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Neuronal ectopic masses induced by prenatal irradiation in the rat

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Summary. Ectopic neuronal masses below the subcortical white matter were seen in the brains of postnatal rats after 200 cGy irradiation at embryonic day 14. In contrast with the laminated organisation of the cortex located above the subcortical white matter, the ectopic masses were formed of confluent nodules composed of pyramidal and non-pyramidal neurons distributed at random, with no laminar organisation. Afferent and efferent fibres to/from the ectopic masses running together with fibres passing the subcortical white matter indicated that the ectopic masses were heavily connected to neighbouring structures. Examination of irradiated embryos revealed that the ectopic masses originated from ectopic periventricular rosettes, composed of germinal cells, which were formed shortly after irradiation. Neuronogenesis in these rosettes did not follow an inside-out gradient, as seen in the laminated cortex; however, early-generated neurons predominated in the external regions, whereas late-generated neurons were mainly located in the middle and internal regions of the ectopic masses.

Key words: Cerebral cortex – Brain malformation – Subcortical ectopia – Irradiation

Introduction

High doses of irradiation during embryogenesis may produce different types of cortical malformation depending on the developmental stage of the embryo. Irradiation in the early stages of cortical neurogenesis may lead to the formation of large neuronal ectopias below the "true" cortex and underlying subcortical white matter (Hicks et al. 1959; Berry and Eayrs 1966; D'Agostino and Brizee 1966; Dekaban 1969; Takeuchi et al. 1976; Schmahl et al. 1979; Ferrer et al. 1984).

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Studies using the Golgi method have shown that these ectopic masses are composed of pyramidal and non-pyramidal neurons (Donoso and Norton 1982; Ferrer et al. 1984), whereas the use of horseradish peroxidase has disclosed that some neurons in the ectopic masses project to the spinal cord (Jensen and Killackey 1984). However, little is known about the distribution of non-pyramidal neurons, and no information exists on the possible connections between the "true" cortex and the ectopic neuronal masses. Furthermore, although some studies have shown that the ectopic masses are derived from germinal subcortical rosettes formed shortly after irradiation, the presence of neuronogenesis in these ectopic masses is not well documented.

We have examined, by means of parvalbumin immunocytochemistry, the distribution of some non-pyramidal neurons in the ectopic masses in the brains of rats exposed to 200 cGy irradiation at embryonic day 14 (E14). In the neocortex and hippocampus, the calcium-binding protein parvalbumin is found in particular types of interneurons which use GABA as a neurotransmitter (Celio 1986, 1990; Kosaka et al. 1987; Demeulemeester et al. 1988, 1989, 1991; Hendry et al. 1989; Van Brederode et al. 1991). The study of nerve fibres between the "true" cortex and the ectopic masses has been carried out using the Golgi method. Finally, the temporal organisation of the neurogenesis in the ectopic masses has been examined with tritiated methyl-thymidine autoradiography.

Materials and methods

Female Wistar rats weighing 150±25 g were mated with normal males. Twice a day (at 7 a.m. and 7 p.m.) vaginal smears were obtained; the presence of sperm indicated embryonic day 0. Rats with positive plugs were housed in individual cages and kept under controlled temperature, humidity and 12 h dark/light cycles. They received a standard diet at libitum and had free access to drinking water. The rats were irradiated at E14, with a single dose of 200 cGy. The source of radiation was a 300 kV(p) Stabilipan with a half-value layer of 3.3 mm copper.

