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Timetables of cytogenesis in the rat subfornical organ

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Timetables of neurogenesis and ependymal cell production in the rat subfornical organ (SFO) were determined by examining the offspring of pregnant rats injected with [^{3}H]thymidine on E13–E14, E14–E15, . . . E21–E22, respectively. The proportion of postmitotic cells originating each embryonic day was determined by analyzing, in the adult offspring, the progressive reduction in the proportion of labeled precursors from the maximum amount seen in the E13–E14 group. Neurogenesis was found to occur over an extended period of time, beginning on E12 and continuing through E21. Ependymal cells were generated E15 through E21. Both neuron and ependymal cell production occurred in a triphasic pattern and followed an anterior (older) to posterior (younger) gradient. The anterior to posterior production gradient may be related to the morphological variation which exists along this plane. A production gradient intrinsic to a particular level was found only in the posterior SFO, where peripheral neurons form earlier than core neurons. That neurogenetic gradient may be related to the core-periphery topographical patterns found in other studies, and suggests that the core neurons, since they are among the last to be formed, may be interneurons.

INTRODUCTION

In 1973, Simpson and Routtenberg[51] stimulated interest in the subfornical organ (SFO) when they demonstrated that this small circumventricular organ appeared to be involved in drinking behavior. Since then, accumulating evidence has implicated the SFO as a critical neural component in the regulation of body fluid levels and arterial pressure. It is now believed that the SFO, in response to blood-borne angiotensin II (AII), mediates central mechanisms associated with the arousal of thirst[16,36,52,26], the central pressor response[32,36], and the secretion of vasopressin from the hypothalamic nuclei[17,24,27,56]. There is also evidence that the SFO may influence the release of oxytocin[17,19] from the posterior pituitary, as well as adrenocorticotropic hormone (ACTH)[18,43], luteinizing hormone (LH)[15], and follicle-stimulating hormone (FSH)[29] from the anterior pituitary. Recent studies indicate that in addition to AII, atrial natriuretic peptide (ANP), may also act on the SFO[9,22,47]. The finding of irregularities in the action of both AII[39,46] and ANP[46] on the SFOs of spontaneously hypertensive rats has suggested a role in genetic hypertension.

There have been numerous morphological[2,12,13,42,53], histochemical[1,20,30,33], electrophysiological[7,8,45,49,56], and neural circuitry[23,25,28,30,35,37,55] studies of the rat SFO. However, there have been relatively few developmental studies[4,10,14,20,57]. To our knowledge there has as yet been no [^{3}H]thymidine autoradiographic study dating SFO cell origin. In view of the increasing interest in the SFO, we have undertaken a study to quantitatively determine the timetable of cytogenesis through the use of long survival, comprehensive [^{3}H]thymidine autoradiography.

MATERIALS AND METHODS

The experimental animals were the offspring of Purdue-Wistar timed-pregnant rats. The day the females were sperm positive was designated embryonic day one (E1). Normally, births occur on E23, which is also designated as postnatal day zero (P0). Two or more pregnant females were given 2 subcutaneous injections of [^{3}H]thymidine (Schwartz-Mann; spec. act. 6.0 Ci/mM; 5 μCi/g b. wt.) to ensure comprehensive cell labeling. The injections (given between 09.00 and 11.00 h) to an individual animal were separated by 24 h. The onset of the [^{3}H]thymidine injections was progressively delayed by 1 day between groups (E13–E14, E14–E15, . . . E21–E22). All animals were perfused through the heart with 10% neutral formalin on P60. The brains were kept for 24 h in Bouin's fixative, then were transferred to 10% neutral formalin. At least 6 animals from each injection group (including males and females) were blocked coronally and were embedded in paraffin. Sections were 6 μm in thickness and every 15th section through the SFO was saved. Slides were coated with Kodak NTB-3 emulsion, exposed for 12 weeks, developed in Kodak D-19, and post-stained with hematoxylin and eosin.

Timetables of cytogenesis were determined for both ependymal cells and neurons at 3 levels along the anterior-posterior plane. At each level peripheral and core neurons were quantified separately (Fig. 1). Neurons were distinguished from glial and endothelial cells by their generally larger size, rounded shape, and clear cytoplasm containing a prominent nucleolus (Fig. 4C). Neurons were considered labeled when silver grains were observed overlying the nucleus in densities above background levels. Non-neuronal elements were excluded from the analysis, and were distinguished from neurons by their smaller size, non-rounded shape, and darker

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cytoplasm. Cells were counted microscopically at X312.5 using an ocular grid (0.085 mm^2). The proportion of labeled cells (labeled neurons/total neurons and labeled ependymal cells/total ependymal cells) was then calculated from these data.

