

THE SUBPLATE, A TRANSIENT NEOCORTICAL STRUCTURE: Its Role in the Development of Connections between Thalamus and Cortex

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KEY WORDS: neurogenesis, pioneer neurons, waiting period, cell death, ocular dominance columns

INTRODUCTION

The functioning of the mammalian brain depends upon the precision and accuracy of its neural connections, and nowhere is this requirement more evident than in the neocortex of the cerebral hemispheres. The neocortex is a structure that is divided both radially, from the pial surface to the white matter into six cell layers, and tangentially into more than 40 different cytoarchitectural areas (Brodmann 1909). For instance, within the cerebral hemispheres, sets of tangential axonal connections link neurons within a given cortical layer to each other and also link neurons of different cortical areas; sets of radial connections link neurons of different layers together. In addition, the major input to the neocortex arises from neurons in the thalamus, which in turn receive a reciprocal set of connections from the cortex. These connections are highly restricted: In the radial domain, thalamic axons make their major projection to the neurons of cortical layer 4, and the neurons of cortical layer 6 project back

to the thalamus. Connections are also restricted tangentially, in that neurons located in specific subdivisions of the thalamus send their axons to specific cortical areas. For instance, neurons in the lateral geniculate nucleus (LGN) of the thalamus connect with primary visual cortex, whereas those situated in the ventrobasal complex connect with somatosensory cortex. There are also local patterns of connections within a given cortical area, for example, the ocular dominance columns in primary visual cortex of higher mammals, or the barrels in rodent somatosensory cortex (Woolsey & van der Loos 1970). The ocular dominance columns are based on the fact that the inputs of LGN axons representing the two eyes are segregated from each other in layer 4 and their terminal arbors are clustered together in patches (LeVay et al 1980).

A primary question is how these sets of connections form during development. The purpose of this review is to consider this question as it pertains specifically to the formation of connections between thalamus and cortex [for a more general review of the formation of connectivity, see Goodman & Shatz (1993)]. Several major steps are involved in this developmental process. First, the constituent neurons of the thalamus and cortex must be generated. Next, axons must grow along the appropriate pathways and select the appropriate targets. In the visual system, this means that LGN axons must grow up through the internal capsule, bypass many other inappropriate cortical areas, and then select visual cortex. Finally, the axons must enter the cortical plate, recognize and terminate within layer 4, and segregate to form ocular dominance columns. Thus, in addition to the general problems of pathfinding and target selection faced by all developing neurons, thalamic neurons are faced with a series of tangential and radial decisions as they form the final pattern of connections within neocortex: they must choose the correct cortical area and the correct layer, and must restrict the extent of their terminal arbors. In addition, similar problems must be solved by the neurons of cortical layer 6 as they grow towards and invade their thalamic targets.

A growing body of evidence suggests that the formation of connections between thalamus and cortex requires the presence of a specific and transient cell type, subplate neurons. These neurons are present early in development, but by adulthood the majority have disappeared. Here we consider their life history and review the evidence for their role in the patterning of connections.

DEVELOPMENT AND MATURATION OF THE SUBPLATE NEURONS

The Preplate is Comprised of Subplate and Marginal Zone Cells

In the development of the cerebral cortex, an early germinal zone, the ventricular zone (VZ), gives rise, through successive rounds of cell division

and migration, to the postmitotic neurons that comprise the adult cortical layers. As Figure 1 shows, during development the histology of the cerebral wall is very different from that of the adult (Boulder Committee 1970). Early in development, in addition to the VZ there is a cellular zone located immediately below the pial surface, which has been termed the preplate (Rickmann et al 1977, Stewart & Pearlman 1987) [or primordial plexiform layer (Marin-Padilla 1971)] (Figure 1b). The Golgi studies of Marin-Padilla (1971) showed that the preplate is filled with loosely packed, polymorphic cells with a neuronal morphology. As the cortex matures, a zone of densely-packed pyramidal cells appears in the middle of the preplate, and this was termed the cortical plate (Figure 1c). These observations led Marin-Padilla to propose that the primordial plexiform layer is split apart by later-forming neurons of the cortical plate. In particular, he suggested that in the adult, the outer neurons form layer 1, the inner neurons form layer 7, and the number of neurons in these two layers remains unchanged during the subsequent maturation and growth of the cortex (reviewed in Marin-Padilla 1988).

In more recent years, ^3H -thymidine labeling experiments have confirmed and extended this idea, and demonstrated that some revision is necessary. For example, in the development of the cat cerebral cortex, such experiments have demonstrated directly that neurons belonging to the preplate are the earliest-generated neurons of the cerebral cortex. By using ^3H -thymidine birthdating and autoradiography, Luskin & Shatz (1985b) determined that genesis of cells in the occipital pole begins after E21 in the cat. When fetuses are ^3H -thymidine-labeled at E24 (but not earlier, at E21) and analyzed at E31, labeled cells, the first postmitotic cells of the visual cortex, can be observed in the preplate. However, if this autoradiographic analysis is performed later, at E40, the early-generated population (labeled at E24) can be seen to have split into two zones, as shown in Figure 1c (Luskin & Shatz 1985b). The deeper of the two zones is called the subplate (SP), a zone first defined by Kostovic & Molliver (1974) that is situated below the cortical plate. The other zone, termed the marginal zone (MZ) (Boulder Committee 1970), is located immediately below the pial surface. In a parallel study focusing on the cortical plate, Luskin & Shatz (1985a) showed that the neurons that eventually comprise the adult cortical layers 2–6 are generated next. Those neurons constituting the deepest cortical layer (layer 6) are generated first, and those occupying the most superficial layers (layers 2 and 3) are generated last (Luskin & Shatz 1985a), as had previously been observed in rodents by Angevine & Sidman (1961).

Taken together, these observations demonstrated that in the cat the early-generated preplate is split in two by the incoming migrating cortical plate neurons, thereby creating a cellular framework consisting of the MZ and SP. That the early-generated population of cells consists of neurons has been confirmed by means of ^3H -thymidine birthdating combined with immunohistochemistry for neuronal markers (Chun et al 1987; Chun & Shatz 1988a,

