Feedforward Inhibitory Connections from Multiple Thalamic Cells to Multiple Regular-Spiking Cells in Layer 4 of the Somatosensory Cortex

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Running Title
Multiple-to-multiple thalamocortical connections

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Abstract

Thalamocortical (TC) cells in the ventrobasal thalamus make direct excitatory connections with regular-spiking (RS) cells in layer 4 of the somatosensory cortex, but also make disynaptic feedforward inhibitory connections with the RS cells via layer 4 fast-spiking (FS) cells. In this study, we investigated connection rules of the feedforward inhibitory circuit from multiple TC cells to multiple RS cells, at the level of synaptic potentials. Using thalamocortical brain slices of young mice (postnatal days 12-16), we made simultaneous patch-clamp recordings from three adjacent cortical cells (two RS cells and one FS cell), combined with minimal stimulation of presumed single TC fibers. We found that nearly all (97%) of TC fibers, which generated excitatory inputs onto RS cells, also generated divergent excitatory inputs onto adjacent FS cells. 44% of TC fibers generated divergent excitatory inputs onto adjacent pairs of RS cells. We then combined the triple patch-clamp recording with multi-site (two to three) minimal stimulation of single TC fibers, and found that 86% of FS cells received convergent inputs from all of stimulated TC fibers. We also found that 68% of FS cells generated divergent inhibitory inputs onto adjacent pairs of RS cells. The results indicate that spikes in TC cells, which excite RS cells, also excite adjacent FS cells with high fidelity. The results also indicate that FS cells receive convergent excitatory inputs from multiple TC cells, and then send divergent inhibitory outputs to multiple RS cells.
Introduction

In the rodent somatosensory system, whisker signals are conveyed to the ventrobasal thalamus, and then transferred to the barrel field of the somatosensory cortex (barrel cortex) (reviewed in Waite 2004). Thalamocortical cells (TC cells) in the ventrobasal thalamus project their axons to layer 4 of the barrel cortex (Jensen and Killackey 1987; Killackey 1973). Functionally, the TC cells are connected to excitatory regular-spiking (RS) cells and inhibitory fast-spiking (FS) cells in cortical layer 4 (Bruno and Simons 2002; Simons 1978; Swadlow 1995).

Feedforward inhibition is a salient feature observed in the somatosensory thalamocortical circuit (reviewed in Swadlow 2003). Using thalamocortically-connected slices (Agmon and Connors 1991; see Fig. 1A), synaptic organization governing the feedforward inhibition has been studied in vitro, at the level of synaptic potentials and currents. Thalamic stimulation produces feedforward IPSPs in layer 4 RS cells (Agmon and Connors 1992). Minimal stimulation of presumed single TC fibers generates all-or-none EPSPs in both RS and FS cells in layer 4 (Beierlein et al. 2003; Gibson et al. 1999; Gil et al. 1999). Action potentials in layer 4 FS cells generate IPSPs in layer 4 RS cells (Beierlein et al. 2003; Sun et al. 2006).

These in vitro studies have revealed a feedforward inhibitory circuit from single TC cells to single RS cells via FS cells. However, it is relatively unclear how the thalamocortical circuit from multiple TC cells to multiple RS cells is synaptically organized. In the barrel cortex, only 10-15% of total neurons are GABAergic inhibitory interneurons (Beaulieu 1993; Lin et al. 1985), and not all GABAergic interneurons are FS cells (Amitai et al. 2002; Gibson et al. 1999). Thus, excitatory RS cells are much larger in number than inhibitory FS cells. In this study, we therefore investigated the
connection rules of the feedforward inhibitory circuit from multiple TC cells to multiple RS cells via FS cells, at the level of synaptic potentials.

A recent study has detected divergent synaptic inputs of TC fibers onto RS and FS cells, in which paired patch-clamp recordings of RS and FS cells were made and the minimal stimulation was then applied to presumed single TC fibers (Gabernet et al. 2005). They observed tightly-correlated occurrence of all-or-none EPSC responses in both RS and FS cells, and concluded that the correlated events were caused by activation of the same TC fiber. Such a correlation-based experiment also has been reported in the lateral geniculate nucleus (LGN) of the thalamus (Blitz and Regehr 2005).

We here used the same correlation-based paradigm, to address the synaptic organization of the multiple-to-multiple thalamocortical feedforward circuit. Our quantitative analysis revealed that; (1) nearly all of the TC fibers which generated excitatory inputs onto RS cells also generated excitatory inputs onto adjacent FS cells; (2) adjacent pairs of RS cells also received divergent excitatory inputs from the same TC fiber; and (3) FS cells received convergent excitatory inputs from multiple TC cells and sent divergent inhibitory outputs to multiple RS cells.
Methods

Slice preparation and electrophysiological recording

Experiments were performed using C57/BL6 mice (postnatal days 12-16). All experimental procedures were in accordance with the institutional guidelines concerning the care and handling of experimental animals, and were approved by the Animal Research Committee of our institute. Animals were anesthetized by halothane (Takeda Chemical Industries, Osaka, Japan) and then killed by decapitation. The brain was rapidly removed and immediately placed in a chilled, artificial cerebrospinal fluid (ACSF). The ACSF contained (in mM): NaCl 124, KCl 2.5, NaH₂PO₄ 1.1, MgSO₄ 1.2, CaCl₂ 2.5, NaHCO₃ 26, and glucose 12. It was oxygenated by a gas mixture containing 95% O₂ and 5% CO₂. Thalamocortical slices (300 µm thickness), where the thalamic ventrobasal region and the barrel cortex were synaptically connected, were made according to Agmon and Connors (1991). The slices were transferred into a holding tank, incubated at 32 ºC for 30 min, and then maintained at room temperature until just before recording.

