Laminar Specificity of Local Circuits in Barrel Cortex of Ephrin-A5 Knockout Mice

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Cortical circuits are characterized by layer-specific axonal arbors. Molecular laminar cues are believed to direct the development of this specificity. We have tested the hypothesis that ephrin-A5 is responsible for preventing layer 2/3 pyramidal cell axons from branching within layer 4 (Castellani et al., 1998) by assessing the laminar specificity of axonal arbors in ephrin-A5 knockout mice. We find that in barrel cortex of knockout mice, layer 2/3 pyramidal neurons form axonal arbors specifically in layers 2/3 and 5, avoiding layer 4. This pattern of arborization is indistinguishable from that of wild-type littermates. Furthermore, we find that in wild-type mice, laminar patterns of ephrin-A5 expression differ between cortical areas despite the similarity of layer-specific local cortical circuits across areas. Most notably, ephrin-A5 is not expressed preferentially in layer 4 of wild-type mouse barrel cortex. We conclude that ephrin-A5 is not responsible for preventing the development of layer 2/3 pyramidal cell axonal arbors in layer 4 of mouse barrel cortex. These observations also suggest that if ephrin-A5 plays a role in the emergence of layer-specific circuits, that role must differ between cortical areas.

Key words: ephrin; eph receptor; local circuits; mouse; barrel cortex; somatosensory cortex

Cortical circuits are characterized by axonal arbors that are highly specific for cortical layers (for review, see Gilbert, 1983; Callaway, 1998a). For example, layer 2/3 pyramidal neurons have extensive local axonal arbors specifically in layers 2/3 and 5, but their axons avoid layers 4 and 6 (Gilbert, 1983; Martin and Whitteridge, 1984; Ojima et al., 1991; Callaway and Wiser, 1996; Gottlieb and Keller, 1997). The development of this laminar specificity is precise from the outset: growing axons arborize initially in the correct layers without making exuberant arbors in incorrect layers (Lund et al., 1977; Katz, 1991; Callaway and Katz, 1992; Callaway and Lieber, 1996; Callaway, 1998b) (for review, see Katz and Callaway, 1992). Laminar specificity can also develop in organotypic cortical slice cultures (Yamamoto et al., 1989; Bolz et al., 1990; Molnar and Blakemore, 1991; Bolz et al., 1992; Yamamoto et al., 1992; Dantzker and Callaway, 1998). These results imply that growing axons are likely to use layer-specific molecular cues and not activity cues to distinguish correct from incorrect cortical layers. Molecular cues that serve this function have not been identified.

A recent report by Castellani et al. (1998) strongly suggests that ephrins may play a role in the development of layer-specific axonal arbors within cortex. Specifically, they found that presumptive layer 2/3 neurons avoided growing on membrane carpets that expressed ephrin-A5. Furthermore, they found that ephrin-A5 was expressed in layer 4 of "sensorimotor" cortex, whereas an ephrin-A receptor, EphA5, is in layers 2/3 and 5. Because interactions between ephrin ligands and their Eph receptors are generally inhibitory (for review, see Flanagan and Vanderhaeghen, 1998; O'Leary et al., 1999), resulting in reduced axonal growth or branching, these observations led to the hypothesis that the ephrin-A5 in layer 4 interacts with the EphA5 receptors of layer 2/3 pyramidal neurons to prevent axonal arborization specifically in layer 4.

We have tested this hypothesis by investigating the laminar specificity of axonal arbors of layer 2/3 pyramidal neurons in S1 barrel cortex of ephrin-A5 knockout mice (Frisen et al., 1998). In addition we have performed *in situ* hybridization to investigate the cortical laminar and areal expression patterns of ephrin-A5 in wild-type and ephrin-A5 knockout mice. Contrary to the hypothesis of Castellani et al. (1998), we find that the laminar specificity of local axonal arbors from layer 2/3 pyramidal neurons is normal in ephrin-A5 knockout mice. The axons branch preferentially in layers 2/3 and 5, avoiding layer 4. Furthermore, we find that in wild-type mice, ephrin-A5 is not expressed preferentially in layer 4 of barrel cortex, although it is expressed in layers 4 and 6 of some other cortical areas. We conclude that ephrin-A5 is not responsible for the prevention of axonal growth by layer 2/3 pyramidal neurons within layer 4 of mouse barrel cortex.

