

# Fine-scale specificity of cortical networks depends on inhibitory cell type and connectivity

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**Excitatory cortical neurons form fine-scale networks of precisely interconnected neurons. Here we tested whether inhibitory cortical neurons in rat visual cortex might also be connected with fine-scale specificity. Using paired intracellular recordings and cross-correlation analyses of photostimulation-evoked synaptic currents, we found that fast-spiking interneurons preferentially connected to neighboring pyramids that provided them with reciprocal excitation. Furthermore, they shared common fine-scale excitatory input with neighboring pyramidal neurons only when the two cells were reciprocally connected, and not when there was no connection or a one-way, inhibitory-to-excitatory connection. Adapting inhibitory neurons shared little or no common input with neighboring pyramids, regardless of their direct connectivity. We conclude that inhibitory connections and also excitatory connections to inhibitory neurons can both be precise on a fine scale. Furthermore, fine-scale specificity depends on the type of inhibitory neuron and on direct connectivity between neighboring pyramidal-inhibitory neuron pairs.**

The precision of cortical connections is manifested at many different levels of organization. The cerebral cortex is parceled into functionally and anatomically distinct areas, each of which connects to distinct subsets of the other areas<sup>1</sup>. And within each area, specific connections create and respect laminar and columnar functional architecture<sup>2–8</sup>. Within any one of these laminar or columnar modules there are numerous inhibitory and excitatory neuron types whose dendritic and axonal arbors are intimately entangled. Yet, even at this level of organization, connections remain specific such that axonal arbors that terminate in a given region selectively connect to some cell types while avoiding others<sup>9–21</sup>. Thus, neighboring neurons of different types receive input from different cortical layers. Neighboring neurons of the same type, however, tend to receive local input from the same cortical layers.

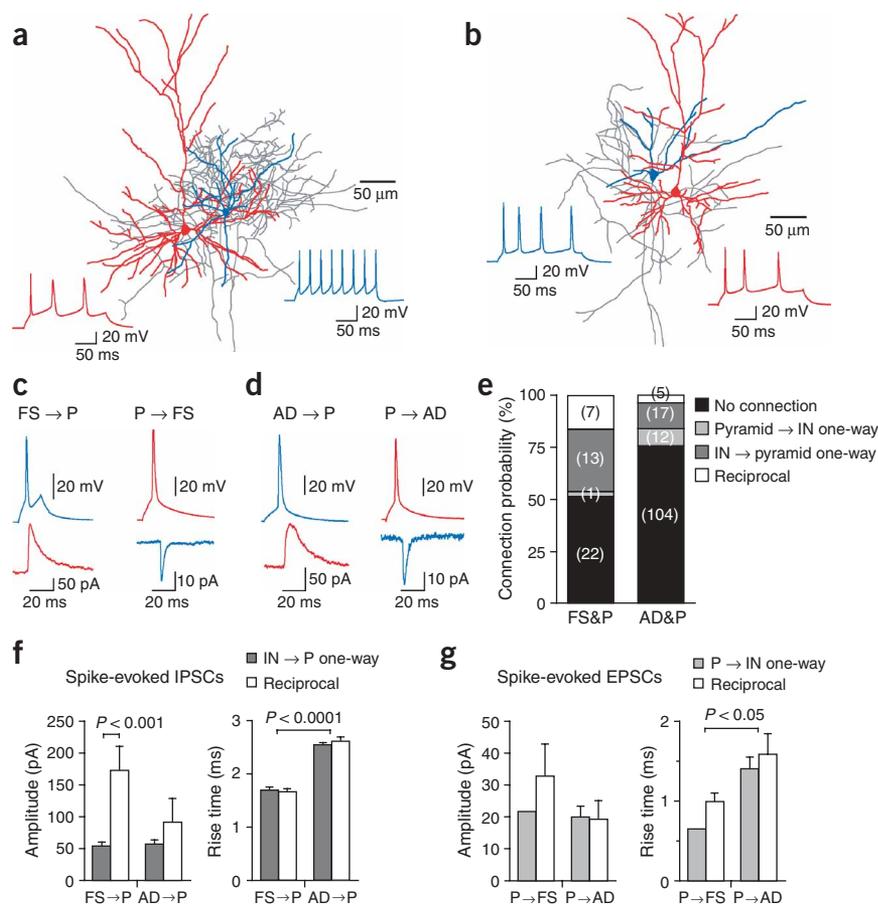
Most recently, it has been found that even neighboring neurons of the same anatomical type can, nevertheless, receive input from different sources<sup>22,23</sup>. This ‘fine-scale’ specificity has been demonstrated using methods that allow differences in input to neighboring neurons to be probed with high resolution. For example, by simultaneously recording from (and stimulating) three or more neighboring layer 5 pyramidal neurons, it was found that when two neurons are connected to each other, the probability that they will share input from a third neuron is higher than expected in a randomly connected network<sup>22</sup>. In previous work, we combined simultaneous recordings from pairs of layer 2/3 pyramidal neurons with laser scanning photostimulation<sup>23</sup>. This stimulation method evokes action potentials in a small, spatially confined population of neurons and can be repeated rapidly at many different stimulation sites. As the population of neurons that is stimulated fires action potentials relatively asynchronously, cross-correlation analyses

of the synaptic currents recorded in neuron pairs can be used to determine whether they share common input on a fine spatial scale. This study showed that fine-scale specificity of inputs to layer 2/3 pyramidal neurons depends on the laminar source of the excitatory input and differs for excitatory versus inhibitory inputs. Layer 2/3 pyramids share common input from layer 4 and from within layer 2/3 only in the minority of cases in which they are directly connected to each other. But they share common inhibitory input and excitatory input from layer 5 regardless of their direct connectivity. The excitatory neurons within layers 4 and 2/3 therefore create preferentially connected subnetworks embedded within the laminar and columnar functional architecture.