Individual slices were transferred to a submerged recording chamber, and were continuously perfused with the ACSF kept at 30-32 ºC. The slices were visualized using infrared differential interference contrast (IR-DIC) microscopy (Axioskop FS, Carl Zeiss, Jena, Germany) equipped with a Newvicon tube camera (C2400-07, Hamamatsu Photonics, Hamamatsu, Japan). Three adjacent cells in cortical layer 4 were randomly selected for electrophysiological recording. The intercellular distance was measured from center to center. The intercellular distance was less than 40 µm.

Membrane potentials of three cortical cells were simultaneously recorded in current-clamp mode using three patch-clamp amplifiers, two Axoclamp-2B amplifiers
(Molecular Devices, Sunnyvale, CA) and an EPC-7 amplifier (HEKA Elektronik, Lambrecht, Germany). Patch pipettes were filled with an intracellular solution containing (in mM): K-methanesulfonate 135, NaCl 6, EGTA 0.2, HEPES 10, Mg-ATP 4, Na3-GTP 0.3, and phosphocreatine-Tris 10 (pH 7.3, 290-295 mOsm). The pipette resistance was 4-6 MΩ with the intracellular solution. Liquid junction potentials were not corrected. All electrical signals were low-pass filtered at 3 kHz, and then digitally sampled at 10 kHz using an ITC-16 interface (Instrutech, Port Washington, NY) that was controlled by IgorPro (Wavemetrics, Lake Oswego, OR).

Only cortical cells with resting membrane potentials more negative than -50 mV and overshooting action potentials were accepted for this study. Characterization of layer 4 cortical cells (RS cells and FS cells) was based on the firing patterns (Fig. 1B). The RS cells had a mean resting membrane potential of -69.0 ± 0.4 mV (n = 104) and a mean membrane input resistance of 432.9 ± 11.9 MΩ (n = 104), whereas the FS cells had a mean resting membrane potential of -65.6 ± 0.5 mV (n = 52) and a mean membrane input resistance of 81.7 ± 3.4 MΩ (n = 52). Thalamocortical EPSPs were measured at the holding potential of -73 mV, whereas FS-evoked IPSPs in RS cells were measured at the holding potential of -53 mV.

**Minimal stimulation of thalamic fibers**

A custom-made bipolar electrode was used for minimal stimulation of presumed single TC axons. The bipolar electrode was made by gluing two monopolar tungsten microelectrodes (FHC, Bowdoinham, ME). The distance between their tips was approximately 250 µm. For local electrical stimulation, the parallel bipolar electrode was placed perpendicular to the surface of the slices: one tip of the bipolar electrode
was pushed into the slices, while the other tip was placed away from the surface of the slices.

The stimulating electrode was placed within the ventrobasal thalamus. Electrical stimulation was applied as a cathodal pulse with 100 µs duration through the electrode tip pushed into the slices. The electrical pulse was generated by a stimulus isolation unit (ISO-Flex, A.M.I., Jerusalem, Israel). The stimulus intensity was adjusted in each experiment of minimal stimulation (range: 40-250 µA), to evoke all-or-none responses with about 50% success rates. At the threshold intensity, 20-60 trials of stimuli (0.1-0.3 Hz) were applied in each experiment of minimal stimulation. We accepted the following responses as minimal stimulation-evoked EPSPs: (1) when the responses were clearly all-or-none, (2) the responses had a constant EPSP latency, and (3) a small change of stimulus intensity changed the success rates of the EPSPs but did not change the EPSP amplitudes.

In the correlation-based paradigm (Fig. 2), “reference” connections were critical for us to judge whether single TC fibers were stimulated or not. Therefore, our criterion to establish the reference connections was that minimal stimulation-evoked responses in cortical cells had EPSP amplitudes larger than 2 mV. Such EPSP responses exhibited clearly distinguishable success and failure events, so that we could easily determine the successes and failures by eye. There were no limitations on EPSP amplitude in “test” connections. When more than one cortical cell could be regarded as reference cells, we regarded both RS and FS cells as reference cells, and then individually analyzed the presence of divergent thalamocortical inputs (see Fig. 4A1 and 4A2).

Data analysis
We quantitatively analyzed the correlation-based paradigm in the following way. Success and failure events in reference cells could be easily determined by eye as described above, while success and failure events in test cells were determined by the threshold obtained from $1.6 \times \text{RMS}$ (root mean square) of the baseline noise in test cells (Markram et al. 1997; Beierlein et al. 2003). In this study, our criterion for correlated responses was that all of the success events in the reference cells were accompanied by the success events in the test cells (i.e., 100% match of the success events). The complete match indicates that we strictly detected the divergence of thalamocortical inputs.

To examine the firing patterns of the cortical cells, repetitive firing was elicited by a 500 ms positive current injection. The injected current was adjusted to evoke about 50 Hz of initial spike-frequency (i.e., the inverse of the interval between 1st and 2nd spike). The spike-frequency adaptation during the repetitive firing was calculated as the ratio of the average of the last four spike-frequencies to the initial spike-frequency. The spike-width of action potentials was calculated at the half amplitude of the 1st spike during the repetitive firing. The amplitude of afterhyperpolarization (AHP) following action potentials was calculated as the minimal membrane potential between the 1st spike and 2nd spike during the repetitive firing, which was subtracted from the membrane potential of the 1st spike threshold.