The determination of the proportion of cells arising (ceasing to divide) on a particular day used a modification of the progressively delayed comprehensive labeling procedure[5] and is described in detail elsewhere[6]. Briefly, a progressive drop in the proportion of labeled neurons from a maximal level (>95%) in a specific population indicates that the precursor cells are producing postmitotic neurons. By analyzing the rate of decline in labeled neurons, one can determine the proportion of neurons originating over blocks of days (or single days) during development. Table I is presented as an illustration of this procedure.

Throughout the quantitative analysis, it was noted that trends in cell labeling within animals were very consistent. However, variability between animals in an injection group was large enough to mask this trend. Therefore, cell labeling patterns were analyzed with the sign test, a nonparametric statistic designed for these types of data[11]. The sign test determines the consistency of sequential

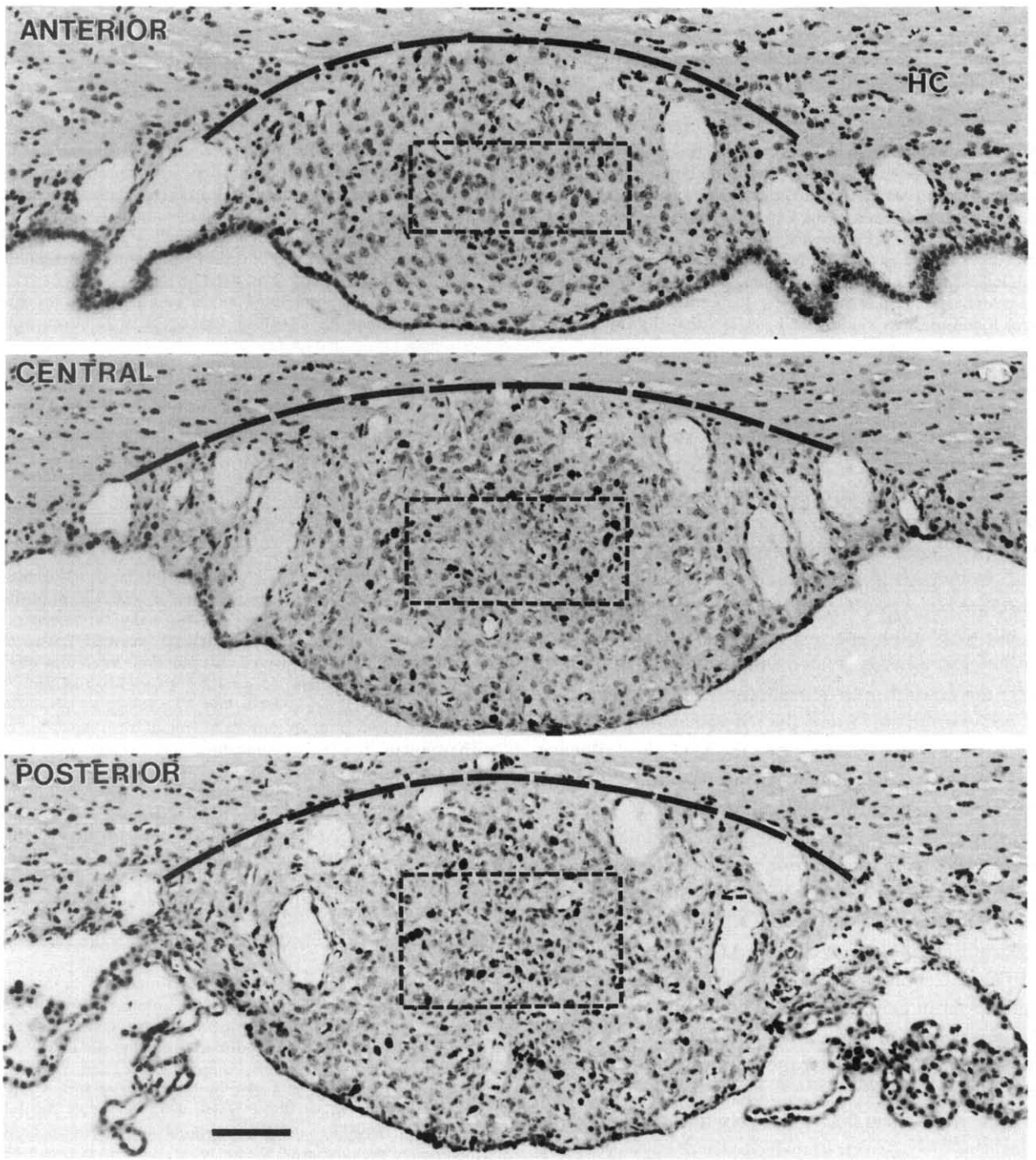

Fig. 1. Coronal sections selected for quantitative analysis. Anterior, central, and posterior levels correspond, respectively, to levels A6.2, A6.0 and A5.8 of Pellegrino et al.[41]. Heavy dashed lines enclose the SFO. Fine dashed lines separate the core and periphery, which were quantified separately. Representative sections are from animal 154 with injection schedule E21-E22. HC = Hippocampal commissure. Scale = 0.1 mm.