In this study, we investigated whether these fine-scale excitatory subnetworks might have precise relationships to inhibitory neurons. Can inhibitory connections be specific on a fine scale? Is there fine-scale specificity of excitatory connections to inhibitory neurons, and does it depend on the inhibitory cell type? Does the direct connectivity between neighboring inhibitory and excitatory neurons depend on whether they are part of the same fine-scale excitatory subnetwork? We found that fast-spiking inhibitory neurons in cortical layer 2/3 connect preferentially to neighboring pyramidal neurons that provide them with reciprocal excitation. We also found that these reciprocally connected fast-spiking interneuron–pyramidal cell pairs shared common fine-scale excitatory input and thus belonged to the same fine-scale subnetworks. Other cell pairs did not share common fine-scale excitatory input: these pairs included fast-spiking interneuron–pyramidal cell pairs with one-way or no connections and all pairs involving adapting neurons. These findings have implications for understanding how connections from different types

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**Figure 1** Direct connections between layer 2/3 inhibitory neuron–pyramidal neuron pairs. **(a,b)** Anatomical reconstructions and intrinsic firing properties for neuron pairs consisting of a simultaneously recorded pyramidal neuron (red dendrites) and inhibitory neuron (blue dendrites and grey axons). Intrinsic firing properties indicated that the inhibitory neuron in **a** was fast-spiking (FS) and the inhibitory neuron in **b** was adapting (AD). Direct connectivity between neuron pairs was assessed by recording postsynaptic currents during voltage clamp in one cell while evoking action potentials during current clamp recording in the other cell **(c,d)**. **(c)** Results from a reciprocally connected pyramidal neuron–FS interneuron pair. **(d)** Results for a pyramidal neuron–AD interneuron pair. Recordings from pyramids are red and from inhibitory neurons are blue. **(e)** Percentage of cell pairs of each type that were either not connected, connected one way from the interneuron to the pyramid, connected one way from the pyramid to the interneuron, or reciprocally connected. Numbers of cell pairs are indicated in parentheses. **(f)** Comparison of amplitudes and rise times (mean  $\pm$  s.e.m.) of inhibitory postsynaptic currents (IPSCs) measured in pyramids after stimulation of FS interneurons or AD interneurons for pairs with one-way versus reciprocal connections. **(g)** Comparison of amplitudes and rise times (mean  $\pm$  s.e.m.) of EPSCs measured in FS interneurons or AD interneurons after stimulation of pyramids, for pairs with one-way versus reciprocal connections.

of inhibitory neurons mediate interactions within and across excitatory subnetworks.

## RESULTS

The methods used in this study were identical to those described previously<sup>23</sup>. Whole-cell recordings were made simultaneously from two neighboring layer 2/3 neurons in rat visual cortex slices. We then tested the two neurons to determine whether they were connected to each other by stimulating one of the neurons and measuring postsynaptic currents in the other. We then used focal uncaging of glutamate (photostimulation) to generate action potentials in small, spatially confined populations of neurons. Control experiments showed that only neurons within about 50  $\mu$ m from the stimulation site fire action potentials<sup>23</sup>. We stimulated hundreds of sites spanning all cortical layers while recording excitatory postsynaptic currents (EPSCs) during voltage clamp. We then conducted cross-correlation analyses on the EPSCs recorded from the two cells. Shifted correlograms (from nonmatched stimulation trials) were subtracted from matched correlograms to determine the number of synchronous EPSCs that could be attributed to common input to the two neurons rather than stimulus-evoked time locking of action potentials in different presynaptic neurons<sup>23,24</sup>. These ‘shared’ EPSCs were then expressed as a percentage of all evoked EPSCs to calculate the ‘correlation probability’ (CP). The CP represents the proportion of all evoked EPSCs that were attributable to common input from the same presynaptic neurons. CPs were calculated separately for EPSCs evoked by photostimulation at sites in each of the cortical layers.

These studies differ from previous studies only in that instead of targeting intracellular recordings to pairs of neighboring pyramidal

neurons, differential interference contrast (DIC) optics were used to target recordings to one pyramidal neuron and one inhibitory interneuron (**Fig. 1a,b**). Inhibitory neurons were classified as either fast-spiking or adapting on the basis of their intrinsic firing properties in response to intracellular current injection (**Fig. 1a,b**; see Methods)<sup>18,25,26</sup>. ‘Fast-spiking’ interneurons are identified by the lack of spike rate adaptation, and many previous studies have shown that these are parvalbumin-expressing basket cells<sup>27</sup>. Adapting interneurons potentially include any of several other distinct cell types, but they are clearly distinguished from fast-spiking inhibitory neurons in terms of their morphology, physiology, gene expression and connectivity<sup>18,25–28</sup>.

### Fast-spiking interneuron–pyramid pairs

Direct connections were tested between 43 layer 2/3 neuron pairs consisting of one pyramidal neuron and one fast-spiking inhibitory neuron (**Fig. 1**). (Photostimulation data were collected for 23 of these same cell pairs; see below.) For 51% of these cell pairs (22 of 43) there were no detectable connections between cells in either the inhibitory-to-excitatory or the excitatory-to-inhibitory direction. Most of the connections that were present in the remaining 21 cell pairs were inhibitory. In particular, fast-spiking inhibitory neurons had a 47% chance (20 of 43) of being connected to each neighboring pyramidal neuron, whereas a connection from the pyramidal neuron onto its fast-spiking neighbor was detected in only 19% of pairs (8 of 43). Notably, however, when there was an excitatory connection from the pyramid to the fast-spiking interneuron, there was almost always (7 of 8 pairs or 88%) a reciprocal connection from the inhibitory neuron to the pyramid.

The probability of an inhibitory connection onto the pyramid was, therefore, highly dependent on whether the same cell pair also had an

**Figure 2** Cross-correlation analyses of photostimulation-evoked EPSCs simultaneously recorded in adjacent layer 2/3 neuron pairs consisting of one pyramidal neuron and one fast-spiking inhibitory neuron (FS). (a) Reciprocally connected pair. (b) Pair with a one-way inhibitory connection. Plots at left of each panel show for each cell (FS or pyramidal) reconstructions of the locations of photostimulation sites (colored squares) relative to the locations of laminar borders and cell bodies of recorded neurons (triangles represent pyramids; circles represent inhibitory neurons). The color of each square indicates the sum of amplitudes of EPSCs that were observed after photostimulation at that site, as indicated by the colored scales beneath each plot. Traces at center of panels show example voltage-clamp recordings for stimulation sites indicated by large numbered squares. Simultaneous recordings from the FS cell (blue) and the pyramidal cell (red) are shown for these representative stimulation sites. Short horizontal lines above each trace indicate the onset of photostimulation. Histograms to the far right of each panel show matched and shifted correlograms computed from data collected after stimulation in each layer. The corresponding CPs computed from these analyses are also indicated.

