DEVELOPMENT OF INVERTED CELLS IN INFRAGRANULAR LAYERS OF THE RABBIT VISUAL CORTEX

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In the adult cerebral cortex, we described previously a population of spiny neurones as typically (1) unfolding their major dendrite (the apical dendrite) towards the white matter, (2) lying in the cortical layers 5 and 6 and (3) axonally projecting to other cortical areas but not to subcortical centres (Bueno-López et al.,1991; Reblet et al.,1992). The conspicuous inverted morphology of these adult cells was similar to that of many early generated cells to the transient (cortical) subplate (Marín-Padilla,1972; Shatz et al., 1988). Although most subplate neurones disappear during late development, some subplate cells seem to remain alive at the deepermost portion of layer 6 and the uppermost one of the white-matter in the adult cortex (Shatz et al., 1988). We asked whether the conspicuous inverted neurones of the adult cortex and cortical subplate are cells generated at the same time during ontogenesis and thus whether the inverted cells are part of a sort of spiny, cortico-cortical projection neurones that have characteristically a morphological phenotipe indeed distinct from that of the most common class of projection neurone of the cortex, namely the typically oriented pyramidal cell.

To find out the generation time of cortical neurones, pregnant rabbits received a single injection of 5-bromo-2´-deoxyuridine (BrdU) in sterile normal saline (70 mg *per* Kg of body weight, i.p.) at different gestation days (GD13, GD14, GD15, GD16, GD17, GD18 and G19). The mating day was considered GD0. Offsprings were studied from weaning on. [Only the offspring of a pregnant rabbit that received the BrdU injection at GD13 was sacrificed during foetal time (GD25)].The rest of the animals were perfused transcardially with a solution of 4% paraformaldehyde in PB 0,1 M pH 7,6. Their brains were then removed from the skulls and postfixed during three hours in the same fixative. After cryoprotection in a solution of PBS-30% sucrose, the brains were frozen and then cut on a cryotome at 30 µm. Sections were "free-floating", immunocytochemically incubated with a monoclonal antibody against BrdU as in Soriano et al. (1991)

To verify the development of the morphological phenotype of the cortico-cortical projection cells, we placed DiI or DiA crystals on the fixed occipital cortices of a number of prenatal rabbits aged GD18, GD21, GD25, GD27 and postnatal (P)0, P1, P3 and adult (P22 on). These brains were fixed as above (except those aged GD18, which were fixed by immersion in 10% neutral formalin). Some animals would receive, instead of DiI or DiA crystals, an *in vivo* injection of 10% Biocitin or 4% Fluorogold in their occipital cortex at P0 or P2 and, after 2 days, perfused as above. Biocitin was then revealed prior to BrdU, both by means of an ABC (Vector) kit and DAB but the latter being Nickel-intensified in the case of BrdU. When successful, we used all these experiments with double labelling (with BrdU at the cell nucleus and the retrogradely transported tracer at the cell somata and dendrites) to correlate the cell generation times to the morphologies of the identified cortico-cortical projection cells.

As early as GD18, two morphological subtypes of cortico-cortical projection cells were retrogradely labelled by the fluorescent dyes at a range of distances from the injection sites. These two projection cell types were either typically or inversely oriented pyramidal neurones (Fig. 1). Cells of both types were labelled at the cortical plate and the uppermost portion of the intermediate zone, the so-called subplate. Some other cells of both types were also labelled deep in the intermediate zone. These findings strongly suggest that both morphological phenotypes of cortico-cortical projection neurones appear during very early development, indeed before these cells go into the cortical plate. Earlier hypothesis favoured instead that the morphologies of the inverted and other atypically oriented cells mature from other cell forms in concordance with extrinsic cues, through a process of cell rotation or differential cell growing (for discussion see Van der Loos, 1977). We have seen no intermediate morphology supporting this morphological continuum.

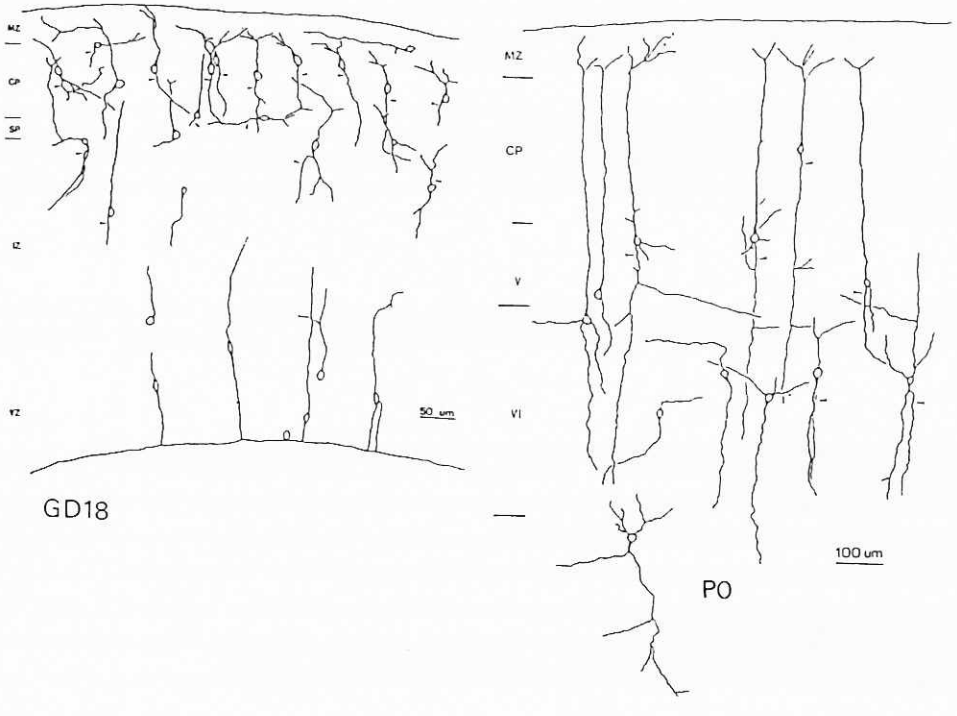

Figure.1. Camera lucida drawings of retrogradely labelled cells at GD18 and P0. The arrowheads point to the cell axons.

