections with multiple labeling were captured sequentially for each label using the appropriate filter for each dye, and the images were merged.

Statistical analysis

The percentage of projection neurons by layer was calculated by dividing the number of projection neurons in layer V or layer VI by the number of projection neurons in both layers for each prefrontal area. We employed linear correlation coefficient Pearson ρ to test the relationship in the paired percentage of layer V projection neurons for each prefrontal area directed to MD and VA/VL, or directed to MD and AM.

RESULTS

Overview of prefrontal cortex

The prefrontal cortex in rhesus monkeys is large and architectonically heterogeneous [reviewed in; Barbas, 2000; Barbas et al., 2002]. It has lateral, basal (orbital) and medial sectors, and each sector has several architectonic areas, listed briefly below to describe their location. References to architectonic areas are according to the map of Barbas and Pandya (1989), modified from the map of Walker (1940).

On the lateral surface area 10 encompasses the entire frontal pole, and is adjoined dorsally by area 9 (D9), ventrally by lateral area 12 (L12), and centrally by area 46. The latter is located within the dorsal bank of the principal sulcus and the adjoining cortex above (D46), and within the ventral bank of the principal sulcus and the adjoining cortex below (V46). Area 46 is bordered by area D9 above and area L12 below, and caudally by area 8, which is parcellated into dorsal (D8)

and ventral (V8) subdivisions, situated respectively within the rostral bank of the upper and lower limbs of the arcuate sulcus and the adjacent lateral cortex.

On the basal surface, the prefrontal cortex includes orbital area 12 (O12), which extends from the inferior prefrontal convexity to the orbital surface. Area O12 is bordered by area 13 in the central part of the orbital surface. Area 13 shares a border with area 11 anteriorly, and with areas orbital proisocortex (dysgranular cortex (OPro)) and area orbital periallocortex (agranular cortex) (OPAll) posteriorly, which are the most caudal orbitofrontal areas [for review see; Barbas and Zikopoulos, 2006; Barbas, 2007]. On the medial part of the orbital surface area 13 abuts area 14 rostrally, which has orbital (O14) and medial (M14) subdivisions. Orbital area 25 (O25) is situated behind area O14 on the basal surface and extends on the medial surface below the corpus callosum (M25). This is the most caudal extent of the medial prefrontal cortex, and is bordered above by the subcallosal part of area 24. The latter skirts the rostral part of the corpus callosum, and shares a border with area 32 anteriorly. Area 32 is situated below medial area 9 (M9), above area M14 and behind area 10.

The entire prefrontal cortex has bidirectional connections with MD, its principal thalamic nucleus, but also with a large number of other thalamic nuclei, including the VA and AM, whose connections are more restricted within the prefrontal cortex, as described below. The nomenclature of the thalamus is according to the map of Jones (1985) as modified from the map of Olszewski (1952).

Injection sites in thalamic nuclei

Data on projection neurons to the above prefrontal cortices were obtained from five animals after injection of distinct retrograde tracers in the VA, MD and AM nuclei, totaling 11 injection sites (Table 1). The tracers were largely restricted to the intended nuclei, with some spread to neighboring nuclei or adjacent tracts in some cases, as elaborated below. None of the affected tracts are connected with the prefrontal cortex. In addition, there was no leakage of tracer in overlying cortical areas in any case, confirmed by the absence of labeled neurons in layers II and III, contrasted by densely distributed labeled neurons in layers V and VI, which project to the thalamus. There was no evidence of spread of the dyes to other subcortical structures. Spread of the tracer within the thalamus was observed in case AZ (injection intended for AM spread to MD) and the injections in VA in cases BE and BG, which spread to VL, another motor-related thalamic nucleus. The VL has a similar pattern of projection as the VA (Kievit and Kuypers, 1977), but its connections with prefrontal cortices are generally sparser and found mostly in ventral lateral nucleus, pars medialis (VLm) (Barbas et al., 1991; Dermon and Barbas, 1994). We conservatively refer to injection sites in VA as VA/VL, unless the VL was not involved. The distribution of labeled projection neurons in the prefrontal cortex after injections in VA/VL, MD, or AM was similar and their relative numbers within layers were comparable among cases.
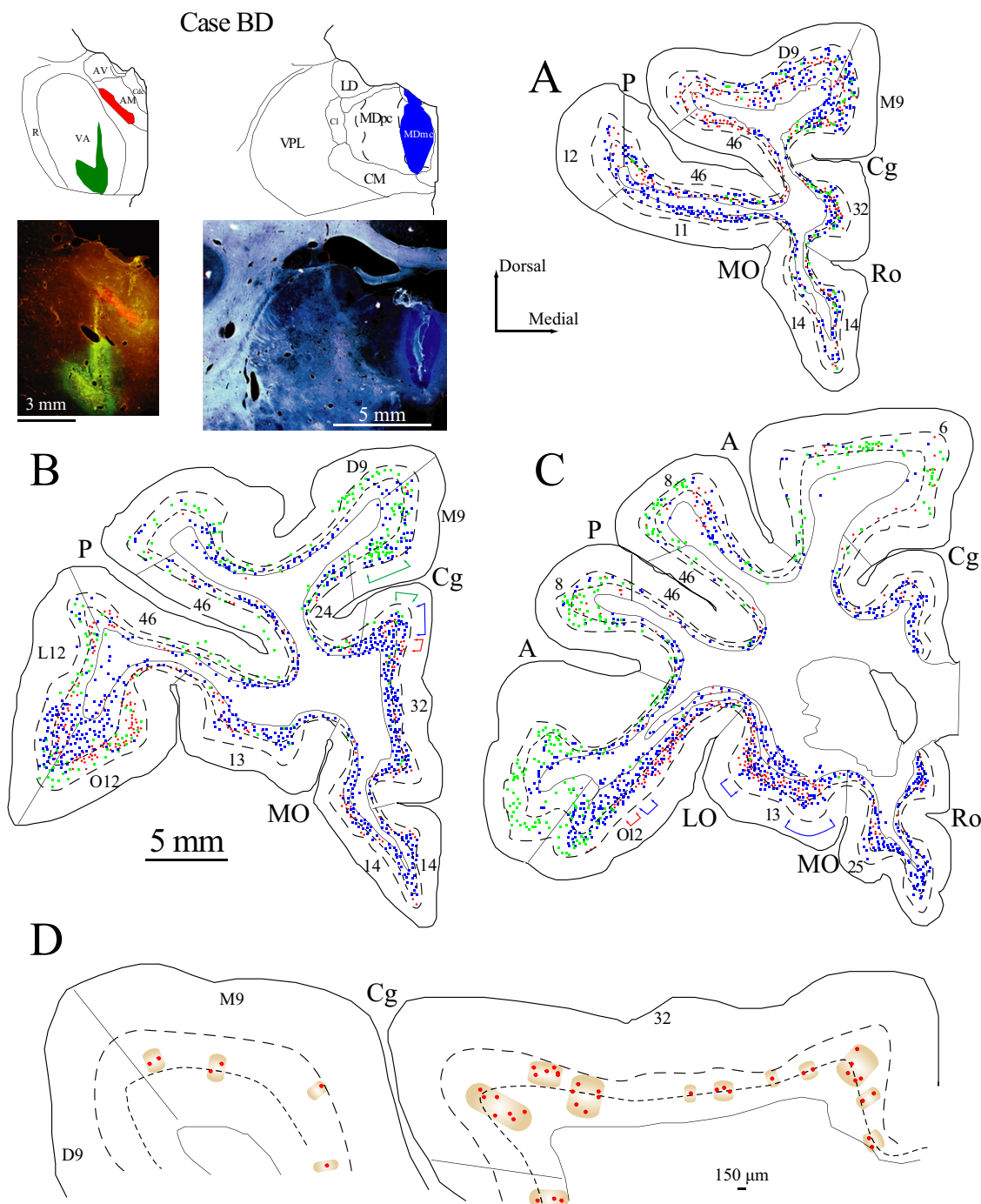
Fig. 1. Projection neurons in prefrontal cortices directed to the VA, AM and MD nuclei. *Inset* (top left), shows maps (top) and photomicrographs of the injection sites in VA, AM and MDmc in two coronal sections through the thalamus (colored areas, case BD). (A–C) Distribution of projection neurons in a series of coronal sections in rostral (A) to caudal (C) prefrontal cortices after injection of Fluoroemerald in the VA (green), Fluororuby in the AM (red) and Fast Blue in MDmc (blue). Each colored dot represents one neuron. Colored brackets in (B–C) show examples of the modular distribution of prefrontal projection neurons to AM (red), VA (green) and MD (blue). (D) Neurons projecting to AM (enlarged from B) are shown separately to illustrate the clustering of neurons into modules of variable widths, separated by at least 150 μm. Dotted lines in A–D show the top of cortical layers V and VI.