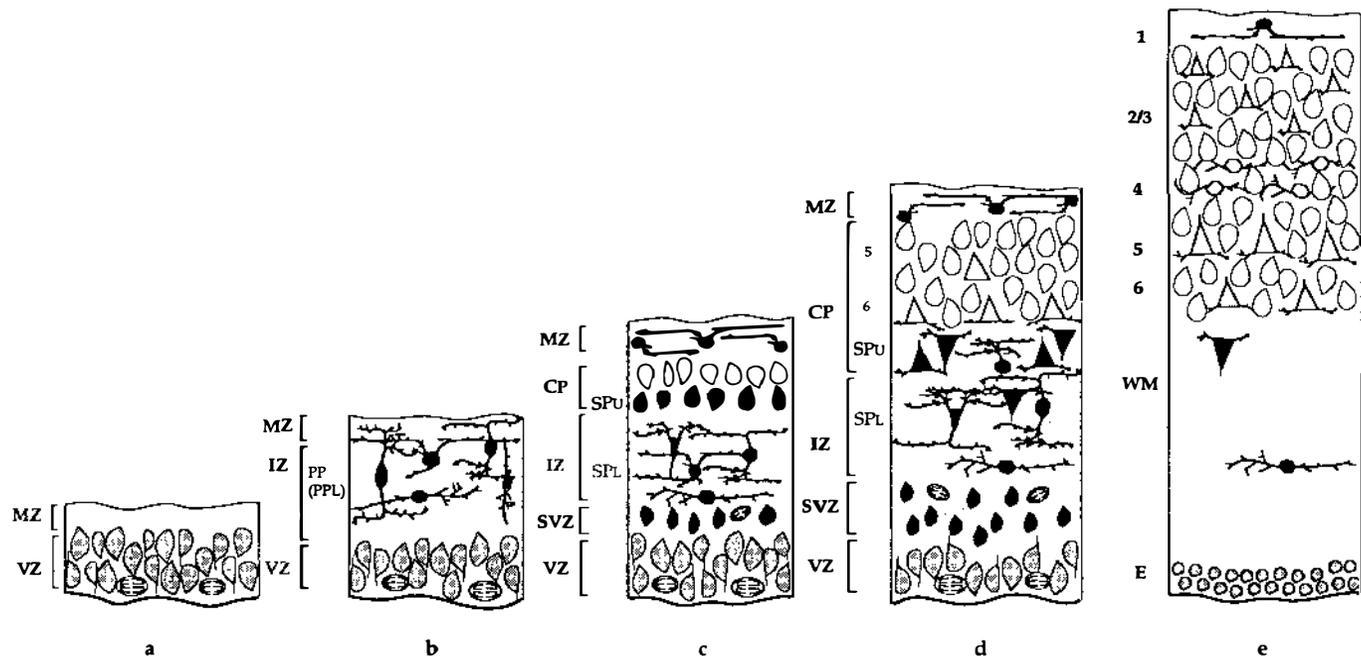


Figure 1 Schematic diagram of the histological changes in the cerebral wall during neocortical development, based on ^3H -thymidine labeling studies combined with morphological studies including Golgi impregnations, DiI labeling, and immunohistochemistry. Diagram updated and modified from Boulder Committee (1970). (a) Initially, the cerebral wall is comprised of a germinal zone, the ventricular zone (VZ), and a marginal zone (MZ). (b) As the first postmitotic cells migrate from the VZ, they settle below the MZ to form the preplate (PP), or primordial plexiform layer (PPL). This is a zone of loosely-packed cells (black), many of which have neuronal morphologies, that is situated within the intermediate zone (IZ) just below the MZ. (c) With ensuing neurogenesis and migration, a cell-dense zone, the cortical plate (CP), forms. PP neurons are split into two populations that comprise the MZ and the SP. In higher mammals, ^3H -thymidine labeling studies show that the SP consists of two subdivisions, upper (SP_U) and lower (SP_L). Cells residing in the SP_U form the base of the cortical plate as histologically defined, but the fact that the majority disappear by adulthood, coupled with their early birthdates, indicates that the SP_U cells are part of the subplate neuron population. Transient cells of the SP_U are shown in black, and permanent neurons that comprise the base of layer 6 are shown in white. (d) At even later times in development, the cortical plate thickens as layers 5 and 6 form, and the MZ and SP neurons achieve maturity. (e) By adulthood, the majority of neurons in the MZ and in both the SP_U and SP_L have disappeared, leaving scattered interstitial neurons in the white matter (WM) and Cajal-Retzius neurons in layer 1; the VZ and subventricular zone (SVZ) have also disappeared, leaving an ependymal layer (E).

1989a); such experiments reveal striking similarities between immunostained early-generated cells and the Golgi drawings of Marin-Padilla (1971, 1988).

In addition, the ^3H -thymidine labeling studies suggest that at least in the cat some of the subplate neurons condense into a layer of cells in the middle of the preplate, as shown in Figure 1c. The appearance of this clear histological layer has traditionally been interpreted as signaling the formation of the cortical plate. Thymidine birthdating, however, reveals that this first accumulation of neurons into a "plate" does not go on to form the base of layer 6 in the adult. Rather, like the neurons that initially reside in the preplate, very few survive (Figure 1e); for example, neurons generated at E24 condense to form the cortical plate by E30, but few if any can be found in adult layer 6 (Luskin & Shatz 1985b) (see Figure 1 and section titled "Death of Subplate Neurons," below). Instead, adult layer 6 is formed by neurons generated later (after E30) that take up positions in the cortical plate directly above the upper subplate (Luskin & Shatz 1985b). Thus, these experiments suggest that subplate neurons reside in two zones, a loosely packed zone below the cortical plate called the lower subplate (Luskin & Shatz 1985b) and a more densely packed zone at the base of the cortical plate called the upper subplate (Luskin & Shatz 1985b) (see Figure 1c, d). A similar subdivision of the subplate into upper and lower portions has also been noted in primates (Kostovic & Rakic 1990) through histological criteria.

Thymidine-labeling studies in rodents also indicate the presence of an early-generated population of neurons that forms a preplate. However, the exact relationship between these early-generated neurons and the permanent neurons of the cortical plate is harder to determine. In higher mammals such as cats, monkeys, and ferrets, where the period of neurogenesis is prolonged, a single injection of ^3H -thymidine can label a subset of neurons within a single cortical layer, whereas in rodents, because of the rapid pace of cortical neurogenesis (see Table 1), a similar injection typically labels cells in multiple layers. Therefore, the ^3H -thymidine labeling technique can be used reliably in higher mammals to identify subplate neurons based on their early birthdates and the fact that many of these earliest-generated cells are not present in the adult, whereas in studies of rodent subplate great care must be taken to ensure that only the earliest-generated cells (rather than the permanent cells of layer 6) are being examined. For example, in studies of neurogenesis in the mouse cortex, Wood et al (1992) pinpointed accurately the first day of neurogenesis of subplate and marginal zone cells as E12 by demonstrating (a) that a single injection of label on E11 produced no heavily labeled cells and (b) that few if any cells labeled at E12 come to reside in the cortical plate, but instead the vast majority were located in the subplate and marginal zones. In addition, the majority of cells labeled at E12 had disappeared by P21 (see Table 1). Woo et al (1991) reached similar conclusions in their studies of the hamster

cortex, in which they carefully followed the fates of only the heavily-labeled cells following a single injection of tritiated thymidine at E9 or E10 (see Table 1). In the rat, Bayer & Altman (1990) used an indirect subtraction method involving injection of thymidine on two consecutive days to calculate the percentage of subplate or marginal zone cells generated on a given day. They concluded that the period of neurogenesis of subplate and marginal zone cells began at E13 (= E12, using E0 as day of insemination) and was rather prolonged. However, they did not examine any animals receiving thymidine injections earlier than E13, so the exact time of onset of subplate neurogenesis was not determined. In addition, they did not follow their thymidine-labeled cells into late postnatal life, so they also could not determine whether these cells disappear in the adult. König & Marty (1981) also birthdated cortical neurons in the rat and showed that no cells could be heavily labeled on E11, which suggests that E12 is the first day on which postmitotic neurons are generated. Valverde et al (1989) have confirmed and extended these results by showing that the majority of the neurons generated on E12 come to reside in layer I and layer 6b, which most likely corresponds to the subplate in the rodent. Al-Ghoul & Miller (1989) gave a single thymidine injection at E12 (see Table 1) to study rat subplate neurons. In their studies, heavily-labeled cells were exclusively restricted to a zone at the base of the cortical plate at birth, but the majority had disappeared by P20, indicating that cells generated

Table 1 Birth and death of subplate neurons in various species

	Mouse	Rat	Hamster	Ferret	Cat	Sheep	Monkey	Human
Birthdate ^a	E12–13	E12–?	E9–10	E20–24	E24–30	E26–31	E38–48	
Waiting period ^b (thalamic)		E16–17		E27–P5 ^c	E36–50		E78–124 ^d	
Death ^e	≥80% by P21	yes	50–80% between P4 & adult		90% by 4 mos.	yes	yes	yes
SP/CP ratio ^f	1:2	1:2			1:1		3:1	4:1

^a Angevine & Sidman 1961, Kostovic & Rakic 1980, König & Marty 1981, Luskin & Shatz 1985a, Al-Ghoul & Miller 1989, Jackson et al 1989, Bayer & Altman 1990, Woo et al 1991, Saunders et al 1992, Wood et al 1992. Based on ³H-thymidine or BrdU labeling.

^b Rakic 1974, Wise & Jones 1978, Catalano et al 1991, Erzurumlu & Jhaveri 1992, Ghosh & Shatz 1992b. Based on direct labeling of thalamic afferents.

^c E27: Johnson & Casagrande 1993; P5: K Herrmann & CJ Shatz. unpublished observations. Estimates based on preliminary Dil labeling.

^d Rakic 1977. By E78, fibers have already reached visual subplate, by E124, many have grown in to layer 4. Times earlier than E78 or between E78 and E124 have not been reported.

^e Al-Ghoul & Miller 1989, Chun & Shatz 1989b, Kostovic & Rakic 1990, Woo et al 1991, Saunders et al 1992, Wood et al 1992. Where quantitation is available, numbers have been included.

^f Kostovic & Rakic 1990. Calculated on the basis of area measurements.

at E12 in the rat cortex are not only the earliest generated, but are also transient. Thus, a consensus is emerging concerning the birthdates of the earliest-generated cells in several species of rodents as well (see Table 1).

Interestingly, in the reeler mouse (Caviness & Sidman 1973, Caviness & Rakic 1978, Goffinet 1992), the preplate layer is not split into two tiers (Ogawa et al 1992); instead, the cortical plate neurons continue to accumulate in an outside-in fashion, such that the cortex forms beneath the early-generated cells with layer 6 distal and layer 2 proximal to the ventricular zone (Caviness & Rakic 1978, Caviness & Frost 1983). Ogawa et al (1992) performed molecular studies of the subplate and marginal zone regions of reeler mice to determine whether interactions between preplate and cortical plate neurons were essential for normal laminar formation. By immunizing reeler mice with homogenates of wild-type fetal cortices, they generated a monoclonal antibody, CR-50, that recognizes a transient cell-surface molecule expressed on the marginal zone cells of wild-type but not reeler embryos. When wild-type embryos are treated with injections of the CR-50 antibody at E11, the cortex at E13 and E16 resembles that of reeler embryos: the preplate is not split by the cortical plate neurons, suggesting that the antigen recognized by CR-50 is instrumental in directing this early split of the preplate into subplate and marginal zone. This observation strengthens the hypothesis that the cellular framework consisting of the early-generated marginal zone and subplate cells plays a role in patterning the subsequent development of the cortical layers.

Subplate Neurons Mature Early and Participate in Functional Neural Circuits

Not only are subplate neurons the earliest-generated neurons of the cortex, they are also the earliest to mature, differentiate, and participate in complex neural circuits during fetal life. In the cat, cells labeled with ^3H -thymidine at E24 and then immunostained have been shown to express MAP-2 (Chun & Shatz 1989a) and peptide neurotransmitters (summarized in Table 2) well before the neurons of the cortical plate do (Chun et al 1987; Chun & Shatz 1989a,b; reviewed in Shatz et al 1988, 1990), demonstrating not only that they are neurons, but also that they acquire their adult neuronal characteristics very early.