TABLE I

Neurogenesis in the central SFO

Injection group	*n*	*% Labeled cells (mean ± S.D.)*	*Day of origin*	*% of cells originating**
			E12	0.15 (100–A)
E13–14	6	(A) 99.85 ± 0.33	E13	1.23 (A–B)
E14–15	6	(B) 98.63 ± 0.60	E14	6.12 (B–C)
E15–16	4	(C) 92.51 ± 2.60	E15	23.60 (C–D)
E16–17	10	(D) 68.91 ± 4.40	E16	15.48 (D–E)
E17–18	7	(E) 53.42 ± 5.20	E17	3.29 (E–F)
E18–19	4	(F) 50.14 ± 3.27	E18	23.17 (F–G)
E19–20	5	(G) 26.96 ± 7.78	E19	14.73 (G–H)
E20–21	7	(H) 12.24 ± 6.41	E20	0.19 (H–I)
E21–22	3	(I) 12.05 ± 6.97	E21	12.05

* Fig. 2B graph.

neuron production between paired locations within individual animals. The comparisons are grouped into 3 categories: (1) $X > Y$ '–' comparison; (2) $X < Y$ '+' comparison; (3) $X = Y$, '0' comparison. The zero comparisons are discarded and, depending on the total number of remaining '+' and '–' comparisons, either a binomial distribution or a normal appoximation is used to calculate probabilities (P). The graphs throughout this report show the variable group data rather than the more consistent trends within individual animals. Consequently, some of the statistically significant neurogenetic gradients are not conspicuous.

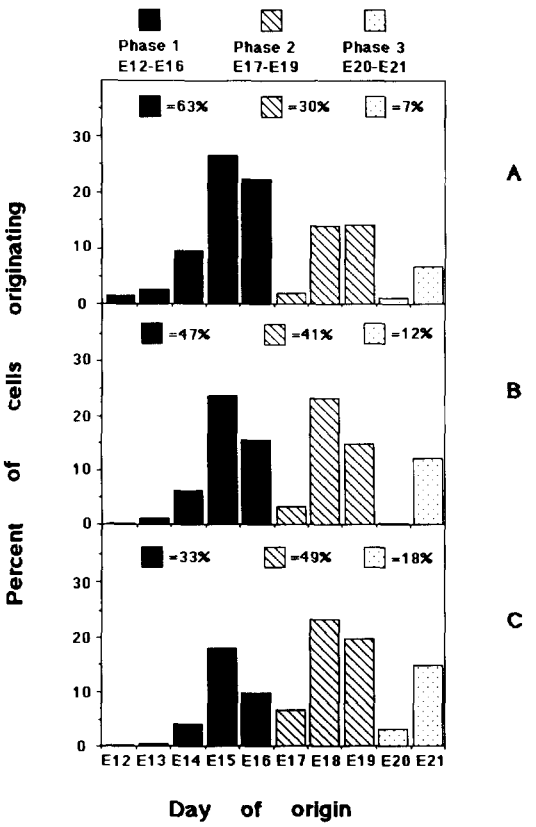

Fig. 2. A: proportion of anterior neurons formed each day E12–E21. B: proportion of central neurons formed each day E12–E21. C: proportion of posterior neurons formed each day E12–E21.