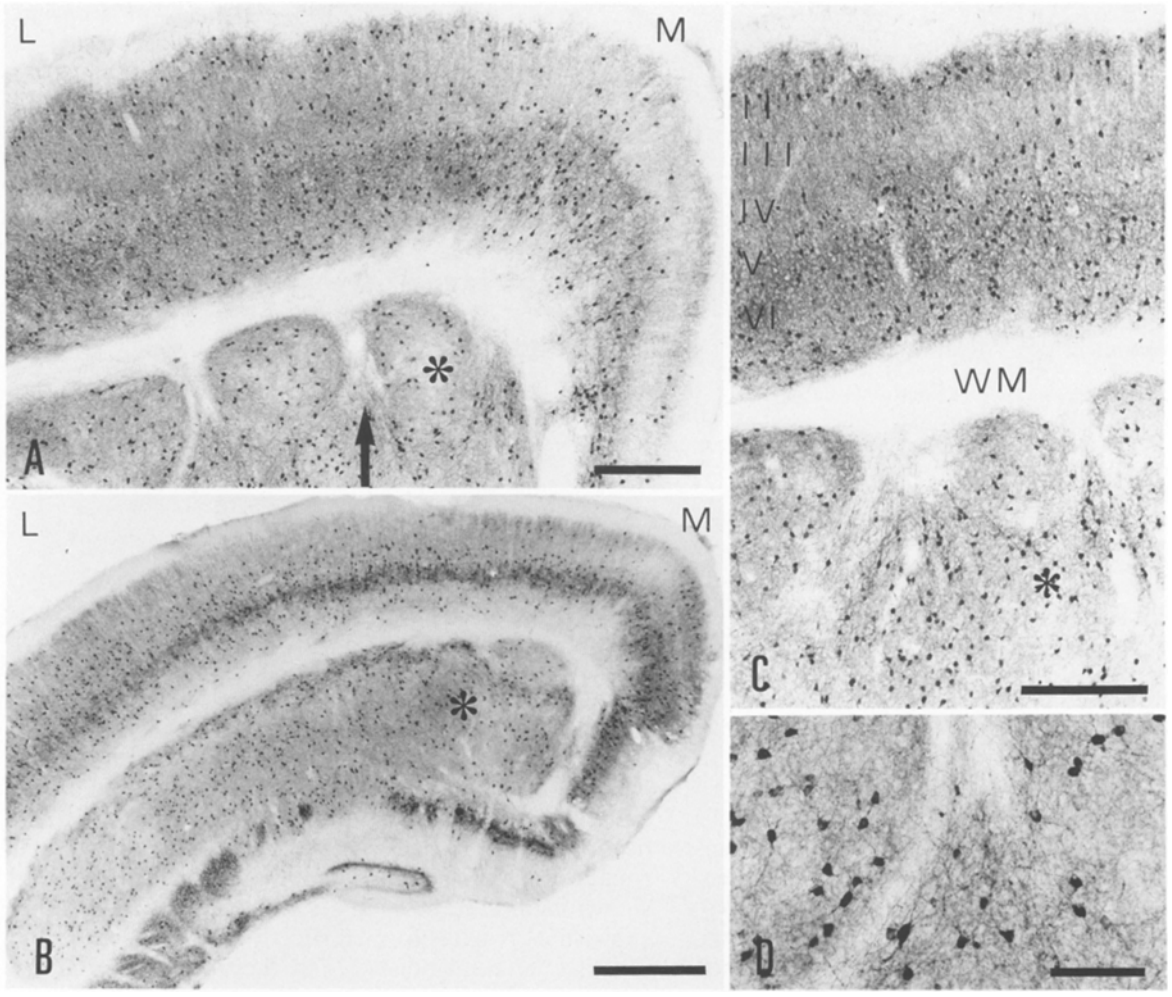

Fig.1 A–D. Parvalbumin immunoreactivity in the cerebral cortex of rat (postnatal day 30: P30) irradiated at embryonic day 14 (E14) with 200 cGy irradiation. **A** Anterior coronal section showing a laminated "true" cerebral cortex and an inner large cortical mass (*asterisk*), formed by nodules separated by tracts of fibres (*arrow*). **B** Posterior coronal section (occipital cortex) showing a normal distribution of parvalbumin-immunoreactive cells in the "true" cortex and a large ectopic mass of neurons (*asterisk*), which is partly composed of parvalbumin-immunoreactive neurons distributed at random. **C** High magnification to show the borders of the "true" cortex, the ectopic mass and the white matter (*WM*); II–VI, cellular layers of the laminated cortex. **D** Parvalbumin-immunoreactive neurons in the ectopic mass with their typical multipolar morphology. *L*, Lateral regions; *M*, medial regions. **A–C**, *bar* = 500 μm; **D**, *bar* = 50 μm

Two pregnant rats were killed at gestational days 15 and 18 with deep diethyl-ether anaesthesia. The brains of the embryos were fixed by immersion in 2% glutaraldehyde and embedded in paraffin. Serial, dewaxed 10-μm-thick sections were stained with cresyl violet.