In case BD, three distinct tracers were injected (Fig. 1, inset). The core of the Fluoroemerald injection was in the ventral anterior thalamic nucleus, magnocellular part (VAmc) and the ventral anterior thalamic nucleus, parvi-

cellular division (VApc). The halo of the injection was in the ventral part of the neighboring motor-related ventral lateral nucleus, pars oralis (VLo). In previous studies we found no evidence of connections between the VLo and prefrontal

cortices (Barbas and Mesulam, 1981; Barbas et al., 1991; Dermon and Barbas, 1994). Injection of a second tracer, Fast Blue, was in the caudal part of the magnocellular portion of the mediodorsal thalamic nucleus (MDmc) and stria medullaris (Sm). The injection of a third tracer, Fluororuby, was restricted to the ventral edge of the AM nucleus. The halo of the injection impinged on the dorsal part of the mammillothalamic tract, and the needle tract passed through the ventral part of the fornix.

In two cases, two distinct tracers were injected in VA and MD. In case BE, the injection of Fluororuby covered the caudal half of VAmc and the ventral part of VLo (Fig. 2, inset). The dye impinged on the medial part of the VLm and the ventral part of the posterior ventral lateral nucleus (VLp). Injection of Fluoroemerald was in the posterior two-thirds of the ventral part of the mediodorsal thalamic nucleus, parvicellular division (MDpc) (Fig. 2, inset). In case BG, the injection of Fluororuby covered the caudal fourth of VApc and the central part of VL (Fig. 3, inset, left). In the same animal the Fast Blue injection site included the central part of MDpc throughout its dorso-ventral extent (Fig. 3, inset, right).

In two other cases, two distinct tracers covered parts of AM and MD. In case BB the core of the injection of Diamidino Yellow was restricted to the central portion of the AM nucleus (Fig. 4, inset, left and center). Injection of Fluoroemerald was in the rostral half of MDmc at the border of MDmc and MDpc (Fig. 4, inset, right). In case AZ (not shown) the core of the injection was in caudal AM and spread to a small portion of the anterior part of MD, the anterior ventral nucleus (AV), the central denso cellular (Cdc), and central superior lateral (Csl) nuclei, and the stria medullaris. Injection of Fluororuby was restricted to the caudal part of MDmc.

Overview of prefrontal cortico-thalamic projections

Injections in thalamic nuclei labeled numerous projection neurons in prefrontal cortices, whose relative distribution within areas varied, consistent with the location of the injection site and the topographic nature of cortico-thalamic projections [reviewed in; Jones, 2007]. The overall number of labeled neurons also varied, which likely is related to the size of the injection site. Detailed accounts of normalized densities of projection neurons in prefrontal cortices directed to AM and VA were reported in previous studies (Xiao and Barbas, 2002b, 2004). Here we provide examples of the density of projection neurons in prefrontal cortices for one case where all three thalamic nuclei were injected with distinct tracers (case BD). In this case with a large injection of Fast Blue in MDmc the highest density of

projection neurons was found in medial prefrontal (area 25, $n=818/mm^3$; area 32, $n=803/mm^3$; area 24, $n=321/mm^3$; M14, $n=240/mm^3$; M9, $n=225/mm^3$) and orbitofrontal areas (area OPro, $n=492/mm^3$; area 11, $n=397/mm^3$; area 13, $n=321/mm^3$; O12, $n=259/mm^3$; O14, $n=250/mm^3$), and the lowest density was found in lateral areas (D9, $n=150/mm^3$; D46, $n=188/mm^3$; L12, $n=135/mm^3$; area 10, $n=150/mm^3$; V46, $n=120/mm^3$; V8, $n=59/mm^3$; D8, $n=26/mm^3$). These findings are consistent with the predominant connections of medial and orbitofrontal cortices with MDmc and lateral areas with MDpc (Goldman-Rakic and Porrino, 1985; Siwek and Pandya, 1991; Barbas et al., 1991; Dermon and Barbas, 1994). In the same case, a small injection of Fluororuby in the thalamic AM nucleus labeled neurons in medial (area 32, $n=195/mm^3$; area 24, $n=108/mm^3$ M9, $n=88/mm^3$; M14, $n=69/mm^3$; area 25, $n=43/mm^3$), orbitofrontal (O12, $n=113/mm^3$; area 13, $n=98/mm^3$; area 11, $n=83/mm^3$; O14, $n=82/mm^3$; area OPro, $n=25/mm^3$), and lateral areas (area 10, $n=105/mm^3$; L12, $n=88/mm^3$; D46, $n=114/mm^3$; V46, $n=145/mm^3$; D9, $n=52/mm^3$; D8, $n=18/mm^3$; V8, $n=18/mm^3$). In the same case an injection of Fluoroemerald in VA labeled neurons in medial areas (M9, $n=84/mm^3$; area 32, $n=58/mm^3$; area 24, $n=29/mm^3$), and lateral areas (V8, $n=86/mm^3$; D8, $n=65/mm^3$; L12, $n=83/mm^3$; D9, $n=62/mm^3$; area 10, $n=28/mm^3$; v46, $n=24/mm^3$; D46, $n=19/mm^3$). In orbitofrontal areas the highest density of projection neurons was noted in area O12 ($n=41/mm^3$), and the density was lower in other areas (OPro, $n=6/mm^3$; area 13, $n=7/mm^3$).

Laminar organization of prefrontal cortico-thalamic projections

Projection neurons directed to the motor-related nuclei VA/VL overlapped with those projecting to MD, especially in dorso-medial and lateral prefrontal areas (e.g. Figs. 1 and 3). Projection neurons to AM and MD overlapped especially in medial and orbitofrontal cortices (e.g., Figs. 1 and 4). Supplementary Fig. 1 shows the regional and laminar distribution of labeled neurons projecting to all three thalamic nuclei on a reconstructed brain (case BD).