The synaptic latency of the EPSP onset was determined as the time from stimulus artifact to 5% of the EPSP amplitude. Amplitudes of individual EPSP responses during the correlation-based paradigm were measured as the individual peak-values (searched during 10 ms just after the EPSP onset), subtracted from the individual baseline values (mean membrane potential during 1 ms just before the EPSP onset). Thalamocortical
EPSP responses were discarded if feedforward IPSPs or polysynaptic EPSPs were observed. All analyses were performed using IgorPro. All pooled data were represented as means ± SEM except as noted.
Results

*Thalamocortical EPSPs in layer 4 RS and FS cells*

We identified excitatory RS cells and inhibitory FS cells in layer 4 of the somatosensory barrel cortex, based on the firing patterns (Gibson et al. 1999; Beierlein et al. 2003). Figure 1B shows an IR-DIC image of a triple patch-clamp recording of cortical cells (two RS cells and one FS cell) and their firing patterns. RS cells displayed strong spike-frequency adaptation during repetitive firing (adaptation ratio: 0.34 ± 0.01, n = 104), broad spike-width of action potentials (1.16 ± 0.02 ms, n = 104), and small AHP amplitudes following action potentials (4.4 ± 0.3 mV, n = 104). To record FS cells, we targeted neurons with relatively large somata (Fig. 1B; Gibson et al. 1999; Simons and Woolsey 1984). FS cells displayed little spike-frequency adaptation (adaptation ratio: 0.90 ± 0.03, n = 52), narrower spike-width (0.45 ± 0.01 ms, n = 52), and deeper AHP (15.9 ± 0.4 mV, n = 52).

A minimal stimulation paradigm has been frequently used to assess the unitary responses in somatosensory thalamocortical synapses (Beierlein et al. 2003; Gabernet et al. 2005; Gibson et al. 1999; Gil et al. 1999). In this paradigm, the extracellular simulation intensity at the thalamus is minimized and adjusted to evoke clear all-or-none synaptic responses in cortical cells. The success events evoked by the threshold stimulation are presumed to originate from activation of a single presynaptic TC fiber. We recorded such all-or-none responses in RS or FS cells, using the minimal stimulation paradigm (Fig. 1C). The minimal stimulation-evoked EPSPs in RS cells had a mean amplitude of 3.7 ± 0.4 mV (n = 33), whereas those in FS cells had a larger amplitude (6.9 ± 0.7 mV, n = 31). The mean synaptic latencies from thalamic stimulation to the EPSP onset in cortical cells were 3 to 4 ms (3.6 ± 0.1 ms in RS cells,
n = 33; 3.2 ± 0.1 ms in FS cells, n = 31), which are consistent with the previous report (Beierlein et al. 2003).

_Detection of functional divergence of thalamic fibers onto multiple cortical cells_

Gabernet et al. (2005) recently detected divergent synaptic inputs of TC fibers onto RS and FS cells. They made paired recordings of RS and FS cells, and then applied the minimal stimulation to presumed single TC fibers. They reported that, in particular examples, the minimal stimulation elicited tightly-correlated occurrence of all-or-none responses in both RS and FS cells, concluding that the RS and FS cells received EPSC inputs from the same TC fibers. In the present study, we quantitatively examined the probability of detecting the divergent synaptic inputs, using the same correlation-based paradigm.

We first made patch-clamp recordings from three adjacent cortical cells, located deeply from the cut surface of the brain slice. We then moved a stimulating electrode within the ventrobasal thalamus, and searched for a spot where clear all-or-none EPSPs were observed in one of the three cortical cells. When we observed all-or-none EPSPs in a cell, we regarded it as the “reference” cell and the two other cells as the “test” cells. Interestingly, we frequently observed all-or-none responses in both the reference and test cells, even though we applied the minimal stimulation only to the reference cell (All in Fig. 2A). In addition to the identical threshold, the occurrence of the all-or-none EPSPs was tightly correlated (Individual in Fig. 2A, and Fig. 2B). This indicates that the reference and test cells received EPSP inputs from the same TC fiber. Figure 2B and 2C shows how to determine whether the EPSP responses in the reference and test cells are correlated or not.
Quantitative analysis of the divergent thalamocortical inputs

Using the correlation-based paradigm, we not only observed thalamocortical divergence onto RS and FS cells (Fig. 2), but also observed thalamocortical divergence onto pairs of RS cells which had not been previously reported (Fig. 3). We then quantitatively examined the connectivity of TC fibers onto RS and FS cells (Fig. 4A) and the connectivity of TC fibers onto pairs of RS cells (Fig. 4B). Since we simultaneously recorded three cortical cells (RS1, RS2, and FS cells) in each experiment, two divergent inputs onto RS and FS cells (RS1-FS pair and RS2-FS pair) and one divergent input onto pairs of RS cells (RS1-RS2 pair) could be analyzed from each experiment. Pairs of cortical cells, neither of which received thalamocortical EPSP inputs, were excluded from the quantitative analysis, because we suspected that TC axons were severed during the slicing procedures.