In the adult, two classes of cortical plate neurons, interneurons and projection neurons, exhibit different transmitter phenotypes: interneurons are immunoreactive for GABA and frequently colocalize a neuropeptide such as somatostatin, NPY, or CCK (Hendry et al 1984), whereas at least some projection neurons are likely to use excitatory amino acids (Baughman & Gilbert 1980, Giuffrida & Rustioni 1989). To investigate whether subplate neurons exhibit similar phenotypes, Antonini & Shatz (1990) injected retrograde tracers into the subplate, cortical plate, or distant targets such as

Table 2 Phenotypes of subplate and marginal zone cells^a

Projection sites	Localization	Species (reference)
Thalamus	SPN	Cat (Gilbert & Kelly 1975, McConnell et al 1989)
	SPN	Ferret (Antonini & Shatz 1990)
	SPN	Rat (De Carlos & O'Leary 1992)
Contralateral hemisphere	SPN	Cat (Chun et al 1987)
	SPN	Ferret (Antonini & Shatz 1990)
Superior colliculus	SPN	Cat (McConnell et al 1989)
Cortical plate	SPN	Cat (Chun et al 1987, Wahle & Meyer 1987, Antonini & Shatz 1990, Friauf et al 1990)
	SPN	Ferret (Antonini & Shatz 1990)
	SPN	Cat (Chun et al 1987, Antonini & Shatz 1990)
Local connections within subplate	SPN	Cat (Chun et al 1987, Antonini & Shatz 1990)
	SPN	Ferret (Antonini & Shatz 1990)
<u>Classical neurotransmitters/receptors</u>		
GABA	SPZ/?	Monkey (Huntley et al 1988, Meinecke & Rakic 1992)
	SPN/MZ	Cat (Chun & Shatz 1989a, 1989b)
—GABA-A receptor	SPN/?	Ferret (Antonini & Shatz 1990)
	SPZ/?	Rat (Lauder et al 1986)
EAA uptake mechanisms	SPZ/?	Monkey (Meinecke & Rakic 1992)
	SPN/?	Cat (Antonini & Shatz 1990)
—glutamate receptors	SPN/?	Ferret (Antonini & Shatz 1990)
	SPN/?	Ferret (Herrmann & Shatz 1992)
<u>Calcium binding protein</u> (CaBP, calbindin)	SPN/?	Cat (Antonini & Shatz 1990)
	SPN/MZ	Ferret (Antonini & Shatz 1990)
<u>MAP2</u>	SPN/MZ	Rat (Liu & Graybiel 1992, Sánchez et al 1992)
	SPN/MZ	Human (Sims et al 1988, Honig et al 1990)
	SPN/MZ	Monkey (Mehra & Hendrickson 1993)
	SPN/MZ	Cat (Chun et al 1987, Chun & Shatz 1989a)
	SPN/MZ	Ferret (Allendoerfer et al 1990)
<u>CR-50</u>	SPN/MZ	Mouse (Crandall et al 1986)
	-/MZ	Mouse (Ogawa et al 1992)
<u>Neuropeptides</u>		
Neuropeptide Y (NPY)	SPN/MZ	Monkey (Huntley et al 1988, Mehra & Hendrickson 1993)
	SPN/-	Cat (Chun et al 1987, Wahle & Meyer 1987, Chun & Shatz 1989a)
	SPN/?	Ferret (Antonini & Shatz 1990)
Cholecystokinin (CCK)	SPN/MZ	Cat (Chun & Shatz 1989a)
	SPZ/?	Monkey (Huntley et al 1988)
Somatostatin (SRIF)	SPN/-	Cat (Chun et al 1987, Chun & Shatz 1989a)
	SPN/?	Ferret (Antonini & Shatz 1990)
	SPZ/?	Rat (Feldman et al 1990)
	SPZ/?	Rat (Gonzalez et al 1989)
	SPN/-	Monkey (Mehra & Hendrickson 1993)
—SRIF receptor	SPZ/?	Rat (Del Rio et al 1991)
Substance P (or SP-like)	SPN/-	Monkey (Mehra & Hendrickson 1993)
	SPZ/-	Rat (Del Rio et al 1991)

able 2 (continued)

rojection sites	Localization	Species (reference)
<u>stative degeneration/death markers</u>		
lz-50	SPZ/- SPN/- SPN/-	Human (Wolozin et al 1988) Cat (Valverde et al 1990) Rat (Al-Ghoul & Miller 1989)
ubplate-1	SPN/-	Cat (Naegele et al 1991)
<u>stative growth factors/receptors</u>		
leurotrophin family-like	SPZ/MZ	Cat (KL Allendoerfer, A Hohn & CJ Shatz, unpublished observations)
.C3	SPZ/MZ SPN/-	Ferret (Allendoerfer & Shatz 1991) Rat (Yu et al 1992)
strogen-R	SPZ/MZ	Rat (Miranda & Toran-Allerand 1992)
75-NGFR	SPZ/- SPZ/MZ SPN/- SPN/- SPZ/- SPZ/?	Human (Kordower & Mufson 1992) Monkey (Meinecke & Rakic 1993) Cat (Allendoerfer et al 1990) Ferret (Allendoerfer et al 1990) Rat (Koh & Higgins 1991) Mouse (Wayne et al 1991)
kB	SPZ/?	Ferret (Allendoerfer et al 1994)
kC	SPZ/?	Ferret (Allendoerfer et al 1994)
<u>hesion molecules</u>		
hondroitin sulfate proteoglycan (CSPG)	SPZ/MZ	Mouse (Bicknese et al 1991, Sheppard et al 1991, Miller et al 1992)
ibronectin	SPN/MZ SPN/MZ	Cat (Chun & Shatz 1988b) Mouse (Pearlman 1987, Sheppard et al 1991, Pearlman et al 1992)
aminin	SPZ/?	Rat (Hunter et al 1992)
.1	SPZ/?	Mouse (Godfraind et al 1988, Chung et al 1991)
ICAM	SPZ/?	Mouse (Godfraind et al 1988, Chung et al 1991), Human (Terkelsen et al 1992)
<u>lasma proteins</u>		
lbumin	SPZ/MZ	Human (Møllgård & Jacobsen 1984)
lpha-fetoprotein	SPZ/MZ	Human (Møllgård & Jacobsen 1984)
etuin/AHSG	SPZ/MZ SPN/MZ	Human (Dziegielewska et al 1987) Sheep (Saunders et al 1992, Dziegielewska et al, 1993)
g-like immunoreactivity	SPZ/MZ	Rat (Fairén et al 1992)
realbumin	SPZ/MZ	Human (Møllgård & Jacobsen 1984)
ransferrin	SPZ/MZ	Human (Møllgård & Jacobsen 1984)

* Abbreviations:

iPN: subplate neurons (marker is associated with subplate neurons, as proven by ³H-thymidine birth-dating studies, colocalization with another known marker, excitotoxic deletion of subplate neurons, or retrograde labeling)

iPZ: subplate zone (marker is located within the subplate zone, but is not proven to be associated with subplate neurons)

MZ: marginal zone

: not found in marginal zone

: the question of whether the marker was located in the marginal zone is not clear or was not addressed in the study

:AA: excitatory amino acids

the thalamus or opposite hemisphere and then immunostained sections for NPY or somatostatin. Results indicated that at least some of the local circuit subplate neurons are peptide or calbindin immunoreactive, whereas subplate neurons with long projections are not. Instead, subplate neurons with thalamic or interhemispheric projections could be retrogradely labeled with ^3H -aspartate, indicating that they may use an excitatory amino acid as a transmitter. These observations suggest that the subplate exhibits basic features of cortical organization later echoed in the layers of the adult cerebral cortex.

Subplate neurons also apparently participate in early functional circuits. Some of the earliest synapses in the telencephalon are found in the subplate and marginal zone, at times well before the onset of synaptogenesis in the cortical plate (Molliver et al 1973; Kostovic & Rakic 1980; König & Marty 1981; Blue & Parnavelas 1983a,b; Chun & Shatz 1988a). To investigate whether subplate neurons are capable of firing action potentials and whether they receive functional synaptic inputs, intracellular microelectrode recordings or current source density recordings were made from subplate neurons in acute slices of fetal and neonatal cat visual cortex (Friauf et al 1990, Friauf & Shatz 1991). As early as E50, subplate neurons received synaptic inputs and fired action potentials in response to electrical stimulation of the optic radiations, indicating that some of the synapses seen in the electron microscope are indeed capable of functional synaptic transmission.

To determine the presynaptic origin of at least some of these synapses, Herrmann et al (1991) injected the anterograde tracer PHAL into the thalamus of neonatal ferrets, and showed with electron microscopy that labeled thalamic axons made synaptic contacts onto subplate neurons. This finding is entirely consistent with many previous light microscope observations showing that in cat, ferret, and primate thalamic axons accumulate and "wait" in the subplate in large numbers before they grow into the cortical plate. (We will consider the subject of the "waiting" period in more detail below.) Subplate neurons are also likely to make synaptic contacts with each other in view of the fact that they extend local axon collaterals within the subplate, as revealed by immunohistochemistry (Wahle & Meyer 1987, Chun & Shatz 1989a) and intracellular injections of biocytin (Friauf et al 1990).

