(Synthecell Vega, Columbia, MD), 200 μM dNTPs (Boehringer Mannheim), and 2 U of Taq polymerase (Perkin-Elmer). An amount of antisense primer end-labeled with [γ-^{32}P]adenosine triphosphate (Amersham, Arlington Heights, IL) corresponding to 500,000 to 1,000,000 cpm/μl was also added. Amplification was performed in the GeneAmp PCR System 9600 (Perkin-Elmer Cetus) with the following incubation times: 30-s denaturation at 94°C (60 s for the first cycle only), 45-s annealing at 60°C (55°C for the Cα primer pair), and 60-s extension at 72°C. Products of amplification were analyzed by electrophoresis in 5%, 29:1 polyacrylamide gels and visualized by autoradiography. The intensity of the radioactive signal for each cytokine was measured with a PhosphorImager (Molecular Dynamics, Sunnyvale, CA). Semiquantitative analysis was performed with a modification of the method described in (*9*). A simple regression curve was fitted for the twofold dilutions of the standard cDNA, and the equation obtained was used to determine the amount of target sequence in the patient samples. Results were expressed as fold increase over the positive control. The positive control corresponds to the last dilution (1:6400) of the standard cDNA in which amplified products (positive signal) may be detected.

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20. To obtain purified CD4$^+$ cell populations, we resuspended 15 × 10^7 PBMCs in phosphate-buffered saline (PBS; ×1) at 20 × 10^6 cells per milliliter, and monoclonal antibodies to the Fc receptor (1 μg per 10^6 cells), CD8 (2 μg per 10^6 cells), and CD19 (5 μg per 10^6 cells) were added at the appropriate concentrations. Cells were incubated in the presence of the different antibodies at 4°C for 45 min. At the end of the incubation, cells were washed three times in cold PBS, resuspended in cold PBS containing 2% fetal bovine serum (FBS) at 5 × 10^6 to 7 × 10^6 cells per milliliter, and 7.5 ml of the cell suspension was plated in petri dishes that were precoated with goat antibody to mouse (Sigma) and incubated at 4°C for 45 min. At the end of the incubation, cells were harvested (petri dishes were washed five times with 5 ml of PBS), washed, and resuspended in RPMI + 20% FBS at 10^7 cells per milliliter. After this negative selective procedure, the percentage of CD4$^+$ cells ranged between 80 and 90%. The contaminating cells included CD8$^+$ cells (<5%) monocytes, B cells, and NK cells. To perform in vitro stimulation, we incubated purified CD4$^+$ cell populations (5 × 10^6 cells per 0.5 ml) with purified antibody to CD3 (8 μg/ml) on ice for 60 min. Then, to induce cross-linking of the CD3 molecule, we added goat antibody to mouse (50 μg/ml) and incubated the cells at 37°C for 4 hours. At the end of the incubation, we prepared cell pellets for the analysis of cytokines by PCR and stored them at −80°C until they were used.

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The Thermal Grill Illusion: Unmasking the Burn of Cold Pain

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In Thunberg's thermal grill illusion, first demonstrated in 1896, a sensation of strong, often painful heat is elicited by touching interlaced warm and cool bars to the skin. Neurophysiological recordings from two classes of ascending spinothalamic tract neurons that are sensitive to innocuous or noxious cold showed differential responses to the grill. On the basis of these results, a simple model of central disinhibition, or unmasking, predicted a quantitative correspondence between grill-evoked pain and cold-evoked pain, which was verified psychophysically. This integration of pain and temperature can explain the thermal grill illusion and the burning sensation of cold pain and may also provide a basis for the cold-evoked, burning pain of the classic thalamic pain syndrome.

The sensations of pain and temperature stem from parallel ascending sensory channels that are regarded as physiologically separate (*1*). However, these sensations can be shown to interact. In 1896, Thunberg reported that innocuous warm and cool stimuli applied simultaneously to the skin by means of interlocking spiral tubes elicited a sensation of strong heat, which he compared to the burning sensation that commonly accompanies cold pain (*2*). We investigated the cause of this illusion with neurophysiological and psychophysical methods.

