Auditory Thalamocortical Synaptic Transmission In Vitro

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Received 3 July 2001; accepted in final form 5 September 2001

Cruikshank, Scott J., Heather J. Rose, and Raju Metherate. Auditory thalamocortical synaptic transmission in vitro. J Neurophysiol 87: 361–384, 2002; 10.1152/jn.00549.2001. To facilitate an understanding of auditory thalamocortical mechanisms, we have developed a mouse brain-slice preparation with a functional connection between the ventral division of the medial geniculate (MGv) and the primary auditory cortex (ACx). Here we present the basic characteristics of the slice in terms of physiology (intracellular and extracellular recordings, including current source density analysis), pharmacology (including glutamate receptor involvement), and anatomy (gross anatomy, Nissl, parvalbumin immunocytochemistry, and tract tracing with 1,1-dioctadecyl-3,3,3',3'-tetramethylindocarbocyanine perchlorate). Thalamocortical transmission in this preparation (the “primary” slice) involves both α-amino-3-hydroxy-5-methylisoxazole-4-proprionic acid/kainate and N-methyl-D-aspartate-type glutamate receptors that appear to mediate monosynaptic inputs to layers 3-4 of ACx. MGv stimulation also initiates disynaptic inhibitory postsynaptic potentials and longer-duration intracortical, polysynaptic activity. Important differences between responses elicited by MGv versus conventional columnar (“on-beam”) stimulation emphasize the necessity of thalamic activation to infer thalamocortical mechanisms. We also introduce a second slice preparation, the “shell” slice, obtained from the brain region immediately ventral to the primary slice, that may contain a nonprimary thalamocortical pathway to temporal cortex. In the shell slice, stimulation of the thalamus or the region immediately ventral to it appears to produce fast activation of synapses in cortical layer 1 followed by robust intracortical polysynaptic activity. The layer 1 responses may result from orthodromic activation of nonprimary thalamocortical pathways; however, a plausible alternative could involve antidromic activation of corticotectal neurons and their layer 1 collaterals. The primary and shell slices will provide useful tools to investigate mechanisms of information processing in the ACx.

INTRODUCTION

The auditory cortex (ACx) integrates and processes information carried along thalamocortical pathways to perform its major functions (for reviews, see de Ribaupierre 1997; Ehret 1997; Phillips 1995). Much is known about auditory thalamocortical pathways from anatomical and physiological studies. The main input to ACx is the primary thalamocortical pathway (often referred to as the lemniscal pathway) that projects from the ventral division of the medial geniculate (MGv) to the middle layers of primary ACx (Caviness and Frost 1980; Romanski and LeDoux 1993; Willard and Ryugo 1983; reviewed in Winer 1992). Physiological recordings from MGv and ACx indicate that this pathway mediates short-latency responses to acoustic stimuli and carries precise information about stimulus frequency, intensity, and timing (thalamus: Bordi and LeDoux 1994; Calford 1983; Edeline et al. 1999; Lennartz and Weinberger 1992; ACx reviewed in: de Ribaupierre 1997; Eggermont 1998; Ehret 1997). There are also nonprimary thalamocortical pathways (referred to as nonlemniscal or adjunct), including projections from the dorsal and medial divisions of the MG (MGd, MGm), the peripeduncular nucleus, and other nonprimary thalamic nuclei (Arnault and Roger 1990; Clerici and Coleman 1990; Herkenham 1980; LeDoux et al. 1985; Linke and Schwegler 2000; Ryugo and Killackey 1974). Typically these projections target superficial layers of temporal cortex. Responses of nonprimary thalamic cells tend to be weaker, have longer latencies, and more variability than MGv (Allon et al. 1981; Bordi and LeDouxy 1994; Calford 1983; Edeline et al. 1999; Lennartz and Weinberger 1992), so their influence on cortex is expected to be more subtle (discussed in McGee et al. 1992; Sukov and Barth 2001; Weinberger 1995). Thus ACx function depends on the integration of both primary and nonprimary inputs.

Despite a large number of anatomical and physiological studies, very little is known about the cellular and synaptic mechanisms by which thalamic inputs are transmitted to, and processed in, ACx. Some progress has been made using in vivo intracellular recordings (e.g., Metherate and Ashe 1993; Mitani and Shimokouchi 1985; Sukov and Barth 2001), but such studies are hampered by the difficulty of making precise electrophysiological and pharmacological measurements in vivo. Yet detailed intracellular information must be obtained to address even seemingly simple questions such as how synaptic integration produces neuronal receptive fields. Brain-slice preparations offer an ideal environment to make precise intracellular and pharmacological manipulations, and considerable information has been obtained from ACx slices (Buonomano and Merzenich 1998; Hefti and Smith 2000; Kudoh and Shibuki 1997; Metherate and Ashe 1994). Nonetheless, the issue of thalamocortical processing has not been addressed directly because thalamocortical axons are severed during the preparation of conventional (coronal) slices and thus cannot be stimulated selectively.

To address similar problems in the somatosensory system, Agmon and Connors (1991) developed a slice preparation that maintained connections from the ventrobasal complex to barrel cortex. Since its development, that preparation has been applied to numerous issues fundamental to the understanding of thalamocortical processing in the somatosensory system. These include determining which cortical cells, as defined by position, morphology, and intrinsic properties, receive direct thalamic input (Agmon and Connors 1992; Gibson et al. 1999;
Porter et al. 2001); what transmitters mediate thalamocortical transmission (Gil and Amitai 1996a); functional differences between intracortical and thalamocortical synapses (Gil and Amitai 1996b; Gil et al. 1997, 1999); and mechanisms of synaptic plasticity (reviewed in Castro-Alamancos and Connors 1997; Feldman et al. 1999).

To facilitate similar progress in the understanding of auditory forebrain mechanisms, we have developed a brain-slice preparation that maintains an intact auditory thalamocortical pathway. In initial studies using slices from rat and mouse, we demonstrated the likely feasibility of such a preparation by showing that stimulation within the thalamus or thalamocortical pathway could elicit cortical responses (Metherate and Cruikshank 1999). More recently, we have focused on the mouse brain because its smaller size permits more intact connections within a slice and for future transgenic studies. We have substantially refined the preparation to the point that we can reliably identify and study the primary auditory thalamocortical pathway in vitro. The present study represents an extensive anatomical and physiological characterization of this more refined preparation. We also introduce a second slice preparation, obtained from the area immediately ventral to the primary preparation, that may contain a nonprimary pathway. Several features of this second preparation are characterized and contrasted with those of the primary preparation. Portions of this study have appeared in abstract form (Cruikshank et al. 1999).

METHODS

Preparation of slices

All procedures followed the University of California, Irvine, animal-use regulations. Slices were taken from postnatal day (P)13–P19 FVB mice and maintained in vitro. Slice planes were nearly horizontal, but with the lateral end typically raised 15°, as illustrated in Fig. IA. This plane was chosen so that thalamocortical axons, and parts of the MGv and ACx, could all be obtained within a single slice (Fig. 1).

Following decapitation under halothane anesthesia, brains were rapidly removed and placed in 0–4°C artificial cerebrospinal fluid (ACSF; in mM: 125 NaCl, 2.5 KCl, 1.25 KH2PO4, 25 NaHCO3, 1.2 MgSO4, 2 CaCl2, and 10 dextrose; bubbled with 95% O2–5% CO2). Before slicing, the brains were blocked by making three cuts with a handheld razor blade while submerged in cold ACSF. First the anterior 25% of the brain was removed with a coronal cut. The remaining brain block was then propped forward to rest on its cut surface, and a second cut was made in a nearly horizontal plane (but with the lateral end typically raised 15° to ensure both MG and ACx to be obtained within a single slice (Fig. 1).

Following decapitation under halothane anesthesia, brains were rapidly removed and placed in 0–4°C artificial cerebrospinal fluid (ACSF; in mM: 125 NaCl, 2.5 KCl, 1.25 KH2PO4, 25 NaHCO3, 1.2 MgSO4, 2 CaCl2, and 10 dextrose; bubbled with 95% O2–5% CO2). Before slicing, the brains were blocked by making three cuts with a handheld razor blade while submerged in cold ACSF. First the anterior 25% of the brain was removed with a coronal cut. The remaining brain block was then propped forward to rest on its cut surface, and a second cut was made in a nearly horizontal plane (but with the lateral end typically raised 15°), splitting the brain into dorsal and ventral portions. The dorsal portion was discarded, then the ventral portion was lifted from the ACSF and glued to the stage of a Vibroslice with its freshly cut surface facing down. The MGv (in primary slice) and a typical position for shell stimulation (in shell slice) are indicated by arrows. Dashed rectangles indicate approximate areas of the fluorescent images in Figs. 8, A1 and B1 (primary), and 10, A1 and B1 (shell).

FIG. 1. Anatomical positions of the primary and shell slices. A: drawing on right shows schematic of coronal mouse brain section at level of medial geniculate (MG) and auditory cortex (adapted from Franklin and Paxinos 1997). The ventral MG (MGv, black) is surrounded by a shell of nonprimary auditory nuclei (gray). Likewise, primary auditory cortex (ACx, black—also indicated by asterisk) is bordered dorsally and ventrally by nonprimary auditory cortical areas (gray). The dotted line shows the approximate 15° angle that allows both MGv and ACx to be obtained within a slice. A, left: the left hemisphere of an actual mouse brain, viewed from the front, illustrating the planes of the primary and shell slices; they are 600 μm thick, and cut at about the angle indicated in the adjacent drawing. Orientation: lateral toward right, dorsal toward top. B: same hemisphere as in A but viewed from lateral side. Orientation: anterior toward left, dorsal toward top. C: same as B except the cortex was dissected away, revealing midbrain and thalamic structures (SC, superior colliculus; IC, inferior colliculus). MG is visible, and its bottom 30–50% appears to be within the primary slice. Shell slice is below MG. The structure immediately anterior to MG is the lateral geniculate (LG). D and E: the primary and shell slices are laid down flat and viewed from the dorsal side (as in recording chamber). Orientation: anterior is toward left, lateral is toward bottom. Typical recording sites in the middle layers of temporal cortex are indicated by asterisks. The MGv (in primary slice) and a typical position for shell stimulation (in shell slice) are indicated by arrows. Dashed rectangles indicate approximate areas of the fluorescent images in Figs. 8, A1 and B1 (primary), and 10, A1 and B1 (shell).

slices were examined under a dissecting microscope (in recording chamber) to determine if they were in appropriate planes. If correctly blocked and sectioned, there would ultimately be two useful slices per brain (the “primary slice” and the “shell slice,” Fig. 1), each of which had distinct physiology and anatomy as presented in RESULTS.

In initial experiments (n = 29 slices), slices were cut at 600 μM to ensure a high probability of intact thalamocortical connections. However, in later experiments, after learning more about the pathway trajectory, slice thickness was successfully reduced to 500 μM (n = ...
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38; also n = 2 @ 400 μM), thus improving chances of maintaining healthy preparations.

Electrophysiology

Recordings took place in an interface chamber (Haas model, Med Systems) maintained at 34°C, with a liberal flow of warmed humidified gas (95% O2-5% CO2) and ACSF passing over the slices. An initial incubation period of 1–2 h preceded data acquisition.