MATERIALS AND METHODS

Intracellular labeling, histology, and anatomical reconstructions. Sagittal slices (400 μm thick) were prepared from the barrel cortex of ephrin-A5 knockout mice (Frisen et al., 1998) and their wild-type littermate controls using methods similar to those described previously (Yabuta and Callaway, 1998). Animals used in these studies were 32–44 d old.

All procedures for intracellular labeling, staining, and anatomical reconstruction were identical to those described previously (Yabuta and Callaway, 1998). Briefly, slices were held in an interface chamber for 1–8

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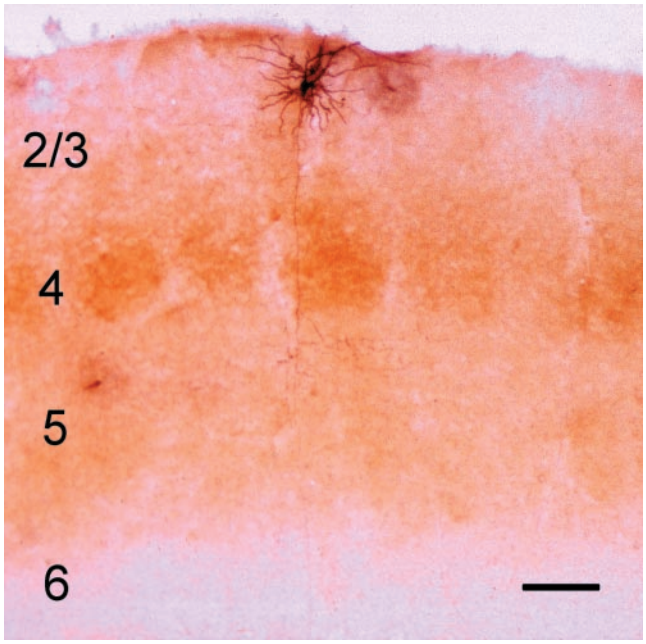

Figure 1. Photomicrograph of a section from a wild-type mouse barrel cortex brain slice containing an intracellularly labeled layer 2/3 pyramidal neuron. The section is double-stained for biocytin, to reveal the labeled neuron, and CO, to reveal laminar boundaries and barrels. The laminar pattern of CO staining (layers indicated by *numbers* to the *left*) and CO dense barrels in layer 4 are clearly visible. The neuron is located in layer 2, has a pyramidal dendritic morphology, and a main descending axon that branches specifically in layers 2/3 and 5, and not in layer 4. The reconstruction of this neuron's axonal and dendritic arbors is illustrated in the *top left panel* of Figure 2. Scale bar, 200 μm.

hr before being moved to a recording chamber where neurons were filled with biocytin during whole-cell recording. Slices were fixed, resectioned, and then double-stained for biocytin and cytochrome oxidase (CO) to yield black neurons against a red/brown background (see Fig. 1). Pyramidal neurons that were located in layer 2/3 of barrel cortex (determined from CO staining; see Fig. 1), and sufficiently well labeled such that their complete axonal arbors within the brain slice could be easily distinguished without fading of axons distant from the cell body, were selected for further analysis. The axonal and dendritic arbors of the pyramidal cells and the borders of the cortical layers were reconstructed using a computerized reconstruction system (Neurolucida, Microbrightfield, Colchester, VT). For each reconstructed neuron, the laminar specificity of axonal arbors was quantified by counting the numbers of axonal branches within each cortical layer (cf. Callaway and Lieber, 1996; Dantzker and Callaway, 1998).