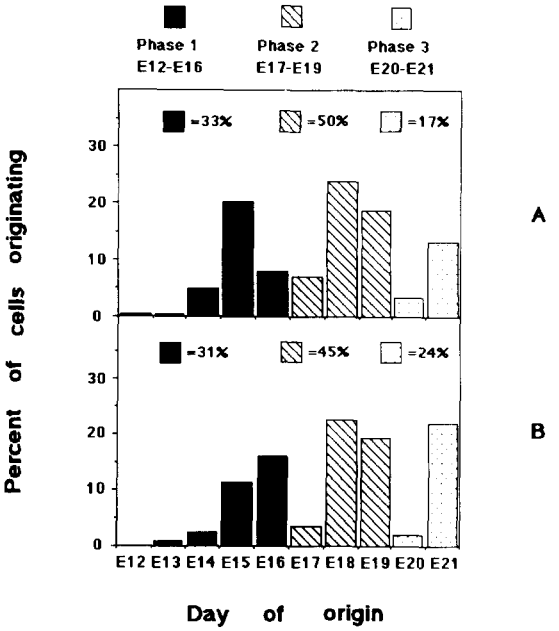

Fig. 3. A: proportion of peripheral neurons formed each day E12–E21 at the posterior level. B: proportion of core neurons formed each day E12–E21 at the posterior level. Due to variability between animals, the group data do not highlight the statistically significant difference found between core and periphery ($P < 0.0001$).

RESULTS

Neurogenesis was found to occur over an extended period of time. As can be seen in Fig. 1, neuronal precursors (labeled cells) are still present as late as E21. The time of origin of neurons throughout three levels of the SFO is presented in Fig. 2. (In this figure data from the core and periphery of each level have been combined in order to provide a more general view of neurogenesis.) Neuron production appears to follow a triphasic pattern. Phase one production occurs before E17, peaking on E15. Phase two begins on E17 and ends on E19. Phase three begins on E20 and presumably ends postnatally.

While the triphasic pattern of neuron generation is consistently seen at each anteroposterior level, the three levels differ in the proportion of neurons generated during each phase. The first phase generates 63% of anterior neurons, 47% of central neurons, and only 33% of posterior neurons. The second phase generates 30% of anterior, 41% of central, and 49% of posterior neurons. The third phase is responsible for 7% of anterior, 12% of central, and 18% of posterior neurons. These differences are significant between anterior and central levels ($P < 0.0001$), and between central and posterior levels ($P < 0.003$). Overall, the anterior neurons tend to be formed earlier than the more posterior neurons.