Sharp intracellular recording microelectrodes were pulled from filamented glass (1.0 mm OD: A-M Systems) on a horizontal puller (p97, Sutter Instruments) and had resistances of 60–140 MΩ when filled with 3 M K-acetate (+10 mM HEPES buffer, pH 7.3). Extracellular recording microelectrodes were also pulled from filamented glass (1.5 mm OD: A-M Systems), on the same puller, and had resistances of 0.5–5.0 MΩ when filled with ACSF. Neural signals were amplified (CyberAmp AT-401 and Axoclamp 2B, Axon Instruments), monitored on oscilloscope (Tektronix), then digitized (5–20 kHz) and stored on computer (PowerMac, Apple Computer). Extracellular electrical stimuli (0.1–0.2 ms, 1–325 μA) were delivered via concentric bipolar electrodes (Ultrasmall, 25 μm inner core, 200 μm overall diameter, F. Haer) and a constant current isolation unit (Axon Instruments). Control of experiments and off-line analysis were done with computers (AxoData and AxoGraph, Axon Instruments; PowerMac, Apple Computer).

Stimulation and recording sites were visualized with a dissecting microscope (Fig. 1, D and E). Stimuli were delivered to the MG (primary slice, Fig. 1D) or the region ventral to the MG (shell slice, Fig. 1E, indicated by arrow). Responses were recorded in temporal cortex, from the cortical column with the largest extracellular response in layer 4 (i.e., the “focus”—see Laminar response profile of primary slice is dominated by middle layer CSD sink). Intracellular recordings were mainly in layers 3–4 (30–50% of the distance from the pia to the white matter; asterisks in Fig. 1, D and E), and simultaneous extracellular recordings were usually in layers 3–4 for the primary slice, and layer 1 for the shell slice (<250 μm lateral distance between intra- and extracellular sites in layers 3–4). To determine the laminar locations of current sinks and sources, one-dimensional current source density (CSD) analysis was performed (Agmon and Connors 1991; Johnston and Wu 1995; Mitzdorf 1985).

First, extracellular responses were recorded at evenly spaced locations (either 125 or 150 μm spacing) beginning two positions above the pia and ending either in the deep cortical layers or below layer 6. The CSD value for a given location (at a given time point) was then calculated by subtracting twice the voltage at that location from the sum of the voltages at the two nearest locations and dividing the result by the square of the distance separating recording sites.

To test for the roles of glutamate receptor subtypes in evoked responses, the N-methyl-d-aspartate (NMDA) receptor antagonist di-2-amino-5-phosphonopentanoic acid (APV; 50 μM) and the α-amino-3-hydroxy-5-methylisoxazole-4-propionic acid (AMPA/K), KA receptor antagonist 6-cyano-7-nitroquinoline-2,3-dione (CNQX, 20 μM) were applied to the bath (both drugs from Research Biochemicals International). CNQX stock solution included dimethyl sulfoxide (DMSO; final concentration 0.4%). In experiments designed to block synaptic transmission, Ca2+ concentration in the ACSF was reduced and replaced with Mg2+ (“low calcium,” in mM: 0.2 CaCl2 and 3.0 MgSO4, all other salts the same as normal ACSF). In experiments designed to selectively suppress polysynaptic responses (see Intracellular recordings in layers 3–4 of the primary slice reveal consistent early response and more irregular late polysynaptic response), ACSF with high concentrations of divalent cations was used (“high divalent ACSF”; in mM: 115 NaCl, 2.5 KCl, 25 NaHCO3, 4.2 MgCl2, 7 CaCl2, and 10 dextrose). Besides the adjustments in Ca2+ and Mg2+ concentrations (which were 3.5 times normal values), sulfates and phosphates were also left out to prevent Ca2+ precipita-

Anatomy

Fixation and sectioning of recorded slices. To examine the exact planes of section, some slices were processed for either Nissl staining or parvalbumin (PV) immunohistochemistry after electrophysiological recording (described in Results). For both types of labeling, whole slices were first fixed by immersion in 4% paraformaldehyde (in 0.1 M Na-phosphate buffer; PB, pH 7.2) for ≥12 h, then sectioned on a vibratome at 50 μM.

PV immunohistochemistry. PV procedures were carried out with free-floating sections at room temperature with agitation, except where specified. Sections were pretreated for 30 min in 0.5% H2O2, rinsed in phosphate-buffered saline (PBS; pH 7.15; 0.1 M PB, 0.5 M NaCl) containing 0.1% Tween 20, then incubated for 2 h in PBS containing 0.3% Triton X-100. Next, nonspecific sites were blocked using an Avidin/Biotin Blocking Kit (Vector Labs), followed by immersion in 10% horse normal serum for 2 h (HNS, Vector Labs). Sections were then incubated at 4°C for 12–48 h in a combined antibody solution (to reduce background staining) (Hirck et al. 1994) containing a 1:500 dilution of monoclonal PV antibodies (pa-235, Sigma), a 1:500 dilution of biotinylated anti-mouse IgG (Vector Labs), 0.1 M PB, 0.5 M NaCl, 0.3% Triton X-100, 2% HNS, and 0.1% mouse normal serum (MNS, Sigma). Standard peroxidase visualization followed. Briefly, sections were rinsed in PBS containing 0.1% Tween-20, incubated in Avidin/Biotin complex for 2 h (Vectorstain Elite ABC-Peroxidase Kit, Vector Labs), rinsed again in PBS, then in 0.05 M Tris buffer (TB; pH 8.0). Next they were preincubated in nickel/DAB solution (0.3% nickel ammonium sulfate, 0.033% diaminobenzidine, TB), then reacted for 2–5 min by adding H2O2 (0.01%). Following rinses in TB and 0.1 M PB, sections were mounted on gelatin-coated slides, air dried, and dehydrated with increasing concentrations of alcohol, cleared with Histo-Clear (National Diagnostics), and coverslipped using Permount (Fisher).

Nissl staining. In the Nissl material, after sectioning at 50 μM (see preceding text), tissue was rinsed in 0.05 M PB, mounted on gelatin-coated slides, then air dried for 12–36 h. Next, the slide-bound sections were immersed for 2–24 h in a solution of 50% chloroform and 50% alcohol, rehydrated in descending concentrations of alcohol in H2O, then immersed in 0.06% cresyl violet stain (0.6 mg/ml cresyl violet acetate, 0.72 mg/ml Na-acetate, 4.9 μl/m 1 N acetic acid, H2O—usually 300 ml) for ~5 min, followed by 30 s of rinsing in H2O. After that, the tissue was dehydrated, cleared, and coverslipped as described for the immunoreacted tissue (see preceding text), except that acetic acid was added to the 95% alcohol dehydration step to accelerate differentiation of the cresyl violet stain (0.5 ml glacial acetic per 200 ml alcohol solution).

Fixation and sectioning of whole brains. To compare Nissl and PV patterns in the coronal plane, sections were cut from whole fixed brains. Under deep anesthesia [50–100 mg/kg pentobarbital sodium (Nembutal)], mice were transcardially perfused with 0.9% saline until most visible blood was flushed from the animal, then with 4% paraformaldehyde (see preceding text) for ~10 min. Solutions were 4–8°C and perfused at 10–15 ml/minute using a peristaltic pump (Fisher). The brains were postfixed overnight at 4°C (same fixative), embedded in 3% agarose (to increase stability during sectioning), then sectioned on a vibratome at 50 μM in the coronal plane. Every third section was Nissl stained (see preceding text), and adjacent sections were immunoreacted for PV (see preceding text) and calbindin (not shown).

Tract tracing with Di-I. The fluorescent lipophilic tracer 11,11-dioctadecyl-3,3,3',3'-tetramethylindocarbocyanine perchlorate (Di-I; Molecular Probes) was used to examine fiber pathways in previously recorded primary and shell slices and in some whole

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brains. First the slices and brains were fixed by immersion and perfusion respectively (described in the preceding text), then allowed to postfix ≥2 days. Next, 25- to 100-µM-diam particles of Di-I were inserted into targets in the still intact slices and brains under a dissecting microscope, using a broken micropipette to manipulate the Di-I (targets indicated in RESULTS). The slices or brains were then placed in small plastic cups and covered in warm liquid agarose (3%) that was made with PB containing 0.1% sodium azide. After the agarose hardened, the cups were filled to the top with PB (including 0.1% sodium azide), sealed with parafilm, and placed in the dark at 30–38°C for 1–4 mo to permit Di-I diffusion. The slices or brains were subsequently sectioned on a vibratome at 50 µM (for whole brains, sectioning was in horizontal plane), rinsed in PB, then mounted on gelatin-coated slides, and coverslipped with Vectashield mounting medium (Vector Laboratories).

SLICE PLANE EXAMINATION IN UNESTAINED WHOLE BRAINS. To compare the slice planes to the positions of the MG and other macroscopic features, six brains were fixed by perfusion (see preceding text) and cut on a vibratome in the planes normally used for physiological experiments. For clarity, no blocking was done so that the slice planes could be seen in the context of the whole brains. For the same reason, only three cuts were made on the vibratome: one cut separated the dorsal part of the brain from the primary slice, a second separated the primary and shell slices, and a third separated the shell slice and the ventral part of the brain (Fig. 1). The slice thicknesses were 600 µM and were in the approximate planes of physiologically recorded primary and shell slices, based on positions and appearances of structures contained within them (Fig. 1, D and E).

MICROSCOPY AND IMAGE ACQUISITION. The Nissl and PV labeling patterns were examined using standard bright-field microscopy at a variety of magnifications (×25–1,000), and images were captured with a digital camera (SPOT; Diagnostic Instruments) attached to an Olympus microscope. Di-I labeling was examined using epifluorescence microscopes, again at a variety of magnifications, and images were captured with digital cameras (Zeiss and Olympus). Photographs of the unstained tissue (Fig. 1) were taken using a Polaroid camera (MicroCam) fitted to a dissecting microscope (WPI), then the photo prints were digitized with a scanner (Color OneScanner, Apple Computer).

For the most part, we adopted the auditory forebrain divisions of the Franklin and Paxinos (1997) mouse atlas. These divisions are in general agreement with others (mouse: Caviness 1975; Willard and Ryugo 1983; Wree et al. 1983; rat: LeDoux et al. 1987; Winer et al. 1999) and our own anatomical studies in the mouse (Cruikshank et al. 2001).

RESULTS

As indicated in METHODS, when the mouse brain was cut along the near horizontal plane illustrated in Fig. 1, A and B, two distinct slice preparations could be obtained, each with unique physiological properties. The dorsal-most of these will be referred to as the “primary slice,” and the more ventral will be called the “shell slice” (Fig. 1). Initially, the two preparations were distinguished based on gross anatomical features and by relating these features to the effective stimulation loci and cortical responses profiles. For example, in the unstained primary slice, the MG can be seen directly, and the lateral geniculate (LG), hippocampus, striatum, and other structures have characteristic shapes (Fig. 1D). When the MG is stimulated in a living primary slice, it results in a strong middle layer response in auditory cortex (described in the following text). The shell slice, and the structures contained within it, also have characteristic shapes. For example, the hippocampus is wider in the medial-lateral direction, giving it a more rounded appearance (Fig. 1E). More importantly, the shell slice is located in a sufficiently ventral plane that it contains no obvious MG nucleus. Thus, in the shell slice, the region just medial to the hippocampus (indicated by an arrow in Fig. 1E) corresponds to the area ventral to the MG nucleus. Stimulation of this region consistently results in an upper layer current sink in temporal cortex (described in the following text), contrasting with the middle layer sink produced by MG stimulation in the primary slice.

Primary slice

ANATOMICAL FEATURES: PRIMARY SLICE CONTAINS MGV AND ACX. Figure 1C provides a lateral view of the mouse brain with part of the neocortex and hippocampus dissected away to facilitate comparison between the positions of the slices and the MG as a whole. The MG appears as a rounded protuberance posterior to the LG and ventral to the superior colliculus (SC). Notice that the primary slice contains the bottom 30–50% of the visible portion of the MG, which mostly consists of the core ventral division of the MG (MGv). Given that the slicing angle was chosen to obtain both the MGv and ACx within the same plane (Fig. 1A, right; see METHODS), it would seem logical that primary slices that contain the MGv would also contain primary ACx. These observed and inferred features of the primary slice, combined with its distinct middle layer response profile, are consistent with the known projection of the MGv to middle layers of primary auditory cortex (Caviness and Frost 1980; Romanski and LeDoux 1993; Willard and Ryugo 1983; reviewed in Winer 1992).