In situ *hybridization.* Seven-day-old ephrin-A5 knockout mice and wild-type littermates were perfused transcardially with 4% paraformaldehyde in 0.1 M borate buffer, pH 9.5. The brains were removed, post-fixed for 24 hr at 4°C, and then cryoprotected in 30% sucrose for 24–48 hr. Coronal sections (30 μm) were cut on a cryostat and stored at −70°C.

Hybridizations of both sense and antisense probes to ephrin-A5 were performed. Antisense probe used for hybridization was a 249 bp ephrin-A5 riboprobe directed against nucleotides 464–713 (Winslow et al., 1995). Plasmids were linearized, and ^{35}S-UTP-labeled riboprobes were transcribed using either T3 (ephrin-A5 antisense) or T7 polymerase (ephrin-A5 sense).

Sections were hybridized as previously described (Simmons et al., 1989) with the following modifications: sections were pretreated with 0.05% Triton X-100 in 0.1 M TEA buffer for 20 min followed by 0.01 mg/ml proteinase K for 5 min at 37°C. Sections were exposed to Kodak Biomax film (4 d) and then dipped in Kodak NTB-2 emulsion diluted 1:1 in water. Sections were exposed for 5 weeks, developed in D19 Kodak developer, fixed in Kodak polymax fix, counterstained with thionin, dehydrated, defatted, and coverslipped.

RESULTS

Laminar specificity of axonal arbors

The laminar specificity of layer 2/3 pyramidal neuron axonal arbors from ephrin-A5 knockout mice and their wild-type littermates was indistinguishable. A total of 16 layer 2/3 pyramidal neurons were intracellularly labeled in barrel cortex, and their axonal and dendritic arbors were reconstructed. Eight of these neurons were from ephrin-A5 knockout mice, and the remaining eight were from their wild-type littermates. A photograph of a typical intracellularly labeled layer 2/3 pyramidal neuron is shown in Figure 1, and computerized Neurolucida reconstructions are shown in Figure 2. Axonal arbors of layer 2/3 pyramidal neurons in barrel cortex of both ephrin-A5 knockout mice (Fig. 2, *right panels*) and their wild-type littermates (Fig. 2, *left panels*) were highly specific for layers 2/3 and 5, avoiding layer 4. This specificity was most striking for neurons located more superficially in layer 2/3 (Fig. 2, *top panels*) the dendrites of which did not extend into layer 4. For both knockout and wild-type mice, neurons located deeper in layer 3 (Fig. 2, *bottom, left panel*) had more axonal branches within layer 4, but they still arborized preferentially within layers 2/3 and 5. (Two of eight neurons from knockout mice and four of eight from wild-type mice had dendritic branches in layer 4.) Nevertheless, the preference of axonal arbors for layers 2/3 and 5 was clear in all cases (Fig. 2). This laminar pattern of axonal arborization is indistinguishable from that described previously in mouse barrel cortex (Gottlieb and Keller, 1997).