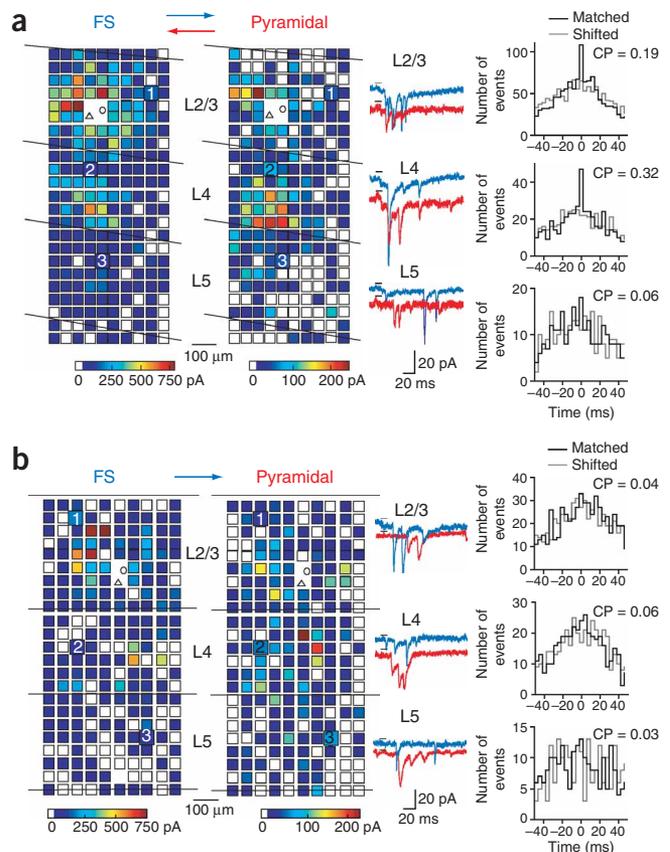
excitatory connection from the pyramid to the inhibitory neuron. In cell pairs without an excitatory connection, the probability that the inhibitory neuron would connect to the pyramid was only 37% (13 of 35 cases with no excitatory connection), but the probability of an inhibitory connection increased significantly, to 88% (7 of 8 cases with an excitatory connection) in cell pairs with an excitatory connection ( $P = 0.017$ ; Fisher Exact Test).

These observations indicate that connections between fast-spiking interneurons and neighboring pyramidal neurons are highly specific and not random. Fast-spiking inhibitory neurons connect relatively promiscuously to their neighboring pyramids, yet they still maintain a strong preference for the minority of pyramidal neurons that provide their direct excitatory input. Further specificity was also reflected in the strength of the connections from fast-spiking interneurons. For the cases in which the connection from the interneuron to the pyramid was not reciprocated, the IPSC amplitudes averaged only  $54.0 \pm 6.0$  pA (mean  $\pm$  s.e.m.; **Fig. 1f**). When there was a reciprocal excitatory connection, however, the IPSC amplitudes were more than threefold larger—a significant increase ( $172.2 \pm 37.9$  pA;  $P < 0.001$ ). Thus, not only are fast-spiking interneurons more likely to connect to neighboring pyramidal cells that reciprocate their connections, but these connections are also far stronger than unreciprocated connections.

### Adapting interneuron–pyramid pairs

We tested direct connectivity between 138 neuron pairs consisting of one adapting inhibitory neuron and one pyramidal neuron (**Fig. 1**). (Photostimulation data were collected for 38 of these same cell pairs; see below). An excitatory connection from the pyramid to the interneuron was detected in 12% (17/138) of pairs, and an inhibitory connection from the interneuron to the pyramid was detected in 16% (22/138) of pairs. Reciprocal connections were rare, occurring in only 3.6% (5/138) of pairs.

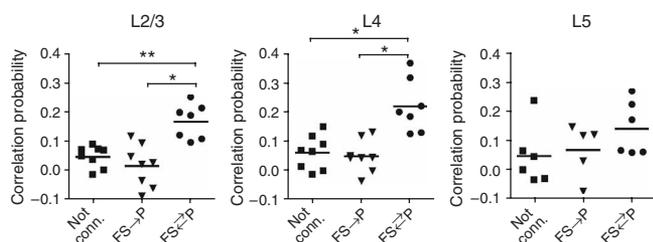
Unlike connections involving fast-spiking interneurons, for adapting interneurons, connections in one direction did not depend significantly on the presence of connections in the other direction. In particular, for the subset of 17 pairs in which there was a connection from the pyramid to the interneuron, it was reciprocated in 29% (5/17) of cases. This proportion does not vary significantly from the proportion of inhibitory connections in the pairs with no excitatory connection (14%, 17/121,  $P > 0.1$  Fisher Exact Test). Thus, adapting interneurons do not show the same preference as fast-spiking interneurons to connect preferentially to pyramids that provide them with excitatory input.



The size and kinetics of synaptic currents measured from direct connections between cell pairs also differed for adapting versus fast-spiking interneurons. Unlike fast-spiking interneuron–pyramid pairs, for pairs involving adapting interneurons, there was no significant difference in IPSC amplitudes between pairs with one-way versus reciprocal connections (**Fig. 1f**). And the synaptic currents for fast-spiking interneuron–pyramid pairs had significantly faster kinetics than those for pairs involving adapting interneurons. The rise times for IPSCs from fast-spiking interneurons were significantly shorter ( $1.7 \pm 0.04$  ms) than those from adapting interneurons ( $2.6 \pm 0.03$  ms;  $P < 0.0001$ ), probably owing to the more proximal location of synapses from fast-spiking basket cells relative to those from some types of adapting interneurons<sup>27</sup>. The rise times for EPSCs onto fast-spiking interneurons were also significantly shorter ( $0.95 \pm 0.10$  ms) than those onto adapting interneurons ( $1.5 \pm 0.13$  ms;  $P < 0.05$ ), probably owing to the presence of glutamate receptors with faster kinetics at the synapses on fast-spiking interneurons<sup>28</sup>.