Further direct anatomical information was also determined...
histologically. After physiological recording and classification, the tissue was processed for Nissl staining or parvalbumin (PV) immunohistochemistry (see Methods), and attempts were made to determine the locations of the tops and bottoms of each slice with respect to thalamic and cortical boundaries. Prior to using the immunohistochemical methods on the near-horizontal slices required for the recordings, the PV pattern was first examined in the more conventional coronal plane to determine the relationship between regional boundaries and the PV pattern (n = 3). Adjacent sections processed for PV and Nissl were directly compared. This is illustrated in Fig. 2, A and B, for a P16 mouse brain. It is obvious that PV labeling is very dense in the MGv and substantially lighter in surrounding subdivisions. Especially noteworthy is the sharp boundary on the ventral border of the MGv. Figure 2B also shows a distinct cortical pattern; there is a relatively strong and laminated pattern of PV labeling in the primary auditory cortex, weaker labeling dorsally and ventrally in the adjacent nonprimary areas, and virtually no labeling more ventrally, in the cortex surrounding the rhinal fissure (Fig. 2B). A detailed description of the PV expression pattern within the auditory forebrain of the adult mouse has recently been presented (Cruikshank et al. 2001). The juvenile pattern is generally similar except there is less overall expression throughout most parts of the brain, and a greater contrast between primary and nonprimary areas that emerges from an earlier development of PV expression in the primary areas (del Rio et al. 1994; Frassoni et al. 1991; reviewed in Hof et al. 1999). The latter makes the PV pattern especially useful for localization in the recorded juvenile slices.

Figure 2C provides an example of a 600-μm-thick recorded primary slice that was resectioned at 50 μM and processed for PV immunohistochemistry. The arrangement of the sections in relation to the original recorded slice is as follows: section 1 was 100 μM from the top of the slice (dorsal side up in recording chamber), section 3 was the bottom section of the slice, and section 2 was 50 μM above section 3. The approximate planes of the sections are depicted by the white lines (labeled 1–3) in Fig. 2B. Note that there is an intensely immunopositive MGv in section 1 but no labeled MGv in section 3. Importantly, section 2, which was just 50 μM from section 3, clearly contains an immunopositive MGv. Thus the PV staining provides for precise localization of the ventral border of the MGv within the recorded slices. Another important feature is the relatively intense and laminated labeling in the auditory cortex for all three sections of Fig. 2C, indicating that this particular slice likely contained primary ACx throughout its thickness. In more ventral sections from other slices, the cortical PV labeling was both less intense and less laminated, presumably reflecting a position in the ventral belt region of auditory cortex, between the primary area and rhinal fissure (Fig. 2B).

Although the PV labeling provided greater precision than the Nissl staining, the overall findings using the two methods were consistent and are presented together (primary slices: n = 13 PV, n = 3 Nissl). The top of the primary slice always contained at least part of the MGv (16/16), and the bottom of the primary slice nearly always reached below the MGv (14/16). The bottom usually ended in the PPD region but sometimes went as low as the substantia nigra (4/16). In terms of the cortex, the tops of the primary slices were always well laminated (16/16) with clear cellular PV labeling (13/13), consistent with a location in primary auditory cortex. For the majority of cases, the cortical PV labeling became weaker and less laminated in the ventral sections (9/13), suggesting that the bottom of the slices might contain nonprimary ACx. Nonetheless, there was always some clear cellular labeling in the PV material even in the most ventral sections of the primary slice, indicating a location above the perirhinal area (Fig. 2B). The cortical findings from the three Nissl slices are consistent with this conclusion.

LAMINAR RESPONSE PROFILE OF PRIMARY SLICE IS DOMINATED BY MIDDLE LAYER CSD SINK. The dominant feature of the cortical response to MG stimulation in the primary slice is a robust negative field potential and associated CSD sink in layers 3–4, as illustrated in Fig. 3A. The example in Fig. 3A illustrates this and other typical features of the laminar response. In an initial procedure, the cortex was “mapped,” by moving the electrode horizontally within layer 4, until finding the anterior-posterior position with the largest fast negative field potential evoked by MG stimulation (i.e., the “focus” of the response). The laminar profile was then determined for the cortical column corresponding to that focus by recording extracellular responses in evenly spaced intervals. Figure 3A1 shows the field potentials and CSD traces resulting from that procedure, conducted in normal ACSF. Following a 100-μA MG stimulus, the largest negative field potential and CSD sink (upward deflection) were recorded at 450 μM from the pia surface (total cortical thickness was 1,275 μM; Fig. 3A). Besides the large middle layer sink, the slice also displayed clear supragranular and infragranular CSD sources (downward deflections) at 150–300 and 750 μM, respectively. In addition, there was a small sink on the cortical surface.

The gray-scale plot in Fig. 3A1 is a representation of the fast portion of the laminar CSD data. The areas under the “raw” CSD traces on the left were measured (initial 20 ms after stimulation), converted to gray values, then plotted at the appropriate cortical depths. Notice that the darkest values correspond with the largest sinks, and lightest values correspond with largest sources (scale in Fig. 3B). This example is replotted in Fig. 3B (arrow), along with 21 other gray-scale plots prepared in the same manner. Each represents the CSD profile from a single slice. They are arranged according to recording date and include all of the primary slices for which laminar analysis was carried out. The length of the bars are normalized to the total cortical thickness (mean thickness was 1,295 ± 12 μM; range = 1,205–1,400). Notice that the position of the major sinks are relatively consistent, with the centers falling between 24 and 50% of the distance from pia to white matter (mean = 38.8 ± 1.4%; Fig. 3B). The main sinks spanned one to three consecutive recording positions (mean = 2.0 ± 0.2 positions; spacing 125–150 μM), which is reflected in the different thicknesses of the dark bands (Fig. 3B).

Thus Fig. 3B shows that all 22 slices had a dominant sink in layers 3–4. More subtle features of the CSD results may not be apparent in the plot and will be briefly mentioned. First, for a number of slices there were clear secondary sinks located either on the cortical surface (6/22) or in infragranular layers (10/22) that were spatially separate from the main middle layer sink (mean infragranular sink location = 72.8 ± 2.0% of distance between pia and white matter). Second, nearly all slices (21/22) had both supragranular and infragranular sources, which generally abutted directly against the main
middle layer sink. Third, in 7/22 cases there was a deep infragranular source, clearly separate from the main infragranular source, located 84.8 ± 1.9% of distance from pia to white matter.

The mean onset latency for the main CSD sink was 4.1 ± 0.3 ms, whereas the mean infragranular sink latency was slightly shorter (3.9 ± 0.4 ms), while the mean supragranular latency was slightly longer (4.7 ± 0.4 ms). These latency differences are roughly proportional to the differences in their corresponding depths and might simply relate to the conduction time required for afferent inputs to reach them; infragranular was closest to the cortical border, supragranular was furthest, and granular was between. Thus the three sinks might all represent direct monosynaptic responses despite the supragranular latency being significantly longer than that of the main sink (P < 0.02, paired t-test). At any rate, the observation of the infragranular sink latency being no longer that the main sink latency (P = 0.93, paired t-test) indicates that the infragranular response is unlikely to be secondary to the main middle layer response.

**Primary Slice Responses Require Synaptic Transmission: Calcium Sensitivity.** Blocking synaptic transmission, by lowering Ca\(^{2+}\) concentrations in the ACSF (replaced with Mg\(^{2+}\); see Methods), profoundly suppressed the MG-evoked responses across all cortical laminae. Figure 3A2 illustrates this effect. After confirming the typical middle layer profile in normal ACSF (Fig. 3A1), low calcium ACSF was perfused over the slice for ~1 h (until reductions in layer 4 field potentials had become asymptotic), then the laminar profile was re-determined. Figure 3A2 shows that the MG-evoked field potential, and CSD traces are almost flat under low calcium conditions, with only extremely weak responses in the lower layers remaining (600–1,200 μM deep). Four other primary slices were tested in this way. All had a virtually complete (and reversible—in normal ACSF) blockade of middle layer responses during low calcium perfusion. For two of those slices, a short latency (3–4.5 ms) spike-like field potential remained (i.e., small and narrow) but only in the lower layers. These results suggest that the majority of the MG-evoked extracellular responses in the primary slice are synaptically mediated. The residual responses that sometimes remain in the lower layers likely result from direct activation of either afferent fibers or antidromically activated cells.

**Intracellular Recordings in Layers 3–4 of the Primary Slice Reveal Consistent Early Response and More Irregular Late Polysynaptic Response.** Given the predominant middle layer CSD sinks and the known projection to this same region from the MGv, it seemed likely that major monosynaptic thalamocortical responses in the primary slice occur at the foci of the fast sinks in the middle cortical layers. Thus as a first step in the intracellular investigation of the thalamocortical responses, it seemed reasonable to direct recordings near those sinks. To do this, the auditory cortex was first mapped with an extracellular electrode to find the “focus” of the MG-evoked response in layers 3–4 (as described for the laminar recordings), then the intracellular recordings were conducted at

![Laminar profiles and low-Ca\(^{2+}\) sensitivity of thalamocortical responses in the primary slice. A1: laminar field potentials and associated current source densities (CSDs) for example slice in normal artificial cerebrospinal fluid (ACSF). One-hundred-fifty-micrometer separation between recordings; numbers on left indicate depth of recording sites within ACx (gray background). “Pia” indicates surface of layer 1, “WM” indicates border between white matter and layer 6. MG stimulus intensity = 100 μA. Traces represent averages of 10 responses (gray-scale plot described in the following text). Notice large fast field potentials and CSD sinks around layers 3–4. Associated sources are immediately above and below. Also note small sink on surface; this was the smallest of the CSD responses classified as “surface sinks” (n = 6; see main text). A2: responses of same slice during perfusion of low-Ca\(^{2+}\) ACSF. Note the near absence of any evoked field potentials or CSDs. Responses recovered after return to normal ACSF (not shown). B: gray-scale CSD profiles of all primary slices, arranged by recording date. Areas under the raw traces were first measured (initial 20 ms after artifact), then gray-scale plots were generated using DeltaGraph (DeltaPoint). Values were normalized to the maximum sink for a given slice (max sink = +1), and plotted from +1 (white) to −1 (black), with 20 linear gray steps between (scale in B). Plot heights normalized to total cortical thickness. Some slices were not recorded to the white matter, so they don’t reach 100%. Note generally consistent sink positions (dark bands) in layers 3–4. For further explanation, see results.\(^{1}\)]
or near that focus (within ~250 μM). Sixty-six cells, located between 30 and 50% of the distance from the pia to white matter, were recorded from 24 primary slices. Mean values of passive membrane properties (±SE) were as follows: resting potential = \(-69.3 \pm 0.9\) mV, input resistance = \(46.8 \pm 2.1\) MΩ, spike threshold (current) = \(0.39 \pm 0.03\) nA, spike threshold (membrane potential) = \(-48.7 \pm 0.5\) mV, spike height = \(64.1 \pm 0.7\) mV, spike width (at half-amplitude) = \(0.87 \pm 0.03\) ms. Three cells had categorically narrower spikes (range: 0.28–0.44 ms) than the other 63 (range 0.64–2.21 ms). They also fired at high rates (±100 Hz for hundreds of milliseconds during depolarizing current injection) and had deep/fast after-hyperpolarizations (AHPs), consistent with the fast spiking cell type (Connors and Gutnick 1990; McCormick et al. 1985; Porter et al. 2001). The majority of cells with wider spikes displayed strong spike frequency adaptation and had more shallow/gradually developing AHPs, consistent with the regular spiking cell type (Connors and Gutnick 1990; McCormick et al. 1985; Porter et al. 2001). We observed no obvious differences in synaptic responses between cell types and will report the data together.