The prevailing explanation of the thermal grill illusion is based on Alrutz's proposal that the perception of "heat" (evoked at temperatures between 45° and 50°C) is not a specific sensation but rather a fusion resulting from the simultaneous activation of specific warm and cold spots (*3*). (Cold spots can be activated by cooling and also, paradoxically, by high temperatures.) The grill was thought to evoke this fusion by the simultaneous activation of sensory channels for warmth and cold by warm and cool, rather than hot, temperatures. In the 1920s, several experimental psychologists concurred with this proposal, whereas others concluded that the sensation evoked by the grill was more tactual, like a pricking sensation. One group questioned whether the illusion was due to suggestion and to the confusion resulting from an unnatural stimulus.

Modern physiological findings have confirmed the existence of specific cutaneous receptors for warm and for cold. However, many warm receptors cease their discharge at temperatures above 45°C and are thus not active at high temperatures (*1*). Instead, specific heat nociceptors have thresholds around 45°C, which is now the accepted threshold for perception of heat pain (*1, 4*). These findings contradict the fusion hypothesis, because nociceptors, but not warm receptors, are activated by high temperatures, whereas warm receptors, but not nociceptors, are activated by the grill.

We considered the alternative hypothesis that the grill illusion results from an unmasking rather than a fusion. Thunberg suggested that fusion could be shown if a selective block of the sensory channel for warmth enabled a hot stimulus to elicit a cold sensation (*2*), but in fact the converse occurs. Selective elimination of sensibility to cold, but not warmth (produced by a pressure block of peripheral, cold-specific, Aδ nerve fibers), actually enables a cold stimulus (at temperatures up to 25°C) to elicit a burning heat sensation (*5*). The primary afferents responsible are probably C polymodal nociceptors, many of which respond to cold as well as to heat and sometimes to pinch (*5, 6*). Thus, we hypothe-

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sized that the heat sensation evoked by the grill reflects the central unmasking (or disinhibition) of the cold-activated C polymodal nociceptive channel because of a reduction in specific cold activity by the simultaneous warm stimulus. This we tested by directly examining the central sensory channels physiologically.

We first characterized the illusion with quantitative psychophysical methods. Thermal stimuli were presented to the palmar surface of the dominant hand of 11 physiologically normal people with a thermode (20 by 14 cm) (Fig. 1) consisting of 15 parallel silver bars, each 1 cm wide (*7*). Using double-blind procedures, we presented three different 50-s stimuli three times each in pseudo-random, counterbalanced order: "Warm," in which the temperature of the entire surface of the skin-thermode interface was raised from the baseline of 34°C to 40°C; "Cool," in which the interface temperature was lowered to 20°C; and "Grill," in which the even-numbered bars were warmed to 40°C and the odd-numbered bars were cooled to 20°C.

During each 3-min intertrial interval, the participants used three visual analog scales (VAS) to independently report sensations of cold, heat, and pain, and they selected words from a list of descriptors (*8*). They rated the Warm stimulus as moderately hot but not painful and the Cool stimulus as moderately cold but not painful (Fig. 2). In contrast, nearly all participants (10 out of 11) rated the Grill stimulus as painful (an average of 12.5 on the 50-mm VAS scale, $P < 0.001$). They also rated Grill as hotter than Warm ($P < 0.03$) and as less cold than Cool ($P < 0.002$). In addition, they assigned more pain-related descriptors to Grill than to Warm or Cool ($P < 0.001$), and words such as stinging, smarting, and burning were used nearly exclusively for Grill (by 10 participants out of 11). Thus, the Thunberg thermal grill illusion is a robust phenomenon. The salient feature of this illusion is that the addition of interlaced warm bars to a cool stimulus results not only in a diminished sensation of cold but also in a sensation of painful heat.

We examined the physiological activity in the ascending thermosensory channels by using the same stimulus paradigm with a smaller thermode (4 by 4 cm) and recording from spinal cord neurons that project to the brain in the spinothalamic tract (STT), which is classically associated with pain and temperature sensation. We focused on STT cells in lamina I of the dorsal horn because: lamina I is the nearly exclusive termination site of cold-sensitive C polymodal primary afferent fibers and (probably) of cold-specific Aδ fibers as well (*9*, *10*); lamina I contains a unique concentration of thermoreceptive-specific neurons (*1*, *10*); and lamina I STT axons ascend in the behaviorally critical location for temperature and pain sensation (*11*). The more commonly studied STT cells in the deep dorsal horn that have a wide dynamic range (as well as nociceptive cells in other locations) differ in all these respects and reportedly respond to cold only at temperatures below 15°C (*12*). We examined all three major types of cat lamina I STT neurons (*10*): nociceptive-specific (NS) cells that are responsive only to noxious heat or pinch (or both) and receive input from heat nociceptors, thermoreceptive-specific (COLD) cells that are responsive to cooling and receive input from specific cold receptors, and multimodal cells that are responsive to noxious heat, pinch, and cold (HPC) and receive input from cold-sensitive C polymodal nociceptors.