We first determined the laminar distribution of cortico-thalamic neurons in prefrontal cortices that projected to each of the three thalamic nuclei for all cases, and the results are shown in Fig. 5. This analysis included data from all injection sites and for all prefrontal areas, except for a few areas that did not have labeled neurons in a sufficient number of cases to conduct statistical analysis (e.g. areas 11 and 14 after injections in VA/VL; or area 11 after injection in MDpc). The prefrontal projection to area

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Abbreviations used in the figures

A	arcuate sulcus	LO	lateral orbital sulcus
Cg	cingulate sulcus	MO	medial orbital sulcus
Cl	central lateral nucleus	Olf	olfactory tubercle
CM	centromedian nucleus	Pcn	paracentral nucleus
D46	dorsal part of area 46	Re	nucleus reuniens
LD	lateral dorsal nucleus	Ro	rostral sulcus
LF	lateral fissure	V46	ventral subdivision of area 46
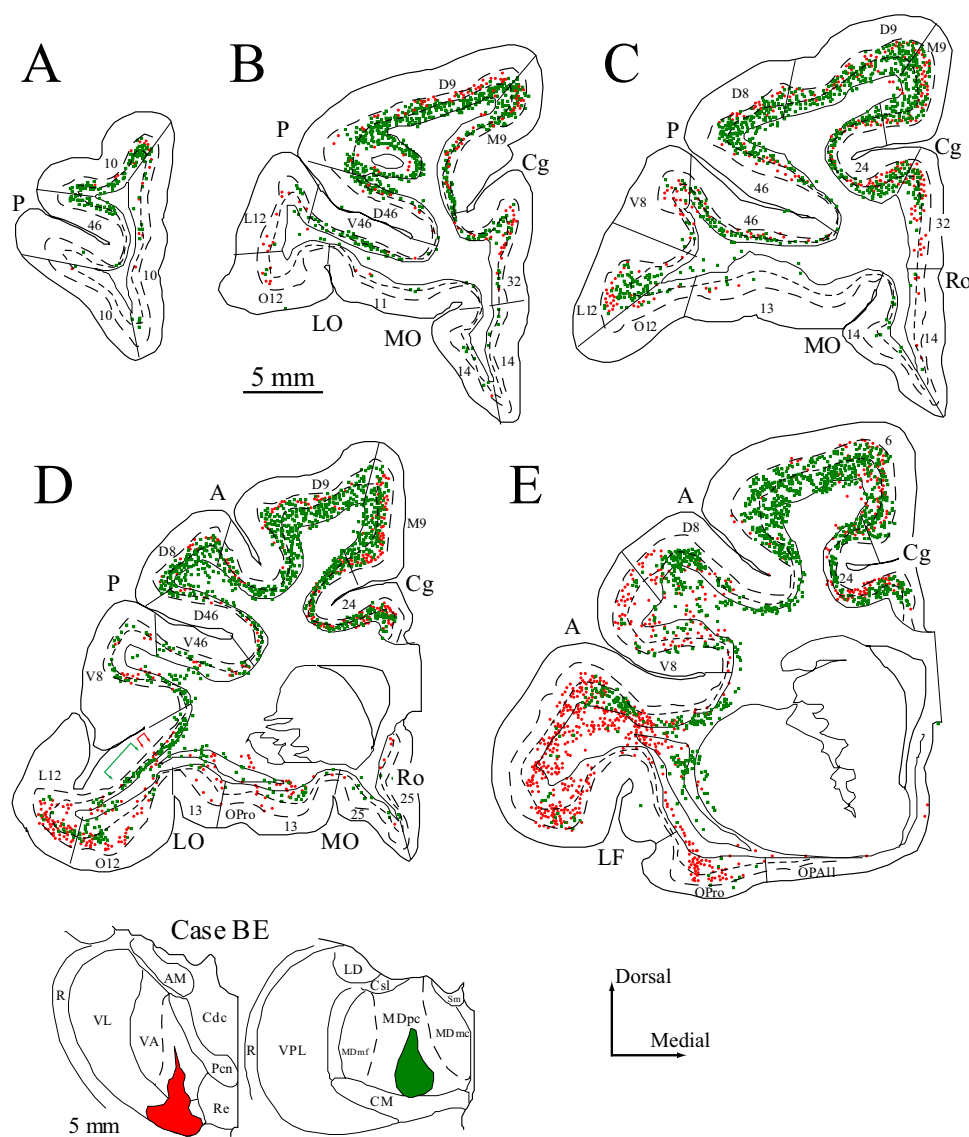
Fig. 2. Projection neurons in prefrontal cortices directed to the VA/VL and MD thalamic nuclei. (A–E) Distribution of projection neurons in a series of coronal sections in rostral (A) to caudal (E) prefrontal cortices after injection of Fluororuby in the VA/VL (red) and diamidino yellow in MDpc (green). Colored brackets in (D) show examples of the modular distribution of prefrontal projection neurons to VA/VL (red), and MD (green). Dotted lines in A–E show the top of cortical layers V and VI. *Inset*, injection sites in VA/VL and MDpc shown in coronal sections through the thalamus (colored areas, case BE).

11 is consistent with its predominant connections with MDmc (Barbas et al., 1991; Dermon and Barbas, 1994), as seen here for one case (case BD).

Most projection neurons in prefrontal cortices were found in layer VI. However, we found significant proportions of projection neurons in layer V that were directed to each of these nuclei as well (Fig. 5). About half of the projection neurons in prefrontal cortices directed to VA/VL originated from layer V (44%) and the rest (56%) were found in layer VI (Fig. 5A). About one fifth of the projection neurons directed to MD or AM originated from layer V and the majority (~80%) originated from layer VI (Fig. 5B, C).

Moreover, the prevalence of projection neurons in layer V varied regionally. There was a higher proportion of projection neurons directed to AM in dorsal and medial

prefrontal areas 24, 25, 32, 14 and 9 (about 30%) than in lateral and orbitofrontal areas (5%–20%, area OPro was an exception). This regional bias was also seen for projections to MD and VA/VL, confirmed by a significant correlation (Pearson $\rho=0.78$, $P<0.01$, Fig. 6A), with dorsal and medial prefrontal areas (9, 32 and 24) showing consistently higher percentages of projection neurons in layer V (Pearson $\rho=0.86$, $P<0.01$, Fig. 6A and inset). The relative proportions of projection neurons from layer V to AM and MD also showed a high correlation for dorso-medial areas 9, 24 and 32 (Pearson $\rho=0.86$, $P<0.01$, Fig. 6B).