Subplate neurons not only have descending axons and collateral branches within the subplate, but many send axons into the cortical plate. Intracellular injections of biocytin into subplate neurons have shown that their axons can terminate within the marginal zone and cortical layer 4, particularly at neonatal ages (Figure 2) (Friauf et al 1990). It is not clear, however, whether subplate neurons that project to distant targets such as the thalamus also have collaterals within the cortical plate, or whether instead some or all of the local circuit subplate neurons are responsible. Subplate neurons receive synaptic inputs from waiting thalamic axons and in turn make axonal projections into the

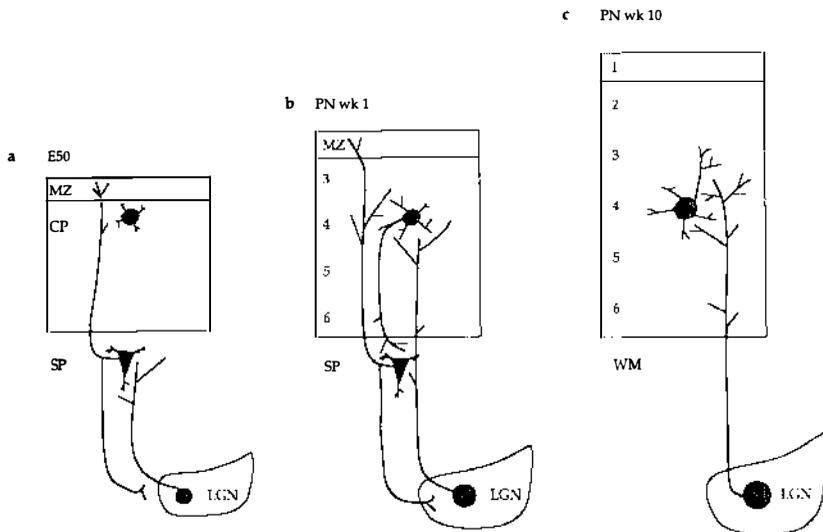


Figure 2 Subplate neurons may participate in a transient synaptic circuit that also includes thalamic axons and neurons of cortical layer 4. (a) E50 cat: While LGN axons accumulate in the subplate (SP) and make synaptic contacts with subplate neurons (Ghosh & Shatz 1992b, Herrmann & Shatz 1992), subplate neurons send axons up into the cortical plate (CP) and marginal zone (MZ) (Friauf et al 1990). They also send a projection back to LGN (McConnell et al 1989). It is not known whether a single subplate neuron can make both of these axonal projections simultaneously. (b) By postnatal week 1 in the cat, LGN axons have invaded layer 4 (Ghosh & Shatz 1992b), and subplate neurons elaborate collaterals within layer 4 (Wahle & Meyer 1987, Friauf et al 1990). In turn, layer 4 neurons send a transient projection into the subplate (Callaway & Katz 1992). Subplate neurons may also project to the LGN, but this has not been confirmed directly. Thus a recurrent pathway between layer 4 and subplate exists. (c) By postnatal week 10, the majority of subplate neurons have disappeared (Chun & Shatz 1989b) and the mature pattern of connectivity between the LGN and layer 4 is present.

cortical plate (Figure 2a), raising the possibility that they may function as a cellular scaffold that forms a crucial but transient link between developing thalamic axons and their ultimate target cells in cortical layer 4 (Figure 2b,c).

ROLE OF SUBPLATE NEURONS

Subplate Neurons Pioneer the Intracortical Pathway to Thalamus

During the development of the brain, growing axons must traverse considerable distances to find their targets. The pathway from LGN to cortical layer 4, or the reciprocal pathway from cortical layer 6 to LGN, is many tens of millimeters by adulthood, and even early in development the sets of neurons

that must ultimately interconnect are many cell body diameters distant from each other. A solution to this difficult problem may be found in the early outgrowth of axons from pioneer neurons (Bate 1976, Kuwada 1986, Klose & Bentley 1989; reviewed in Goodman & Shatz 1993) when distances between neurons and their targets are small and local environmental cues appear sufficient to point growth cones in the right direction. To examine the earliest efferent projections from the cerebral cortex, several investigators (McConnell et al 1989, Blakemore & Molnár 1990, De Carlos & O'Leary 1992, Erzurumlu & Jhaveri 1992; SK McConnell, A Ghosh & CJ Shatz, submitted) have used the lipophilic fluorescent tracer 1,1-dioctadecyl-3,3,3',3'-tetramethyl indocarbocyanine perchlorate (DiI) to trace connections in fetal brains. McConnell et al (1989) found that when DiI was placed in the internal capsule at E30 in the cat, the injection retrogradely labeled exclusively neurons in the preplate, which at this early age is composed only of subplate and marginal zone neurons. At later ages, after the preplate has split into subplate and marginal zone, similar injections labeled only subplate, but not marginal zone, neurons. Hence, it is likely that subplate but few if any marginal zone neurons supply the early axons to the internal capsule. DiI injections into the preplate itself at E30 labeled axons traveling through the intermediate zone and entering the internal capsule (McConnell et al 1989), showing that some subplate neurons had extended descending axons very early, before many of the neurons of layers 5 and 6, which form the descending projection in the adult, had even become postmitotic and begun to migrate (Luskin & Shatz 1985a). Moreover, at E26, DiI injections into the preplate never retrogradely labeled any neurons outside of cortex. Placement of DiI directly into the thalamus starting at E30 retrogradely labeled subplate neurons in temporal cortex, and then, by E36, also the subplate neurons underlying visual cortex. Thus, these observations collectively indicate that the axons of subplate neurons are the first to traverse the pathway from cortex through the internal capsule in the cat—that is, subplate axons “pioneer” these pathways in the classic sense of the word (Bate 1976).

In rodents, subplate neurons perform a similar pioneering function *in vivo* for the corticothalamic projection (De Carlos & O'Leary 1992, Erzurumlu & Jhaveri 1992). Unlike McConnell et al's (1989) findings for carnivores, however, De Carlos & O'Leary (1992) did not find any evidence that subplate neurons extended axons beyond the thalamus to the superior colliculus. In addition, in rodents, subplate neurons apparently do not send axons into the spinal cord or even into the midbrain superior peduncle (De Carlos & O'Leary 1992). The observation that subplate axons do project to superior colliculus in carnivores but do not in rodents could be a result of a species difference; the pathway taken by corticotectal axons in the cat is a dorsal one, extending posteriorly past the LGN and through the pretectum (SK McConnell, A Ghosh

& CJ Shatz, submitted), whereas in the rat, the colliculus is innervated by collaterals that branch dorsally from ventral spinally-projecting axons (O'Leary et al 1990). Thus the mechanism for layer 5 axon pathfinding toward the superior colliculus may differ between rodents and carnivores. On the other hand, given the sensitivity of this type of experiment to the exact placement of the DiI injection, it is possible that the DiI was placed in such a way in the rodent as to miss the small population of subplate axons that project to the superior colliculus; alternately, dye leakage could conceivably have labeled subplate neurons in the cat that project only to the posterior thalamus. Future experiments are necessary to resolve this question definitively.

In contrast, ascending axons from subcortical structures such as the brainstem do not invade the cortex until much later, after the reciprocal connections between cortex and thalamus have already begun to form in the rodent (De Carlos & O'Leary 1992). Immunohistochemical studies of the monoaminergic and catecholaminergic pathways to cortex (Schlumpf et al 1980) have indicated that these axons traverse the intermediate zone at E16 at the earliest. A similar conclusion has been reached by Erzurumlu & Jhaveri (1992), in a study of rat cortical development in which the first brainstem neurons were not retrogradely labeled with DiI injections into the cortex until E17, well after subplate neurons could be labeled with an injection in the internal capsule. All of these considerations suggest that the first axon pathway laid down within the intermediate zone of the cerebral cortex derives from the subplate neurons. If so, this raises the possibility that as in invertebrates the later-growing efferent axon systems, such as those arising from cortical layers 5 and 6, travel along the pathway originally laid down by the subplate axons, and that this early pathway is necessary for target selection.

A good way to test directly whether the subplate neurons play an essential role in subsequent pathfinding and target selection by the axons of cortical layers 5 and 6 would be to ablate subplate neurons at very early times in development, before their axons have reached the internal capsule, and then to examine the consequences for the corticothalamic or corticotectal projections. At present, no methods are available that permit the selective ablation of subplate neurons at these ages. However, in the cat, by E37 kainic acid injections selectively remove subplate neurons, leaving the neurons of the cortical plate essentially intact (Chun & Shatz 1988b, Ghosh et al 1990; SK McConnell, A Ghosh & CJ Shatz, submitted). At this time, although subplate axons have traversed the internal capsule and invaded the thalamus, cortical plate axons have just begun to elongate within the intermediate zone and have not yet reached the internal capsule (SK McConnell, A Ghosh & CJ Shatz, submitted). The effects of subplate ablation on cortical axogenesis were assessed by allowing lesioned animals to develop until around birth, when

^3H -leucine was injected into the visual cortex to trace the pattern of the descending projection. In half of the lesioned animals, descending projections formed normally. In the other half, however, the labeled axons traversed the internal capsule and then gathered at the antero-medial borders of the LGN (in the region of the perigeniculate nucleus), where they failed to grow into the body of the LGN. This situation contrasts markedly to that in normal animals at comparable ages, in which cortical axons have already grown well into the LGN and are concentrated in interlaminar regions.