While a clear anterior to posterior neurogenetic

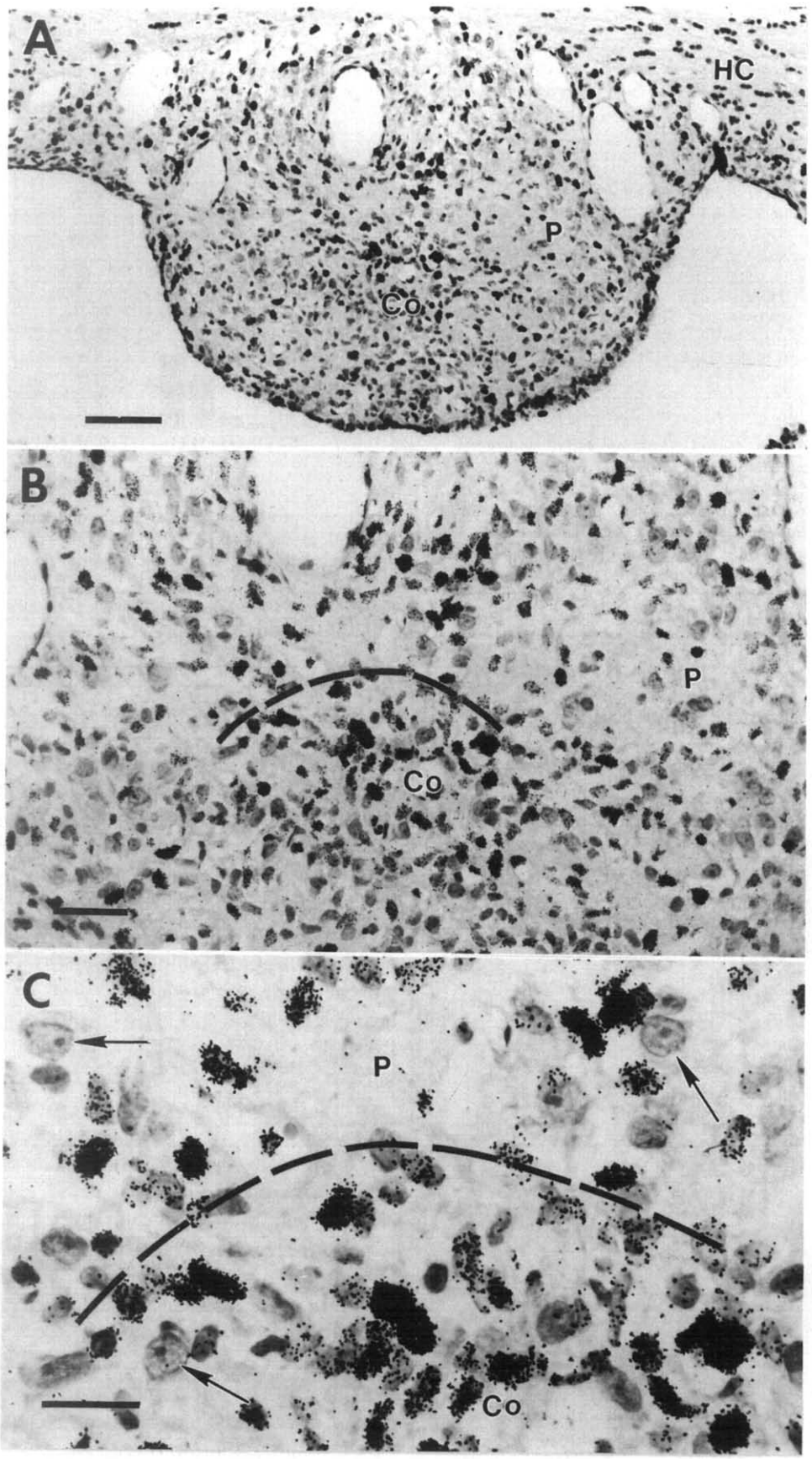
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Fig. 4. Posterior SFO from animal #171 with injection schedule E19–E20. A: low magnification view of SFO. Note the higher proportion of labeled cells clustered in the core (Co) compared to the periphery (P). HC = Hippocampal commissure. Scale = 0.1 mm. B: intermediate magnification view of part of 4A. 47% of core cells are unlabeled (postmitotic), compared to 69% of the peripheral cells. Dashed line indicates part of the border between core and periphery. Scale = 50 μm. C: high magnification view of the border shown in 4B. Note the decrease in cell labeling upon entering the periphery. Arrows point to some unlabeled neurons. Neurons may be identified by their relatively large size, rounded shape, and relatively clear cytoplasm. Scale = 25 μm.

gradient exists throughout the SFO, only the posterior level showed an intrinsic gradient. Group data (Fig. 3) show that the earlier production phases generate more peripheral neurons (Fig. 3A) than core neurons (Fig. 3B). Together, the first two phases generate 83% of peripheral neurons compared to 76% of core neurons. Peripheral neuron production peaks one day earlier than core production during phase 1. The most striking difference is found during phase 3, where 13% of peripheral neurons form on E21, compared to 22% of the core neurons. It is important to note that, due to variation among animals, the group data pictured in Fig. 3 do not highlight the statistically significant difference between core and periphery. However, within individual animals the consistency of sequential production was found to be significant ($P < 0.0001$). Fig. 4 presents a section from the posterior level of an animal injected on E19–E20. As can be seen in Fig. 4A,B, the proportion of unlabeled (postmitotic) neurons is greater in the periphery than in the core. Fig. 4C, a high magnification view of the junction between core and periphery, shows a decrease in cell label upon entering the periphery. Note that most of the unlabeled cells are quite large.