Rats irradiated at E14 were killed at postnatal days 1, 7 or 30 (P1, P7, P30) with deep diethyl-ether anaesthesia. The animals were immediately perfused through the heart with saline followed by 4% paraformaldehyde. The brains were then removed from the skull and immersed in a similar solution of paraformaldehyde at 4° C for 24 h. After this, they were cut into slabs which were cryoprotected with 30% saccharose, frozen in liquid nitrogen and stored at −80° C until use. The brains of some irradiated animals were embedded in paraffin, and serial 10-μm-thick sections were dewaxed and stained with haematoxylin and eosin or with cresyl violet. The brains of eight rats were processed for parvalbumin immunocytochemistry. Serial 50-μm-thick sections were obtained with a cryostat and processed free-floating following the avidin and biotin procedure (ABC, Vectastain, Vector). Well-characterized monoclonal antibodies against parvalbumin (Sigma, clone PA-235) were used at a dilution of 1:3000. Peroxidase was visualized with 0.05% diaminobenzidine and 0.01% hydrogen peroxide. False-positive immunoreactions were ruled out by incubating a few tissue sections without using the primary antibody.

Some irradiated rats aged 30 days were processed following the rapid Golgi method. Briefly, brain slabs were fixed with 3% potassium dichromate and 1% osmium tetroxide (4:1) for 6 days, and later washed with 0.75% silver nitrate and immersed in 0.75% silver nitrate for 48 h. Double or even triple impregnations were carried out in some cases. Sections 100 μm thick were obtained with a sliding microtome and mounted on glass slides.

Finally, a group of pregnant rats irradiated at E14 received a single intraperitoneal injection of 5 μg/g body weight of tritiated methyl-thymidine (Amersham) at gestational days 15, 16, 17 or 19. Animals born to these mothers were killed at P21 and their brains were processed for autoradiography. Serial 10-μm-thick sections were dewaxed, dipped in Kodak NTB2 photographic emulsion, air-dried, stored dry in dark boxes for 1 month and later developed with Microdol X. The sections were counterstained with cresyl violet. Only cells with more than 20 silver grains in their nucleus were considered the product of a first generation, and the day of thymidine injection was the day these cells were born (Sid-

man 1970). Normal animals were processed in the same manner and used as controls.

Results

Animals irradiated at E14 and examined postnatally had large neuronal ectopic masses separated from the "true" cortex by the subcortical white matter. Bundles of fibres in continuity with the subcortical white matter penetrated the ectopic masses and delimited irregular nodules of variable sizes. The "true" cortex had a reduced thickness, but the different cortical layers were clearly defined. These findings were also seen with parvalbumin immunocytochemistry (Fig. 1). Immunoreactive cells in the laminated cortex ("true" cortex) were found in all cortical layers, excepting the molecular layer, but predominated in layers II, V and VI. Immunoreactive terminal buttons, most of them surrounding the soma of unlabelled cells, stained the cortical neuropil of the same layers homogeneously. These characteristics were similar in the anterior and posterior regions of the neocortex (Fig. 1A, B).

Parvalbumin-immunoreactive cells were distributed at random in the ectopic masses, but their morphology,