We recorded from 5 NS, 10 COLD, and 7 HPC lamina I STT cells in the lumbosacral cord of anesthetized cats that had receptive fields on the glabrous hind paw (*13*). As was consistent with their thermal stimulus-response functions (Fig. 3A), none of these cells were activated by the Warm stimulus; rather, the Warm stimulus partially inhibited the baseline (34°C) ongoing discharge of COLD cells. As expected, NS cells were unaffected by the Warm, Cool, or Grill stimuli. Also as expected, both COLD and HPC lamina I STT cells responded briskly to the Cool stimulus (Fig. 3, B through D). However, the responses of COLD and HPC cells to the Grill stimulus differed significantly. The responses of COLD cells to Grill were strongly reduced from their responses to Cool (to 51%), but the responses of HPC cells to Grill remained nearly the same as their responses to Cool (84%, $P < 0.003$) (*14*). Thus, in

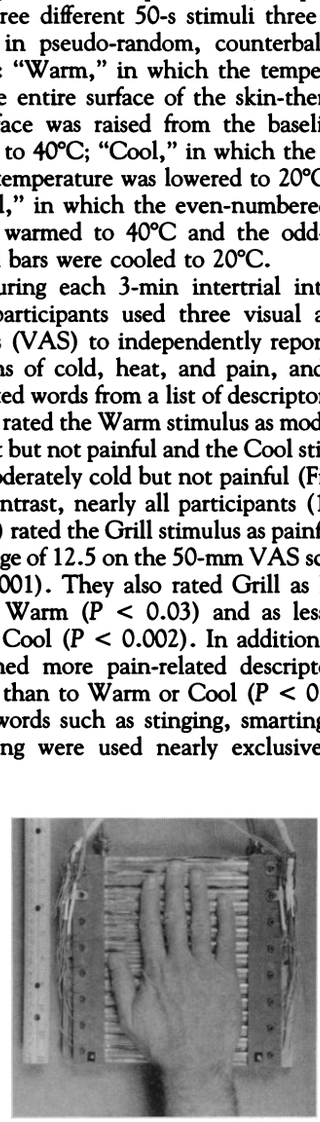

Fig. 1. The thermode used with human participants. The stimulus surface (20 by 14 cm) was made of 15 1-cm-wide sterling silver bars, set about 3 mm apart. Underneath each bar were three longitudinally spaced thermoelectric (Peltier) elements (1 cm^2), and a thermocouple was located on top of each bar. Alternate (even- and odd-numbered) bars could be controlled independently.

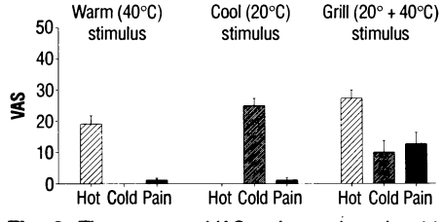

Fig. 2. The average VAS ratings given by 11 participants in response to the Warm, Cool, and Grill stimuli. Ratings were made immediately after each stimulus on a 50-mm scale for each of the three sensations: heat ("not at all warm" to "extremely hot"), cold ("not at all cool" to "extremely cold"), and pain ("not at all painful" to "extremely painful").