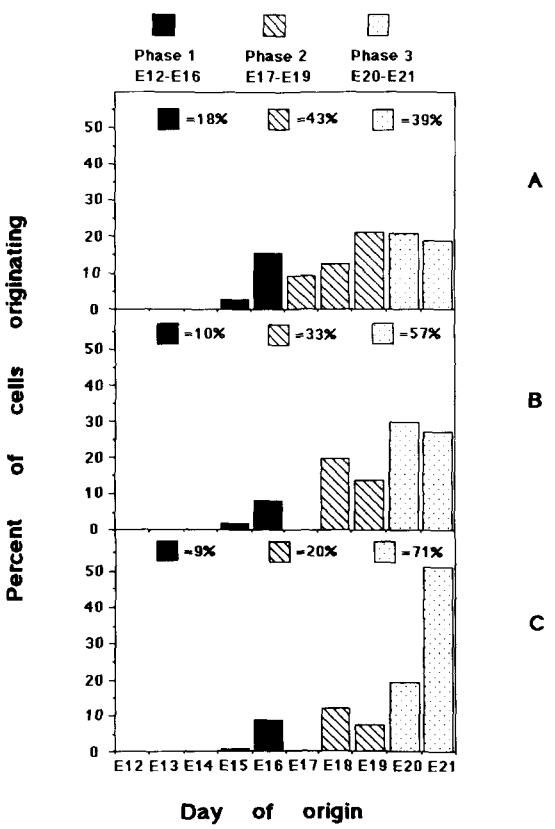

Fig. 5. A: proportion of anterior ependymal cells formed each day E15–E21. B: proportion of central ependymal cells formed each day E15–E21. C: proportion of posterior ependymal cells formed each day E15–E21.

Time of origin data were also obtained for the ependymal cells of the SFO (Fig. 5). At each level substantial production occurs during the latter part of phase 2 and during phase 3. The proportion of cells generated during the last phase progressively increases as one proceeds posteriorly. 39% of anterior, 57% of central, and 71% of posterior cells are formed during phase three. The anterior ependymal cells are significantly older than central cells ($P < 0.0001$). Central ependymal cells are slightly older than posterior cells ($P < 0.044$). Further analysis of data showed that within each level neurons are generated before ependymal cells ($P < 0.0001$).

DISCUSSION

Timetable of neurogenesis

The data indicate that neurons in the SFO are generated during an extended period, beginning on E12 and continuing through E21. The substantial amount of neurogenesis occurring on E21, especially at the posterior level, suggests the possibility of postnatal neurogenesis. Our finding that neurons are still being produced as late as E21 is consistent with the observation of Dellmann and Stahl[14] that undifferentiated cells are seen as late as postnatal day 2. It may be that these undifferentiated cells are the late forming neurons observed in the present study.

The extended period of cell production observed in the SFO is not typical of neurons. This is not surprising since the SFO, one of the circumventricular organs, is not typical neural tissue. The concept of circumventricular organs (CVO) was introduced by Hofer in 1959 to classify structures, located along the midline ventricular surface, with morphology atypical to the central nervous system (as referenced by Dellmann and Simpson[13]). In a summary of structural and functional aspects of the CVOs, Palkovits[40] lists their common features which

include: dense vascularization; fenestrated capillaries surrounded by labyrinth-like pericapillary spaces; specialized ependyma with active pinocytosis; and neuronal afferents which not only synapse with cells, but also can terminate on the basement membrane of the pericapillary spaces. Various anatomical characteristics have contributed to specialized biochemical properties in the SFO and other CVOs[54]. The results of the present study suggest that an extended period of neurogenesis may be yet another feature — a developmental feature — with which the CVOs distinguish themselves. Indeed, Altman and Bayer[3] found that another rat CVO, the area postrema, is unique among the regions of the lower medulla in that mitotic precursors continue to take up label each day between E17 and E22. Whether an extended period of neurogenesis is indeed a distinguishing characteristic of CVOs remains to be determined. In a study of the developing subcommissural organ (SCO) in the mouse, Rakic and Sidman[44] found that cells are formed between embryonic days 11 and 15 inclusive. Since Rakic and Sidman used a slightly different methodology and investigated the mouse rather than the rat, it is difficult to directly compare their findings with those of the present study.

Patterns of neurogenesis

A triphasic pattern of neuron production was observed in the SFO. Substantial production was seen on E14, E15, and E16 (phase 1), on E18 and E19 (phase 2), and again on E21 (phase 3). Analysis at 3 levels along the anterior-posterior plane revealed a neurogenetic gradient, with anterior neurons forming before central neurons, and central neurons forming before posterior neurons. Ependymal cell production also followed an anterior (older) to posterior (younger) gradient. Consistent with our findings, a study of acetylcholinesterase (AChE) activity in the developing rat SFO found that anterior AChE tracts appear several days earlier than posterior AChE tracts[20].