In these same animals where the LGN projection was abnormal, cortical projections to and within other subcortical regions (e.g. the superior colliculus) were also missing, although intracortical pathways such as the projections to the claustrum and across the corpus callosum appeared normal. These observations imply that subplate neurons are involved in the process of target invasion by cortical axons, but that there may be additional cues available as well. Similar conclusions have been drawn from experiments in lower vertebrates (Kuwada 1986) and invertebrates (Bastiani et al 1985) when pioneer neurons have been ablated genetically or with a laser.

Taken together, these observations imply a critical role for subplate pioneers in the normal innervation of subcortical targets by cortical plate neurons. Surprisingly, however, in the absence of subplate neurons, cortical plate neurons are apparently able to navigate more or less correctly through the internal capsule and grow toward the appropriate thalamic target, although they do not innervate it. A simple hypothesis is that cortical plate axons employ subplate axons as a substrate for growth through the internal capsule and into the subcortical targets. At the earliest times of their outgrowth from the cortical plate, these axons would have easy access to subplate axons and could grow along them. The subplate neurons were ablated in this study at the earliest at E37, when some cortical plate outgrowth has begun. These early interactions between subplate and cortical plate axons may be sufficient to start the cortical plate axons down the correct path. It is also possible that once the pioneer pathway has been laid down by the initial subplate neuron outgrowth, some residual molecular cues remain in the internal capsule which serve to guide the cortical axons in a crude manner; and it is likely that additional environmental cues are present in the extracellular matrix, independent of subplate neurons. *In vitro* studies have shown that axons can employ more than one extracellular cue for growth, and that elimination of any single cue can be insufficient to block outgrowth (Neugebauer et al 1988, Tomaselli et al 1988). The failure of cortical plate neurons to innervate their subcortical targets in half of the ablated animals implies that target recognition involves two distinct steps, pathfinding and innervation, and suggests an additional role for the subplate axons in the second. The absence of a projection to the superior colliculus in these animals implies that in the cat,

at least, there is indeed some role for the subplate neurons in pioneering not only the corticothalamic pathway but also the corticotectal pathway. It would also be of great interest to perform similar experiments in other species to examine the generality of these observations.

Several groups have cultured slices of rat cortex either alone or in conjunction with a target slice, such as LGN or another slice of cortex (Yamamoto et al 1989, 1992; Bolz et al 1990; Molnár & Blakemore 1991) with the goal of further investigating pathfinding mechanisms. Bolz et al (1990) and Yamamoto et al (1992) did not comment on the projections of the subplate neurons in their cortical slices to the thalamic or other cortical explants in their cultures; nonetheless, the thalamic slices were innervated by the appropriate projection neurons (in the deep layers) of cortical explants, whereas the cortical slices were appropriately innervated by neurons in more superficial layers. Bolz et al (1990) therefore concluded that the projections of subplate neurons are not necessary for the formation of corticothalamic projections *in vitro*. However, the presence or absence of subplate neurons was not confirmed directly in these slices by labeling with ^3H -thymidine or other markers (see Table 2). In addition, the cocultures involved slices of cortex taken at P1, after connections between thalamus and cortex have already been established *in vivo*, raising the possibility that the growing cortical axons are responding to cues laid down much earlier. Finally, it may be that the subplate neurons are required for the targeting of cortical axons to the correct thalamic nucleus, rather than for the generic ingrowth of cortical axons into thalamus, which is being assessed in the coculture studies. Further experiments are required to resolve these issues and to establish the appropriateness of this *in vitro* approach for elucidating mechanisms of pathfinding as opposed to target selection. For example, it would be of great interest to repeat these coculture experiments with "naive" cortex and thalamus taken from fetal rats, particularly because the subplate deletion experiments considered above were performed in cat much earlier in development than these *in vitro* studies.

Are Subplate Neurons Required for Thalamocortical Pathfinding?

As described above, the axons of subplate neurons may create or themselves serve as a scaffold on which the descending corticothalamic projection is built. This subplate neuron scaffold may also serve an additional purpose in the establishment of the ascending thalamocortical projections. Thalamus and cortex must specifically connect with each other in two different ways: they must first find their appropriate modality-specific cortical area, and then must grow into their appropriate laminar targets, cortical layers 4 and 6 [for a recent review, see also O'Leary & Koester (1993)]. This specificity of

connections could come about by several mechanisms, including selective fasciculation, timing of axon outgrowth, extracellular matrix or cell-surface cues within the cortical plate and subplate, chemotropic guidance mechanisms, or even competition between ingrowing thalamocortical axons for common target neurons located in the subplate or cortical plate.

In considering mechanisms by which thalamic axons might grow to their appropriate cortical target areas, one possibility is that thalamic axons require subplate axons. This idea arises from observations, based on DiI labeling of subplate and thalamus, that the first growth cones of LGN neurons and visual subplate neurons are seen within the internal capsule at the same time (E30 in cat: McConnell et al 1989, Ghosh & Shatz 1992b; E14–15 in rat: Blakemore & Molnár 1990, De Carlos & O'Leary 1992), and likewise for axons from somatosensory thalamus and corresponding subplate in rat that were labeled with DiI and DiA and visualized simultaneously at E15 (Erzurumlu & Jhaveri 1992). These observations raise the possibility that thalamic and subplate axons from corresponding regions might meet in the internal capsule and subsequently fasciculate on each other as they make their final traverse to the appropriate targets within thalamus or cortex, and that this fasciculation could form the basis for the establishment of specific connections between thalamus and cortex (Blakemore & Molnár 1990, Ghosh & Shatz 1993). Such selective fasciculation between anterior- and posterior-growing axons has been observed in the development of longitudinal pathways in the grasshopper embryo. Moreover, in these embryos, laser ablation of two specific posterior-growing axons prevents the anterior-growing axon from growing normally and reaching its target (Bastiani et al 1986).

However, an apparent contradiction with the suggestion that selective fasciculation underlies the process of pathfinding by thalamocortical and corticothalamic axons is that at later times, DiI-labeled axon bundles from the cortex and from the thalamus apparently run in separate fascicles, with the afferent thalamocortical axons traveling more superficially in the subplate (Bicknese et al 1991, Miller et al 1991a), and the efferent corticothalamic axons deep in the intermediate zone (Shatz & Rakic 1981, Bicknese et al 1991, Miller et al 1991a; SK McConnell, A Ghosh & CJ Shatz, submitted; in all of these studies, the location of subplate axons in relation to the afferent and efferent pathways was not determined). It may be important that these two pathways are also different with regard to cell-surface molecules. Two chondroitin sulfate proteoglycan (CSPG) core proteins are apparently restricted to the afferent thalamocortical pathway, but not the corticothalamic pathway (Bicknese et al 1991, Sheppard et al 1991, Miller et al 1992) (see Table 2), suggesting that specific cortical pathways can be marked with different molecules. Even though it appears that at later times there are spatially distinct pathways to and from cortex, it may be that the earliest thalamic axons and

subplate axons do fasciculate with each other, but the later-growing thalamic or cortical axons prefer to grow on like axons. This would result in the formation of two separate pathways, but with the axons of subplate neurons and the earliest growing LGN neurons located at the interface. Some support for this suggestion comes from preliminary studies in which one fluorescent dye was used to label the corticothalamic axons and another to label the thalamocortical axons, and the two axons pathways were seen to be apposed to each other in the intermediate zone (Blakemore & Molnár 1990) or internal capsule (Bicknese & Pearlman 1992). More definitive evidence is obviously required here, including: (a) examining these pathways at very early ages, (b) showing at the ultrastructural level that thalamic axons fasciculate on identified subplate axons, and (c) demonstrating that deleting subplate neurons before thalamic axons traverse their intracortical pathways prevents them from arriving at their correct cortical targets.

Subplate Neurons are Required for Target Selection and Ingrowth by Thalamic Axons

Once axons from the thalamus have successfully navigated through the internal capsule and intermediate zone, they must stop at the appropriate cortical target and grow into the cortical plate. Early studies of the development of connections between geniculocortical axons and their ultimate targets, neurons of cortical layer 4, used relatively low resolution tract tracing techniques such as transneuronal labeling with ^3H -proline (Rakic 1977, Shatz & Luskin 1986) or thalamic lesions followed by examination of degenerating axon terminals (Lund & Mustari 1977). These studies showed that label representing thalamic axons accumulated within the subplate for an extended period of time, from three days in rats (Lund & Mustari 1977) to several weeks in cats and primates (Rakic 1977, Shatz & Luskin 1986), before invading the cortical plate. From these observations developed the concept of a "waiting period" (Rakic 1977), in which axons accumulated and paused in the subplate for a period of time before growing into layer 4. In addition to the geniculocortical axons from visual thalamus, other afferent axonal systems, including thalamocortical projections from somatosensory thalamus (Wise & Jones 1978) and inter-hemispheric connections via the corpus callosum (Wise & Jones 1976, 1978; Innocenti 1981), appear to "wait" in the subplate before invading the cortical plate, suggesting that waiting periods may be a general feature of the development of corticopetal axonal systems.