### Shared input to FS interneuron–pyramid pairs depends on connectivity

As detailed above, fast-spiking (FS) interneurons connected preferentially to neighboring pyramidal neurons that provided them with reciprocal excitatory connections. Here we show that these same reciprocally connected cell pairs shared excitatory input from the same neurons in layers 2/3 and 4, whereas unconnected pairs and pairs with one-way inhibitory connections did not. We combined laser scanning photostimulation with paired whole-cell recordings to assess fine-scale specificity of connections (**Fig. 2**). **Figure 2a** illustrates an example for a cell pair consisting of a fast-spiking interneuron and a reciprocally connected pyramidal neuron. **Figure 2b** shows an example



**Figure 3** Correlation probabilities for EPSCs measured simultaneously in fast-spiking interneuron–pyramidal neuron pairs. Correlation probabilities are shown separately for photostimulation sites in each cortical layer and for cell pairs that either were not connected (squares), had a one-way inhibitory connection (inverted triangles) or were reciprocally connected (circles). Cell pairs with one-way excitatory connections were not sampled using photostimulation. Mean values for each group are indicated by horizontal lines. Brackets highlight statistically significant differences between groups (\*,  $P < 0.005$ ; \*\*,  $P < 0.0005$ ; Mann-Whitney test).

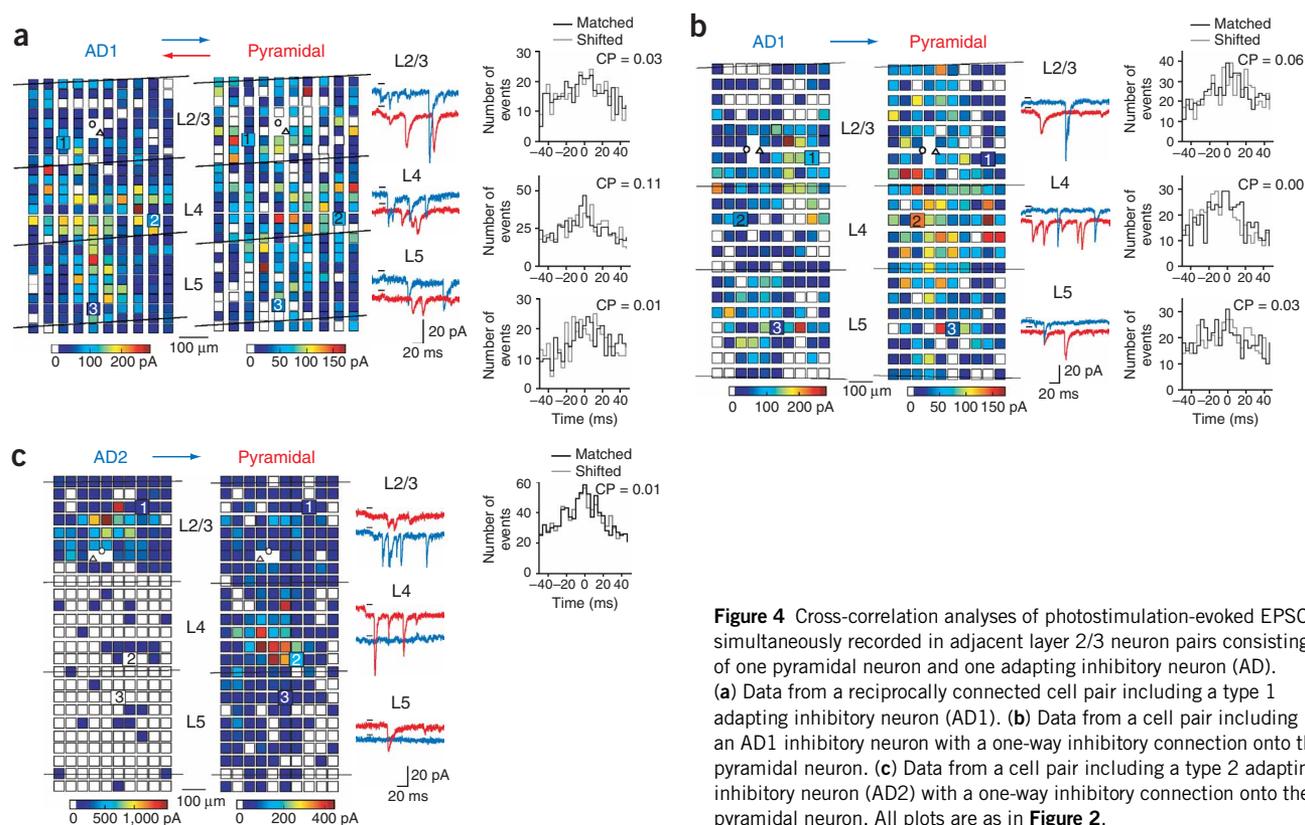
from a cell pair consisting of a fast-spiking neuron whose connection to the pyramidal neuron was not reciprocated. In both cases, photostimulation was used to generate action potentials at hundreds of sites across the cortical layers. Examples of photostimulation-evoked EPSCs for all four neurons from the example cell pairs are illustrated in **Figure 2**. Cross-correlation analyses show that some EPSCs occur nearly synchronously within the two simultaneously recorded neurons (see ‘zero’ bin in histograms to the right of **Fig. 2**), whereas some EPSCs do not (non-zero bins). Of those EPSCs that do occur synchronously, some result from common input to the two cells: that is, a single stimulated neuron that fired an action potential was connected to both recorded neurons. Other EPSCs occur nearly synchronously because

two different stimulated neurons, each connecting to a different recorded neuron, fired action potentials nearly synchronously.

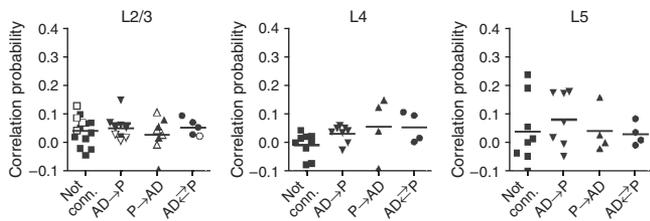
To determine how many of the synchronous EPSCs were due to common input, as opposed to synchronous action potentials in neurons providing non-shared inputs, we computed shifted correlograms and subtracted them from correlograms based on matched stimulation trials (see Methods)<sup>24</sup>. Previously published control experiments demonstrate that shifted correlograms accurately estimate the correlation of spike times in presynaptic, photostimulated neurons (see supplementary material in ref. 23). The remaining EPSCs in the zero bin can be attributed to shared input to the two recorded neurons. These remaining EPSCs were then compared with the total number of evoked EPSCs to calculate the CP; that is, the proportion of evoked EPSCs that come from inputs shared by both cells. For example, a CP of 0.19 from stimulation sites in layer 2/3 for the reciprocally connected cell pair in **Figure 2a** indicates that these two cells share 19% of their layer 2/3 excitatory inputs.