The anterior to posterior production gradients observed in the present study are likely related to morphological variation which exists along the anterior-posterior plane. In studies of the rat SFO, regional differences in both surface and internal morphology have been observed[12,42,53]. Phillips et al.[42] describe three morphologically distinct 'zones' along the ventricular surface. (Zones 1, 2 and 3 correspond, respectively, to the anterior, central, and posterior levels of the present study.) While the lateral anterior zone is covered with cilia-like structures, the central zone lacks these structures, and is further characterized by flat ependymal cells. In contrast, bulging of the ependymal cell surface is a notable feature of the posterior zone, which has clumps of cilia-like structures. These findings were confirmed by Dellmann and Simpson[12] in a study which also observed distinct internal 'regions'. (The rostral, rostral 2/3 central, and caudal 1/3 central regions of Dellmann and Simpson correspond, respectively, to the anterior, central, and posterior levels of the present study.) Dellmann and Simpson found the anterior level to have relatively few neurons and capillaries, while numerous neurons and dense vascularization were observed at central and posterior levels. At the central level fenestrated capillaries are surrounded by wide pericapillary spaces; small pericapillary spaces were found more posteriorly. Neurons located immediately underneath the ependymal surface are especially numerous at the posterior level, causing ependymal cells to bulge markedly into the ventricular lumen. In a study of the capillary network of the SFO, Sposito and Gross[53] confirmed many of Dellmann and Simpson's findings. They noted that the area of lowest capillary density was the rostral SFO. Moreover, the rostral capillaries were not fenestrated and had no pericapillary spaces. Sposito and Gross suggest that the capillary morphology of the rostral SFO may make it a suitable location for the specialized 'osmosensitive' neurons which have been shown to exist in the SFO[21,50].

The varying morphology within the SFO underlies a range of functions of its neurons. These neurons receive a variety of signals — neural, blood-borne, and possibly cerebrospinal fluid-borne. Time of origin differences along the anterior-posterior plane may be related to a range in function along this plane. The early forming anterior neurons, situated away from the main capillary plexus, may include the osmosensitive neurons but are not likely to be major targets of blood-borne AII and ANP. The later forming central neurons probably are such targets, as they are most closely associated with the dense capillary plexus, wide pericapillary spaces, and fenestrated endothelia. Indeed, van Houten et al.[58] found the greatest concentration of binding sites for blood-borne AII occurred in the central SFO. The latest forming posterior neurons include cells best situated for detection of potential CSF-borne signals, since many lie directly beneath the bulging ependymal cells of the posterior SFO.

In addition to the overall anterior-posterior production gradients, the present study found an intrinsic gradient at the posterior level. At that level peripheral neurons form earlier than core neurons. Time of origin of peripheral and core cells was examined separately because several studies had observed a pattern of differences between those two locations[1,25,30,33,34,38,48]. It has been noted that many afferent fibers terminate preferentially in either the core or periphery, instead of terminating uniformly

throughout the SFO[31]. AChE afferents[1,28,31] terminate predominantly in the periphery; afferents immunoreactive for angiotensin[34] and serotonin[30] terminate mainly in the core. It has also been noted that the majority of the SFO's efferent pathways emerge from the peripheral SFO, while many SFO afferents terminate in the core[31]. In several studies this topographical pattern was localized to the more posterior body of the SFO[1,25,30,33,38,48].

The intrinsic neurogenetic gradient found in the more posterior SFO may be a reflection of a segregation between output cells in the periphery and short axon cells in the core. Electrophysiological studies[7,8] have presented evidence for the existence of interneurons (though not necessarily in the core). Since afferent and efferent connections are largely topographically segregated, core neurons could presumably integrate incoming neural and blood-borne signals before relaying information to peripheral output cells. The fact that the core neurons tend to be the last formed is consistent with the suggestion that they may be interneurons. In many neural systems the interneurons are among the latest produced cells[5].

In summary, the time of origin of neurons and ependymal cells of the rat SFO was investigated. The SFO was found to have an unusually long period of neurogenesis, with cell production following an anterior (older) to posterior (younger) gradient. This anterior to posterior gradient may be related to morphological and functional variation along the same plane. At the posterior level, core neurons form after peripheral neurons. This production pattern is consistent with the suggestion that core neurons may be interneurons.

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