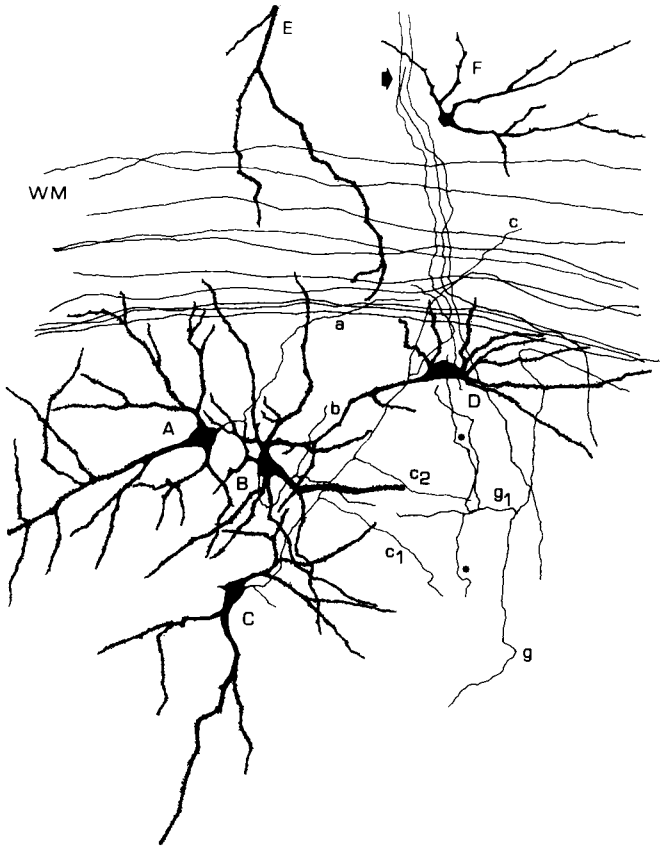

Fig. 2. Golgi-impregnated neurons in the ectopic mass of rat irradiated at E14 and killed at P30. Pyramidal neurons *A, B* and *C* have their apical dendrites directed towards the centre of the mass. The axons of these cells (*a, b* and *c*) emerge from the basal region of the cell body and penetrate into the subcortical white matter (*WM*) giving off several collaterals (*c1, c2*) before penetrating it. An ascending axon of unknown origin (*g*) also sends out a collateral (*g1*) before being incorporated in the white matter. Other fibres (*arrows*) pass across the white matter. *D* is a neuron in the ectopic mass; *E*, dendrite; and *F*, non-pyramidal neuron in the inner levels of the laminated cortex. Golgi method, camera lucida drawing, *bar* = 50 μm

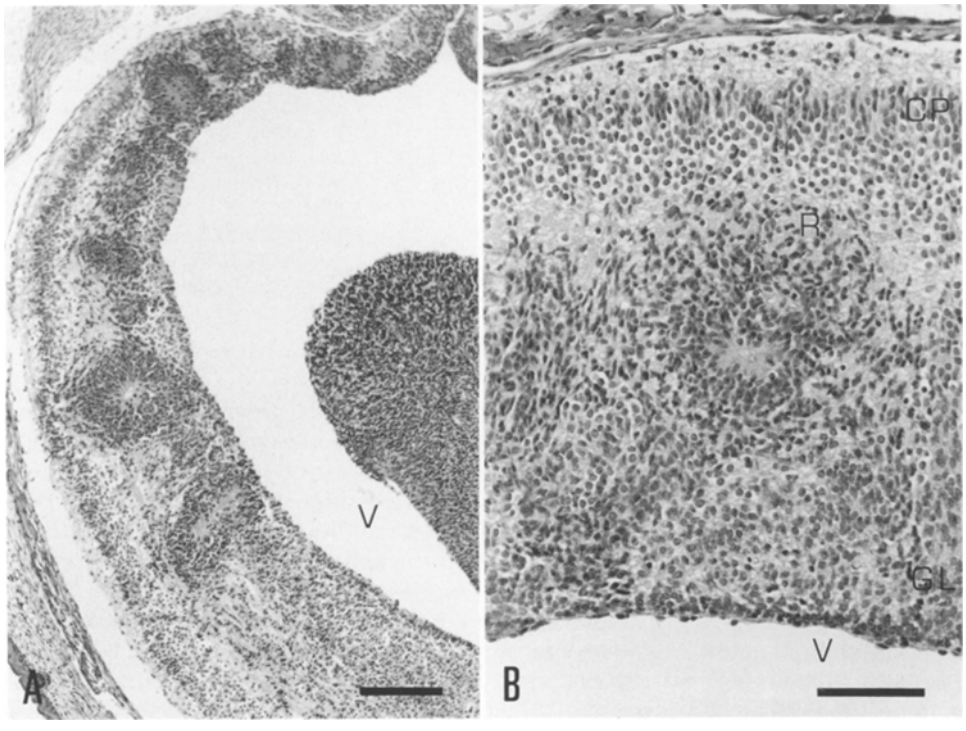

Fig. 3. A Coronal view of the telencephalic mantle of a rat embryo irradiated at E14 and killed at E18. Germinal rosettes are seen near the periventricular region. **B** High magnification to show the structure of the abnormal telencephalic mantle. *V*, Ventricle; *CP*, cortical plate; *GL*, periventricular germinal layer; *R*, germinal rosette. **A**, *bar* = 400; **B**, *bar* = 100 μm; H & E