We then compared the relative proportion of neurons in layers V and VI for each injection site within each prefrontal area, which revealed quantitative differences in the laminar distribution of projection neurons directed to AM, MD, and
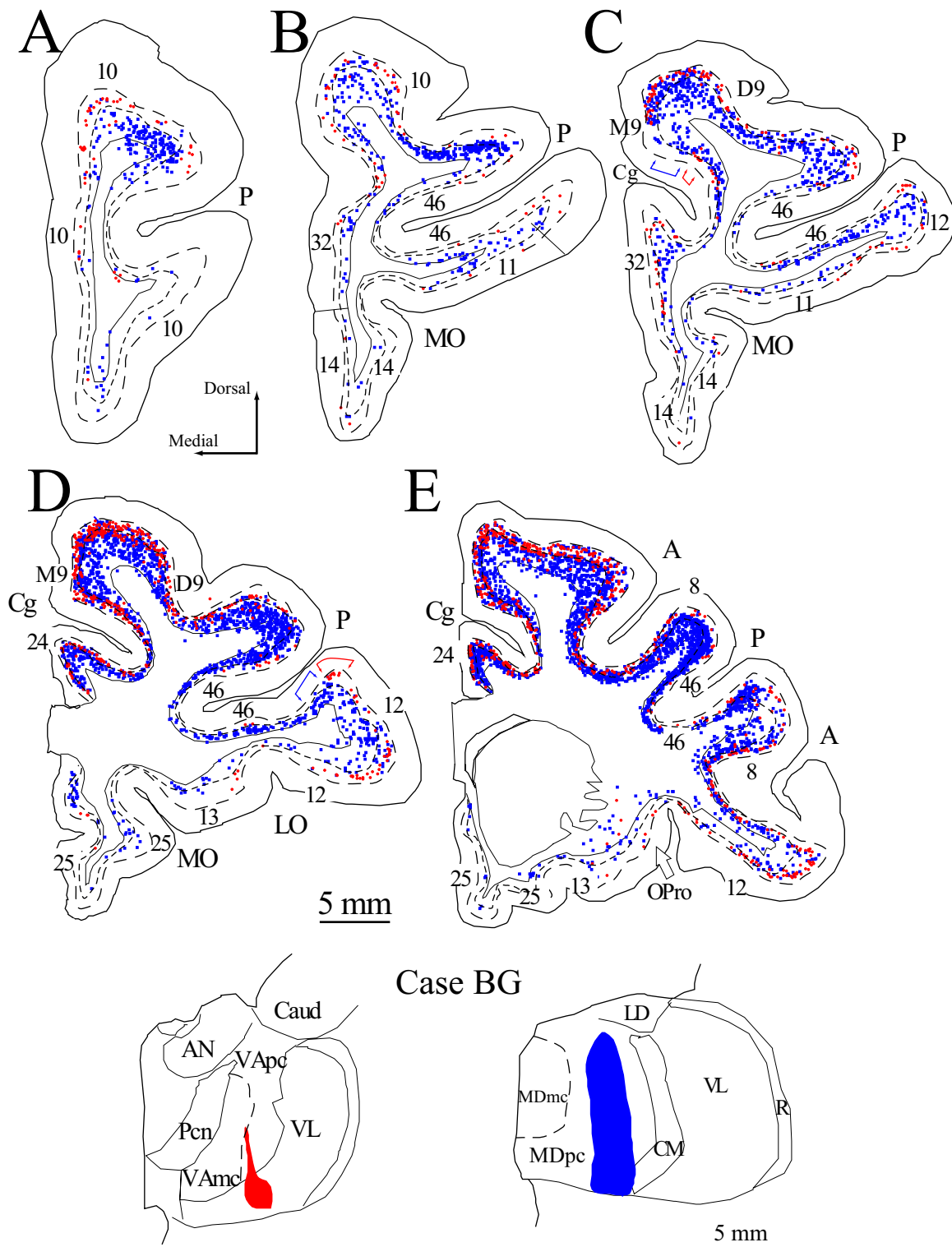
Fig. 3. Projection neurons in prefrontal cortices directed to the VA/VL and MD nuclei. (A–E) Distribution of projection neurons in a series of coronal sections in rostral (A) to caudal (E) prefrontal cortices after injection of Fluororuby in the VA/VL (red) and Fast Blue in MDpc (blue). Colored brackets in (C, D) show examples of the modular distribution of prefrontal projection neurons to VA/VL (red), and MD (blue). Dotted lines in A–E show the top of cortical layers V and VI. *Inset*, injection sites in VA/VL and MDpc shown in coronal sections through the thalamus (colored areas, case BG).
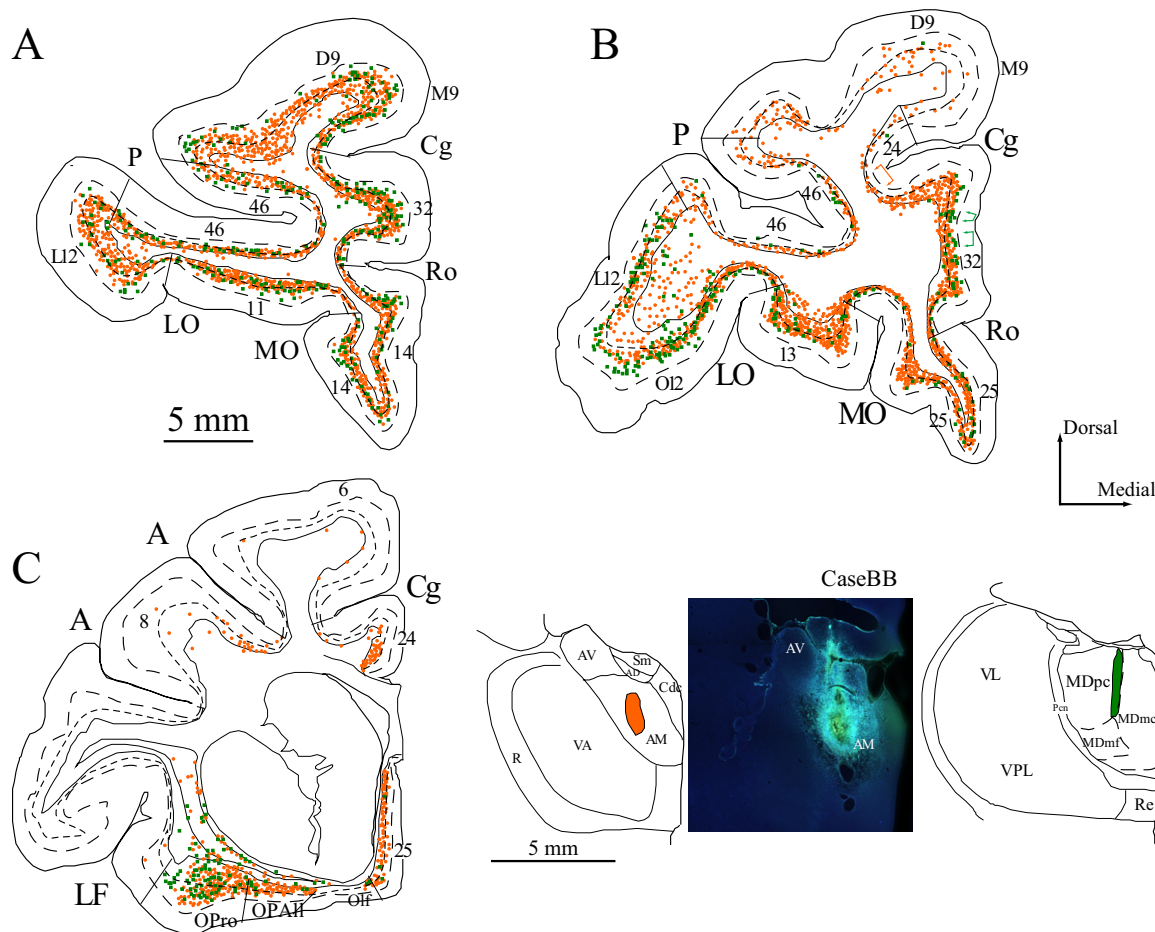
Fig. 4. Projection neurons in prefrontal cortices directed to the AM and MD thalamic nuclei. (A–C) Distribution of projection neurons in a series of coronal sections in rostral (A) to caudal (C) prefrontal cortices after injection of Diamidino Yellow in the AM (orange) and Fluoroemerald in MD (green). Colored brackets in (B) show examples of the modular distribution of prefrontal projection neurons to AM (orange), and MD (green). Dotted lines in A–C show the top of cortical layers V and VI. *Inset*, injection sites in AM (left and center) and MD (right) in coronal sections through the thalamus (colored areas, case BB).