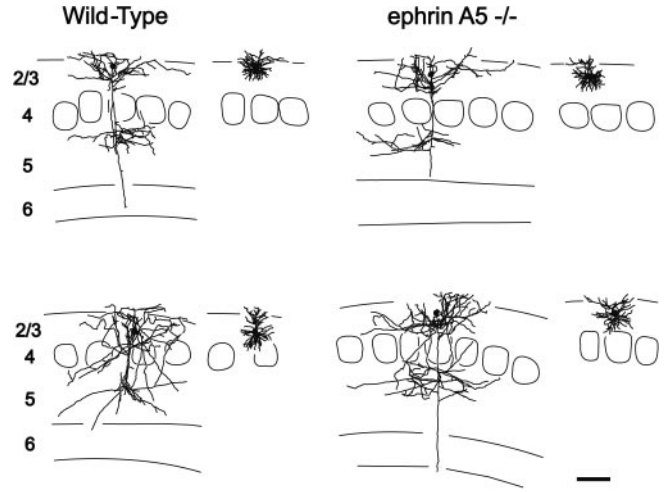

Figure 2. Reconstructions of the axonal and dendritic arbors of layer 2/3 pyramidal neurons in the barrel cortex of wild-type (*left panels*) and ephrin-A5 −/− mice (*right panels*). Each neuron has extensive axonal arbors in layers 2/3 and 5, and few or no axonal branches in layer 4. Reconstructions of the axonal and dendritic arbors of each neuron are illustrated separately, with the dendritic arbors shown to the *right* of the corresponding axonal arbors. The locations of CO-dense barrels are indicated by *outlines* in layer 4; these also indicate the upper and lower borders of layer 4. Other laminar borders are indicated by *horizontal lines*. The layers are identified by *numbers* at the *left* of each reconstruction. Scale bar (applies to all four reconstructions): 200 μm.

The similarity of axonal arbors from wild-type and ephrin-A5 knockout mice is also apparent from quantitative analyses of the number of axonal branches in each cortical layer. For each layer 2/3 pyramidal neuron, the percentage of axonal branches in each cortical layer was calculated. These values were pooled for all neurons within each group (knockout and wild-type), and the pooled values are illustrated in Figure 3. Neurons from both groups branch preferentially in layers 2/3 and 5 rather than layer 4. Most notably there are no significant differences between groups (wild-type vs knockout) in the percentage of their axonal branches in layers 2/3, 4, or 5 ($p > 0.1$, Student's t test, two-tailed). Furthermore, for the knockout mice, there are significantly fewer axonal branches in layer 4 (12.3 ± 3.3%) than in either layer 2/3 (55.3 ± 3.8%) or layer 5 (26.1 ± 2.5%; $p < 0.05$).

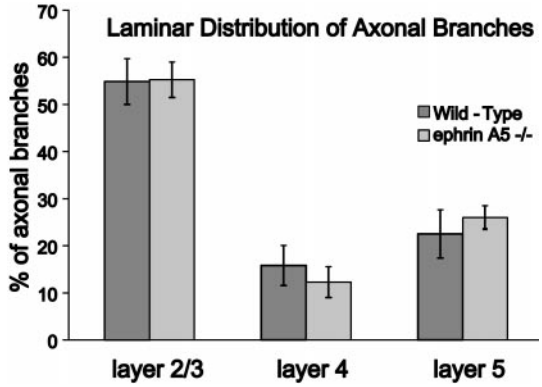

Figure 3. Histogram illustrating the laminar distributions of axonal arbors of layer 2/3 pyramidal neurons in the barrel cortex of wild-type (*dark bars*) and ephrin-A5 −/− (*light bars*) mice. The height of each bar indicates the mean (±SEM) percentage of axonal branches in cortical layers 2/3, 4, and 5. The laminar distributions of axonal arbors from wild-type and ephrin-A5 −/− mice are indistinguishable. For both populations, axonal branches are located preferentially in layers 2/3 and 5, with fewer branches in layer 4.