The divergent inputs from TC fibers to RS and FS cells were first analyzed (Fig. 4A). When we regarded RS cells as the reference cells (Fig. 4A1), 96.6% (28/29) of the RS-FS pairs received EPSP inputs from the same TC fibers (filled circles in Fig. 4A1), whereas we found that in only 3.4% (1/29) of the RS-FS pairs, the reference RS cell alone received EPSP inputs (an open circle in Fig. 4A1). When we regarded FS cells as the reference cells (Fig. 4A2), 53.7% (29/54) of the RS-FS pairs received EPSP inputs from the same TC fibers (filled circles in Fig. 4A2), whereas 46.3% (25/54) of the RS-FS pairs received EPSP inputs onto only the reference FS cells (open circles in Fig. 4A2). These results indicate that nearly all of the TC fibers generated EPSP inputs onto both RS and FS cells (filled circles in Fig. 4A1 and 4A2) or onto only FS cells (open circles in Fig. 4A2), but not onto only RS cells (an open circle in Fig. 4A1). These
results also indicate that, when a TC fiber generated excitatory inputs onto RS cells, the TC fiber also generated divergent excitatory inputs onto adjacent FS cells with high fidelity (96.6%; Fig. 4A). The divergent inputs from TC fibers to adjacent pairs of RS cells were then examined (Fig. 4B). We found that adjacent RS pairs also exhibited tightly-correlated EPSP responses using the correlation-based paradigm (Fig. 3). However, the probability of detecting the divergent inputs was not high. In 43.5% (10/23) of the RS-RS pairs, two RS cells received EPSP inputs from the same TC fibers (filled circles in Fig. 4B). In the rest of the RS-RS pairs (56.5%; 13/23), only one of the RS-RS pairs received EPSP inputs (open circles in Fig. 4B). These results indicate that adjacent RS cells received divergent excitatory inputs from the same TC fiber. However, these results also indicate that the probability of the RS-RS pairs receiving the divergent inputs (43.5%; Fig. 4B) was lower than the probability of the RS-FS pairs receiving the divergent inputs (96.6%; Fig. 4A), when we regarded RS cells as the reference cells.

**Convergent synaptic inputs from multiple thalamic fibers onto single FS cells**

Figure 4A shows that, even if a TC fiber did not generate EPSP inputs onto RS cells, the TC fiber generated EPSP inputs onto FS cells. This implies a high probability of convergent excitatory inputs from multiple TC cells onto FS cells. To directly address this issue, we repeatedly used the correlation-based paradigm in the same slice (Fig. 5). We first made simultaneous patch-clamp recordings from three cortical cells (two RS cells and one FS cell) within a close distance (Patch-Clamp in Fig. 5A). We then moved a stimulating electrode within the thalamus, applied the correlation-based paradigm if a reference connection onto the RS1 or RS2 cell was established, and examined the
connectivity of the stimulated TC fiber (termed TC1) onto the test FS cell (*TC1 Stim.* in Fig. 5A and 5B1). We moved the stimulating electrode to a different spot within the thalamus, applied the correlation-based paradigm again if we found another reference connection onto the RS1 or RS2 cell, and examined the connectivity of the different TC fiber (termed TC2) onto the test FS cell (*TC2 Stim.* in Fig. 5A and 5B1). TC1-evoked EPSPs onto the RS1 and RS2 cells had different amplitudes from TC2-evoked EPSPs onto the RS1 and RS2 cells (Fig 5B1 and 5B2), indicating that we stimulated different TC fibers. By repeating the procedure, we stimulated several TC fibers separately, so that we were able to examine the convergent synaptic inputs from multiple TC fibers onto test FS cells.

Using this method, we quantitatively examined the convergent thalamocortical inputs onto FS cells (Fig. 5C). We recorded from 7 triplets (two RS cells and one FS cell), and stimulated at least two (two to three) TC fibers in each triplet. We confirmed the independent stimulation of different TC fibers, based on whether the correlation-based paradigm at different spots produced different patterns of EPSP amplitudes in RS1 and RS2 cells (see Fig. 5B2 and the legends for details). Six of 7 FS cells we examined (85.7%) received EPSP inputs from all of the stimulated TC fibers (Fig. 5C). The rest one FS cell received EPSP inputs from two of three stimulated TC fibers (#7 in Fig. 5C). These results indicate a high probability of convergent synaptic inputs from multiple TC fibers onto FS cells.

**Divergent synaptic inputs from single FS cells to multiple RS cells**

Inhibitory connections from FS cells to RS cells are vital parts of the feedforward inhibitory circuit from the thalamus to layer 4 of the barrel cortex. Previous reports
using paired whole-cell recordings have revealed inhibitory connections from single FS cells to single RS cells (Beierlein et al. 2003; Gabernet et al. 2005; Sun et al. 2006). We finally examined the divergent synaptic inputs from single FS cells to adjacent pairs of RS cells quantitatively, using simultaneous whole-cell recordings from three cells (Fig. 6).

We obtained voltage records from 52 triples, each of which consisted of two RS cells and one FS cell. In 41 of 52 triplets, action potentials in presynaptic FS cells evoked unitary IPSPs in at least one of the two RS cells. Divergent inhibitory inputs onto both the two RS cells were detected in 68.3% (28/41) of the triplets (Fig. 6A1, and filled circles in Fig. 6B), whereas we could not observe the divergent inputs in 31.7% (13/41) of the triplets (Fig. 6A2, and open circles in Fig. 6B). We also found that the IPSP amplitudes in the two RS cells were positively correlated (filled circles in Fig. 6B; $r = 0.77$). The mean amplitudes of unitary IPSPs were 2.7 ± 0.2 mV ($n = 69$). The probability of detecting unitary IPSPs was 66.3% (69 of 104 FS-RS pairs from 52 triplets). These results indicate a high probability of divergent synaptic inputs from FS cells to adjacent RS cells.
Discussion

In this study, we investigated the connection rule of the feedforward inhibitory circuit from multiple TC cells to multiple RS cells via FS cells. We found that nearly all of TC fibers which generated excitatory inputs onto RS cells also generated divergent excitatory inputs onto adjacent FS cells (Figs. 2 and 4). Adjacent pairs of RS cells received divergent excitatory inputs from the same TC fiber (Figs. 3 and 4). There was a high probability of convergent excitatory inputs from multiple TC fibers to FS cells (Fig. 5). We also found a high probability of divergent inhibitory inputs from single FS cells onto adjacent pairs of RS cells (Fig. 6). These results are summarized in Fig. 6C.