VA/VL (Fig. 7). As shown in Fig. 7, MD was targeted heavily by layer VI neurons (steeper slope; Fig. 7B), VA/VL was targeted equally by both layers V and VI (smallest slope; Fig. 7C), and AM was targeted mainly by layer VI but also a significant number of layer V neurons (intermediate slope; Fig. 7A).

Sublaminar distribution of prefrontal cortico-thalamic projection neurons in layer V. We found that prefrontal neurons that projected to the three thalamic nuclei showed a spatial bias within layer V. The significant overlap of prefrontal cortico-thalamic neurons projecting to different thalamic nuclei made it possible to directly compare their sublaminar distribution within layer V. As shown in Figs. 1–4 and summarized on a reconstructed brain in Supplementary Fig. 1, the projection systems to the three thalamic nuclei overlapped, especially in lateral areas 9, 10, 12, 46 and 8, and in medial areas 24, 32, 14 and 9. In these areas, projection neurons in layer V directed to VA/VL were found mostly superficial to projection neurons directed to MD, as shown in photomicrographs in Fig. 8, and in the maps of coronal sections through the prefrontal cortex (Figs. 1–3). Similarly, pro-

jection neurons directed to VA/VL were found above neurons projecting to AM (Fig. 1A–C). In general, projection neurons directed to AM and MD were found at similar laminar depths, with the vast majority found in the deep parts of layer V, close to layer VI (Fig. 4). There was no apparent sublaminar organization of labeled neurons in layer VI.

Modular organization of prefrontal cortico-thalamic projection neurons

The above evidence shows a distinct laminar and sublaminar distribution of projection neurons within prefrontal areas directed to the three thalamic nuclei. In addition, within each prefrontal area the three projection systems showed a modular organization, which was apparent throughout the prefrontal cortex. Cortico-thalamic projections to VA/VL and MD were organized in interdigitated modules perpendicular to the pial surface, extending through layers VI and V. Fig. 1D illustrates at a higher magnification the modules for a single projection (red labeled neurons projecting to AM) in area 32 (from Fig. 1B). The modules are seen in maps of projection neurons on diagrams of coronal sections through
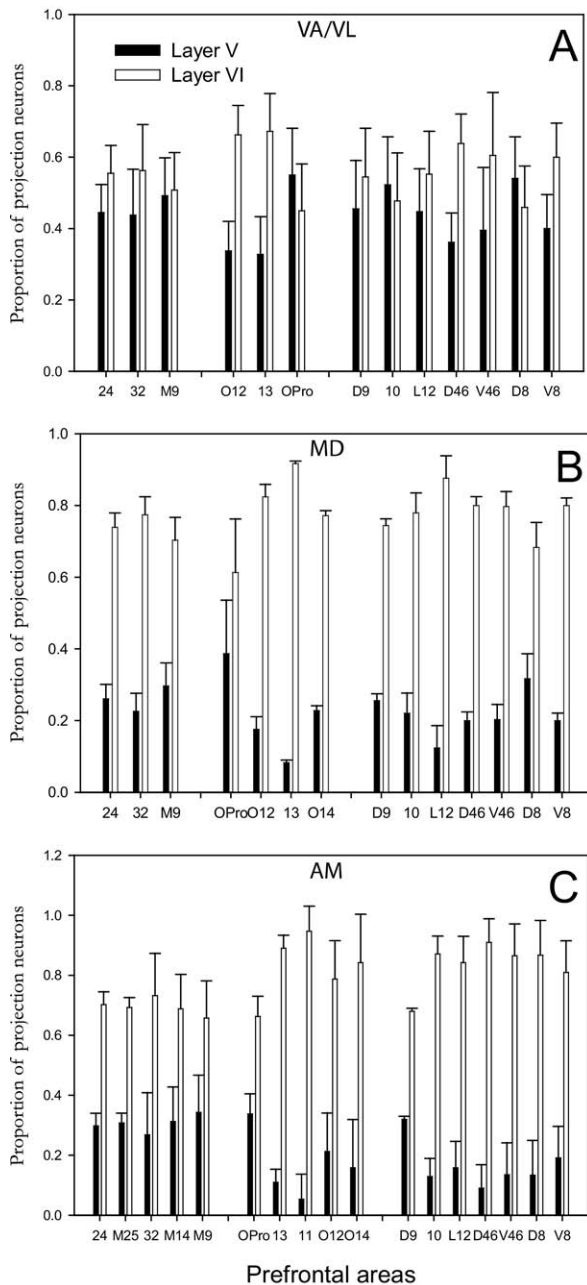
Fig. 5. Laminar origin of projection neurons in prefrontal cortices directed to three thalamic nuclei. (A) Proportion of projection neurons in layer V and layer VI directed to VA (or VA/VL). (B) Proportion of projection neurons in layer V and layer VI directed to MD. (C) Proportion of projection neurons in layer V and layer VI directed to AM. Vertical lines on bars show standard errors. Prefrontal areas are grouped based on their topography: Medial areas, left; orbital areas, middle; dorsolateral areas, right.

the prefrontal cortex (colored brackets in Fig. 1B and Fig. 2D show examples in areas 32, M9 and L12; Fig. 3C and D plots show examples in area M9 and area 46). Photomicrographs in Fig. 8A and B show examples of modules of labeled neurons in areas 24 and dorsal 9.

The modular organization was also evident for projection neurons directed to AM and MD, which were found at the same laminar depth (Figs. 1 and 4 and 8). For example, Fast Blue–labeled neurons to MD were found in modules next to clusters of Fluororuby-labeled neurons projecting to AM (colored brackets in Fig. 1B and C show examples in areas 32 and O12). Similarly, Fluoroemerald-labeled neurons directed to MD were found in large numbers in orbital and medial prefrontal areas, where they were interdigitated with Diamidino Yellow–labeled neurons directed to AM (colored brackets in Fig. 4B show examples from area 32).
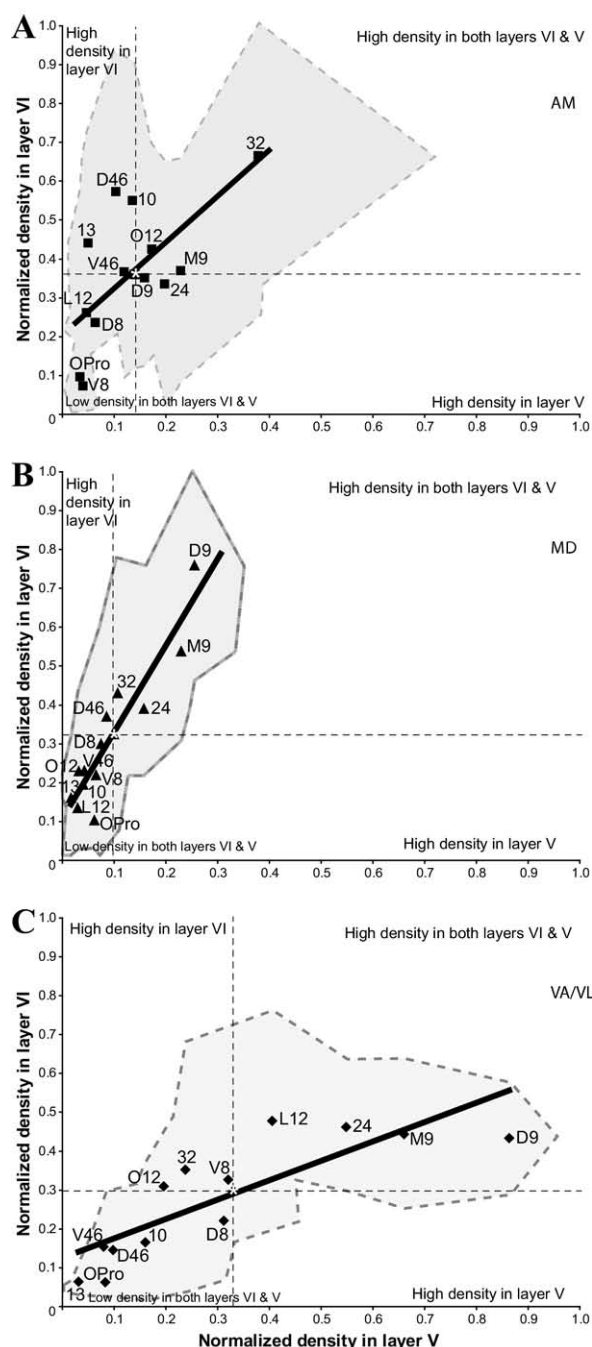
Module sizes of projection neurons in each prefrontal area targeting MD, AM, and VA/VL varied. Modules targeting VA/VL had generally smaller width, with the exception of modules in area 12. The average width of modules was MD, 660 (mean±30 μm (SEM); range, 160–2020 μm); AM, 560 (mean±40 μm; range, 170–1230 μm); and of VA/VL, 490 (mean±30, range 170−1160 μm).