For the reciprocally connected cell pair (**Fig. 2a**), there were sharp peaks in the zero bin of the matched correlograms for stimulation sites in both layer 2/3 and layer 4. The CPs for this cell pair were 0.19 and 0.32 for layer 2/3 and layer 4, respectively. For layer 5 stimulation sites, there was not a sharp peak in the zero bin of the matched correlogram, and the CP was 0.06. For the cell pair with a one-way inhibitory-to-excitatory connection (**Fig. 2b**), the results from the cross-correlation analyses were very different: there were not sharp peaks in the zero bin of the matched correlograms for stimulation sites in any of the cortical layers. Thus, for this cell pair, CPs for all layers were relatively small (0.04 for layer 2/3, 0.06 for layer 4 and 0.03 for layer 5).

The trends illustrated for these two example cell pairs were representative of all 23 fast-spiking–pyramid pairs for which photostimulation data were collected. Reciprocally connected cell pairs shared more



**Figure 4** Cross-correlation analyses of photostimulation-evoked EPSCs simultaneously recorded in adjacent layer 2/3 neuron pairs consisting of one pyramidal neuron and one adapting inhibitory neuron (AD). (a) Data from a reciprocally connected cell pair including a type 1 adapting inhibitory neuron (AD1). (b) Data from a cell pair including an AD1 inhibitory neuron with a one-way inhibitory connection onto the pyramidal neuron. (c) Data from a cell pair including a type 2 adapting inhibitory neuron (AD2) with a one-way inhibitory connection onto the pyramidal neuron. All plots are as in **Figure 2**.



**Figure 5** Correlation probabilities for EPSCs measured simultaneously in adapting interneuron–pyramidal neuron pairs. Correlation probabilities are shown separately for photostimulation sites in each cortical layer and for cell pairs that either were not connected (squares), had a one-way inhibitory connection (inverted triangles), had a one-way excitatory connection (triangles) or were reciprocally connected (circles). Filled symbols indicate cell pairs including type 1 adapting interneurons, and open symbols indicate cell pairs including type 2 adapting interneurons. Mean values for each group are indicated by horizontal lines.

common input than did both unconnected cell pairs and cell pairs with one-way inhibitory connections (Fig. 3). For stimulation sites in layer 2/3, CPs for reciprocally connected cell pairs were high, averaging  $0.17 \pm 0.02$  (mean  $\pm$  s.e.m.,  $n = 7$  pairs), whereas the CPs were significantly lower for unconnected pairs (CP =  $0.05 \pm 0.01$ ,  $n = 8$ ,  $P < 0.0005$ , Mann-Whitney U test) and for pairs with one-way inhibitory connections (CP =  $0.01 \pm 0.03$ ,  $n = 8$ ,  $P < 0.005$ ). Results for layer 4 stimulation sites were similar to those for layer 2/3. CPs were high for reciprocally connected cell pairs (CP =  $0.22 \pm 0.03$ ,  $n = 7$ ) and significantly lower for unconnected pairs (CP =  $0.06 \pm 0.02$ ,  $n = 8$ ,  $P < 0.005$ ) and for pairs with one-way inhibitory connections (CP =  $0.05 \pm 0.02$ ,  $n = 8$ ,  $P < 0.005$ ). For stimulation sites in layer 5, CPs were calculated only when both cells in the pair received significant evoked excitatory input from that layer (see Methods). For stimulation sites in layer 5, the CPs for reciprocally connected cell pairs were larger than for other cell pairs (CP =  $0.14 \pm 0.04$ ,  $n = 6$  versus  $0.05 \pm 0.04$ ,  $n = 6$  for unconnected pairs and  $0.07 \pm 0.04$ ,  $n = 5$  for one-way inhibitory pairs), but the difference was not statistically significant ( $P > 0.09$ ).

### Adapting interneuron–pyramid pairs share little common input

We collected photostimulation data from 38 adapting interneuron–pyramidal pairs and found that, unlike pairs involving fast-spiking interneurons, these cell pairs did not share much common excitatory input, regardless of their direct connectivity. We separated adapting interneurons into two groups on the basis of the relative strength of excitatory input from different cortical layers<sup>18</sup>. Analysis of results from this data set (data not shown) confirmed previous published observations<sup>18</sup>, indicating that the laminar organization of local excitatory inputs to adapting interneurons distinguishes two nonoverlapping populations: type 1 adapting interneurons (AD1; 26 of 38 cells), which received more balanced input from layers 2/3, 4 and 5 (Fig. 4a,b), and type 2 adapting interneurons (AD2; 12 of 38 cells), which received the great majority of their excitatory input from layer 2/3 and very little (if any) from layer 4 or layer 5 (Fig. 4c). Therefore, for cell pairs including AD2 neurons, we calculated CPs only for stimulation sites in layer 2/3.

For adapting interneuron–pyramidal cell pairs, there were never sharp peaks in the zero bins of the matched correlograms, regardless of the adapting interneuron type, connectivity to the pyramidal neuron or the layer stimulated (see representative cross-correlation analyses of photostimulation-evoked EPSCs in Fig. 4). We plotted CPs according to the type of direct connectivity between the recorded neurons (unconnected, one-way inhibitory, one-way excitatory, reciprocal

and the layer stimulated (Fig. 5). Average CPs never exceeded 0.10 for any group. For stimulation sites in layer 2/3, the CPs of pairs including AD1 versus AD2 interneurons were indistinguishable ( $0.04 \pm 0.01$ ,  $n = 24$  pairs versus  $0.05 \pm 0.01$ ,  $n = 12$ ). Therefore, we combined data for AD1 and AD2 neurons for subsequent analyses. Analyzed according to direct connectivity between neuron pairs, CPs for photostimulation sites in layer 2/3 were  $0.04 \pm 0.01$  (mean  $\pm$  s.e.m.,  $n = 14$  cell pairs) for unconnected cell pairs,  $0.05 \pm 0.01$  ( $n = 9$ ) for pairs with one-way inhibitory connections,  $0.03 \pm 0.02$  ( $n = 8$ ) for pairs with one-way excitatory connections, and  $0.05 \pm 0.01$  ( $n = 5$ ) for pairs with reciprocal connections. For stimulation sites in layer 4 the corresponding CPs were  $-0.008 \pm 0.01$ ,  $n = 9$ ;  $0.03 \pm 0.01$ ,  $n = 8$ ;  $0.06 \pm 0.05$ ,  $n = 4$ ; and  $0.06 \pm 0.03$ ,  $n = 4$ ). For stimulation sites in layer 5 the CPs were  $0.04 \pm 0.04$ ,  $n = 8$ ;  $0.08 \pm 0.04$ ,  $n = 7$ ;  $0.04 \pm 0.04$ ,  $n = 4$ ; and  $0.03 \pm 0.02$ ,  $n = 4$ .