Technical considerations

The correlation-based paradigm in this study has been used to examine whether two postsynaptic cells received divergent synaptic inputs from the same presynaptic fiber. Using this paradigm, Gabernet et al. (2005) reported that RS and FS cells in the barrel cortex received divergent EPSC inputs from the same TC fiber. In the LGN of the thalamus, Blitz and Regehr (2005) examined whether direct EPSCs and feedforward IPSCs in a thalamic relay cell originated from the same retinogeniculate fiber. Thus, the correlation-based paradigm has been recognized as a useful technique to detect divergent synaptic inputs.

In general, synaptic connectivity would be underestimated in slice preparations, since some axons are severed during the slicing procedure. Long-range TC axons in thalamocortical slices (Agmon and Connors 1991) may be especially exposed to the axonal cutting. Despite this potential for underestimation, we observed an extremely high probability (96.6%) of divergent inputs from TC fibers onto RS and FS cells, when
TC→RS connections were established (Fig. 4A1). This may be attributable to our correlation-based paradigm in which we established the reference connections. We did not use slice preparations where we could not find any reference connections. The procedures enabled us to exclude the slices in which thalamocortical axons were severed, which consequently minimized the underestimation.

We also detected divergent synaptic inputs from TC fibers onto pairs of RS cells (Figs. 3 and 4B). However, the connectivity was not high (43.5%). Although it remains unclear whether the connectivity onto pairs of RS cells is underestimated or biologically low, we suspect a possibility of the underestimation based on following two reasons. First, thalamocortical synapses are distributed on both proximal and distal dendrites of spiny stellate cells (a major type of RS cell), but on the somata of inhibitory interneurons (Benshalom and White 1986; Keller and White 1987; White et al. 1984). Distally-located synapses onto RS cells might be subject to the axonal cutting. Second, the correlation-based paradigm in the present study could not detect small (roughly smaller than 1 mV) EPSPs as observed in RS cells (e.g., RS2 in Fig. 2A and 2C), which also would result in an underestimation of the connectivity.

The probability of detecting EPSP inputs onto RS cells was 53.7% when FS cells were the reference cells (Fig. 4A2), but was lower when RS cells were the reference cells (43.5%; Fig. 4B). As discussed above, we suspect that the different probabilities were also derived from our correlation-based paradigm which could not detect small EPSP amplitudes. In the case of FS cells as reference cells (Fig. 4A2), EPSP amplitudes in only the test RS cells have to be large enough to be judged as correlated responses (i.e., roughly larger than 1 mV). On the other hand, in the case of RS cells as reference cells (Fig. 4B), EPSP amplitudes in both of the reference and test RS cells must be large
In this study, we placed an extracellular stimulating electrode in the thalamus, to activate TC fibers. Extracellular thalamic stimulation not only activates TC fibers orthodromically, but also activates corticothalamic (CT) fibers antidromically (reviewed in Castro-Alamancos 2004). Thus, there is a possibility that some of thalamus-evoked EPSPs in our recordings are derived from the activation of CT fibers. However, Beierlein et al. (2003) reported that the TC fiber-evoked EPSPs had synaptic latencies of approximately 3 ms, whereas the CT fiber-evoked EPSPs had longer synaptic latencies of 6 to 7 ms. The synaptic latencies of the EPSPs we recorded (3 to 4 ms) were consistent with those of the TC fiber-evoked EPSPs, suggesting that we mainly stimulated TC fibers.

The stimulus intensity of the minimal stimulation in this study (range: 40-250 µA) was relatively higher than that in previous reports (range: 5-100 µA, see Beierlein et al. 2003; Gabernet et al. 2005). Nevertheless, limited numbers of TC fibers appeared to be locally stimulated in our recordings for the following reasons. When the minimal stimulation was applied to one of three simultaneously-recorded cells (see RS1 Reference in Fig. 2A), we frequently observed correlated EPSP responses in the other two cells (see RS2 and FS in Fig. 2A). However, we could not observe any additional synaptic responses in the other two cells. If many TC fibers were stimulated, we must have observed such additional synaptic responses in the other two cells.

Synaptic organization of the thalamocortical connection

In this study, we frequently observed that adjacent cortical cells received divergent inputs from the same TC fiber (Figs. 2-4). These divergent thalamocortical inputs, based
on our electrophysiological study, are consistent with previous morphological studies. Single thalamic axons are extensively ramified within single barrels (extent of the axon arbors, ca. 200-500 µm in rats; Jensen and Killackey 1987). The dendritic field span of rat layer 4 neurons (spiny stellate cells and FS cells) is about 200 µm (Amitai et al. 2002; Lübke et al. 2000; Simons and Woolsey 1984). Thus, the extensive ramification of single TC axons is consistent with our finding that adjacent cortical cells frequently received divergent inputs from the same TC fiber.