DISCUSSION

Projections from prefrontal cortex to MD, the principal thalamic nucleus for the prefrontal cortex, the motor-related
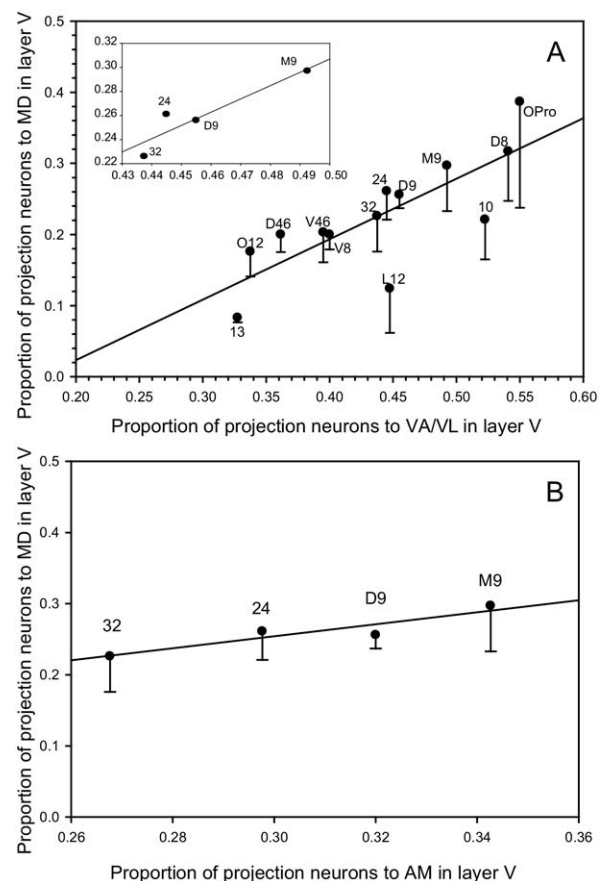
Fig. 6. Relationship of the proportions of projection neurons in layer V in prefrontal cortices directed to pairs of thalamic nuclei. (A) Correlation of the proportion of layer V projection neurons in prefrontal areas directed to VA/VL and MD (Pearson ρ=0.78); the correlation was higher for dorso-medial prefrontal areas considered separately (Pearson ρ=0.86, inset). (B) Correlation of the proportion of layer V projection neurons in prefrontal areas directed to AM and MD in dorso-medial prefrontal areas (Pearson ρ=0.86). Vertical lines show standard error.

Fig. 7. Laminar comparison of the relative density of prefrontal neurons projecting to three thalamic nuclei: (A), AM; (B), MD; (C), VA/VL. Asterisks in each scatter plot indicate the position of the average relative density of projection neurons in layers V and VI for all prefrontal areas, separating the plot area in quadrants. Prefrontal areas that cluster in the top left or bottom right quadrant included a higher than average density of projection neurons in layer VI or V, respectively. Prefrontal areas that cluster in the top right or bottom left quadrant included respectively a higher or a lower than average density of projection neurons in both layers VI and V. The slope of the fit (solid lines) shows that MD was targeted heavily by layer VI neurons (steeper slope), VA was targeted equally by both layers V and VI (smallest slope), and AM was targeted mainly by layer VI but also by a significant number of layer V neurons (intermediate slope). The shaded areas in each plot indicate the variation in the density of projection neurons in layer V (X-axis) and layer VI (Y-axis) for each prefrontal area.

nuclei VA/VL, and the limbic nucleus AM, showed a dual organization into modules and layers. Prefrontal cortices issued projections in significant numbers from layer V, especially when directed to the VA/VL nuclei. Moreover, there was consistently a higher proportion of projection neurons in layer V in dorsal and medial than in ventral prefrontal areas.

Organization of prefrontal cortico-thalamic projections into modules and layers

Projection neurons to the three thalamic nuclei originated from many prefrontal areas, and overlapped extensively, especially in dorso-medial and lateral prefrontal areas, which issued robust projections to MD and VA/VL, and in medial and orbitofrontal cortices, which projected to all three nuclei. In the areas of overlap, projection neurons to MD, VA/VL and AM were organized into interdigitated modules extending through layers VI and V, suggesting some degree of segregation of prefrontal projections to distinct thalamic nuclei. The modular organization and sizes found are in agreement with previous observations regarding ocular dominance columns in areas 17 and 18, which ranged between 500 and 2000 μm in width (Friedman et al., 1989), or the width of afferent fiber columns in the prefrontal cortex, which was reported to average 685 μm (Bugbee and Goldman-Rakic, 1983), and the width of interdigitated columns of contralateral and ipsilateral projections from the parietal cortex to the prefrontal cortex, which ranged from 300 to 750 μm (Goldman-Rakic and Schwartz, 1982; Goldman-Rakic, 1984).

Projection neurons to the three thalamic nuclei were also organized in layers, as summarized in Fig. 9. Most projection neurons in all cases were found in layer VI, which gives rise to cortico-thalamic projections in all cortical systems [reviewed in; Steriade et al., 1997; Jones, 2007]. However, significant proportions of prefrontal cortico-thalamic neurons were found in layer V, in a pattern that differed depending on thalamic destination. Among the nuclei studied, the proportion of projection neurons from layer V directed to the VA/VL was higher than to MD or AM. Moreover, within layer V, projection neurons directed to the VA/VL occupied primarily the upper part of layer V, whereas projection neurons directed to MD or AM occupied the deep part of layer V, showing a sublaminar organization.