More recent studies that reexamined the waiting period with DiI tracing techniques provide further evidence for potential interactions between the "waiting" axons and cellular elements in the subplate. In the cat, Ghosh & Shatz (1992b) found that the geniculocortical axons arrived earlier in the subplate (E36 rather than E40) and invaded the cortical plate earlier (E50

rather than E55) than expected based on data from ^3H -proline autoradiography (Shatz & Luskin 1986). During the intervening time between E36 and E50, the majority of LGN axons accumulate within the subplate beneath the visual cortex, forming extensive terminal branches confined to the visual subplate. In addition, as axons grow through the optic radiations on their way to visual cortex, they send out interstitial collaterals into the subplate of inappropriate areas (e.g. the auditory subplate) as if the axons were “sampling” a variety of cortical areas. Naegele et al (1988), studying geniculocortical development in the hamster by using HRP tracing, also showed that between P3 and P5 multiple short collaterals with no terminal arbors were extended into the subplate and deeper portions of the cortical plate. In both species, these collaterals appear to be a transient feature of the developing geniculocortical pathway, since they are absent by P7 (Ghosh & Shatz 1992b) in cat and during the second postnatal week in the hamster (Naegele et al 1988). The existence of collaterals and terminal branches within the subplate during the waiting period contrasts with the simple morphology of thalamocortical axons—consisting of a single parent axon tipped with a growth cone—at earlier times in development. The existence of these collaterals in a zone also filled with subplate neurons and many synapses implies that thalamocortical axons may be involved in ongoing dynamic interactions both as they grow towards their appropriate area and while they are “waiting” within the subplate appropriate for their thalamic nucleus of origin.

Although there is no dispute concerning the existence of a long waiting period in cats and primates, where the pace of development is comparatively slow and it is possible to define a clear period in which thalamocortical axons are confined to the subplate, **in rodents the very rapid pace of development has led to some controversy about whether there is a waiting period and if so, how long (Wise & Jones 1978, Catalano et al 1991). This controversy is based on more recent experiments in which DiI has been used to label somatosensory thalamocortical axons in the rat, and then the timecourse of their ingrowth into the cortical plate has been assessed by inspecting fluorescence micrographs (Catalano et al 1991).** Such experiments suggest that thalamic axons proceed directly from the subplate into the cortical plate without a significant delay (e.g. days to weeks). However, as mentioned above, Naegele et al (1988) have shown that thalamic axons make modest branches in the rodent subplate, suggesting that interactions, albeit much more limited in extent and duration than in higher mammals, may still take place. **Clarification of this point would be aided if the precise border between the subplate and the cortical plate could be pinpointed with more accuracy in rodents, perhaps by combining axon labeling studies with ^3H -thymidine birthdating, in order to determine exactly when thalamic axons leave the subplate.**

The role of the interactions between subplate neurons and thalamocortical axons was tested by deleting them during the waiting period (Ghosh et al 1990; Ghosh & Shatz 1992b, 1993). Visual subplate neurons in the cat were deleted with an injection of kainic acid at E42, and the histology of the subplate zone and the morphology of the LGN axons were examined at E60. In subplate-ablated animals, the entire zone that normally contains the subplate neurons had collapsed, and the LGN axons no longer arborized below the cortical plate. Instead of fanning out and branching in the subplate, the axons grew past the visual cortex in a tight fascicle. These observations suggest that subplate neurons are necessary for LGN axons to stop and arborize below their normal cortical target before they grow into the cortical plate. If the cortex is observed later, at P5, the axons still have not grown in, even though normally they would have done so more than two weeks earlier; in fact, they grow past visual cortex and into the white matter underlying the adjacent cingulate gyrus. This phenomenon is not specific for visual subplate. If a deletion is made in the subplate beneath the auditory cortex and DiI is injected into the MGN, it can also be seen that MGN axons grow past auditory cortex (Ghosh & Shatz 1993). It remains to be seen whether at later ages these axons are then able to innervate layer 4 in other, non-auditory areas, which still contain subplate neurons. Such findings could shed light on the issue of whether intrinsic differences among subplate neurons specify the identity of overlying cortical areas, or whether instead there are competitive interactions within the subplate, coupled with timing of arrival of axons at their correct cortical target areas. Whatever the case, these experiments imply that the cortical plate alone does not contain sufficient information to promote axon ingrowth; interactions in the subplate are necessary.

Although the nature of these interactions is unknown, several lines of evidence suggest that thalamocortical axons exhibit an affinity for the earliest-generated subplate neurons. For example, in the reeler mouse, where the preplate fails to split (Caviness & Rakic 1978, Ogawa et al 1992) and consequently subplate is located above the cortical plate as a kind of "superplate", the geniculocortical axons still initially grow into the superplate at E15 (Molnár & Blakemore 1992). They traverse the cortical plate in diagonal fascicles toward the early-generated superplate cells, wait on top of the developing cortical plate (Molnár & Blakemore 1992), and finally loop down to innervate cortical layer 4 (Frost & Caviness 1980, Caviness & Frost 1983, Molnár & Blakemore 1992). Thus, thalamocortical axons may select subplate neurons regardless of the radial position of these neurons within cortex, again implying close interactions.

It is possible that the affinity of thalamic axons for subplate neurons could be mediated by adhesive interactions. An array of adhesion and extracellular matrix molecules have been shown to be localized to the subplate and marginal

zone, but not the cortical plate, early in development (see Table 2). For example, L1, a cell surface glycoprotein, and J1, a secreted glycoprotein, were detected in the subplate and marginal zone of developing mouse embryos (Godfraind et al 1988). Additionally, fibronectin is found in these zones in both cat and rodent (Chun & Shatz 1988b, Pearlman et al 1992), and at least some of it is thought to be associated with the subplate neurons themselves (Chun & Shatz 1988b, Pearlman et al 1992). Moreover, deletion of subplate neurons abolishes the fibronectin immunoreactivity, implicating a possible role for this matrix molecule during the period in which axons normally wait in the subplate. Not only might there be permissive influences on axon growth, but preliminary evidence from coculture experiments suggests that the cortical plate itself may be nonpermissive for thalamic axon ingrowth. Götz et al (1992) demonstrated that when thalamic explants were cocultured with E16 cortex, rather than P1 cortex, there was no innervation of the cortical slice. In a separate *in vitro* assay, it was then shown that membranes from E16 but not P7 cortex cause growth cone collapse (Hubener et al 1992), suggesting that repulsive influences from embryonic cortex might also contribute to the waiting period. If so, the relative affinities of the thalamic axons for subplate and cortical plate would be expected to change at the end of the waiting period and the onset of invasion of the cortical plate.

Do Subplate Neurons Play a Role in the Specification of Cortical Areas?

Much discussion has addressed the issue of how cortical areas are specified during development. One view is that cortical areas have intrinsic differences that are mapped out very early in development, perhaps even in the ventricular zone (Rakic 1988). Alternatively, cortical areas may emerge gradually from an undifferentiated "protocortex" through epigenetic influences such as interactions with afferent inputs (O'Leary 1989, Shatz 1992). The results of Ghosh et al (1990) and Ghosh & Shatz (1993) indicate that subplate neurons stand as a crucial link in cortical target recognition, whichever strategy is used. The observation that LGN axons grow past the visual cortex in the absence of subplate neurons indicates that the cortical plate alone has insufficient information to allow the ingrowth of appropriate axons.

Several pieces of evidence suggest, however, that thalamic axons are not guided to their appropriate target areas solely by molecular "labels" present on subplate neurons in different cortical areas. In coculture experiments, axons from LGN explants appear to terminate appropriately in layer 4 of visual cortical explants and to form functional connections, as revealed by DiI labeling (Molnár & Blakemore 1991, Yamamoto et al 1992) and current source density analysis (Yamamoto et al 1989, 1992). However, when LGN explants are challenged to make a decision between cortical explants derived from both

appropriate and inappropriate regions (i.e. visual vs frontal), the thalamic explants are able to innervate both cortical explants with appropriate laminar specificity, regardless of the area of origin (Molnár & Blakemore 1991, Yamamoto et al 1992). In vivo experiments also suggest that different areas of cortex have multiple developmental potentials and their ultimate fate is dependent on the afferents they receive. Schlaggar & O'Leary (1991) transplanted occipital (visual) cortex to the presumptive barrel field of parietal (somatosensory) cortex, and found that barrel-like morphologies developed in the occipital-derived transplant. Therefore, the ability to form barrels is not unique to "somatosensory" cortex, the region that forms barrels normally, but is also possessed by the embryonic "visual" cortex.

These experiments would seem to suggest that the subplate does not possess intrinsic positional information that marks an area as appropriate for ingrowth, or thalamic explants should have exhibited a preference in vitro, and/or might not innervate a transplant from another cortical area that contained inappropriate subplate neurons. However, in the case of the transplant studies, the transplants were made in newborn rats, when VB axons had already begun to grow into the somatosensory cortex in vivo. The axons were thus cut during the transplantation experiment and presumably regrew from the thalamic radiations into the transplant. They did not have to renavigate the entire pathway from thalamus to cortex. Thus the cues needed may have been present in the subplate early on when the VB axons were first growing towards cortex but were perhaps no longer relevant, or even present, after birth.