Properties of neuronal firing have been used for classification of cortical inhibitory interneurons (Kawaguchi 1995). In layer 4 of the rodent barrel cortex, non-adapting inhibitory interneurons are classified as FS cells, whereas two types of adapting inhibitory interneurons have been reported: low-threshold spiking (LTS) cells (Beierlein et al. 2003; Deans et al. 2001; Gibson et al. 1999) and regular spiking non-pyramidal (RSNP) cells (Beierlein et al. 2002; Porter et al. 2001; Sun et al. 2006). However, these previous studies also suggested that FS cells are major mediators of thalamocortical feedforward inhibition. Thalamocortical EPSPs in LTS cells are much smaller in amplitude than those in FS cells (Beierlein et al. 2003; Gibson et al. 1999). Although thalamocortical EPSPs in RSNP cells have amplitudes comparable to those in FS cells (Porter et al. 2001), unitary IPSPs from RSNP cells to spiny cells have small amplitudes (Sun et al. 2006).

Connectivity between thalamic and cortical cells has been well studied using in vivo extracellular recordings, in which thalamic and cortical spikes were simultaneously recorded and then the connectivity was assessed by cross-correlation analysis of the spikes. The cross-correlation analysis revealed that connectivity between TC and FS cells was significantly higher than that between TC and RS cells (Bruno and Simons
2002), and that FS cells received highly convergent inputs from multiple TC cells (Swadlow and Gusev 2002). However, thalamocortical convergence has not been studied at the finer level of synaptic potentials. Our present study using in vitro intracellular recordings (Fig. 5) was consistent with previous studies using in vivo extracellular recordings.

In this study, we examined divergent thalamocortical inputs onto nearby (~40 µm) cortical cells, but did not examine those onto distant cortical cells throughout single barrels (ca. 150-300 µm in diameter in mice; Woolsey and van der Loos 1970). Thus, our studies provide information about local- and fine-scale thalamocortical connectivity, rather than large-scale connectivity (e.g., studies of the size of whisker receptive fields in vivo: Armstrong-James and Fox 1987; Moore and Nelson 1998; Zhu and Connors 1999). Our approach might contribute to elucidating thalamocortical projection to the minicolumns, which are subdivisions in the barrel structures with transverse diameters of 40-50 µm (Bruno et al. 2003; reviewed in Mountcastle 2003).

One unsolved issue is that we studied the thalamocortical circuit in a developmental stage. Our thalamocortical slices were obtained from young mice (postnatal days 12-16). It therefore remains unclear how the thalamocortical circuit we studied reflects mature thalamocortical circuits. We should address this issue in the future, although it is not technically easy to apply the correlation-based paradigm to adult mice (> 4 weeks old).

**Synaptic organization of the intracortical inhibition**

As shown in Fig. 6, we observed a high probability of FS→RS inhibitory connections. In thalamocortical transmission, the FS→RS inhibitory connections could be used for feedforward inhibition if TC fibers connect onto both the RS and FS cells,
and also used for lateral inhibition if TC fibers connect onto only FS cells. It is an important issue to determine which the FS→RS connections are preferentially used for. However, as shown in RS2 and FS cells of Fig. 5B1, we frequently observed the coexistence of the divergent TC→RS/FS connections (TC2 Stim. and TC3 Stim. in Fig. 5B1) and the non-divergent TC→FS connections (TC1 Stim. in Fig. 5B1) onto the same pairs of RS and FS cells (FS→RS2 IPSPs were observed in Fig. 5B1; data not shown). Thus, it is likely that individual FS→RS inhibitory connections are used for both feedforward and lateral inhibition, depending on the individual TC fibers.

Figure 6B shows that the amplitudes of unitary IPSPs onto two RS cells were strongly correlated. This correlation indicates that FS cells, which generate larger IPSPs onto one RS cells, also generate larger IPSPs onto the other RS cells. Thus, it is likely that the amplitudes of FS-evoked IPSPs onto RS cells are determined by presynaptic FS cells, rather than by postsynaptic RS cells. This finding functionally implies that the strength of feedforward inhibition is determined by which FS cells are activated.

We reported a high probability of detecting FS→RS inhibitory connections (66.3%; Fig. 6), which is consistent with a previous study using young (2-3 weeks old) mice (approximately 50%; Gabernet et al. 2005). On the other hand, the probability is lower in 2 weeks old rats (44%; Beierlein et al. 2003), and even lower in older (3-5 weeks old) rats (21%; Sun et al. 2006). Thus, the high connectivity in our recordings seems to be due to both species differences and age differences.

**Functional implication**

A recent study using thalamocortical slices has shown that thalamic stimulation evoked highly synchronous IPSCs in adjacent spiny stellate cells in layer 4 (Sun et al. 2006).
One of the likely mechanisms for the synchronous IPSCs is derived from a group of FS cells which were mutually connected with electrical and chemical synapses (Gibson et al. 1999). In the present study, we found that adjacent RS cells frequently received divergent inhibitory inputs from the same FS cell (Fig. 6). Thus, it is likely that the inhibitory divergence of FS cells to RS cells may also participate in the thalamus-evoked synchronous IPSCs in RS cells.