In our study, the typically oriented cells retrogradely labelled as cortico-cortical projection neurones at any cortical or subcortical depth, had at GD18 an apical dendrite that proceed until reaching the marginal zone (presumptive cortical layer 1) (Fig. 1). The inversely oriented cortico-cortical projection cells had in turn a major (apical) dendrite that went deep into the cortical plate or the intermediate zone below it. Some of these inverted cells had additionally a slender dendrite or an axonal branch that proceeded towards the marginal zone and occasionally went into it. Whereas the typically oriented cells either could kept their contact with layer 1 for life or loose it after birth (in the

case of some infragranular neurones), as occurs in the rat too (Koester and O´Leary, 1992), the inversely oriented cells with external processes shortened their thin ascending dendrite or bent their axon, in order to go into the intermediate zone, well before birth (Fig. 1). We think these evidences support that the inversely and typically oriented neurones are two distinct subclasses among the cortico-cortical projection cell class, possibly maturing from a characteristic genotype each and under distinct local influences.

Furthermore, the fates of inversely and typically oriented cells as cortico-cortical projection neurones were unlike in our study. Typically and inversely oriented projection neurones probably play different functional roles in the adult cortex. During prenatal development, cells with inverted morphologies were labelled in presumptive layers 5 and 6 as cortico-cortical projection cells. When the topographic adult pattern of cortico-cortical projection emerged, from P0 onwards, the inverted cells were labelled mostly in layers 5b and 6a as originating widespread axonal projections both into the extrinsic (inter-areal) and intrinsic (intra-areal) connection systems (Fig. 2A). In turn, typically oriented cells were labelled as origin of more restricted extrinsic and intrinsic projections and found throughout layers 2, 3, 5 and 6 (the cells labelled at the deepermost portion of layer 6 and the subplate furnishing only the intrinsic projection system but in a less widespread way than those inverted cells labelled at the layer 5-6 border). This raises questions on the ultimate destiny of the inversely oriented cells we found at deep layer 6 as origin of cortico-cortical projections during prenatal development. Inversely oriented cells lying in this radial position project to the claustrum in the brain cortex of adult rabbits (Bueno-López et al., 1992) and therefore the possibility exists that these deep-located cells change their axon targets or restrict them to the claustrum during late prenatal development. We cannot rule out however that a number of deep-located inverted cells die during development.

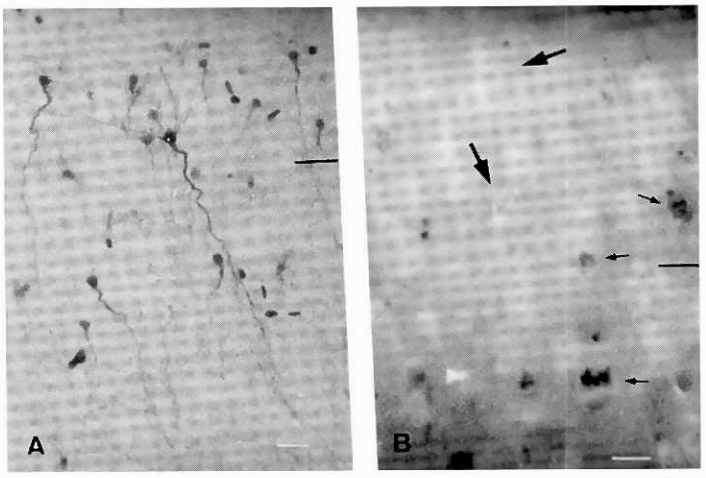

Figure 2.A. Retrogradely labelled cells in area18 following an injection tracer in area 17 at P1. B. Double-labelled cells (Fluorogold on somata and dendrites and BrdU on nucleii) in area 18 of an adult brain that received a pulse of BrdU at GD17. Notice double-labelled inversely and typically oriented cells (big arrows). Other double-labelled cells are out of phocus (small arrows). Black bar shows the layer 5-6 border. White calibration bar = 50 m.

If they are two distinct classes of neurones they can be generated at different times. In the present study, we have checked the neurogenesis of the subplate and infragranular layers, which is from GD13 to GD19 in the rabbit. At GD13 there were a great many BrdU labelled nucleus at the subplate and presumptive layer 1 and fewer at presumptive layer 6. In the following days the nuclear label appeared on cells of the same layers but the number of labelled cells increased at presumptive layers 6 and 5. This indicating an inside-out gradient as it occurs in the rat (Bayer and Altman, 1991). Also as it occurs in the rat, there was great coincidence in the generation time of subplate and infragranular cells in the rabbit occipital cortex. In the case of double-labelled cortices we saw double-labelled cells of both morphologies (Fig. 2B). This suggest that probably most cortico-cortical cells lying in infragranular layers are generated in the same period, i.e., between GD13 and GD19.

We conclude that the distiction between the inversely and typically oriented, cortico-cortical projection cells cannot be explained by differences in their time of generation. Rather, the distinct morphologies of these cells are a consequence of their early neuritic polarization, which is established before their going into the cortical plate. We suggest that, because the inverted cells shorten their connecton with the marginal zone later but before birth, the inverted cells additionally mature under cortical influences different from those acting on typically oriented pyramidal cells.

Akcnowledgments

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