### DISCUSSION

We have shown that both inhibitory connections and excitatory connections to inhibitory neurons can be precise on a fine scale (Supplementary Fig. 1), using analysis of direct connections measured during paired recordings and cross-correlation analyses of photostimulation-evoked EPSCs. The fine-scale organization of connections between inhibitory and excitatory neurons depends, however, both on the inhibitory cell type and the organization of other connections within the network.

Analyses of direct connectivity during paired recordings showed that fast-spiking inhibitory neurons in layer 2/3 connect to their neighboring pyramids with a moderately high probability (47%). But this probability is highly dependent on whether the pyramid makes an excitatory connection to the fast-spiking interneuron. Usually the pyramid does not connect to the fast-spiking interneuron, and in these cases there is a one-way inhibitory connection in only 37% of the cell pairs. But when the pyramidal neuron does connect to the fast-spiking interneuron, the connection is nearly always (seven of eight pairs) reciprocated. In view of the fact that some connections are cut during preparation of brain slices, it could be that the true probability of reciprocity is closer to 100%. Not only was the probability of an inhibitory connection increased by more than twofold when there was an excitatory connection, but in the reciprocally connected cases, the IPSCs were more than threefold larger than in the case of one-way connections. Together these differences make the inhibition from fast-spiking interneurons strongly biased (by about sixfold) toward the same layer 2/3 pyramidal neurons that provide them with excitatory input.

A previously published study of connections between 243 fast-spiking interneuron–pyramidal neuron pairs showed no difference in the probability of inhibitory connections regardless of whether a cell pair had an excitatory connection<sup>29</sup>. Furthermore, in that study, excitatory connections were observed in more than half of the cell pairs tested (exact numbers were not reported), far more than the rate of 19% that we observe. These differences could be due to the use of recordings from a different cortical area, as data were combined for recordings made from ‘visual and somatosensory neocortical areas’ in that study. We believe it is more likely, however, that the difference is due to the immature state of the cortex in that study (postnatal days 14–16, compared with postnatal days 21–26 in our study). Age-dependent refinement of the specificity of synaptic connections has been extensively documented in many systems, including the visual cortex<sup>30</sup>.

Not only do fast-spiking inhibitory neurons connect preferentially to the same layer 2/3 pyramids that excite them, but these reciprocally connected pairs are also part of the same subnetworks of excitatory neurons (Supplementary Fig. 1). Our cross-correlation analyses of

photostimulation-evoked EPSCs show that reciprocally connected fast-spiking interneuron–pyramid pairs share common excitatory input from layer 4 and from within layer 2/3. Therefore, not only does the fine-scale specificity of excitatory connections create preferentially connected subnetworks of excitatory neurons<sup>23</sup>, but these subnetworks also preferentially select the same fast-spiking interneurons that provide them with reciprocal inhibition.

In contrast to results for fast-spiking interneurons, we observed no evidence for fine-scale specificity of connections involving adapting interneurons. For paired recordings involving an adapting interneuron and a pyramid, reciprocal connections were not observed with greater frequency than expected from chance coincidence on the basis of the probability of one-way connections. Moreover, cross-correlation analyses of photostimulation-evoked EPSCs in these same cell pairs indicated that they share little common excitatory input, regardless of whether they are directly connected. Nevertheless, our adapting interneurons probably included multiple inhibitory neuron types that could not be distinguished with the methods we used, and there are probably some relatively rare inhibitory cell types that were not sampled in our experiments. Thus, given the small sample size in some of our adapting interneuron groups (such as only five reciprocally connected pairs), it is possible that a subset of adapting interneuron types does actually connect to other neurons with fine-scale specificity.

It is very unlikely that the systematic differences in CPs that we observe between neuron pairs, on the basis of either their direct connectivity or on the type of inhibitory neuron, can be attributed to effects of cutting during brain slice preparation. This is because the recorded neurons were sufficiently deep in the brain slices such that their recipient dendritic arbors were preserved, regardless of cell type. Cutting might, however, have reduced the extent of the differences in CP that we observe to be correlated with connectivity between fast-spiking inhibitory neuron–pyramidal neuron pairs. This is because cutting could have affected the direct connectivity between pairs such that pairs that were reciprocally connected before cutting had only a one-way connection or no direct connection at all after cutting. But these previously connected pairs would still share common input resulting in a high value for CP. This potential artifact could explain the higher CP values for a small subset of pairs with one-way connections (Fig. 3).

We have previously reported fine-scale specificity of excitatory connections to pyramidal neurons in rat visual cortex<sup>23</sup>. Pairs of neighboring layer 2/3 pyramidal neurons that were directly connected to each other shared common excitatory input from the same presynaptic neurons in layer 4 and within layer 2/3, whereas unconnected pairs shared little common excitatory input from these layers. For these same cell pairs, however, excitatory input from layer 5 and photostimulation-evoked inhibitory connections from layer 2/3 and layer 4 followed different rules. The cell pairs shared common input from these sources regardless of whether they were directly interconnected. These observations suggested that excitatory connections from layer 4 to layer 2/3 and within layer 2/3 are precise on a fine scale, but inhibitory connections do not respect the specificity of these fine scale subnetworks. The present study, however, demonstrates that inhibitory connections from fast-spiking inhibitory neurons can be specific on a fine scale.