In conclusion, we found that (1) FS cells receive highly convergent excitatory inputs from multiple TC cells and then send divergent inhibitory outputs to multiple RS cells, and that (2) TC fibers generating excitatory inputs onto RS cells also generate divergent excitatory inputs onto adjacent FS cells. The former finding implies the feedforward inhibition with a low activation threshold: a relatively small number of TC cells activate action potentials in FS cells, which in turn inhibit multiple RS cells. The latter finding implies the precise feedforward inhibition: individual TC→RS excitatory connections are tightly coupled to feedforward TC→FS→RS inhibitory connections. It will be interesting to directly demonstrate these implications, by uncovering the dynamic regulation of ascending signals in the feedforward inhibitory circuit.
Acknowledgements

We thank Dr. Yasuo Kawaguchi for comments on an earlier version of the manuscript.

Grants

This work was supported in part by research grants from the Ministry of Education, Culture, Sports, Science and Technology in Japan.
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**Gabernet L, Jadhav SP, Feldman DE, Carandini M, and Scanziani M.** Somatosensory integration controlled by dynamic thalamocortical feed-forward


**Lin CS, Lu SM, and Schmechel DE.** Glutamic acid decarboxylase immunoreactivity


Figure legends

Figure 1. Thalamocortical EPSPs in layer 4 RS and FS cells

(A) A thalamocortical slice. The ventrobasal thalamus (T) and layer 4 of the barrel cortex (L4 Cx; indicated by square brackets) were visualized by cytochrome oxidase staining, based on the method of Wong-Riley (1979).

(B) An IR-DIC image of three simultaneously-recorded cortical cells (left) and their firing patterns (right).

(C) Thalamocortical all-or-none EPSPs in an RS cell and an FS cell, elicited by the minimal stimulation paradigm. A stimulating electrode was placed in the ventrobasal thalamus, and membrane potentials of RS or FS cells were postsynaptically recorded. 41 responses (RS) or 46 responses (FS) to threshold stimulation were superimposed in each cell. Thalamic stimuli were applied at asterisks.

Figure 2. Detection of functional divergence of thalamic fibers onto multiple cortical cells

(A) Representative voltage records from three cortical cells, when we evoked all-or-none EPSP responses onto the Reference RS1 cell (correlation-based paradigm). A stimulating electrode was placed in the thalamus. 39 responses to threshold stimulation are superimposed in All, and four consecutive responses are shown below Individual. Thalamic stimuli were applied at asterisks. Occurrence of EPSP responses in these cortical cells was correlated.

(B) The two-dimensional plot of individual EPSP responses in the RS1 cell (Reference) and the FS cell (Test), during the correlation-based paradigm. This plot shows the
highly-correlated occurrence of EPSP responses in the Reference RS1 and Test FS cells. Successes and failures of the EPSP responses in the Reference RS1 cell were indicated by Ref Success and Ref Failure, respectively. A gray dotted line indicates the threshold for successes and failures of the EPSP responses in the Test FS cell, obtained from $1.6 \times$ RMS of the baseline noise in the Test FS cell. Note that 100% (23/23) of the success events in the Reference RS1 cell were accompanied by the success events in the Test FS cell. See Methods for the detailed analysis of the correlation-based paradigm. Mean EPSP amplitudes in reference and test cells, shown in Figs. 4 and 5, were calculated as the average of EPSP responses in the case of Ref Success.

(C) The two-dimensional plot of individual EPSP responses in the RS1 cell (Reference) and the RS2 cell (Test), during the correlation-based paradigm. This is the same analysis as in (B), except that the Test cell is the RS2 cell. The two-dimensional plot shows that EPSP responses in the Reference RS1 and the Test RS2 cells appeared to be correlated. However, 91% (21/23) of the success events in the Reference RS1 cell were accompanied by the success events in the Test RS2 cell, and therefore those responses were not regarded as correlated responses in this study (see Methods). This means that we strictly detected the divergent synaptic inputs, but the strict criterion also would lead to an underestimation of the connectivity of thalamocortical divergence. A gray dotted line indicates $1.6 \times$ RMS of the baseline noise in the Test RS2 cell.

Figure 3. Divergent synaptic inputs from thalamic fibers onto a pair of layer 4 RS cells
(A) Representative voltage records from a pair of RS cells, showing highly-correlated occurrence of EPSP responses during the correlation-based paradigm. 51 responses to threshold stimulation are superimposed here. Both RS cells exhibit all-or-none responses. Although an FS cell was also simultaneously recorded, the voltage records are not shown.

(B) The two-dimensional plot of individual EPSP responses in the RS1 cell (Reference) and the RS2 cell (Test). All-or-none responses in the RS1 and RS2 cells were highly correlated. A gray dotted line indicates 1.6 × RMS of the baseline noise in the Test RS2 cell.

Figure 4. Quantitative analysis of the divergent thalamocortical inputs

(A1) Summary data of the divergent thalamocortical inputs onto 29 pairs of RS and FS cells, when we regarded the RS cells as reference cells. Thus, we only analyzed RS-FS pairs in which the RS cells received EPSPs larger than 2 mV. Filled circles (28/29; 96.6%) indicate RS-FS pairs which received EPSP inputs from the same TC fibers. An open circle (1/29; 3.4%) indicates an RS-FS pair which received EPSP inputs onto only the RS cell. Note that nearly all of the TC fibers generating EPSP inputs onto RS cells also generated EPSP inputs onto FS cells. See the legend of Fig. 2B to calculate the mean EPSP amplitudes on the horizontal and vertical axes.

(A2) Summary data of the divergent thalamocortical inputs onto 54 pairs of RS and FS cells, when we regarded the FS cells as reference cells. Thus, we only analyzed RS-FS pairs in which the FS cells received EPSPs larger than 2 mV. Filled circles (29/54; 53.7%) indicate RS-FS pairs which received EPSP inputs from the same TC fibers. Open circles (25/54; 46.3%) indicate RS-FS pairs which received EPSP
inputs onto only the FS cells.