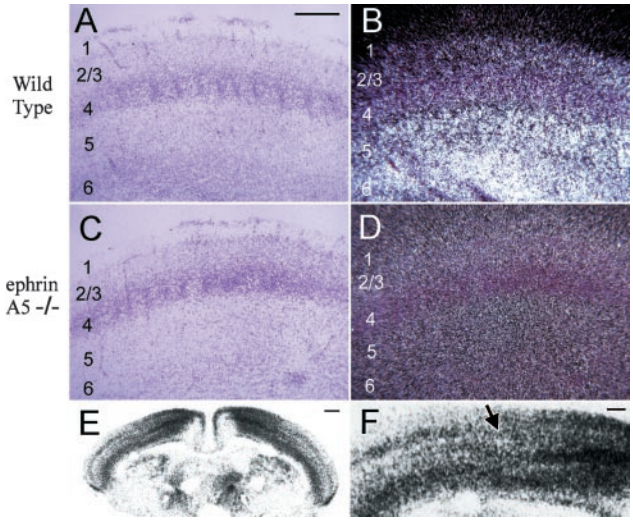

Figure 4. Expression of ephrin-A5 in the cortex of 7-d-old wild-type (*A, B, E, F*) and ephrin-A5 −/− mice (*C, D*). *A–D* are photomicrographs of sections from barrel cortex. *A* and *C* are bright-field photographs of the Nissl-stained sections in which the cortical lamination and cell-dense barrel septa within layer 4 are visible. Dark-field photographs illustrating ephrin-A5 expression in the same sections are shown in *B* and *D*. In the wild-type mouse barrel cortex, the highest levels of ephrin-A5 expression are in the deep layers, 5 and 6 (*B*); there is a sharp transition at the layer 4/layer 5 border where ephrin-A5 expression decreases. In the ephrin-A5 −/− barrel cortex, the expression of ephrin-A5 is at background levels (*D*). *E* and *F* are film autoradiograms of a coronal section through the mouse brain illustrating the expression of ephrin-A5 at low and high magnification, respectively. Two distinct laminar patterns of ephrin-A5 expression are visible in cortex. Medially, expression is highest in layers 4 and 6, whereas laterally, expression is lowest in layer 4. The transition between the expression patterns is indicated by the *arrow* in *F*. Scale bars (shown in *A* for *A–D*): 250 μm; *E*, 500 μm; *F*, 200 μm.

Laminar and areal expression of ephrin-A5

In situ hybridization was used to assess the expression patterns of ephrin-A5 mRNA in the cortex of wild-type and ephrin-A5 knockout mice. These expression patterns are illustrated in Figure 4. The *top four panels* of Figure 4*A–D* show sections through barrel cortex. The *panels* on the *left* (Fig. 4*A*,*C*) are bright-field photographs of the Nissl-stained sections in which the laminar borders are apparent, as are the cell-dense barrel "septa" characteristic of layer 4 of barrel cortex. Dark-field photographs showing the patterns of ephrin-A5 expression in the same sections are shown in the *panels* to the *right* (Fig. 4*B*,*D*). As expected, ephrin-A5 was not detectable above background levels in the cortex of ephrin-A5 knockout mice (Fig. 4*D*). We were surprised, however, to find that in the barrel cortex of wild-type mice, ephrin-A5 was expressed most intensely in the deep cortical layers (layers 5 and 6) and not layer 4 (Fig. 4*B*).

This observation contrasts with that of Castellani et al. (1998) who reported ephrin-A5 expression specifically in layers 4 and 6 of mouse sensorimotor cortex. To show that this difference is likely attributable to areal differences in laminar expression of ephrin-A5, we illustrate such areal differences in wild-type mouse cortex in Figure 4, *E* and *F*. These are low-power (Fig. 4*E*) and high-power (Fig. 4*F*) photographs of a film autoradiogram from a coronal section including the expected location of somatosensory cortex plus adjacent cortex. In the low-power view (Fig. 4*E*), it can be seen that the laminar pattern of ephrin-A5 expression changes abruptly in both cortical hemispheres, such that the pattern medially differs from the pattern laterally. This is more apparent in the higher-power view of the transition zone (Fig. 4*F*). The *left side* of the Figure corresponds to the expected location of somatosensory cortex. Here the darkest label (highest expression) is in deep layers (layers 5 and 6) with a light layer just above (layer 4) followed by another moderately labeled layer (layer 3) and finally another band of light label more superficially. Alignment with an adjacent Nissl-stained section (data not shown) reveals that the lightly labeled layer that is flanked by darker label corresponds to layer 4. This contrasts with the laminar pattern in the immediately adjacent cortex (Fig. 4*F*, *right*), in which dark label is found specifically in layer 4 and also in layer 6. Because this pattern is similar to the pattern described by Castellani et al. (1998) in sensorimotor cortex, it appears likely that their observations were made at a similar location and not in barrel cortex. We also find a similar expression pattern (layers 4 and 6) in Nissl-stained cortex adjacent to the area identified as barrel cortex and illustrated in Figure 4, *A* and *B* (data not shown).