Cortical layer V neurons make up a small proportion of the population of neurons projecting to high-order thalamic nuclei, and also project to motor-related subcortical structures (Guillery, 1995). The upper part of layer V in prefrontal cortex includes the majority of projection neurons directed to the striatum in monkeys, cats and dogs (Selemon and Goldman-Rakic, 1985; Arikuni and Kubota, 1986; Goldman-Rakic and Selemon, 1986; Tanaka, 1987; Yeterian and Pandya, 1994; Thomson and Bannister, 2003). The upper part of layer V also projects to the motor-related VA/VL nuclei of the thalamus, as shown here, providing additional evidence for the preferential involvement of this sub-layer in motor circuits. On the other hand, neurons in the deep part of layer V of association cortices project to
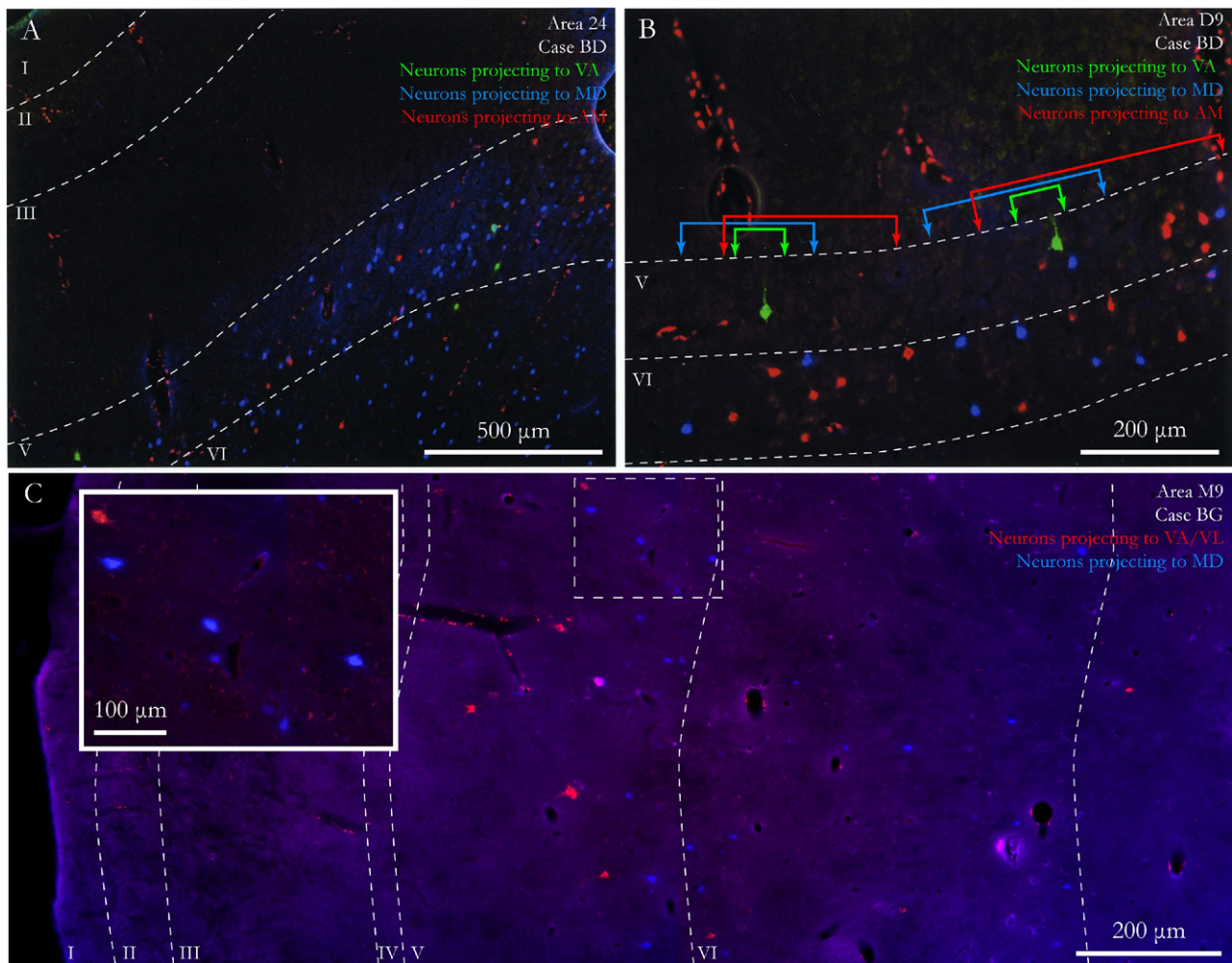
Fig. 8. Modular and laminar organization of prefrontal cortico-thalamic projection neurons. (A) Low power photomicrograph of all layers of area 24 from pia to white matter (case BD) shows labeled prefrontal neurons in layers V and VI projecting to VA (green), AM (red), and MD (blue). (B) Laminar and modular organization of projection neurons in area D9 (case BD). Modules of neurons projecting to each thalamic nucleus are separated by a minimum distance of 150 μm and are delineated by colored lines with arrowheads. (C) Photomontage of area M9 (case BG) shows the laminar distribution of prefrontal neurons projecting to VA/VL (red) and MD (blue). Layer V neurons projecting to VA/VL (red) are found mainly in the upper part, while neurons projecting to MD (blue) are found deeper within layer V. *Inset*, higher magnification of delineated site at the bottom of layer V.

several thalamic nuclei, such as the intralaminar, which then project widely to several cortical areas (Catsman-Berrevoets and Kuypers, 1978; Thomson and Bannister, 2003). This evidence suggests specificity in layer V projection neurons directed to VA/VL, on one hand, and to MD and AM, on the other hand, which may be differentially recruited in behavior.

Circuit differences of laminar-specific projections

Connections within cortical columns. The significance of the differences in the relative distribution of prefrontal cortico-thalamic projection neurons within layers VI and V that were directed to MD, VA/VL and AM is based on the specific anatomic interactions of each of these layers within cortical columns, on one hand, and in circuits linking the cortex with the thalamus, on the other hand. At the level of cortical columns, pyramidal neurons in the upper part of cortical layer V, which included the majority of