(B) Summary data of the divergent thalamocortical inputs onto 23 pairs of RS1 and RS2 cells. RS cells, which received larger thalamocortical EPSPs, were considered RS1 cells and were treated as reference cells. Filled circles (10/23; 43.5%) indicate RS-RS pairs which received EPSP inputs from the same TC fibers, whereas open circles (13/23; 56.5%) indicate RS-RS pairs which received EPSP inputs onto only one member of the RS-RS pairs.

Figure 5. Convergent synaptic inputs from multiple thalamic fibers onto layer 4 FS cells

(A) Experimental design for detecting convergent synaptic inputs from multiple TC fibers onto FS cells in the thalamocortical slices. After simultaneous patch-clamp recording from three cortical cells (two RS cells and one FS cell), the correlation-based paradigm was repeatedly applied at different spots inside the ventrobasal thalamus, to stimulate different TC fibers. $T$, ventrobasal thalamus; $Cx$, somatosensory cortex.

(B1) Thalamocortical inputs onto three cortical cells (RS1, RS2, and FS), evoked by stimulation of three TC fibers (TC1 - TC3). The three TC fibers were separately stimulated by repeating the correlation-based paradigm three times at different spots inside the ventrobasal thalamus. In each correlation-based paradigm (i.e., each TC stimulation), reference connections onto the RS1 or RS2 cells were established (indicated by asterisks), and the connectivity onto the test FS cells was examined. Thus, the two RS cells were used just for reference connections. Note that the FS cell received EPSP inputs from all three of the TC fibers (averaged responses in the
case of *Ref Success* or *Ref Failure* are shown). EPSP amplitudes in the RS1 and RS2 cells were different across each correlation-based paradigm (*TC1 Stim. - TC3 Stim.*), indicating that we stimulated three different TC fibers independently.

(B2) EPSP amplitudes in the RS1 and RS2 cells, evoked by different TC fibers (TC1 - TC3). These were obtained from (B1). Each data point represents the mean ± SD of individual EPSP amplitudes in the case of *Ref Success*. EPSP amplitudes in the RS1 and RS2 cells are indicated by squares (TC1), circles (TC2), and triangles (TC3). Patterns of the EPSP amplitudes in the RS1 and RS2 were different between TC1 and TC2, between TC2 and TC3, and between TC3 and TC1 (*P* < 0.05 for each comparison, two-way repeated measures ANOVA), confirming the independence of the stimulated TC fibers.

(C) Amplitude distribution of convergent thalamocortical inputs onto FS cells, obtained from 7 RS-RS-FS triplets (#1 to #7). Multiple (two to three) thalamocortical inputs were examined onto each FS cell. Circles and crosses indicate the presence and absence of thalamocortical inputs onto FS cells, respectively.

**Figure 6. Divergent synaptic inputs from FS cells onto adjacent pairs of RS cells**

(A) Representative records of unitary IPSPs onto adjacent pairs of RS cells, elicited by single action potentials in presynaptic FS cells. About 15 responses are superimposed (left) and averaged (right) in both (A1) and (A2). Two RS cells received divergent inhibitory inputs in (A1), but not in (A2).

(B) Summary data of unitary IPSPs onto 41 pairs of RS1 and RS2 cells, elicited by single action potentials in FS cells. RS cells, which received larger IPSPs, were considered RS1 cells. Of the 41 pairs, 28 pairs (68.3%) received divergent
inhibitory inputs from the same FS cells (indicated by filled circles). Open circles indicate RS-RS pairs, only one of which received IPSPs from FS cells. The IPSP amplitudes in RS1 and RS2 cells were correlated, and the correlation coefficient was 0.77.

(C) Schematic diagram of the feedforward inhibitory circuit in the somatosensory thalamocortical pathway. FS cells receive convergent excitatory inputs from multiple TC cells, and send divergent inhibitory outputs to multiple RS cells. In addition, spikes in TC cells, which generate EPSPs in RS cells, also generate EPSPs in adjacent FS cells with high fidelity. T, ventrobasal thalamus; Cx, somatosensory cortex.
Figure 1:
Figure 2:

A

Minimal Stimulation to Reference Neuron (RS1)

All

Individual

RS1 (Reference)

RS2

FS

2 mV

1 mV

2 mV

4 ms

B

C

Test FS EPSP (mV)

Reference RS1 EPSP (mV)

Test RS2 EPSP (mV)

Reference RS1 EPSP (mV)
Figure 3:

A

Minimal Stim. to RS1

RS1 (Ref)

RS2

* 2 mV

10 ms

B

Reference RS1 EPSP (mV)

Test RS2 EPSP (mV)

Ref Failure

Ref Success
Figure 4:

A1: Reference: RS cell
A2: Reference: FS cell
B: Reference: RS1 cell

[Scatter plots showing relationships between Mean FS EPSP (mV) and Mean RS EPSP (mV).]
Figure 5:

A

Patch-Clamp

TC1 Stim.

TC2 Stim.

Repeat

B

TC1 Stim.  TC2 Stim.  TC3 Stim.

RS1

RS2

FS

B1

C

Mean FS EPSP (mV) vs. Cell number
Figure 6:

A1 — Divergence (Average) — A2 — No divergence (Average)

FS

RS1

RS2

B

C

RS2 unitary IPSP (mV)

RS1 unitary IPSP (mV)

Divergence

RS1 Only

excitatory

inhibitory