Intracellular responses to MG stimulation typically began with a short-latency depolarizing potential that will be referred to as the “early response.” This response was predominantly produced by glutamate-mediated excitatory postsynaptic potentials (EPSPs) but could also contain a fast inhibitory postsynaptic potential (IPSP; see following text). The onset latencies of the early responses averaged 4.0 \pm 0.1 ms (n = 58 cells with measurable latencies), which generally matched the simultaneously recorded layer 3–4 field potentials and mean CSD sink latencies (mean sink = 4.1 \pm 0.3 ms; P = 0.81, unpaired t-test, intracellular vs. sink onset latencies). These early intracellular responses usually peaked, or at least reached a point of inflection (described in the following text), within 20 ms of the stimulus. As stimulus intensities were adjusted from threshold to higher values (mean threshold = 39.3 \pm 5.5 μA, n = 42), initial slopes and peak amplitudes generally increased sharply at first, then often approached asymptote at higher intensities. Figure 4A illustrates a typical stimulus versus response function for an individual cell, and B plots the average amplitudes of the early responses across all cells, for a broad range of MG stimulus intensities (peak amplitudes measured within 20 ms of stimulation). Although the maximum group value was 4.0 \pm 0.5 mV, responses of some individual cells were larger (e.g., Fig. 4A), ranging \(\leq 9.5\) mV. When such cells were depolarized (with steady intracellular current) to a few millivolts below spike threshold, the MG-evoked early responses could usually trigger action potentials, indicating the presence of EPSPs; however, these early EPSPs did not generally elicit spikes from the resting potential.

Notice in Fig. 4A that at low stimulus intensities (12–25 μA), the responses had smooth rising and decay phases, consistent with those expected for monosynaptic EPSPs. In contrast, at the higher stimulus intensities, late depolarizing components (with irregular inflections and spiking) began to emerge (Fig. 4A). Unlike the early response, which generally had consistent onsets and shapes from trial to trial, the late components were not precisely time-locked to the stimulus and were not always present on every trial (see Fig. 4A, inset). However, when the late response components were present on a given trial in a layer 3–4 intracellular recording, they were also nearly always present in adjacent extracellular recordings (not shown), consistent with them being multineuron phenomena. These responses generally lasted for 300–900 ms but could sometimes continue over a second. In contrast, most of the isolated early responses decayed close to baseline levels within \(~ 150\) ms (Fig. 4A). Virtually identical late responses have been the focus of previous studies, and it was concluded that they reflect polysynaptic intracortical activity (Bai et al. 2000; Hsieh et al. 2000; Metherate and Cruikshank 1999; also see Agmon et al. 1996; Luhmann and Prince 1990 for similar responses in younger animals); further support for this conclusion is presented in the following text. These long duration, presumed polysynaptic responses, will hereafter be referred to as the “late responses.”

Consistent with a polysynaptic nature, the late responses were very sensitive to stimulus rate and were often weak, and/or had a low probability of occurring, with the 0.1- to 0.2-Hz stimulation rate routinely employed in the present study. This sensitivity is evident in Fig. 4, when comparing the response to 0.1- versus 0.033-Hz stimulation (Fig. 4A “200 μA” was \(\pm 0.1\) Hz, while Fig. 4C “control ACSF” was \(\pm 0.033\) Hz; all conditions except stimulus rate were identical). Notice how the slower rate (Fig. 4C) produced a much stronger average late response, which was largely a reflection of higher trial-to-trial probability; late responses occurred in 10/10 trials at the slow stimulus rate, but in only 3/10 trials at the fast rate (the Fig. 4A, inset, shows 2 of the late responses produced during fast stimulation). Frequency sensitivity was tested for seven cells and eight field potentials that expressed clear late responses at 0.05 Hz. It was found that 0.2-Hz stimulation strongly suppressed the late responses for 5/7 cells and 5/8 field potentials (nearly a complete blockade), and 1-Hz stimulation strongly suppressed the others. In contrast, mean amplitudes of early responses remained 80 and 60% of control values with 0.2- and 1-Hz stimulation, respectively.

To further test the nature of the early and late responses, we perfused slices with ACSF containing high concentrations of divalent cations (“high divalents”; see METHODS). High divalents have traditionally been used to selectively suppress polysynaptic activity by raising spike thresholds (e.g., Crepel and Ben-Ari 1996; Luhmann and Prince 1990; Sah and Nicoll 1991; reviewed in Berry and Pentreath 1976). The rationale is that as spike thresholds are raised, intercalated neurons located in a synaptic chain between the stimulus and recording sites will fail; consequently, synaptic responses recorded downstream from the intercalated cells (i.e., “polysynaptic responses”) would also fail. The example in Fig. 4C (top) shows an effect of high divalents on a layer 4 cell. Note how the high divalent ACSF suppresses the late portion of the response, leaving the early peak approximately at control levels. The bottom of Fig. 4C illustrates the simultaneously recorded layer 3–4 field potentials with similar results. The effects of high-divalent ACSF was tested on five field potential and two intracellular responses in primary slices. In all cases, the late responses were almost completely blocked. In contrast, most of the early responses were left at approximately control levels. Together with the effects of stimulus intensity and rate, these results suggest that the early responses were more “secure” than the late responses, consistent with fewer intercalated synapses at which failure could occur. The remainder of the primary slice data will focus on the early responses. For a more extensive
discussion of the late responses, see Metherate and Cruikshank (1999).

EARLY MG-EVOKED RESPONSE IN ACX CONTAINS FAST AMPA AND SLOWER NMDA EPSPS. Within the early response, there were multiple components that could be dissociated physiologically and pharmacologically. Figure 5A illustrates this for a cell in layer 4. The MG was stimulated at 0.1 Hz, which largely exhausted the late response discussed in the preceding text (not shown: note the difference in time scale relative to Fig. 4), and the remaining early response was recorded while the steady-state membrane potential of the neuron was varied with intracellular current injection. Because of unique voltage dependencies, the different subcomponents of the early response were most distinct at depolarized steady-state potentials (Fig. 5A, top). Following an MG stimulus in control solution (Fig. 5A1, top), there was an initial depolarization that peaked quickly (labeled fast EPSP), then a sharp negative deflection to below the baseline membrane potential (IPSP; discussed in the next 2 sections), and finally a second depolarizing peak (slow EPSP). Both the fast and slow EPSPs could trigger action potentials when the cell was “held” a few millivolts below threshold (shown for slow EPSP). The slow EPSP had clear nonconventional voltage dependence, being more prominent at depolarized potentials, suggesting the involvement of NMDA receptors.

To assess which glutamate receptors mediate the fast and slow thalamocortical EPSPs, the AMPA/KA receptor antagonist CNQX (20 μM) and the NMDA receptor antagonist APV (50 μM), were applied to the bath while recording MG-evoked responses. The cell in Fig. 5A illustrates the results. CNQX completely blocked the fast EPSP (and IPSP; discussed in the following text), leaving an isolated slow EPSP that exhibited clear nonconventional voltage dependence (larger responses at depolarized potentials) and was subsequently blocked by the addition of APV. The effects of APV reversed (not shown), but the cell was lost before recovery from CNQX. Figure 5B presents the effects of the antagonists on the simultaneously

FIG. 4. Early and late responses of cells in layers 3–4 of the primary slice. A: responses of example cell to increasing intensities (in μA: 12, 25, 50, 100, 200). Each trace is average of 8–10 responses. Interstimulus interval (ISI) = 10 s. Note increases in slopes and amplitudes with increasing intensities. The apparent differences in spike thresholds are artifacts of averaging. Inset: 4 individual responses for 200-μA stimulus (2 with early and late responses, 2 with early responses only). Note that failure of late response results in smooth decay phase. Spikes are truncated. B: average early response amplitudes as a function of intensity. The “n” represent the number of cells for each intensity. Note the graded increases in peak amplitudes with increasing intensities. C: effects of “high divalent ACSF” (see METHODS) on late responses. Top: intracellular (same cell as in A), bottom: simultaneous middle layer field potential. Stimulus intensity = 200 μA, ISI = 30 s. Average of 10 trials. Note that average control late response at this slower stimulus rate is larger than average in A. Intracellular and extracellular late responses are strongly suppressed by high divalent ACSF, but the early response peaks are similar to control amplitudes. Explained further in RESULTS.
recorded layer 4 field potentials, with similar results, but including a nearly complete recovery in normal ACSF. The effects of CNQX and APV were tested on nine cells and nine simultaneous field potentials, and the results are presented in the Fig. 5C. Together with the time course and voltage-dependence information, these pharmacological data indicate that the fast EPSP is mediated by AMPA and/or KA receptors while the slow EPSP is mediated by NMDA receptors.

**NATURE OF THE FAST IPSP.** The cell in Fig. 5 expressed a clear IPSP in control solution. For example, while both the fast and slow EPSPs could trigger spikes, there were never action potentials during the IPSP, even when strong depolarizing current was injected into the cell to produce “spontaneous” action potentials (not shown). The IPSP had distinct voltage dependence, being more prominent at depolarized steady-state membrane potentials, and reversing at approximately \(-62\) mV.

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**Intracellular**

A 1. Control

-68 mV

2. CNQX

3. CNQX + APV

**Field Potentials**

B 1. Control

2. CNQX

3. CNQX + APV

4. Recovery

**Group**

C 1. Intracellular Fast EPSP

2. Intracellular Slow EPSP

3. Field Potentials (Fast & Slow)
MG-evoked response differs from response produced by conventional columnar (on-beam) stimulation. Aside from recent studies with intact thalamocortical slices (see introduction), in vitro studies have generally employed stimulation of layer 6, or the white matter just below layer 6, in an attempt to activate thalamocortical axons projecting to cells recorded in the cortical column above (discussed in Agmon and Connors 1991; Kenan-Vakin and Teyler 1994; Kirkwood and Bear 1994). This conventional white matter/layer 6 stimulation will be referred to as “on-beam” stimulation. Here we compared responses in primary slices evoked by MG and on-beam stimulation and found clear differences between them relating to IPSP strengths and CSD distributions. These two results will each be described in turn.

Although IPSPs could clearly be evoked using MG stimulation, they appeared to be relatively weak compared with previous studies of auditory cortex employing on-beam stimuli (e.g., Buonomano and Merzenich 1998; Metherate and Ashe 1994). To test whether this was related to focus of stimulation or some other variable, fast IPSPs evoked by on-beam versus MG stimulation were directly compared within individual cells. For 10/12 such cells, the IPSPs evoked by the on-beam stimuli were strongest; in the remaining two cells, the MG- and on-beam-evoked IPSPs were not clearly different.