projection neurons to the VA/VL, are generally large, and have long apical dendrites that ascend to layer I, and long horizontal dendrites that stretch laterally over several millimeters within layer V (Mountcastle, 1997). In the visual cortex, horizontal connections across cortical columns are thought to integrate contour information of objects and bind visual inputs to form much larger receptive fields (Wiesel and Gilbert, 1989; Stettler et al., 2002). The axonal branches of neurons in layer V also ascend vertically to all layers above. The lower part of layer V, where most layer V neurons directed to MD and AM were found, is populated mostly by small pyramidal neurons, whose apical dendrites ascend to layer III, and their branched axon terminals project vertically to layers II, III, IV and V. Small pyramidal neurons are also found in layer VI, and their apical dendrites ascend to the upper part of layer IV, which receives the majority of thalamo-cortical projections. Some axonal branches of layer VI neurons project to layers III–VI
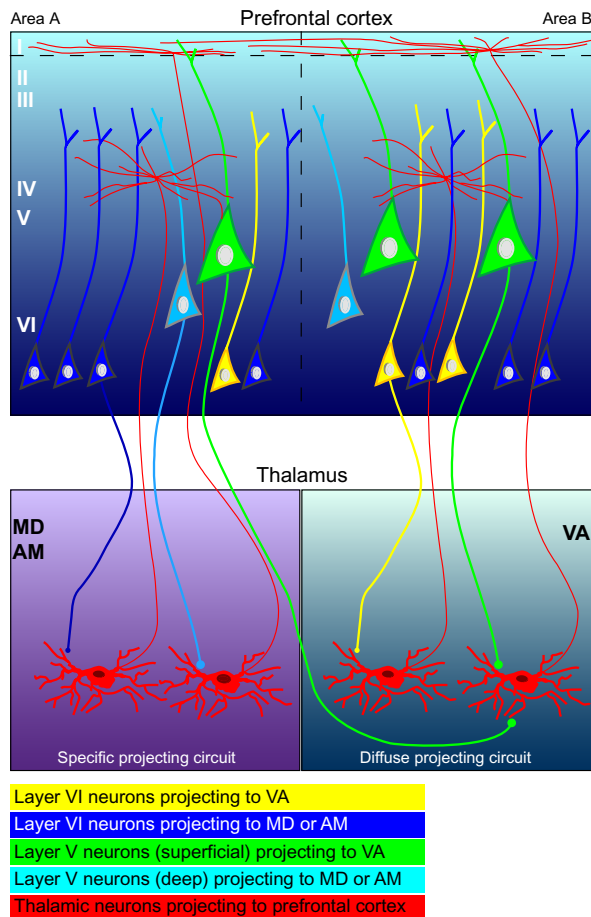
Fig. 9. Schematic diagram summarizes the laminar origin of prefrontal cortico-thalamic pathways to MD, AM, and VA and the reciprocal thalamo-cortical projections. Prefrontal projection neurons directed to all three thalamic nuclei originate from interdigitated modules of pyramidal neurons spanning layers VI and V. *Left panels*, projection neurons directed to MD and AM originate mainly from layer VI pyramidal neurons (dark blue), which have been shown, in previous studies to terminate through small boutons onto thalamic neurons that project focally to the middle cortical layers (left red neuron in MD/AM panel). Most layer V projection neurons directed to MD and AM are pyramidal neurons situated in the deep part of the layer (light blue), and have been shown to terminate through small and large boutons onto thalamic neurons that project to the superficial cortical layers (right red neuron in MD/AM panel). *Right panels*, the VA receives projections from a higher proportion of layer V neurons than MD or AM, which constitute nearly as many as from layer VI. Projections to VA (right panels) that originate from layer VI pyramidal neurons (yellow) have also been shown, in previous studies, to terminate through small boutons onto thalamic neurons that project focally to the middle cortical layers (left red neuron in VA panel). The majority of layer V pyramidal neurons projecting to VA are situated in the upper part of the layer (green). These large layer V pyramidal neurons terminate through small and large boutons onto thalamic neurons that send widespread projections to the superficial layers of the prefrontal cortex (right red neuron in VA panel). Widespread thalamic projections to layer I impinge on the apical dendrites of pyramidal neurons in neighboring columns or areas (areas B and A), and may thus spread activation across multiple areas.

(Thomson and Bannister, 2003). The sublaminar segregation of prefrontal projections into upper layer V, directed to the VA/VL, and into deep layer V, directed to MD or AM,

suggests differential effects on the cortical microarchitecture of columns by each of these layer V projection systems.

Connections with the thalamus. Cortical neurons in layers V and VI also have distinct patterns of projection to the thalamus. Neurons from layer V of sensory association cortices terminate as large and round clusters of terminals in sensory thalamic nuclei and innervate the proximal dendrites of thalamic neurons (Jones and Hendry, 1989; Guillery, 1995; Rockland, 1996; Jones, 1998a,b; Rockland et al., 1999; Rouiller and Welker, 2000; Guillery and Sherman, 2002). Projection neurons directed to the motor-related VA/VL nuclei, half of which originate from layer V, terminate mainly through small but also some large terminals that synapse with calbindin positive thalamic projection neurons with perisynaptic ionotropic glutamate receptors, which, in turn, project to cortical layer I [Fig. 9; Zikopoulos and Barbas, 2007].

In contrast, axons from neurons in layer VI terminate as small boutons in continuous distributions and innervate mainly the distal dendrites of thalamic neurons, forming synapses predominantly with parvalbumin positive thalamic projection neurons enriched with metabotropic glutamate receptors (Reichova and Sherman, 2004; Zikopoulos and Barbas, 2007). In turn, these thalamo-cortical neurons project to the middle cortical layers, including layer IV. Our findings of differences in the prevalence of layer V prefrontal neurons projecting to different thalamic nuclei also suggest differences in recruitment of thalamo-cortical pathways.

Functional implications of laminar-specific connections

The prevalence of layer V cortico-thalamic projections varied regionally, being higher in dorsal prefrontal areas (e.g. area 9) and medial areas (e.g. areas 24 and 32). Area 9 is situated in front of the premotor areas and has a role in planning sequential tasks requiring response monitoring and attentional control (Goldman-Rakic, 1987; Fuster, 1993; Petrides, 1995, 1996; Hikosaka et al., 1999; Sakai et al., 1999; Levy and Goldman-Rakic, 2000). Anterior cingulate areas (24 and 32) are engaged when cognitive demands are high, such as in monitoring performance and errors during conflicting events (Devinsky et al., 1995; Botvinick et al., 1999; Carter et al., 2000; Miller and Cohen, 2001; Paus, 2001; Milham et al., 2001; Walton et al., 2007; Brown and Braver, 2008). Neurons from layer V of area 32 in rhesus monkeys project to lateral prefrontal cortices (Barbas and Rempel-Clower, 1997). In dorsolateral area 9 axons from area 32 form synapses with spines of excitatory neurons and also innervate inhibitory neurons through large terminals (Medalla and Barbas, 2009). The high proportion of projection neurons in layer V in dorsal and medial prefrontal areas may have a role in neuronal processing of sequential events and motor outcomes.

The significant proportion of projection neurons in layer V directed to the VA/VL, MD, and AM thalamic nuclei provides a mechanism for interlinking prefrontal areas through the thalamus (Haber and McFarland, 2001;

McFarland and Haber, 2002; Zikopoulos and Barbas, 2007). This linkage may be effected through thalamo-cortical projections to layer I, which stretch parallel to the pial surface over long distances, where they impinge on the apical dendrites of layer V neurons and likely activate them (von Stein et al., 2000; Larkum et al., 2004). Through this linkage, fast firing driver neurons in layer V may transmit signals across several cortical areas and induce oscillations (Guillery, 1995; Sherman and Guillery, 1998; Rouiller and Welker, 2000; Guillery and Sherman, 2002). There is evidence that in prefrontal cortex, activation of layer V pyramidal neurons may initiate slow wave oscillations that propagate vertically to other layers (Sanchez-Vives and McCormick, 2000; Ulbert et al., 2004). Through these extensive horizontal thalamo-cortical projections to layer I of prefrontal cortex, circuits through the VA may interact with circuits through MD and AM to process information in the context of emotion and memory.

In addition, the prefrontal cortex receives projections from MDmc, VAmc and AM in both hemispheres (Preuss and Goldman-Rakic, 1987a; Dermon and Barbas, 1994), which are part of the circuits through the basal ganglia and the prefrontal cortex (Groenewegen et al., 1990; Xiao and Barbas, 2002b). Bilateral connections between prefrontal cortex and these thalamic nuclei may coordinate bilateral motor activity through communication within local circuits in the prefrontal cortex. Moreover, within the context of the modular and laminar organization of cortico-thalamic projections, reciprocal connections between prefrontal cortices and thalamic nuclei may initiate brain wave oscillations, associated with consciousness, attention and executive control (Smythies, 1997; Steriade, 2000; Llinas and Steriade, 2006; Vertes, 2006; Schiff, 2008). Disturbance in this transthalamic corticocortical communication may underlie the pathology in psychiatric diseases (Llinas et al., 1999; Lewis et al., 2001; Rubenstein and Merzenich, 2003) and movement disorders (Goldman-Rakic et al., 1992; Hoover and Strick, 1993; Bergman et al., 1998; Sabatini et al., 2000; Garcia-Cabezas et al., 2007), affecting specific nodes in this intricate circuitry.

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APPENDIX

Supplementary data