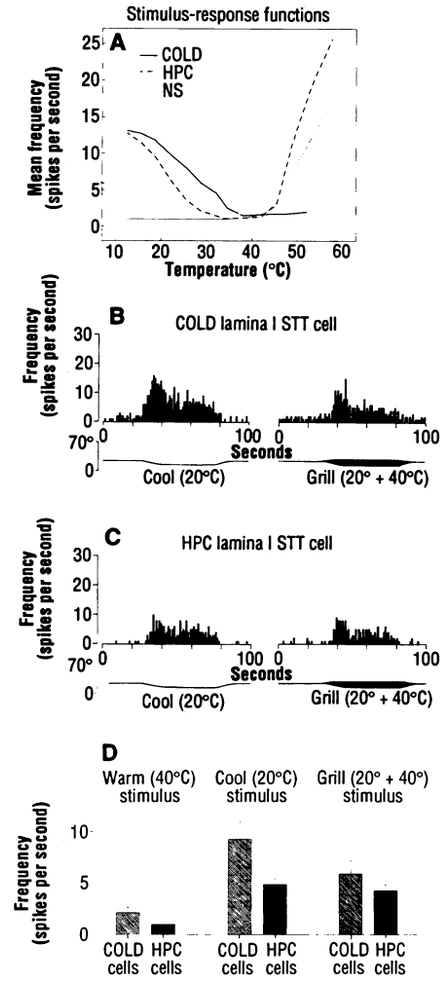

Fig. 3. The physiological characteristics of the recorded lamina I STT neurons. (**A**) Quantitative thermal stimulus-response functions obtained with computer-controlled temperature steps applied with a Peltier stimulator (4 by 4 cm) and plotted against the skin-thermode interface temperature. (**B** and **C**) Single-event peristimulus time histograms showing the activity evoked in a COLD lamina I STT cell and in an HPC lamina I STT cell by the Cool and Grill stimuli (bins, 1 s; stimulus durations, 50 s). (**D**) The average discharge rates of COLD and HPC cells, which show the Grill-induced reduction in COLD cell discharge and the minimal change in HPC cell activity.

parallel with the psychophysical findings, the salient physiological feature of the grill is that the addition of interlaced warm bars to a cool stimulus not only reduces the activity in the cold-specific channel but also shifts the relative pattern of activity in favor of the HPC (C polymodal) channel.

The grill-induced reduction of the COLD channel activity results in a moderate amount of HPC cell activity in the presence of a relatively low amount of COLD cell activity. This is the pattern of activity normally evoked by a moderately strong heat stimulus (Fig. 3A), which is consistent with the sensation of heat that is evoked by the grill (and also with the cool-evoked sensation of heat felt after peripheral nerve block). This pattern of HPC channel and COLD channel activity also resembles that caused by a painfully cold stimulus, albeit at lower absolute levels, because HPC cells become more active at low temperatures and COLD cell activity plateaus at temperatures below 15°C (Fig. 3A) (*15*). Thus, these results are consistent with Thunberg's inference that the grill-evoked sensation of heat resembles the burning sensation elicited by noxious cold, albeit without the intense cold component. These results are supported by the observation that systemic morphine suppresses cold and heat pain and inhibits the activity of HPC and NS lamina I STT cells but does not affect perception of coolness and actually enhances the activity of COLD cells (*16*).

A simple integrative model that compares HPC and COLD activity can explain the grill illusion. The difference in HPC cell activity and COLD cell activity (HPC minus COLD) forms a U-shaped curve that is monotonically increasing in the noxious heat range and again in the noxious cold range, where it is a linear function of decreasing temperature from a threshold of about 25°C (Fig. 4A). The grill produces a shift along this integrative function. Such integration could be performed by cells in the thalamus or cortex that are excited by HPC activity and inhibited by COLD activity (*17*). Thus, our findings indicate that the thermal grill illusion is a central disinhibitory phenomenon in which the reduction of inhibition induced by the COLD channel exposes (or unmasks) the cold-sensitive activity of the HPC channel.

To test our model, we plotted the mean (HPC minus COLD) shift for the Grill stimulus in order to obtain the uniform low temperature that would naturally evoke this difference (Fig. 4A). The model predicts that a stimulus at this temperature should produce a sensation with a pain component equivalent to that evoked by the grill—that is, a sensation of tingling, stinging, smarting, or burning. Extrapolation from the physiological data predicted this temperature to be about 11°C. We then examined the psychophysical responses of the same participants to a series of uniform cold stimuli presented with the same thermode (*18*). Their VAS ratings of cold pain increased significantly at each temperature below 18°C (Fig. 4B), and they consistently used the above descriptors at those temperatures. Interpolation from these cold-pain ratings indicated that a temperature of about 10°C produced an average pain rating (12.5) corresponding to that produced by the thermal grill. Thus, the prediction made by the integrative physiological model was verified.