A potential problem with the above comparison is that the apparent “excitatory drive” was not always equal for the two pathways. In fact, the maximum drive for each cell, as estimated by EPSP amplitudes and slopes, was usually largest for the on-beam stimulus. This is important because the thalamocortical IPSPs are presumed to be polysynaptic phenomena, so the strength of IPSPs are expected to depend (at least somewhat) on the strength of excitatory input from the thalamus. To control for this potential problem, we separately compared IPSPs for the seven cells in which EPSPs evoked by MG stimulation were equal to, or stronger than, on-beam EPSPs (for at least a subset of stimulus intensities). Of these, 5/7 cells still had clearly stronger IPSPs for on-beam stimulation; the other 2 cells showed no clear difference between pathways. Example responses from a cell with stronger on-beam IPSPs are presented in Fig. 6A. When recorded at the resting potential (−71 to −73 mV), the IPSPs were positive going and added with the EPSPs to produce a net depolarizing PSP. Notice that the initial slopes were approximately equal, and in this case,

FIG. 5. Differential effects of glutamate receptor antagonists on the 3 components of the primary slice early response: fast excitatory postsynaptic potential (EPSP), fast inhibitory PSP (IPSP), slow EPSP. A1: example middle layer cell showing 3 components of early response. Resting potential = −68 mV. Steady-state potentials adjusted by injecting intracellular current (in nA, from bottom trace to top: −0.2, 0, 0.2, 0.4). MG stimulus time indicated by arrow. Stimulus intensity = 150 µA. Response components are labeled at most depolarized steady-state level. Traces at 3 most hyperpolarized steady-state potentials are averages of 7–10 responses. Individual responses presented for depolarized potential to illustrate trials with and without action potentials (AP) initiated by slow EPSP. For other trials, the fast EPSP could induce a spike. Scale bars apply to all intracellular traces (A, I–3). A2: 20 µM 6-cyano-7-nitroquinolxaline-2,3-dione (CNQX) blocked the fast EPSP and fast IPSP, leaving a slow EPSP with nonconventional voltage dependence. A3: the combination of 20 µM CNQX + 50 µM dl-2-amino-5-phosphonopentanoic acid (APV) blocked all the MG evoked responses for the cell (the slow EPSP recovered after removal of APV, not shown). B: field potential from same slice as in A (simultaneous, layer 3–4). CNQX blocked the large fast potential, leaving a smaller slow potential (B2). Addition of APV (CNQX + APV) blocked the remaining slow potential (B3), which recovered on subsequent removal of APV (B4, CNQX). A large portion of the fast potential recovered during 2.5-h rinse in normal ACSF (B4, ACSF). C: group effects of glutamate antagonists on fast EPSP (1), slow EPSP (2), and fast and slow field potentials (3; 9 cells, 9 field potentials). Fast EPSPs and field potentials were measured at latency corresponding to peak (initial 20 ms) in control conditions. Slow EPSPs and field potentials were measured at latency corresponding to peak of response during CNQX (at depolarized steady-state potentials for intracellular; the most depolarized value that was still sub-threshold for spiking was used). While the fast EPSP was nearly abolished by CNQX, the slow EPSP was only moderately suppressed; the latter effect was weaker for depolarized steady-state potentials than at resting potentials. Addition of APV reversibly blocked the slow EPSP (completely) even at depolarized potentials. The fast and slow group field potentials had similar effects as the corresponding EPSPs plus partial recovery in normal ACSF.

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AUDITORY THALAMOCORTICAL SYNAPTIC TRANSMISSION IN VITRO

This data, together with the fast time-course, suggest a GABA<sub>A</sub> receptor-mediated mechanism (Avoli 1986; Connors et al. 1988; Cox et al. 1992; Hefti and Smith 2000). Note that the IPSP was blocked during CNQX application, consistent with the interpretation that glutamatergic synapses drive the interneurons responsible for the IPSP (see discussion).

To characterize the incidence of fast IPSPs across cells, responses to MG stimulation were measured at resting potentials and one or more other steady-state potentials (always including a depolarized value near spike threshold) to record on both sides of the GABA<sub>A</sub> IPSP reversal potential. Of the cells tested this way, 15/20 displayed IPSPs (always following initial EPSPs). For eight of these, the IPSPs were fast (onsets <11 ms) and fairly robust, such that the negative going PSPs, recorded at depolarized potentials, reached below the depolarized steady-state levels (included among these is the example cell in Fig. 5A1). For six cells, the IPSPs were fast but weaker, indicated by deflections that did not reach below the steady-state levels. These weaker IPSPs either cut off the EPSPs, causing them to be narrower when recorded at depolarized than at hyperpolarized steady-state potentials, or they created notches in the EPSPs when depolarized (the latter is illustrated in Fig. 6A: also see text in MG-evoked response differs from response produced by conventional columnar (on-beam) stimulation). In addition to the 14 cells with fast IPSPs, one cell exhibited a robust IPSP with a longer latency (onset = 17 ms; but still a reversal potential of −64 mV, consistent with GABA<sub>A</sub>), and 5 cells displayed no apparent IPSPs. Minimum fast IPSP latencies range from 4.8 to 10.8 ms (mean = 6.8 ± 0.4 ms). The latencies between the onset of the EPSPs and IPSPs for these responses ranged from 1.4 to 6.2 ms (mean = 2.8 ± 0.3 ms). In addition to the fast IPSPs, we occasionally observed slower hyperpolarizing potentials with reversal potentials more negative than rest. These are likely to be GABA<sub>B</sub> receptor-mediated IPSPs. Their amplitudes were very small, and they may have been partly masked by overlapping slow EPSPs and/or late polysynaptic responses, so they were not systematically examined here. However, these slow IPSPs may be more clearly illuminated in future studies with specific pharmacological and physiological manipulations (Connors et al. 1988; Metherate and Ashe 1994).
The MG-evoked depolarization was actually larger than the on-beam response. Despite the apparently equal or greater synaptic drive, when the cell was depolarized with intracellular current injection (to about \(-48\) mV with +1.0 nA), the MG stimulus evoked a weaker IPSP than the on-beam stimulus. While the large on-beam IPSP hyperpolarized the cell several millivolts below the steady-state membrane potential, the MG-evoked IPSP was weak, only cutting a small notch in the EPSP (\(-1\) mV deflection), and never reaching down to the steady-state potential.

The IPSP differences indicate that on-beam and MG stimuli evoke middle layer responses with different excitatory-inhibitory balances, suggesting that they activate different intracortical cell groups. To examine this on a larger spatial scale, we compared the laminar profiles produced by MG versus on-beam stimulation within six primary slices (Fig. 6B). Stimulus intensities at the two sites were adjusted in an attempt to equalize their respective layer 4 field responses, and the on-beam and MG stimuli were interleaved.

The thalamocortical profiles of the six slices were among those presented in Fig. 3, therefore the MG-evoked CSDs were all fairly typical, with largest sinks in the middle layers (30–50% of distance between pia and white matter). In contrast, the laminar profiles evoked by on-beam stimulation were more variable from slice to slice, with maximum sinks at a variety of positions, including supragranular and infragranular layers. The cortical depths of the largest on-beam sinks, expressed as a percentage of the distance from the pia to white matter, were: 18, 20, 30, 44, and 70%. The sixth slice had no clear cortical sink produced by the on-beam stimulus. In 5/6 slices, the positions of the largest MG and on-beam evoked sinks mismatched by a minimum of two recording positions (\(\geq 250\) \(\mu\)M; e.g., Fig. 6B). The differences in laminar profiles between MG and on-beam stimuli further indicate that the two stimulation sites do not generally produce the same patterns of cortical activation.

**TRACT TRACING IN THE PRIMARY SLICE: ANATOMICAL CONNECTIONS BETWEEN THE MG V AND ACX.** To anatomically investigate the thalamocortical pathway in the primary slice, we performed tract tracing studies using the lipophilic dye Di-I. Following physiological recording, slices were fixed, then a particle of Di-I (\(\sim 50\) \(\mu\)M diam) was placed either in the MG near the stimulation site (\(n = 8\) slices) or in the ACx near the focus of the middle layer response (\(n = 5\) slices). The dye was allowed to diffuse along the pathway(s) for 1–2 mo (30–38°C), then the slices were re-sectioned into 50-\(\mu\)M-thick segments and imaged using conventional epifluorescence microscopy (see METHODS).

Figure 7A shows the results for a slice in which Di-I was placed in the MG. The two major directions of dye movement were toward the tectum (posterior, then out of the slice plane) and toward the auditory cortex (Fig. 7A1; described in the following text), consistent with the known connections of the MG (Bartlett and Smith 1999; Willard and Ryugo 1983; Winer 1992). Figure 7A1 shows the intensely labeled thalamocortical pathway projecting initially anterior-lateral along the medial edge of the LG (at this point the pathway is part of the superior thalamic radiation; for orientation see Figs. 1D and 2C), then curving posterior-lateral around the hippocampus, toward the cortex. At the approximate position where the pathway crosses the reticular thalamic nucleus, there was an increase in labeling density, possibly involving axons connecting the MG and reticular thalamic nucleus (Fig. 7A1). On reaching the auditory cortical border, the fibers turned abruptly, ascending to layers...
3–4, where they formed a dense terminal plexus (Figs. 7A, 2 and 3). There was also an intense zone of labeling in the lower cortical layers (Fig. 7A2), which included not only axons but also pyramidal somata and dendrites (not shown, but clear in other sections from same slice). Finally, a few axons reached layer 1. Some of these can be seen in A3 (note: the bright labeling on the cortical surface is an artifact, produced by reflectance off loose tissue).

Of the eight primary slices with Di-I applications in the MG, six had terminal-like zones of intense labeling in layers 3–4 of auditory cortex; the remaining two slices had labeled axons in layers 3–4, but they were sparse, and did not form the type of “plexus” seen in Fig. 7A. The infragranular labeling included four slices with many pyramidal cells and dense processes, two slices with a few pyramidal cells and sparse processes, and two slices with no labeled somata (but weakly labeled processes). When present, the pyramidal somata were mostly in layer 6 (75% of the distance from pia to white matter) or around the layer 5/6 border. Finally, in addition to the example, one other slice had clear axons in layer 1. The density of this labeling was considerably higher than in the example and included many axons oriented parallel with the cortical surface.

Of the five primary slices with Di-I particles placed in the ACx, 4/5 had retrograde labeling of cell bodies in the MG.
Figure 7B presents an example. Notice that pathway appears to be interrupted (Fig. 7B1); the middle region of the pathway is actually labeled in more ventral sections of the slice (not shown). This was a common feature and indicates that the thalamocortical pathway is curved in the dorsal-ventral plane. It is most ventral in the middle region, near the reticular thalamic nucleus, and curves dorsally toward both extremities (near the MG and ACx; see Fig. 2C for orientation). Consistent with this, labeled cell bodies in MG were always located near the dorsal surface of the slice. It appears that the ventral dip in the middle of the pathway is necessary so that the thalamocortical fibers can pass under the fimbria; this can be best appreciated by examining the structures present at different dorsal-ventral levels in the parvalbumin material (Fig. 2C).

Taken together, the anterograde and retrograde labeling indicates that the primary slice contains anatomically connected axonal pathways linking the MG and ACx. Furthermore the projections from the MG seem to end predominantly in cortical layers 3–4, although axons are also usually present in the lower layers, and occasionally in layer 1. These three projections could contribute, respectively, to the middle layer, lower layer, and surface CSD sinks characterized previously (laminar response profile of primary slice is dominated by middle layer CSD sink, Fig. 3).

Shell slice
ANATOMICAL FEATURES OF THE SHELL SLICE. The shell slice was located ventral to the primary slice (Fig. 1). Gross anatomical observations (e.g., Fig. 1, C and E) and knowledge of the ventral boundaries of the primary slice (see preceding text) indicate that the thalamic portions of the shell slice are located below the MG proper. This region contains nonprimary or “shell” auditory thalamic subdivisions (e.g., the PPD and PIN; Figs. 1A and 2A) and more ventral nonauditory structures (e.g., substantia nigra, cerebral peduncle; Figs. 1A and 2A). Similar observations and reasoning suggest that the shell slice may contain portions of the “belt” auditory cortical area, ventral to primary ACx.

Direct histological information was also obtained following recordings from shell slices (Nissl stain: n = 17; PV-immunohistochemistry: n = 1). For 12/18 shell slices in which thalamic regions were examined, the top 50–100 μM appeared to be within the PPD; for the remaining 6/18, the tops were located more ventrally, in the cerebral peduncle or substantia nigra (for orientation, see Fig. 2, A and B). For 5/12 shell slices in which cortices were examined (6 removed because of low-quality histology), the temporal cortices were well laminated on the top 50–100 μM. For the other 7/12, lamination was poor (even in the most dorsal sections), indicating localization ventral to primary ACx. The bottoms of the shell slices always appeared to be located below the PPD and primary ACx.