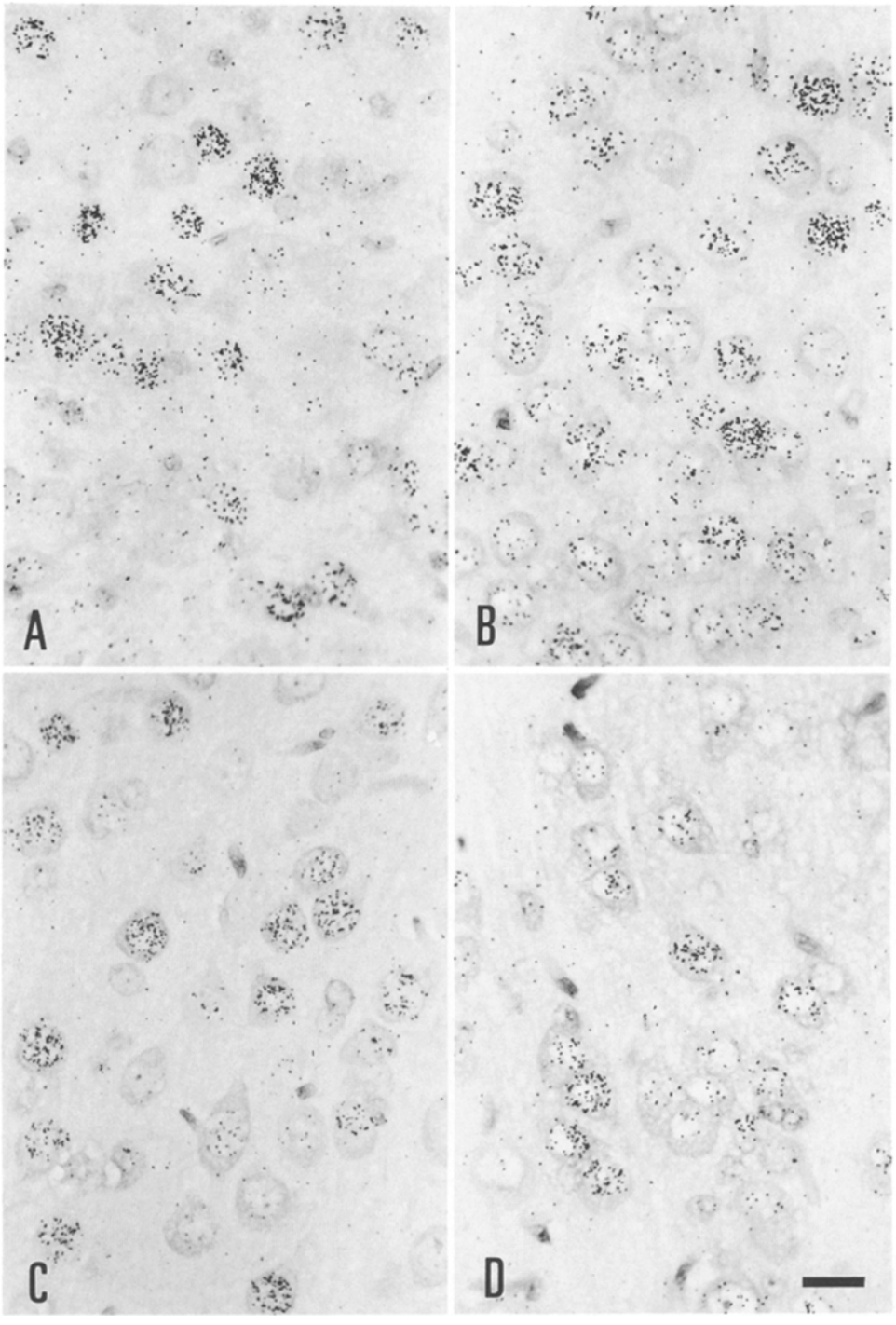

Fig. 4A–D. Labelled neurons in the ectopic masses of rats irradiated at E14 and killed at P30, which received a single pulse of tritiated methyl-thymidine at different embryonic days. **A** Thymidine injection at E15 mainly labels neurons in the periphery of the ectopic mass. **B**, **C** Thymidine injection at E17 labels neurons located in the peripheral (**B**) and middle (**C**) regions of the ectopic mass. **D** A single pulse of thymidine at E19 mainly shows labelled neurons in the medial and inner regions of the ectopic mass. Sections counterstained with cresyl violet; *bar* = 20 μm

most often bitufted or multipolar, was the same as in the laminated cortex (Fig. 1C, D).