Visual Subplate Neurons Are Involved in the Formation of Ocular Dominance Columns

In the visual cortex of higher mammals, thalamic axons from the LGN are segregated in cortical layer 4 according to eye preference into alternating patches of input that represent the anatomical basis for the system of ocular dominance columns (Hubel & Wiesel 1977). The segregation of LGN axons into the ocular dominance columns within cortical layer 4 represents the end-point of their developmental history, and it occurs shortly after LGN axons have left the subplate and invaded layer 4. Axons representing both eyes are initially intermixed with each other in layer 4, and then through a process of remodeling and selective growth, segregate to form eye-specific patches. In the cat and monkey visual system, segregation is complete at about six weeks postnatal (LeVay et al 1978, 1980); in cat, the process begins at about three weeks postnatal, whereas in the monkey this process begins prenatally (Rakic 1977).

Several lines of evidence suggest that subplate neurons may play a role in this final stage of thalamocortical development, at least in the visual system. As shown in the schematic diagram of Figure 2b, intracellular injections of

biocytin into subplate neurons during the first postnatal week in the cat visual system, a time when LGN axons have already invaded layer 4 but before the onset of segregation, indicate that some subplate neurons can send extensive axonal collaterals within layer 4 of the cortical plate (Friauf et al 1990). At the same time, similar intracellular injection techniques have revealed that many layer 4 neurons send a transient axon collateral that traverses layers 5 and 6 to branch within the subplate (Callaway & Katz 1992). These observations suggest that a transient and reciprocal synaptic circuit between the subplate and layer 4 may be present at the onset of ocular dominance column formation. Although future physiological experiments and ultrastructural studies are necessary to confirm the presence of this proposed circuit, electron microscopic studies following PHAL injections into the cortical plate have shown that in neonatal cats some descending cortical axons can make synaptic contacts within the subplate (Lowenstein & Shering 1992).

More direct evidence for a role for subplate neurons in the segregation of LGN axons comes from an experiment in which kainic acid was injected into the subplate during the first postnatal week in the cat to ablate subplate neurons, and the consequences were examined six to eight weeks later, when ocular dominance columns in layer 4 should have normally formed. Results obtained by using the technique of transneuronal transport following intraocular injection of ^3H -amino acids into one eye showed that in the region of visual cortex corresponding to the subplate ablation, LGN axons had failed to segregate into eye-specific patches within layer 4, whereas elsewhere columns had formed normally (Ghosh & Shatz 1992a). This observation suggests that subplate neurons play a role not only at early times in the formation of thalamocortical connections, but also in the final patterning of the geniculocortical projection within cortical layer 4. Although this type of ablation experiment cannot shed light on the underlying cellular interactions that operate normally to produce ocular dominance columns, it is worthwhile to consider whether a similar manipulation in rodent somatosensory cortex might disrupt barrel formation. If so, this would indicate a broad role for subplate neurons in the final patterning of thalamocortical projections within layer 4.

DEATH OF SUBPLATE NEURONS

In the adult, it has long been known that the white matter and layer 1 contain only a few scattered neurons. These are the interstitial cells in the white matter (Ramón y Cajal 1911) and the Cajal-Retzius cells of layer 1 (Marin-Padilla 1971, 1988). Birth-dating studies have proven that many if not all of these neurons are derived from the subplate and marginal zone neurons, because they can only be labeled by injections of ^3H -thymidine at the earliest ages of

cortical neurogenesis (Chun & Shatz 1989b, Woo et al 1991). The scarcity of neurons in the adult in what was a dense neuropil during development suggests that many of the subplate and marginal zone cells are eliminated by cell death. Several lines of evidence strongly support this possibility. First, degenerating neurons with the appropriate interstitial or Cajal-Retzius cell morphology have been observed in the subplate (Kostovic & Rakic 1980, Valverde & Facal-Valverde 1988) and marginal zone (Shoukimas & Hinds 1978, Derer & Derer 1990) of neonates by using electron microscopy. These cells exhibit a swelling of the endoplasmic reticulum and Golgi followed by a progressive darkening of the cytoplasm, a type of degeneration classified as "cytoplasmic" (Pilar & Landmesser 1976, Cunningham 1982) and thought to be characteristic of dying mature neurons. Wahle & Meyer (1987) also observed degenerating NPY immunoreactive "axonal loop" cells in the neonatal cat white matter.

Moreover, the density of subplate neurons immunostained for neuropeptides or for MAP2 in the cat decreases dramatically during the first four postnatal weeks until adult levels are reached (Chun & Shatz 1989b). For example, the density of MAP2-immunoreactive neurons remaining in the white matter of the cat lateral gyrus at 25 weeks postnatal is only about 10–20% of the density at birth. In contrast, the area of the white matter increases by about twofold at most, indicating that growth alone cannot account for this decrease in density. In two rodent studies (Woo et al 1991, Wood et al 1992) a more detailed statistical analysis was performed to account for apparent dilution of subplate neurons by growth of the cortex. In the hamster (Woo et al 1991), a ^3H -thymidine labeled subplate population was compared with a ^3H -thymidine labeled "reference" population in layer 6 directly adjacent to the subplate. Woo et al (1991) showed that the ratio of subplate neurons to layer 6 neurons decreased approximately fourfold between P4 and adulthood. A similar analysis was also performed by comparing the subplate neurons with a reference population of LGN neurons, with nearly identical results. In the mouse, Wood et al (1992) estimated that a greater than 75-fold decrease occurred in labeled subplate neurons cells per unit volume between birth and P21, whereas the upper limit for increase in white matter volume was only 15-fold. They found no E12-labeled cells remaining in the marginal zone at P21, although some cells labeled at E12 were present in the marginal zone at E17. Thus, again it is not possible to account for the fall in subplate neuron number entirely on the basis of growth of the white matter: a major portion of the subplate and marginal zone neurons must be eliminated by cell death during early postnatal life. Although developmentally regulated cell death appears to be a universal feature of subplate neurons in all species where it has been examined, the extent of subplate neuron death is almost certainly not the same in all species, nor is it likely to be uniform between cortical

areas or among the different subplate neuron phenotypes in a given species (Chun & Shatz 1989b, Mehra & Hendrickson 1993).

Molecular Changes Accompany Subplate Neuron Death

The mechanism by which subplate neurons die remains an open question. Immunohistochemical studies have provided evidence that subplate neurons undergo molecular changes during the period of cell death. Naegele et al (1991) isolated an antibody, SP-1, that recognizes a 56-kDa polypeptide expressed transiently and exclusively within subplate neurons (but not marginal zone cells) just after birth in the cat, a time that corresponds to the peak of cell death. Many of the neurons expressing the SP-1 antigen possess an inverted pyramidal morphology, project to thalamus, and are glutamatergic; only a few SP-1⁺ cells express GABA or peptides. The identity of this antigen is unknown, but it is thought to be intracellular.

Staining with Alz-50, a monoclonal antibody isolated from a screen directed against neurofibrillary tangles in the brains of Alzheimer's patients (Wolozin et al 1986), also labels subplate neurons in rat (Al-Ghoul & Miller 1989), cat (Valverde et al 1990), and human (Wolozin et al 1988) during a period of active cell death. The Alz-50 epitope is now known to correspond to an abnormally phosphorylated form of the microtubule-associated structural protein tau (Ueda et al 1990, Lee et al 1991). The SP-1 antigen appears to be distinct from Alz-50 in immunoblots, and exhibits an overlapping, but more restricted, pattern of cellular localization (Naegele et al 1991). In addition to its expression in Alzheimer's disease, Down's syndrome, and developmentally regulated cell death, Alz-50 can be induced in neurons by environmental insults such as axotomy (Miller et al 1991b) or by the elevation of protein kinase C (Mattson 1991), and is thought to be a general marker for dying neurons.

The expression of antigens or proteins unique to the death process suggests that the subplate neurons may actively turn on a program of "death genes." This view of neuron death as an active process that requires transcription and protein synthesis derives from studies of cultured sympathetic neurons (Martin et al 1988) or PC12 cells (DiBenedetto et al 1992) deprived of NGF, where the death of these NGF-deprived neurons is inhibited by transcription and protein-synthesis inhibitors. Another possibility, not mutually exclusive, is that certain genes are turned off during the death process. For example, the proto-oncogene *bcl-2* is expressed developmentally in lymphocytes that do not die (Korsmeyer 1992) and, in the immune system, is thought of as a repressor of cell death. It would be interesting to know whether the interstitial neurons and Cajal-Retzius cells that survive into adulthood express *bcl-2* or other relevant proteins.