LAMINAR RESPONSE PROFILE OF SHELL SLICE IS DOMINATED BY A SURFACE CSD SINK. The dominant evoked response in the shell slice, to stimulation of the region ventral to the MG (indicated by arrow in Fig. 1E; hereafter referred to as the “shell region”), was a strong CSD sink on the surface of cortical layer 1 (Fig. 8). The field potential at the position of the sink was predominantly negative in polarity and had a similar time course as the sink. In contrast, the field potentials in the middle and lower layers were nearly always biphasic with a fast negative phase that preceded the surface sink and a slower positive phase having a similar latency as the surface sink. The example in Fig. 8A illustrates these and other characteristics of the shell slice laminar response (conventions and methodology as in primary slice experiments: Fig. 3). In
addition to the surface sink, there was also a CSD source immediately below the sink (150 μM depth; Fig. 8A1). The gray-scale representation of this CSD profile is drawn to the right of traces and in the group plot (Fig. 8B).

The CSD profiles of all 13 shell slices that underwent laminar analysis are presented in Fig. 8B. Notice that each had a major sink on the cortical surface, which in 12/13 cases was the largest for the slice (Fig. 8B). The surface sinks were narrow, never spanning more than one recording position (spacing: 125–150 μM). Also apparent in the gray-scale plots are several infragranular CSD sinks. These “secondary” sinks, observed in 8/13 slices, had an average location of 58.1 ± 2.8% of the distance from the pia to white matter. In addition to sinks, all 13 slices expressed supragranular CSD sources (sometimes difficult to see in gray scale, but clear in “raw” traces; e.g., Fig. 8A1). In most cases, these sources were present at the recording site immediately below the surface sink (11/13). Finally, for four slices, there were clear infragranular CSD sources centered 68.1 ± 4.8% of the distance from the pia to white matter.

The mean onset latency for the shell slice surface sink was 6.0 ± 0.2 ms, while the mean infragranular sink latency was 4.4 ± 0.4 ms (P < 0.02, paired t-test), suggesting that the infragranular sink is not a secondary phenomenon that follows the main surface sink.

SURFACE CSD SINK IN THE SHELL SLICE REQUIRES SYNAPTIC TRANSMISSION. Blocking synaptic transmission, by lowering extracellular Ca2+ concentrations, strongly suppressed responses evoked by shell region stimulation (Fig. 8A2). Across slices (n = 5), all major sinks and sources were essentially eliminated. The only consistent CSD feature still present in low-Ca2+ ACSF was a small source on the cortical surface (Fig. 8A2, ↑); while this source could sometimes be seen even in control conditions, its duration became longer in low-Ca2+ ACSF, probably due to the removal of the large overlapping sink (Fig. 8A).

Consistent with the loss of CSD sinks, the negative field potentials on the surface of the slice were largely absent in low-calcium ACSF. Likewise, the slower positive field potentials in the middle and lower layers were also eliminated, or nearly so. However, reasonably large fractions of the faster negative field potentials in the middle/lower layers remained (≥50%, 5/5 slices; Fig. 8A2). Furthermore, the durations of these negative potentials generally increased (4/5 slices). Despite the increased durations, these potentials produced no appreciable middle or deep layer CSD sinks (1 of the largest sinks is observed in the example, at 300 μM).

The effects of low Ca2+ indicate that the major CSD sinks and sources in the shell slice, including the dominant surface sink (and associated field potentials), represent synaptically mediated responses. In contrast, at least a portion of the faster middle/lower layer negative field responses are apparently nonsynaptic. It is noteworthy that the latter are unevenly distributed through the middle/lower laminae, being largest in layers 5 and 4 (e.g., Fig. 8A2), suggesting direct activation of a specific cell group in that region.

In addition to calcium manipulations, for two shell slices, CNQX (20 μM) was added to the bath (normal Ca2+) to test for the involvement of AMPA/KA receptors in the synaptic responses. In both cases, all major sinks and sources were profoundly suppressed. This left a modest surface source (similar to low Ca2+) and a very small late sink. In one slice, the NMDA receptor antagonist APV (50 μM) was subsequently added, and this blocked the residual late sink (but not the source). While limited, these antagonist data suggest the involvement of glutamate receptors in the shell slice synaptic responses.

INTRACELLULAR RECORDINGS IN SHELL SLICE REVEAL EARLY AND LATE RESPONSES: EARLY RESPONSE TIME COURSE SIMILAR TO SURFACE CSD SINK. Intracellular recordings were made from nine cells in three shell slices; eight of these cells were located in layers 3–4, and one cell was recorded from approximately layer 6. Mean values of passive membrane properties were as follows: resting potential = −65.0 ± 2.3 (SE) mV, input resistance = 44.1 ± 5.7 MΩ, spike threshold (current) = 0.31 ± 0.04 nA, spike threshold (membrane potential) = −47.3 ± 0.9 mV, spike height = 60.3 ± 1.1 mV, spike width (at half-amplitude) = 1.04 ± 0.05 ms. The range of spike widths was 0.82–1.36 ms, and they all exhibited strong spike frequency adaptation, consistent with the regular spiking cell type (Connors and Gutnick 1990).

Intracellular responses evoked by stimulation of the shell region (arrow in Fig. 1E) generally consisted of an early depolarizing response (probable EPSP) that peaked within 20 ms of the stimulus, followed by a deflection in the hyperpolarizing direction, and finally a large depolarizing late response. These features are illustrated in Fig. 9, A and B, for two cells recorded in layers 3–4; also shown are the paired field potentials, recorded from the surface of layer 1.

The early intracellular responses of the shell slice differed from those of the primary slice in both amplitude and latency. The shell slice responses were typically smaller, which can be seen by comparing the mean amplitudes in Figs. 9C versus 4B. For example, the average amplitudes to 100 μA stimulation were 3.5 ± 0.4 mV for the primary slice and only 1.1 ± 0.3 mV for the shell slice (P < 0.03, unpaired t-test). The latencies of the early intracellular responses in the shell slice tended to be longer than those of the primary slice (mean onsets: primary = 4.0 ± 0.1 ms, shell = 6.2 ± 0.5 ms; P < 0.0001, unpaired t-test) and matched the latencies of field potentials in layer 1 rather than those in layers 3–4 (Fig. 9, A and B; also see LAMINAR RESPONSE PROFILE OF SHELL SLICE IS DOMINATED BY A SURFACE CSD SINK and Fig. 8). The shell intracellular latencies also closely matched the surface CSD sinks (mean onsets: surface sink = 6.0 ± 0.2 ms, layer 3–4 intracellular = 6.2 ± 0.5 ms; P = 0.68, unpaired t-test). Together, these data suggest that the early intracellular PSPs in the shell slice might be produced by activation of synapses in layer 1, perhaps onto apical dendrites of the recorded cells.

The late responses of the shell slices were generally similar to those of the primary slices, and to those in previous studies (Bai et al. 2000; Hsieh et al. 2000; Metherate and Cruikshank 1999). For example, they contained irregular fluctuations that varied from trial to trial. They also had similar laminar profiles, with negative polarity field potentials in the deep and middle layers and positive polarity in the upper layers (Figs. 4C, 8A1, 9, A and B; upper layers not shown for primary). Despite the similarities, late responses tended to be easier to elicit in the shell slice. Although not formally quantified, it was qualitatively apparent that these responses could be maintained at.
early responses, even at relatively high stimulus intensities (e.g., 4/5 cells @ 200 μA).

For six middle layer intracellular recordings, the responses were examined for the presence of IPSPs (see NATURE OF THE FAST IPSP for procedures and definitions). For two of these cells, clear fast IPSPs were observed at the latency of the inflection that separated the early and late responses. Figure 9A shows the response of one of these cells at the resting potential. The response of a third cell appeared to have a weak IPSP at a similar position; it never caused a true hyperpolarizing response but did appear to cutoff the EPSP, making it narrower when recorded at depolarized than at hyperpolarized steady-state potentials (Fig. 9B shows the response of this cell at the resting potential). The fourth cell had a clear slow IPSP (onset ~150-ms poststimulus) but no clear fast IPSP. For the last two cells, no IPSPs were observed.

Besides the middle layer cells, one deep layer cell was recorded. With low-intensity stimulation (15 μA), the response was comparable to the other cells; there was an early response with a fast peak (9 ms), then a downward deflection clearly caused by a fast IPSP, and finally a weak late response. At higher intensities (≥25 μA), there was a short latency evoked spike that appeared to be produced antidromically: it had no apparent preceding EPSP, virtually no latency jitter, and when the cell was hyperpolarized with intracellular current, a small initial-segment spike could be observed in place of the full-blown action potential.

TRACT TRACING IN THE SHELL SLICE: TRANSPORT OF D I-FROM STIMULATION SITE LABELS FIBERS IN LAYER 1 AND PYRAMIDAL CELLS IN LAYER 5. Tracing studies were conducted in shell slices using the same methods as in the primary slices (see TRACT TRACING IN THE PRIMARY SLICE; ANATOMICAL CONNECTIONS BETWEEN THE MG,v AND ACA). Di-I was placed either near the site of stimulation in the shell region (n = 5) or near the focus of the recordings in the temporal cortex (n = 3). Figure 10A illustrates the results for a slice with application near the stimulation site (asterisk). Panel 1 shows that the main route of dye movement was anterior-lateral, initially along the edge adjacent to the hippocampus, then across the striatum to the temporal cortex (Fig. 10A1). There was also clear posterior-going Di-I flow that eventually curved medially before leaving the slice plane (Fig. 10A1). Panel 2 focuses on the pattern of cortical labeling (same slice, different section). The most obvious feature was a dense band of pyramidal cells and processes in layer 5 (shown at higher magnification in 3). A more subtle aspect of the cortical labeling was the dense network of processes in layer 1. They are most readily seen at high magnification (4–6). Panel 4 shows a concentration of these fine processes in the most superficial aspects of layer 1. Panels 5 and 6 focus on individual superficial axons; notice that many other large and small caliber processes can also be seen, mostly behind the focal plane of the images. These included both spiny dendrites and axons with beady varicosities (not shown).

Of the five slices with Di-I application at the shell stimulation site, all of them had processes in superficial layer 1, including discernable axons with varicosities. For 4/5, these processes were at least as dense as the example in Fig. 10A, but for 1/5, they were sparse. In addition, all shell slices displayed robust labeling in layer 5, including pyramidal somata, dendrites, and axons. This labeling was more intense and more
dominant (relative to other layers) than the analogous infra-granular labeling in the primary slice. It should also be noted that although there were apical dendrites and ascending axons passing through all cortical layers, there was no zone of intense terminal-like labeling in the middle layers as commonly seen in the primary slices. In fact, across the five shell slices studied, the middle layers appeared to be the region with lowest density of labeled processes.

Of the three shell slices with Di-I particles placed in the ACx, only one had retrograde labeling of cell bodies in the thalamus (likely in the PPD). In addition, for six pilot experiments (in which Di-I was first dissolved into alcohol, then injected into the cortex), only a few small cells in two slices were ever retrogradely labeled in the shell area. Furthermore, the path of labeling did not appear to lead to a nucleus. Instead it labeled a narrow fiber pathway that passed through the stimulation area and continued past it, along the lateral edge of the thalamus/midbrain (Fig. 10B). This narrow labeled region correlated well with the narrow region for effective stimulation (cf. Fig. 10A1, asterisk); more internal/medial positions were generally ineffective. Based on histological observations (see preceding text), the labeled pathway appears to correspond to the cerebral peduncle. The strong labeling of this narrow fiber pathway through the shell stimulation site, but a low incidence of back-filled thalamic cells, suggests that the cells in the “shell” thalamic nuclei may not be the major source of input to...
the cortex in the shell slice. The next section addresses this possibility.