The likely reason for this difference between the present study and our previous report is that photostimulation-evoked IPSCs almost certainly reflect inputs from multiple inhibitory neuron types that are activated indiscriminately during photostimulation<sup>23</sup>. In particular, our analyses of the rise times of photostimulation-evoked IPSCs measured in the previous study, compared with those from paired

cell stimulation in the present study, suggest that the lack of specificity reflected a contribution of IPSCs from adapting interneurons that masked the specificity of connections involving fast-spiking interneurons. In the present study we have found that the rise times of IPSCs (onto pyramids) that came from fast-spiking interneurons were significantly shorter (1.7 ms) than the IPSCs that came from adapting interneurons (2.6 ms; Fig. 1f). In the previously published study<sup>23</sup>, the rise times of IPSCs evoked in layer 2/3 pyramids by photostimulation were, on average, intermediate in duration (2.0 ms). Another factor that is likely to have contributed to the lack of specificity of inhibitory input observed in our previous study is that there are also some promiscuous connections from fast-spiking interneurons that connect to pyramidal neurons outside of their own fine-scale subnetwork (Supplementary Fig. 1). These promiscuous connections are, however, only about one-sixth the strength of within-network inhibition from fast-spiking interneurons (see above).

In classical terms, the inhibitory connections that we observe from layer 2/3 interneurons onto layer 2/3 pyramids can be considered as providing feed-forward, feedback or lateral inhibition (or combinations of all three). The nature of these connections depends on the sources of excitatory input to the inhibitory neurons that in turn provide inhibition to the layer 2/3 pyramids. Here we refer to these types of connections with reference to the laminar flow of information within the cortex as well as to the fine-scale excitatory subnetworks. Because layer 4 provides the dominant feed-forward excitation to both pyramidal and fast-spiking inhibitory neurons in layer 2/3 (ref. 18), we consider it to be a source of feed-forward excitation<sup>5,6</sup>. If an inhibitory neuron in layer 2/3 connects to its neighboring pyramid and these cells share common excitatory input from layer 4, then the inhibitory neuron contributes feed-forward inhibition. We classify excitatory connections between layer 2/3 pyramids as 'recurrent'<sup>31,32</sup>, and when layer 2/3 neurons excite the same interneurons that in turn inhibit them, this is feedback inhibition. Finally, we consider lateral inhibition with respect to the fine-scale excitatory subnetworks. Inhibition that impinges on the subnetwork but that is driven by excitation from outside the subnetwork is 'lateral' with respect to these subnetworks.

In terms of these definitions, our findings show that fast-spiking interneurons provide all three types of inhibition to their neighboring pyramids, but lateral inhibition from this cell type is much weaker than inhibition within the subnetwork. The presence of feed-forward inhibition is apparent from the shared excitatory input from layer 4 for reciprocally connected cell pairs, and similarly, shared input from within layer 2/3 is evidence of feedback inhibition. Lateral inhibition is apparent from the observation that layer 2/3 pyramidal neurons receive inhibition from fast-spiking interneurons to which they do not provide excitation and with which they do not share common excitatory input from layer 4 or within layer 2/3. But these nonreciprocated, lateral inhibitory connections are about sixfold weaker than the feedforward and feedback inhibition within the subnetwork (see above).

In contrast to inhibition from fast-spiking interneurons, inhibition from adapting neurons is most consistent with lateral forms of inhibition. These inhibitory cells do not belong to the same fine-scale subnetworks as their neighboring pyramidal neurons, regardless of whether they inhibit them directly. Thus, they cannot provide either strong feed-forward or strong feedback inhibition. It is also important to consider, however, that layer 2/3 adapting interneurons also differ from fast-spiking interneurons in their sources of excitatory input. They typically receive their strongest excitation from sources other than layer 4 (ref. 18) and can also receive strong excitation from outside the local cortical region<sup>12</sup>. Thus, the inhibition from adapting interneurons onto

layer 2/3 pyramids is not as easily classified as that from fast-spiking interneurons and may contribute to other more subtle functions.

Feed-forward and/or feedback inhibition that also has a lateral component has been suggested as an important element of cortical processing in several experimental and theoretical studies<sup>32–35</sup>. Our results show that this organization is present with respect to fine-scale neuronal subnetworks and differs according to inhibitory cell type. The results also provide quantitative measures of the relationships between these inhibitory components.

The presence of distinct types of cortical inhibitory neurons, both in the hippocampus and in the cerebral cortex, suggests that each type may have a unique role in the cortical network<sup>36,37</sup>. The unique properties of fast-spiking interneurons<sup>28</sup> as well as studies of their functional interactions with excitatory neurons<sup>38,39</sup> have suggested that they are important in precisely regulating the timing of cortical activity, particularly at high frequencies. In particular, the combination of gap junction and synaptic coupling between fast-spiking interneurons<sup>10,40</sup> results in entrainment at gamma frequencies<sup>39</sup>, and the *in vivo* spike timing of fast-spiking, parvalbumin-positive basket cells is strongly correlated with local field potentials during gamma frequency and high-frequency ripple oscillations<sup>38</sup>. Our observation that reciprocally connected fast-spiking interneuron–pyramid pairs share common excitatory input on a fine scale suggests that these networks are also likely to regulate the precise timing of activity within fine-scale cortical subnetworks. In this context, fine-scale subnetworks may contribute to the synchronization of activity within particular neuronal subpopulations.

## METHODS

The methods used for both the collection and analyses of data in this study were the same as those described previously<sup>23</sup>, except that recordings were made from pairs of neurons consisting of one pyramidal neuron and one inhibitory neuron instead of two pyramidal neurons. All experimental procedures involving live animals were approved by the Salk Institute Animal Care and Use Committee.