We conclude that the thermal grill illusion demonstrates the central integration of ascending pain and temperature sensory channels. Whereas our model can be further tested by examination of integration in thalamic and cortical cells and by extension of the cross-species comparison to primates, this study supports growing evidence that innocuous cold inhibits central pain processing (*5*, *19*), a finding that has everyday clinical importance. This integrative model also provides a testable hypothesis to explain the burning pain and cold allodynia that are characteristic of classic thalamic (central post-stroke) pain syndrome, which, similar to the thermal grill, is nearly always characterized by a reduction or even a complete loss of specific cold sensation (*20*).

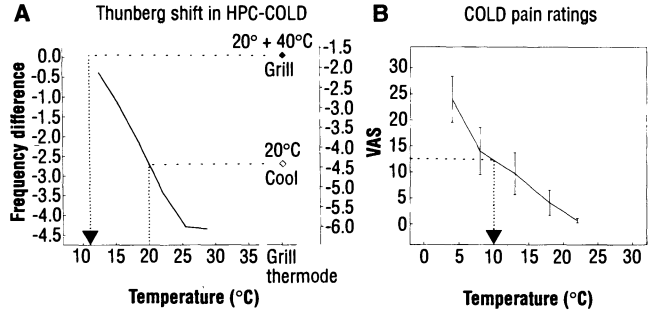

Fig. 4. Verification of the prediction of the integrative model by the observed psychophysical results. (**A**) The empirical difference function (HPC minus COLD) generated by the physiological model, based on the data obtained with the Peltier thermode (4 by 4 cm) (scale at left). The (HPC minus COLD) difference recorded with the grill thermode (a discontinuous surface) was mapped onto this function by equating the (HPC minus COLD) difference obtained at 20°C (scale at right). Extrapolation predicts that a uniform temperature of about 11°C would produce the same (HPC minus COLD) difference as the Grill stimulus. (**B**) The average pain ratings given by participants after 50-s cold stimuli, plotted against the skin-thermode interface temperature. The average pain rating of 12.5 (out of 50) given for Grill is indicated. Interpolation shows that a temperature of about 10°C would produce this average rating, which confirms the prediction of the physiological model.

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7. Participants were six males and five females between 21 and 55 years old who gave informed consent. The temperatures of alternate (even- and odd-numbered) sets of bars were separately controlled by computer-driven thermoelectric (Peltier) elements; the temperature of the skin-thermode interface was monitored with thermocouples. In the Grill stimulus, warming preceded cooling by 5 s, which Thunberg showed enhances the illusion (*2*).
8. Participants were given a common definition of pain: any uncomfortable sensation, such as pricking, stinging, or burning, even if the stimulus is tolerable. They were given a list of 15 words from the McGill Pain Questionnaire [R. Melzack, *Pain* **1**, 277 (1975)] and asked to choose any or no words to describe their sensation. The VAS ratings were averaged across the three presentations of each stimulus condition and within-subject analyses of variance were calculated.
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13. The recording methods have been described previously (*10*, *16*). Quantitative thermal stimuli were applied with a computer-controlled Peltier element (4 by 4 cm). The Cool, Warm, and Grill stimuli were applied with a thermode that had two Peltier elements driving an intervening interlaced

set of 3-mm-wide silver bars (separated by 1 mm) over a surface (4 by 4 cm) by passive thermal conduction. The skin-thermode interface temperature was used for analysis.
14. Response measures were normalized with (Grill-Warm)/(Cool-Warm). The cause of the selective decrease in COLD cell activity is presently undetermined but could reflect inhibition of COLD cells by the activity of warm afferent fibers.
15. The illustrated stimulus-response functions of the HPC and COLD lamina I STT cells studied with the grill show that, at low temperatures, the rate of firing of COLD cells approaches an asymptote and the rate of firing of HPC cells monotonically increases. Unpublished data obtained from other cells with colder probes indicate that HPC activity indeed continues to increase, whereas COLD activity does not, which is consistent with the behavior of C polymodal and cold-specific afferents, respectively (*1, 6*).
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Rearrangements of Synaptic Connections in Visual Cortex Revealed by Laser Photostimulation