**Layer 5 Cells Project Through the Shell Stimulation Region to the Inferior Colliculus and Appear to Have Collateral Projections to Layer 1.** One possible source of input to layers 1 and 5 of the shell slice that does not involve direct projections from the thalamus could be axon collaterals emanating from pyramidal cells in layer 5. Given the dense retrograde labeling of layer 5 cells following Di-I application in the shell area, it is clear their axons pass through the effective stimulation sites. Thus it is possible that these cells could be antidromically activated by the shell stimuli. If they also have collaterals projecting to layers 1 and 5, such antidromic activation could subsequently produce the observed synaptic sinks in those layers.

The preceding scenario requires that the axons labeled in layers 1 and 5 (following Di-I application in the shell region) were actually collaterals of the layer 5 cells that were “filled” in a secondary manner; first the dye would diffuse backward along the main axon to the cell bodies or collateral branching points, then forward to the ends of the collaterals. This scenario would also predict a similar pattern of labeling (i.e., somata in layer 5, axons in layers 1 and 5) if Di-I could be placed at the termination of the main axons, wherever that might be. Because the back-filled layer 5 cells in the shell tracing experiments appeared to be at the depth of cortico-tectal cells, we hypothesized that they might ultimately project to the tectum and that the effective stimulation site in the shell slice was actually part of the cortico-tectal path. To test this hypothesis, we placed particles of Di-I (50–100 μM diam) bilaterally in the inferior colliculus of five whole fixed brains, allowed 2–4 mo for diffusion (30–35°C), then sectioned them at 50 μM (horizontal plane) and compared the patterns to those found previously in the shell slice.

The results are illustrated in Fig. 11. **Panel A** shows a section at a level similar to the shell slice examples in Fig. 10. **A1** and **B**. Notice the strong labeling of the pathway through the area typically effective for stimulation in the shell slices (arrow). The pattern of temporal cortical labeling is shown in **B**, where there are clearly filled pyramidal somata in layer 5, at approximately the same depth as in the shell slice (Fig. 10A2). Some of these cells, and cortical layers 1–4 above them, are shown at higher magnification in **C**. Besides somata, many labeled processes can be seen in layer 5; higher magnification inspection revealed that some had beady varicosities and were probably axons (not shown). In addition, some processes project toward the upper layers, and one can be seen reaching superficial layer 1. Part of layers 1–2 for this section (outlined in **C**) is shown at still higher magnification in the **F**. In addition to the end of the large process mentioned in the preceding text, several other axons can be seen ascending into layer 1. Furthermore a matrix of small caliber, lightly fluorescenting processes can be seen in the very superficial parts of layer 1. **D** and **E** illustrate upper layer 1 axons more clearly (these sections were adjacent to **F**).

Each of the five brains tested exhibited clear labeling of the pathway through the shell area, and many layer 5 pyramidal cells in temporal cortex. Furthermore, 4/5 brains had clear axons in layers 1 and 5. These data confirm the hypothesis that axons belonging to layer 5 cortico-tectal cells project through the shell stimulation region on their way to the inferior colliculus. Further, the pattern of intracortical axonal labeling is consistent with the hypothesis that the same layer 5 cells with axons passing through the shell region also have collaterals to layers 1 and 5. Thus it is possible that antidromic activation of these cells could contribute to the synaptic CSD sinks observed in the shell slice, via contacts made by their axon collaterals.

**Discussion**

The major finding of this study is that an in vitro preparation can be obtained from the mouse brain that contains functionally intact thalamocortical connections from the MGV to the ACX. Stimulation of the MGV in this preparation (i.e., the primary slice) activates not only thalamocortical synapses but also appears capable of initiating polysynaptic activity, indicating the presence of at least partially intact intracortical connectivity. A second major finding is that auditory thalamocortical transmission involves both AMPA and NMDA types of glutamate receptors. Third, this paper presents (for the first time, to our knowledge) a detailed description of laminar CSD profiles in ACX evoked by MG stimulation. Fourth, we observed that these MG-evoked profiles, and the intracellular responses in layer 4, differ from those produced by conventional columnar stimulation, indicating that columnar stimulation may not be a reliable means of selectively activating thalamocortical inputs. Finally, a second in vitro preparation (the shell slice) is presented in which subcortical stimulation appears to selectively activate synapses in superficial layer 1 of temporal cortex. This observation is consistent with known projections of nonprimary auditory thalamic nuclei near the stimulation site, suggesting that the preparation may provide a tool for investigating mechanisms of a nonprimary system; however, alternative interpretations are also presented. Each of these findings will be elaborated in the following text.

*Primary slice includes functionally intact thalamocortical connections from the MGV to ACX: histology, tract tracing, and laminar physiology*

**Major Connection Is from MGV to Middle Layers of ACX.** Gross anatomical observations, Nissl stains, and PV-immunohistochemistry all indicated that parts of the MGV and primary ACX were contained in the primary slice. Consistent with this, the Di-I tracing experiments in the primary slice showed major projections from the MG to cortical layers 3–4, the principal target zone of MGV axons (Caviness and Frost 1980; Romanski and LeDoux 1993; Willard and Ryugo 1983; reviewed in Winer 1992). Furthermore, stimulation of the MG-evoked responses in auditory cortex that were dominated by fast CSD sinks in layers 3–4. Together these data strongly support the hypothesis that the primary slice contains functional projections from MGV to middle layers of primary ACX, and that activation of these projections was the predominant cause of the observed responses. Additional support comes from the observation that similar middle layer responses could also be obtained from stimulation of the superior thalamic radiation (Fig. 2C) or “downstream” parts of the thalamocortical pathway (e.g., in the striatum; Fig. 7A1), but could not be evoked a mere 100–200 μM outside of the pathway (e.g., in the hippocampus, LG, or ventral-basal nucleus) even when these
Ineffective regions were closer to the cortex than the effective sites. Thus nonspecific activation, or spread of stimulation current to the cortex itself, is highly unlikely (Agmon and Connors 1991). It should be made clear that stimulation outside the MG (in primary slice experiments) was limited to preliminary characterization of the slices before formal data collection; responses described in RESULTS were exclusively evoked by stimulation of the MG proper or the border between the MG and superior thalamic radiation but never further downstream (see Metherate and Cruikshank 1999 for effective loci along the pathway).

SECONDARY INPUTS TO LAYERS 1 AND 5–6. In addition to the middle layer CSD sinks, the primary slices also sometimes had fast secondary sinks in the deep layers and/or at the surface of layer 1. Based on onset latency comparisons with the main sink, it was suggested in RESULTS that these secondary sinks might represent monosynaptic responses to direct thalamic inputs (LAMINAR RESPONSE PROFILE OF PRIMARY SLICE IS DOMINATED BY MIDDLE LAYER CSD SINK). Consistent with such direct inputs, Di-I application in the MG often resulted in labeled axons in layers 5–6 and occasionally in layer 1 (in addition to the middle layers—discussed in the preceding text). Cells in the MGv could be the origin of some of these secondary inputs. For example, MGv projections are known to branch in the infragranular cortical layers around the junction between layers 5 and 6 (Romanski and LeDoux 1993; Willard and Ryugo 1993).
synaptic contributions. In fact, virtually no responses remained in all layers, indicating little antidromic or other non-
stimulations designed to suppress polysynaptic activity; all of them depressed the late responses much more than the early responses, consistent with the late responses being predominantly polysynaptic intracortical phenomenon, and the early responses depending on fewer synaptic links. Exactly how many links are involved in the early response remains to be determined. The correlation between the onsets of the early PSP and the fast sink (and field potential) combined with the observed and known projections of the MGv to layers 3–4, suggests that the fastest parts of the early EPSPs are monosynaptic. However, it is clear that the IPSPs within the early response must be di- or polysynaptic (discussed in the following text). Experiments are underway to address the issue of synaptic order. As alluded to at the beginning of the discussion, the presence of long-lasting polysynaptic activity indicates that some functionally intact intracortical networks are preserved in the preparation, and that they can be activated by thalamic inputs. This broadens the utility of the primary slice preparation. For a more extensive discussion of these late responses, see Metherate and Cruikshank (1999).

**Early responses were composed of fast and slow EPSPs and fast IPSPs.** The relatively “secure” early responses were themselves composed of three subcomponents. First, there was a fast AMPA/KA receptor-mediated EPSP, which typically had a weak conventional voltage dependence (Fig. 5C1) (Cox et al. 1992; Jones and Baughman 1988). A second component, present in most cells, was a fast IPSP. In cases where this IPSP was robust, it usually had a reversal potential between the resting membrane potential and spike threshold (Fig. 5A), consistent with a GABA_A receptor mechanism (Avoli 1986; Connors et al. 1988; Cox et al. 1992; Hefti and Smith 2000). On average, the IPSPs followed the onset of the fast EPSP by ~3 ms, enough time for at least one synaptic delay. This is supported by the observation that IPSPs were blocked by CNQX (see also Hablitz and Sutor 1990; Metherate and Ashe 1994); if the IPSPs had been the result of direct inhibitory projections from the thalamus, then antagonism of AMPA/KA receptors could not have blocked them. Instead, it is likely that excitatory thalamic projections drove cortical inhibitory interneurons to fire and those interneurons synapsed on the recorded cells, producing the IPSPs (discussed in Castro-Alamancos and Connors 1997; Douglas and Martin 1991; Porter et al. 2001). This provides additional confidence that the preparation behaves in the expected way for a thalamocortical system. The third major component of the early response was a slow NMDA receptor-mediated EPSP. The pharmacologically isolated slow EPSPs typically had nonconventional voltage dependencies, gradual rising and decay phases, and relatively late peaks, all of which distinguished them from the fast EPSPs. In some cases, the isolated slow EPSP also appeared to have a longer onset latency than the fast EPSP (e.g., Fig. 5A), suggesting the possibility that the recorded slow EPSPs may not have been monosynaptic.