Neurotrophic Factors May Control Survival and Death of Subplate Neurons

It is also possible that neurotrophic factors may play a critical role in subplate neuron survival. Subplate neurons express the low-affinity NGF receptor (p75^{LNGFR}) soon after they become postmitotic (Allendoerfer et al 1990). Expression of p75^{LNGFR} remains high during maturation of the subplate neurons and the waiting period for thalamic axons (Allendoerfer et al 1990, Koh & Higgins 1991, Wayne et al 1991, Kordower & Mufson 1992, Meinecke & Rakic 1993). Subplate neurons are also immunoreactive for a member of the neurotrophin family, based on immunostaining with a pan-neurotrophin antibody (Allendoerfer & Shatz 1991) at the same time that the p75^{LNGFR} staining is strong. This staining for neurotrophin, also observed in basal forebrain neurons that are known to retrogradely transport NGF in the adult (Seiler & Schwab 1984) and during development (Allendoerfer & Shatz 1991), suggests that the subplate neurons could also internalize and transport NGF or a related neurotrophin (Allendoerfer & Shatz 1991). Just before the subplate neurons begin to die, they lose p75^{LNGFR} immunoreactivity, suggesting that they die because they are no longer able to respond to the ligand (Allendoerfer et al 1990). Several days later, the neurotrophin immunoreactivity also disappears from the subplate (Allendoerfer & Shatz 1991).

Although death resulting from trophic factor deprivation is an intriguing hypothesis, the identity of such a factor for subplate neurons has so far been elusive. The p75^{LNGFR} was identified first on neurons that retrogradely transport ¹²⁵I-NGF from their target tissues, such as basal forebrain neurons (Seiler & Schwab 1984, Yan & Johnson 1989, Ferguson et al 1991); however, retrograde transport of ¹²⁵I-NGF to subplate neurons is not observed when labeled factor is injected into any subplate neuron targets, such as thalamus or internal capsule, nor when it is injected directly into the subplate itself (Allendoerfer & Shatz 1991). Moreover, the addition of NGF to E30 ferret subplate neurons in culture does not enhance their survival (KL Allendoerfer & CJ Shatz, unpublished observations). Since p75^{LNGFR} is able to bind equally well to other members of the neurotrophin family, such as BDNF and NT-3 (Rodriguez-Tébar et al 1990, 1992), the possibility remains that a neurotrophin distinct from NGF supports subplate neurons. High-affinity receptors for different neurotrophins, including trkB, truncated trkB, and trkC, are also present in the subplate zone, both early, when the subplate neurons first become postmitotic, and later, as they are dying (Allendoerfer et al 1994). Furthermore, the elegant model of neurotrophin action developed for sympathetic and sensory neurons in which a population of neurons projects to a single target, from which it derives trophic support, may not apply to a heterogeneous population of neurons such as the subplate, which has a number

of different transmitter phenotypes and projection targets both local and distant—subplate neurons project into the cortical plate, across the corpus callosum, back to thalamus, and locally, to one another (see Table 2). The neurotrophin immunoreactivity within the subplate may not result from retrogradely transported neurotrophin but rather from neurotrophin synthesized locally within the subplate that could act in an autocrine fashion, as has been hypothesized for the peripheral nervous system during development (Schechterson & Bothwell 1992).

In addition to neurotrophins, other putative growth factors have also been shown either to be present in the subplate neurons or to have receptors there. For example, Yu & Bottenstein (1991) generated an antibody (AC3) to an uncharacterized growth factor that increases O-2A progenitor cell proliferation; this antibody stains neurons in the subplate zone of fetal and early postnatal rat and has been shown to colocalize with p75^{NGFR} staining in both the subplate and septum (Yu et al 1992). Miranda & Toran-Allerand (1992) have shown that estrogen receptor mRNA and ¹²⁵I-estrogen binding sites are also present in the subplate and marginal zone as these neurons mature. Thus, estrogen may be important in the maturation of the subplate zone and/or subplate neurons, either alone or in concert with one or more growth factors.

Additional Mechanisms May Play a Role in Subplate Cell Death

It is also possible that growth factors serve a maturation and/or differentiation function, but not a survival function within the subplate, and that neuron death takes place through an excitotoxic mechanism. As the subplate neurons mature, they develop a sensitivity to kainate toxicity, and as mentioned earlier this early sensitivity has been used in experimental manipulations to delete subplate neurons selectively (Chun & Shatz 1988b, Ghosh et al 1990; McConnell et al, submitted). In addition, subplate neurons also undergo increases in intracellular Ca²⁺ in response to application of glutamate or agonists in acute slice preparations (Herrmann & Shatz 1992), indicating that developing subplate neurons also possess an assortment of glutamate receptors, including kainate, NMDA, quisqualate, and metabotropic (Herrmann and Shatz 1992; K Herrmann, unpublished observations). Thus the subplate neurons are likely to be vulnerable to glutamate neurotoxicity (Choi 1992) during the cell death period. Molnár et al (1991) proposed that glutamate released by the waiting thalamic axons normally plays a role in the death of subplate neurons. They labeled rat subplate neurons with ³H-thymidine at E12, and then mechanically lesioned the internal capsule at E16, thereby depriving the subplate of all innervation. When they counted ³H-thymidine-labeled cells postnatally, they found that the number on the lesioned side was

greater than that on the control side. They suggested that the increased subplate neuron survival results from the absence of "excitotoxic input" during the period when subplate neurons would normally receive synaptic contacts from the waiting thalamic axons. Unfortunately, this interpretation is somewhat at odds with the presence of a prolonged waiting period observed in "higher" mammals such as cats and monkeys, where the thalamic axons make synaptic contacts with the subplate neurons for weeks or months, without any signs of excitotoxic damage. On the other hand, the sensitivity of subplate neurons to glutamate may continue to increase throughout the waiting period, only becoming sufficient for excitotoxic damage very near the end. It is also possible that it is actually the departure of the thalamic axons from the subplate or their ingrowth into layer 4 that triggers subplate neuron death. The afferents may induce dependence in the subplate cells on a trophic factor derived from LGN neurons, which is withdrawn as they leave the subplate. Obviously, many additional experiments are required to resolve these issues or reveal other possible mechanisms governing survival and death of subplate neurons.

CONCLUSIONS

Many lines of evidence suggest that the subplate neurons play a functional role in setting up connections between cortex and thalamus during development. Experiments have revealed that subplate axons are early pioneers of the corticothalamic pathway, that subplate neurons may play a role in the subsequent waiting period and ingrowth of thalamocortical axons into the cortical plate, and that subplate neurons may even be important for the formation of ocular dominance columns in the visual cortex. However, many of the molecular and mechanistic questions still remain to be addressed. An important tool for investigations of subplate neuron function has been the ability to delete them selectively with kainic acid. The ability to perform such lesions even earlier, before either the thalamocortical or corticothalamic axons have even begun to grow out, would permit a true test of the pioneer hypothesis. In both the cortex and the thalamus, the subplate neurons appear at least *in vivo* to be required for innervation of appropriate targets. Do the local interactions between subplate neurons and either the waiting axons or postsynaptic cells of the cortical plate create a permissive environment for ingrowth? And if so, why are growth cones of subplate neurons themselves immune to this need for a permissive environment? It is likely that molecular interactions between the subplate axon growth cones and their target cells and/or extracellular matrix will be involved.

For this and other reasons, further molecular characterization of subplate neurons will be essential. Neocortical subplate neurons may not possess unique

area-specific molecular labels that direct ingrowing afferents to the appropriate cytoarchitectonic area. How do specific areal subdivisions then emerge if subplate neurons are not intrinsically "marked"? It may be that again, competitive interactions between different thalamic axons will enable inputs to sort out into specific patterns of innervation. In addition, further quantitation of the molecular and anatomical phenotypes that are already known to exist would be useful for understanding which cells are responsible for the diverse roles attributed to subplate neurons; for example, what percentage of subplate neurons project to the thalamus, superior colliculus, or cortical plate, and what percentage are local circuit neurons? What percentage die with respect to transmitter phenotype and projection site, and is there anything unique in a molecular or projection field sense about the subplate neurons that survive into adulthood?

The question of why subplate neurons die could have implications for both developmental brain defects and neurodegenerative disease. Subplate neurons, which are present throughout the neocortex, reach a high degree of functional and morphological maturity early in life and may be uniquely susceptible at this point to the effects of trophic factor deprivation or excitotoxicity. The timely elimination of subplate neurons is a normal developmental process, but if certain aspects of this process are reactivated abnormally in other parts of the brain during aging, an understanding of subplate neuron death may ultimately lead to an understanding of neuron death at any age. In addition, subplate neurons may be uniquely sensitive to prenatal or perinatal trauma owing to their high degree of maturity; even a small subplate lesion during fetal life could conceivably lead to large axon targeting errors during subsequent development.

Finally, the number of subplate neurons and the size of the subplate zone relative to the cortical plate increases dramatically as one ascends the phylogenetic scale (see Table 1); in rodents, the ratio of subplate area to cortical plate area is 1:2, whereas in human it reaches 4:1 (Mrzljak et al 1988, Kostovic & Rakic 1990). Not only is the subplate larger in extent in primates and cats than in rodents, but it persists for a much longer developmental period as well. Thus interactions that occur within the subplate during development may be a basis for the increased complexity in the radial and tangential organization present in the neocortex of higher mammals.

ACKNOWLEDGMENTS

The authors thank Dr. Susan McConnell and Dr. Dennis O'Leary for their critical reading of the manuscript. Research from the authors' laboratory was supported by NIH R37 EY02858 and the Alzheimer's and Related Disorders Association to C.J.S. and NIH NS07158 to K.L.A.

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