**Slice preparation, photostimulation and recordings.** A vibratome was used to cut 300- $\mu$ m-thick coronal brain slices from the primary visual cortex of postnatal day (P) 21–26 Long-Evans rats. Slices were cut in artificial cerebral spinal fluid (ACSF; composition in mM: 124 NaCl, 5 KCl, 1.25  $\text{KH}_2\text{PO}_4$ , 1.3  $\text{MgSO}_4$ , 3.2  $\text{CaCl}_2$ , 26  $\text{NaHCO}_3$  and 10 glucose) and stored in an interface chamber at  $\sim 34^\circ\text{C}$  for at least 1 h until they were transferred to a room-temperature (20–24  $^\circ\text{C}$ ) recording chamber containing ACSF with 60–80  $\mu\text{M}$  ‘caged’ glutamate ( $\gamma$ -( $\alpha$ -carboxy-2-nitrobenzyl)ester, trifluoroacetate, L-glutamic acid). An infrared Olympus DIC microscope with a 40 $\times$ , 0.8 NA water-immersion lens was used to visualize and target recording electrodes to pairs of layer 2/3 pyramidal neurons with somata separated by, on average, about 50  $\mu\text{m}$  for whole-cell recordings. The distances (mean  $\pm$  s.e.m.) between recorded cells were  $49.0 \pm 3.7$   $\mu\text{m}$  for fast-spiking interneuron–pyramid pairs that were not connected;  $57.5 \pm 7.7$   $\mu\text{m}$  for fast-spiking interneuron–pyramid pairs with one-way inhibitory connections;  $45.0 \pm 8.3$   $\mu\text{m}$  for fast-spiking interneuron–pyramid pairs with reciprocal connections;  $42.7 \pm 5.1$   $\mu\text{m}$  for adapting interneuron–pyramid pairs that were not connected;  $43.7 \pm 6.6$   $\mu\text{m}$  for adapting interneuron–pyramid pairs with one-way inhibitory connections;  $47.6 \pm 7.1$   $\mu\text{m}$  for adapting interneuron–pyramid pairs with one-way excitatory connections; and  $52.7 \pm 6.5$   $\mu\text{m}$  for adapting interneuron–pyramid pairs with reciprocal connections. Cell bodies of recorded neurons were at least 50  $\mu\text{m}$  from the surface of the slice. Glass recording electrodes (4–6  $\text{M}\Omega$  resistance) were filled with an intracellular solution consisting of 130 mM potassium gluconate, 6 mM KCl, 2 mM  $\text{MgCl}_2$ , 0.2 mM EGTA, 10 mM HEPES, 2.5 mM  $\text{Na}_2\text{ATP}$ , 0.5 mM  $\text{Na}_2\text{GTP}$ , 10 mM potassium phosphocreatine and 0.3% biocytin. pH was adjusted to 7.25 with KOH. All intracellular recordings had access resistances less than 20  $\text{M}\Omega$ . In all paired recordings, connections between neuron pairs were assessed by injecting current to evoke action potentials in one of the cells recorded in current-clamp while testing for

PSCs during voltage-clamp recording in the other cell (Fig. 1c,d). For detection of excitatory input, inhibitory cells were voltage clamped at  $-65$  mV. For detection of inhibitory input, pyramidal cells were voltage clamped at 0 mV. For each pair, connections were tested for at least 50 trials generating single action potentials in the presynaptic neuron, for each direction. When connections were not detected with this procedure, they were also tested by stimulating in trains of 4–5 action potentials at 50 Hz to induce possible potentiation of weak connections. Inhibitory neurons were classified as either adapting (Fig. 1b) or ‘fast spiking’ (nonadapting; Fig. 1a) on the basis of temporal patterns of action potentials evoked by intracellular current injection<sup>18,25,26</sup>. Also, identification of inhibitory neurons versus pyramidal neurons was always confirmed on the basis of biocytin staining of the intracellularly labeled neurons.

Photostimulation was achieved by uncaging glutamate with 10-ms duration flashes of ultraviolet light from an argon-ion laser focused through the 40 $\times$  microscope objective. This results in generation of action potentials only in neurons with cell bodies within 100  $\mu\text{m}$  and usually less than 50  $\mu\text{m}$  from the site of uncaging<sup>23</sup>. Photostimulation-evoked excitatory postsynaptic currents were measured from recorded neurons in voltage-clamp mode with the holding potentials at  $-65$  mV to isolate EPSCs. Spontaneous EPSCs were also recorded in interleaved trials with no stimulation. Photostimulation-evoked IPSCs were not measured.

**Data analysis.** Maps of photostimulation sites were aligned to laminar borders in fixed and stained tissue (Figs. 2 and 4) and each site was assigned a laminar identity. Sites within 50  $\mu\text{m}$  of laminar borders were discarded from further analyses in order to limit the number of evoked synaptic currents arising from neurons with cell bodies possibly outside the stimulated layer. The electrical recordings from trials with and without (‘control’) photostimulation were analyzed using peak analysis software from Synaptosoft and other custom software developed by the authors. The times of onset and amplitudes of all EPSCs or IPSCs occurring within 150 ms of stimulation were marked. Rise times of PSCs were calculated as the time from 10% to 90% of the amplitude from baseline to peak. Analyses of the laminar sources and strengths of excitatory and inhibitory input to layer 2/3 pyramidal neurons (data not shown) gave results indistinguishable from those described previously<sup>18</sup> and no systematic differences that correlated with results from cross-correlation analyses.

Cross-correlograms of EPSCs were computed for each pair of simultaneously recorded layer 2/3 neurons with separate correlograms being computed for stimulation sites from each cortical layer. For some cells certain layers provided weak or no input to recorded neurons preventing evoked EPSCs from being clearly distinguished from spontaneous EPSCs. In these cases, correlograms were not computed for the corresponding layers (see Results). Correlograms were also computed for spontaneous EPSCs. Cross-correlation data were binned into histograms with 4-ms bins, with the central bin including values of  $0 \pm 2$  ms. Data from the stimulation trials (from the same layer) were also used to create shifted correlograms for each layer and cell pair<sup>24</sup>. To compute correlation probability (CP), the shifted correlogram was subtracted from the unshifted correlogram for the corresponding layer, and then the value in the central bin was divided by the average for the two cells of the estimated total number of evoked EPSCs observed for all trials in the relevant layer. The average number of evoked EPSCs was calculated as the total number of measured EPSCs for cell A minus the expected number of spontaneous EPSCs for that cell, plus the same value calculated for cell B, divided by two. Correlation probabilities for spontaneous EPSCs were invariably small ( $0.003 \pm 0.003$  for fast-spiking interneuron–pyramid pairs;  $0.017 \pm 0.003$  for adapting interneuron–pyramid pairs) and thus have a negligible influence on the CP values calculated for evoked EPSCs.

**Statistical analysis.** Statistical comparisons between correlation probabilities from different groups of neuron pairs (Fig. 3) used the non-parametric Mann-Whitney U test because there was no information available about the normality of these distributions. Comparisons of the proportions of cell pairs with direct inhibitory connections for pairs with excitatory connections versus those without excitatory connections used the Fisher exact test, which is designed specifically for this type of data set and yields an exact measurement of *p*. Values reported are two-tailed.

Note: Supplementary information is available on the Nature Neuroscience website.

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#### COMPETING INTERESTS STATEMENT

The authors declare that they have no competing financial interests.

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