**Transmission at thalamocortical synapses appears to be mediated by both NMDA and AMPA/KA receptors.** The latency issue just mentioned appears to complicate the pharmacological data, and so some additional explanation will be presented regarding what these data might say about transmission at thalamocortical synapses. The basic protocol after re-

 Intracellular recordings in layers 3–4 reveal that auditory thalamocortical transmission is mediated by both AMPA and NMDA receptors and that thalamic stimuli can initiate intracortical activity in vitro

**Layer 3–4 cells have early and late intracellular responses.** Intracellular recordings were made from neurons located near the major sink and zone of thalamocortical termination in layers 3–4. This location was chosen to optimize chances of recording from cells that received direct thalamic input. There were two major temporal components to the MG-evoked responses in these cells that were referred to as the early and late responses. The early responses had short latencies and durations that generally matched the local field potentials and CSD sinks, suggesting they were part of the underlying basis of the sinks. They also had consistent shapes from trial to trial. In contrast, the late responses had long durations and were far less consistent from trial to trial, sometimes failing entirely. Early and late responses were further differentiated by their sensitivities to a number of manipulations designed to suppress polysynaptic activity; all of them depressed the late responses much more than the early responses, consistent with the late responses being predominantly polysynaptic intracortical phenomenon, and the early responses depending on fewer synaptic links. Exactly how many links are involved in the early response remains to be determined. The correlation between the onsets of the early PSP and the fast sink (and field potential) combined with the observed and known projections of the MGv to layers 3–4, suggests that the fastest parts of the early EPSPs are monosynaptic. However, it is clear that the IPSPs within the early response must be di- or polysynaptic (discussed in the following text). Experiments are underway to address the issue of synaptic order. As alluded to at the beginning of the discussion, the presence of long-lasting polysynaptic activity indicates that some functionally intact intracortical networks are preserved in the preparation, and that they can be activated by thalamic inputs. This broadens the utility of the primary slice preparation. For a more extensive discussion of these late responses, see Metherate and Cruikshank (1999).
cording control responses was to first apply CNQX alone, then CNQX + APV, followed by CNQX alone again, and finally control ACSF (order illustrated in Fig. 5C3). Importantly, it was found that combined application of CNQX + APV completely blocked the middle layer responses in both intracellular and extracellular recordings. This indicates, with near certainty, that transmission at the middle layer thalamocortical synapses is mediated by NMDA receptors or AMPA/KA receptors or both. Because CNQX by itself left a clear slow component that was reversibly blocked by APV, it is highly probable that part of the monosynaptic thalamocortical response is mediated by NMDA receptors, even if the recorded responses were not definitively monosynaptic (i.e., hence the sometimes longer latency in CNQX). This subtractive logic cannot be used to implicate AMPA/KA receptors because APV was never applied by itself. However, if one assumes that the fast EPSP in the middle layers is a monosynaptic event, then the virtually complete suppression of the fast EPSP by CNQX (RESULTS; Fig. 5) does implicate AMPA/KA receptors in transmission at thalamocortical synapses. Although it is difficult to prove that a response is monosynaptic, the fast EPSPs and the fast field potentials were recorded at the cortical focus, which in the pharmacological experiments was confirmed to be the position with the shortest latency middle layer field potential. Finally, other studies point to the involvement of AMPA/KA receptors in thalamocortical transmission (Armstrong-James et al. 1993; Gil and Amitai 1996a; Hagihara et al. 1988; Salt et al. 1995; reviewed in Castro-Alamancos and Connors 1997).

Differences in IPSPs and laminar profiles produced by MG vs. conventional stimulation; relationship to acoustic responses in vivo

Generally, the IPSPs produced by MG stimulation were weaker than expected based on previous ACx slice studies in which stimuli were delivered in a conventional fashion, to white matter/layer 6 below the recording site (on-beam) (Buonomano and Merzenich 1998; Metherate and Ashe 1994). The natural question that emerged was whether this was due to differences between the inputs activated by the two types of stimuli or if some other variable(s) produced the lower than expected inhibitory drive in the present study. For example, it is known that some aspects of cortical inhibitory systems are not fully developed in juvenile rodents (e.g., Dunning et al. 1999; Luhmann and Prince 1991; reviewed in Sutor and Luhmann 1995). In fact, this was indicated by the relatively modest cortical PV labeling observed here; PV is a marker for a class of cortical inhibitory neurons and is expressed at much higher levels in the adult mouse ACx (Cruikshank et al. 2001; del Rio et al. 1994). Another possibility was that the slicing procedure or some other aspect of the preparation compromised inhibition. To control for these potential preparation-dependent effects, we directly compared on-beam- and MG-evoked IPSPs within the same slices. On-beam stimulation generally produced robust IPSPs. Thus although the inhibition might not be fully developed in these juvenile slices, this did not preclude clear expression of inhibitory responses using conventional stimulation. In contrast, for the same neuron, MG-evoked IPSPs were weaker even when EPSP strengths were matched. These findings indicated that MG and on-beam stimuli do not activate the same sets of synapses in cortex. This conclusion was further supported by the observation that MG and on-beam stimuli evoked different laminar profiles. While the MG stimuli produced expected profiles with dominant middle layer CSD sinks, on-beam stimuli evoked profiles that were highly variable between slices and whose largest sinks rarely appeared in the middle layers. The laminar profiles undoubtedly depend on the origins and targets of the cells and axons near the stimulating electrodes; it is possible that these factors differ between slices more for the on-beam stimuli than the MG stimuli, contributing to the greater response variability.

It remains to be determined whether or not the cortical activation (e.g., IPSP magnitude and laminar profile) produced by MG stimulation in the primary slice represents a “natural” pattern, similar to that produced by acoustic stimulation in vivo. Some basis for optimism emerges by comparing the present results with the laminar patterns of click-evoked responses in vivo. Recordings from ACx of rodents and primates indicate that the fastest evoked CSD sinks occur most consistently in layers 3–4, and in some cases, about equally fast smaller sinks occur in layers 5–6, similar to the MG-evoked pattern here (Barth and Di 1990; Muller-Preuss and Mitzdorf 1984; Steinschneider et al. 1992). On the other hand, intracerebral sound-evoked responses of ACx cells in vivo have clear IPSPs (de Ribaupierre et al. 1972; Metherate and Ashe 1996; Volkov and Galazyuk 1992), which may differ from the present results. However, it is difficult to compare IPSPs across studies because the degree to which they can be observed, and their amplitudes, depends heavily on methodology and criteria. These issues might best be resolved by combining in vivo and in vitro approaches within a single study e.g., in vivo recordings of responses evoked by both sound and MG stimulation, combined with in vitro thalamocortical experiments from the same brains.

Shell slice

SHELL NUCLEI OF THE AUDITORY THALAMUS ARE DISTINCT FROM MGv. The rodent auditory thalamus includes a constellation of nonprimary nuclei that surround the primary MGv in a shell-like fashion (Cruikshank et al. 2001). As indicated in the INTRODUCTION, these nonprimary nuclei are distinguished from the MGv by their distinct physiological and anatomical properties, including strong projections to layer 1 (Herkenham 1980; Linke and Schwegler 2000; Ryugo and Killackey 1974; Willard and Ryugo 1983; reviewed in Winer 1992). Few groups have investigated the functional effects of the nonprimary inputs to ACx (Weinberger et al. 1995b), but discoveries by Barth and colleagues are intriguing. They found that stimulation of one of the subdivisions (the posterior intralaminar nucleus—they refer to it as PIL, but we will use the more conventional PIN) induces high-frequency (gamma band) oscillations in ACx (Barth and MacDonald 1996; Sukov and Barth 2001). In addition, prolonged stimulation of PIN produced steady increases in cortical cell excitability, depolarization, and spiking. Based on laminar analysis, the inputs responsible for these effects were hypothesized to be on layer 1 dendrites (Sukov and Barth 1998). To investigate cellular mechanisms by which nonlemniscal systems regulate cortical activity, we began to look at the feasibility of obtaining a slice preparation with intact nonlemniscal projections, and so the shell slice was developed.
MANY FEATURES OF THE SHELL SLICE SUGGEST AN INTACT PROJECTION FROM PPD TO LAYER 1. The shell slice was taken from the region of the brain immediately below the primary slice and, in the majority of cases, appeared to contain the PPD and nonprimary auditory cortex. These two regions have been shown to be interconnected via projections from PPD to layer 1 (Linke and Schwegler 2000). Stimulation of the region in or near the PPD (referred to as the shell region) produced a dominant sink at the surface of layer 1 in nearly every case. That sink was mediated by synaptic transmission as indicated by the effects of low calcium and glutamate antagonists. The latency of the surface sink matched the fast intracellular PSPs recorded from middle layer cells, suggesting that the PSPs might be produced by synapses in layer 1 on apical dendrites of the recorded cells. Consistent with this scenario, application of tracer in the shell stimulation region produced relatively dense fiber labeling in superficial layer 1 and very little in layers 3–4. Shell stimulation also produced long-duration late responses with large fluctuations. The late responses appeared to be induced more easily with shell stimulation than with MG stimulation (in the primary slice). In a previous study, we showed that virtually identical late potentials had fluctuations with significant power in the gamma band (Metherate and Cruikshank 1999). These observations are broadly consistent with the finding by Barth and colleagues that stimuli presumed to activate nonlemniscal pathways evoke cortical gamma band activity whereas lemniscal stimulation does not (Barth and MacDonald 1996). In summary, the data from the shell slice reviewed thus far support the hypothesis that the preparation contains a nonlemniscal pathway from PPD to layer 1 of temporal cortex, and that stimulation of this pathway caused the observed responses.

SOME OR ALL OF THE SHELL SLICE RESPONSES MIGHT BE MEDIATED BY AXON COLLATERALS OF ANTIDROMICALLY ACTIVATED CORTICOTECTAL CELLS. Although about two-thirds of the shell slices contained at least part of the PPD, the other one-third appeared to be located more ventrally, entirely below any of the auditory thalamic nuclei (ANATOMICAL FEATURES OF THE SHELL SLICE), indicating that the presence of these nuclei were not required for shell-type responses. In addition, the effective stimulation sites, and the subcortical components labeled after cortical Di-I applications, mostly involved a narrow white matter tract rather than auditory thalamic nuclei (see preceding text). Finally, there was a low incidence of retrograde filling of thalamic somata after cortical Di-I application. These results hinted that the nonprimary auditory thalamic nuclei may not be a major source of cortical input within the shell slice, leading to a search for an alternative explanation. The high incidence of retrograde-filled layer 5 pyramidal cells seen after dye application at the shell stimulation site provided a clue: because they were labeled, their axons must have passed through the stimulation site (Clerici and Coleman 1990; Rouiller and Welker 1991). Given this, it seemed likely that shell stimuli could activate these cells antidromically. If they also had axon collaterals to layer 1, they might contribute to the surface sink. Such a mechanism would be consistent with the conspicuous nonsynaptic field potentials in the lower and middle layers of the shell slice; the lower layer component could result from somatic spikes and the middle layers potentials could be produced by depolarization and/or spiking in proximal apical dendrites (Stuart and Sakmann 1994). Along these lines, the single infragranular cell recorded in this study appeared to display antidromic spiking. The laminar position of the back-labeled cells suggested that they might project to the tectum, so control tracing experiments were conducted in which Di-I was placed in the inferior colliculus to determine whether or not axons of auditory corticotectal cells passed through the shell stimulation area and if these cells had collateral branches to layer 1. Both questions were answered positively; therefore it is possible that this indirect mode of activation contributed to the layer 1 sink.

The implications of the last “control results” are worth additional emphasis. They indicate that a structure exists that could produce the layer 1 sink without a direct orthodromic projection from thalamus. However, it does not prove that this hypothetical mode of activation actually occurs or that direct input from the nonprimary auditory thalamus had no role. It simply means that the “layer 5 cell” alternative cannot, at present, be ruled out. Future experiments, possibly employing tectal stimulation combined with MG inactivation in vivo (Edeline et al. 2001), could resolve this issue.

Conclusions

In summary, the primary slice is an in vitro preparation containing functionally intact portions of the primary auditory thalamocortical system. Just as the analogous somatosensory thalamocortical slice has proven an invaluable tool for elucidating fundamental mechanisms in that system (see INTRODUCTION), so too should the primary slice for the auditory thalamocortical system. In fact, this has already begun with the present work (cf., the pharmacological and laminar results) and with previously published studies conducted in parallel (Hsiaei et al. 2000; Metherate and Cruikshank 1999; Rose and Metherate 2001). In contrast, the shell slice may constitute a “nonprimary” in vitro system. This possibility was supported by a reasonable body of evidence, although a plausible alternative interpretation was presented. Thus at present it should be used with caution. At any rate, the shell slice does appear to provide a novel way of selectively activating layer 1 synapses with extrinsic stimulation (Caulier and Connors 1994; Hess and Donoghue 1999). In closing, it should be mentioned that there may be an alternative to the shell slice for activating a nonprimary pathway in vitro. This would involve stimulation of the MGm within the primary slice. The MGm was often included within the primary slice. Assuming its projections to the ACx are intact, this preparation would have the added asset of containing both primary and nonprimary systems within the same slice, allowing for the study of their interaction.

This work was supported by National Institutes of Health Grants DC-02967 and DA-12929 and by California Tobacco-Related Disease Research Program Grant 8RT-0059.

REFERENCES