In the Golgi studies it was evident that the morphology of pyramidal and non-pyramidal neurons was similar in the laminated cortex of irradiated rats and corresponding layers in age-matched controls. Different neuronal types were seen in the ectopic masses. Pyramidal cells were distributed at random and their apical dendrites were not aggregated in clusters, although most of them were oriented towards the centre of the nodule. The axon emerged from the side opposite to that of the apical dendrite and moved towards the underlying white matter, giving off several collaterals before penetrating it (Fig. 2). Several types of non-pyramidal neurons, including bitufted, bipolar and multipolar cells were stained as well. In addition, large numbers of fibres were seen between the laminated cortex and the ectopic masses with the Golgi method (Fig. 2).

Rats irradiated at E14 and killed at E16 and E18 showed germinal cell rosettes in the vicinity of the periventricular layer (Fig. 3A). These rosettes were composed of oval cells whose long axis was oriented towards

the centre of the rosette (Fig. 3B). As development proceeded, these structures were transformed into confluent, subcortical masses in postnatal rats.

Neurogenesis in the laminated neocortex followed a normal inside-out gradient as neurons generated at E15 and E16 were located in the inner levels of the cortex, whereas neurons generated at E17 and E19 were encountered, respectively, in cortical layers III and II in mature animals. No similar pattern of neuronal migration was recognised in the ectopic masses. Most labelled neurons in rats which received a pulse of tritiated methyl-thymidine at E15 were seen in the periphery of these masses (Fig. 4A), although labelled cells were also encountered in the centre of the masses. Pulses at E16 and E17 showed labelled neurons located in the periphery (Fig. 4B) and medial (Fig. 4C) areas. Injection of tritiated methyl-thymidine at E19 produced labelled neurons located mainly in the medial and inner zones of the ectopic masses (Fig. 4D), although labelled cells were also observed in the peripheral zones.

Discussion

Cortical ectopias, defined as large masses of nerve cells located below the "true" or laminated cortex and underlying white matter, were produced after 200 cGy irradiation at E14. Similar large subcortical masses are produced following irradiation at E13 (Hicks and D'Amato 1966; Ferrer et al. 1984). The present findings, together with studies in embryos irradiated at E13 and E14 and killed at different intervals, have shown that ectopic masses derive from periventricular rosettes which result from the reorganisation of the injured neuroepithelium following irradiation (Hicks et al. 1959; D'Agostino and Brizee 1966; Takeuchi et al. 1976; Ferrer et al. 1984).

The present observations, using the Golgi method and immunocytochemistry with anti-parvalbumin antibodies, have shown that pyramidal and non-pyramidal neurons are intermingled and distributed at random in the ectopic masses. This indicates that germinal rosettes are the source of mixed populations of cortical neurons, including pyramidal and non-pyramidal cells. This would also suggest that irradiation does not selectively affect a particular type of nerve cell precursor.

Previous studies using injections of horseradish peroxidase into the spinal cord in adult rats irradiated at E14 have labelled neurons in the ectopic masses retrogradely (Jensen and Killackey 1984), suggesting that ectopic neurons project to the spinal cord. Our observations have shown that the apical dendrites of most pyramidal neurons are oriented towards the centre of the cortical nodules and that the axons of these cells penetrate into the subcortical white matter after giving off several intra-ectopic collaterals. Our observations with the Golgi method have also shown the presence of large numbers of nerve fibres between the laminated cortex and the ectopic masses.

The present autoradiographic studies have shown that neuroblast migration follows an inside-out gradient in the layered or "true" cortex, which is similar to normal rats (Berry et al. 1964; Berry and Rogers 1965; Hicks and D'Amato 1968; Raedler and Raedler 1978; Miller 1988). No similar gradient was recognised in the ectopic masses. In contrast, a roughly outside-in gradient of neuroblast migration probably occurs in these ectopic masses. Since cortical neurons migrate along radial glial fibres, and normal cortical migration depends on the integrity of these fibres (Pinto-Lord et al. 1982; Ferrer et al. 1992), it may be suggested that radial glial fibres are involved in the abnormal structural organisation of ectopic masses. Studies are now in progress to elucidate this possibility.

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