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Assessing patterns of synaptic connections in the developing mammalian neocortex has relied primarily on anatomical studies. In a physiological approach described here, the patterns of synaptic connections in slices of developing ferret visual cortex were determined with scanning laser photostimulation. Functional synaptic inputs to pyramidal cells in cortical layers 2 and 3 originating from sites close to the neuronal cell body appeared at least 2 weeks before eye opening, prior to the formation of long-range horizontal connections. Extensive long-range horizontal connections appeared in the next 10 days of development. The number of local connections peaked at the time of eye opening; the number of these connections subsequently declined to the level found in the adult while the specificity of long-distance connections increased. Thus, the relative influence of local connections on the activity of layer 2 and layer 3 neurons declines as the cortex matures while the influence of longer range connections increases substantially.

Although neuronal activity participates in the development of circuitry in the visual system (*1*), most insights into the organization of local cortical circuits and their development have derived from anatomical approaches (*2–5*) rather than from the direct assessment of functioning synaptic connections (*6*). During development, axonal branches are unstable (*7*) and the locations of synapses are difficult to determine, even with electron microscopy (*8*). Thus, although the basic anatomical features of local circuits in developing visual cortex are well established (*4, 5*), the relation between these patterns of anatomical projections and the functional interactions among individual neurons remains speculative.

Our investigations focused on the development of horizontal connections in layer 2 and layer 3 of ferret visual cortex. In adult visual cortex, horizontal projections link regions with similar functional properties, forming clusters of axon collaterals in specific regions (*2, 9*). These horizontal connections originate primarily from pyramidal neurons, form excitatory synapses on other pyramidal neurons and interneurons (*10*), and extend several millimeters in the tangential plane of the cortical plate. Anatomical studies have demonstrated that the characteristic patchy patterns of horizontal projecting axons in visual cortex are not present initially but emerge gradually from a more diffuse state by activity-dependent mechanisms. These mechanisms involve the growth of long, unbranched axons; this is followed by the elaboration of collaterals in appropriate locations and the selective retraction of collaterals in inappropriate regions (*4, 11*). However, the locations of functional synapses, if any, along these collaterals are unknown. We developed scanning laser photostimulation to determine the number, position, and relative strength of functional horizontal connections at different stages of development.

Scanning laser photostimulation is based on highly localized laser photolysis of caged neurotransmitters (*12*) in brain slices (*13, 14*). We recorded from single neurons using whole cell, patch-clamp techniques in tangential cortical brain slices (350 μm thick), while the slices were continuously perfused with artificial cerebrospinal fluid containing "caged" glutamate, which is inactive until photolyzed by ultraviolet light (UV, 330 to 380 nm). The localized uncaging of glutamate at any x, y, z coordinate in the slice causes a small number of neurons in the region of the laser spot (≈15 μm in diameter) to generate action potentials; if any of these neurons form synapses with the recorded cell, a monosynaptic postsynaptic current (PSC) is generated (*15*). Photouncaging at a large array of locations throughout the brain slice (up to 1500 sites, 50 μm apart, covering approximately 3.8 mm^2) produces a map of the locations that generate PSCs in the recorded cell (Fig. 1) without contamination by fibers of passage.

We examined the development of local intracolumnar (≤0.5 mm from the electrode) synaptic connections and long-distance intercolumnar (>0.5 mm) synaptic connections in a sample of 27 neurons from tangential brain slices of layer 2 and layer 3 of ferret primary visual cortex. Animals ranged in age from postnatal day 17 (P17, birth = P0) to adult (>P55). Experiments were carried out in ferret brain slices from three age groups, on the basis of the developmental state of the visual system. The first group (P17 through P26) corresponded to the period before eye opening when some layer 2 and layer 3 cells were still migrating (n = 8 cells), the second group (P27 through P40) was from the period just before and shortly after eye opening (n = 12 cells), and the third group (mature, P41 through adult) consisted of slices obtained after eye opening and included adults (n = 7 cells).

In mature cells, stimulation at most sites (82.4 ± 3.6%) did not elicit PSCs in the postsynaptic cell (Fig. 1) (*16*). However, stimulation at several zones approximately 1 mm from the cell body evoked PSCs in the postsynaptic neuron. Because of their size, spacing, and location, these groups of functional synaptic inputs are likely to originate from the clustered axonal arbors of pyramidal cells that have been anatomically

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