DISCUSSION

Laminar specificity of axonal arbors is a hallmark of local cortical circuits. This specificity has been characterized most extensively in primary sensory areas such as the primary visual cortex of cats (cf. Gilbert 1983) and primates (cf. Callaway, 1998a), cat auditory cortex (Ojima et al., 1991, 1992), and mouse barrel cortex (Gottlieb and Keller, 1997). Each of these cortical areas is characterized by similar patterns of laminar specificity of local cortical circuits, suggesting that the development of this specificity is regulated by common mechanisms across areas and across species.

We tested the specific hypothesis (Castellani et al., 1998) that ephrin-A5 is responsible for the prevention of axonal branching within layer 4 by layer 2/3 pyramidal neurons. We have provided strong evidence that this is not the mechanism by which this laminar specificity emerges in mouse barrel cortex. Specifically, ephrin-A5 is not preferentially expressed in layer 4 of developing mouse barrel cortex, and layer 2/3 pyramidal neurons develop normal laminar specificity in the barrel cortex of ephrin-A5 knockout mice.

Insofar as the mechanisms directing axonal laminar specificity are common across cortical areas, these observations also suggest that ephrin-A5 is not necessary for layer-specific growth of layer 2/3 pyramidal neurons in other cortical areas. Nevertheless, because both ephrins and their receptors are expressed in layer-specific patterns in cortex, it seems likely that the ephrins will affect the laminar patterns of axonal growth of neurons expressing receptors. Thus it is possible that in cortical areas where ephrin-A5 *is* expressed in layer 4, it *does* influence the layer-specific development of layer 2/3 pyramidal neurons.

For example, in ferret visual cortex, ephrin-A5 is expressed preferentially in layers 4 and 6 of Area 18 but is absent in layer 4 of Area 17 (A. K. Butler and E. M. Callaway, unpublished observations). Despite this difference in ephrin-A5 expression, layer 2/3 pyramidal neurons do not have local axonal branches in layer 4 in either Area 17 or Area 18 (E. M. Callaway, unpublished observations). It is therefore possible that ephrin-A5 could act to prevent layer 2/3 pyramidal neurons from branching locally in layer 4 of Area 18, but a different mechanism would be required in Area 17.

Assessment of the likelihood of such a scenario is best considered in the context of the full complement of layer-specific circuitry within the cortex. Although local circuits are similar across cortical areas, these same areas differ in the laminar organization of their connections with each other (Felleman and Van Essen, 1991) and with subcortical structures. For example, in monkey primary visual cortex, the same layer 2/3 pyramidal neurons that specifically avoid layer 4 when making local connections specifically target layer 4 of other cortical areas [e.g., areas V2, V3 or MT (for review, see Felleman and Van Essen, 1991)]. Thus, if these neurons express a receptor for a ligand that prevents their growth within layer 4 of area V1, the ability of these same neurons to target layer 4 in area V2 would require either different patterns of laminar expression of ligand in V1 and V2, different patterns of expression of the receptor along the extent of the growing axon, or closely regulated timing of axon growth and ligand expression in different areas.

This example points out that what might seem a sensible design for a developing cortical region when considering only specificity of intrinsic connections may not be the best design when considering the full complement of layer-specific circuits. It is therefore a reasonable possibility that different cortical areas could develop the same local circuits by using the same sets of molecules but in different laminar patterns. The differences could be necessary to correctly establish the extrinsic connectivity that differs between areas.

Indeed, the observation that different cortical areas express ephrin-A5 in different laminar patterns implies that the precise role of this molecule is likely to vary between areas. The fact that different cortical areas are able to develop similar local cortical circuits even in the face of different laminar patterns of ephrin expression suggests that ephrin expression may, in some cases, represent a problem that must be overcome by compensatory mechanisms rather than a solution. Further studies will be required to determine whether differences in the laminar patterns of ephrin-A5 expression are responsible for regulating the growth of axons that differ between areas (e.g., afferent input) or the same ligand plays different roles in the development of local circuits in different cortical areas.

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