NEUROGENESIS IN THE OLFACTORY TUBERCLE AND ISLANDS OF CALLEJA IN THE RAT

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Abstract—Neurogenesis in the rat olfactory tubercle and islands of Calleja was examined with [3H]thymidine autoradiography. Animals in the prenatal groups were the offspring of pregnant females given an injection of [3H]thymidine on two consecutive gestational days. Ten groups of embryos (E) were exposed to [3H]thymidine on E12-E13, E13-E14, . . . , E21-E22, respectively. Three groups of postnatal animals (P) were given four consecutive injections of [3H]thymidine on P0-P3, P2-P5, and P4-P7, respectively. On P60, the percentage of labeled cells and the proportion of cells originating during either 24 or 48 h periods were quantified at several anatomical levels. Three populations of neurons were studied: (1) large cells in layer III, (2) small to medium-sized cells in layers II and III, and in the striatal bridges, (3) granule cells in the islands of Calleja. Neurogenesis is sequential between these three populations with No. 1 oldest and No. 3 youngest. The large neurons in layer III originate mainly between E13 and E16 in a strong lateral-to-medial gradient. Neurons in population No. 2 are generated between E15 and E20, also in a lateral-to-medial gradient; neurogenesis is simultaneous along the superficial-deep plane. Granule cells in the smaller islands of Calleja are generated between E17 and E22 in combined lateral-to-medial and superficial-to-deep gradients. Neurons in the large island of Calleja are generated mainly between E19 and E22 in a strong rostral-to-caudal gradient. Neurogenesis is reduced to <10% in both populations No. 2 and No. 3, occurring mainly between P0 and P4. The neurogenic patterns in populations No. 2 and No. 3 are similar to those in the striatum, while neurogenesis in population No. 1 fits into the pattern of the globus pallidus and substantia innominata. These developmental patterns indicate that the olfactory tubercle is a mixed striato-pallidal system rather than an olfactory cortical area. The lateral-to-medial neurogenic gradient shown in each neuronal population in the olfactory tubercle correlates with both differential anatomical projections and differential neurochemical characteristics along the lateral-medial plane.

Key words: Olfactory tubercle, Islands of Calleja, Ventral striatum, [3H]thymidine autoradiography, Neurogenesis.

The classification of the olfactory tubercle has been the focus of controversy in the anatomical literature. In macrosmatic animals, such as the rat, the olfactory tubercle is a prominent structure in the rostral basal telencephalon. One of its distinguishing features is the presence of dense clusters of granule cells called islands of Calleja. The areas surrounding the islands in the olfactory tubercle have a laminated appearance and receive direct input from the main olfactory bulb. For these reasons the olfactory tubercle has generally been considered as part of the olfactory cortex. However, there are bridges of cells linking it to the overlying striatum, and many of the small to medium-sized neurons throughout the tubercle have a typical striatal appearance. Often, patterns of development are powerful aids in grouping various brain structures into functional systems. This study will present evidence that neurogenetic patterns in the olfactory tubercle can be linked to neurogenetic patterns in both the striatum and the pallidum.

Pulse labeling with a single injection of [3H]thymidine has been used to qualitatively establish approximate times for neuronal birthdays in the olfactory tubercle of the hamster and mouse. The present study uses comprehensive labeling with multiple injections of [3H]thymidine. This method allows an accurate delineation of both the onset and cessation of neurogenesis as well as the determination of the proportion of neurons that originate during single days of embryonic life. The quantitative data to be reported will be correlated with chronologies of neurogenesis in other brain structures.

METHODS

Since neurogenesis in the rat olfactory tubercle and islands of Calleja extends beyond the day of birth, both prenatal and postnatal developmental series were used. All series contained groups of Purdue–Wistar rats given successive daily (between 9 and 11 a.m.) s.c. injections of [3H]-
thymidine (Schwarz-Mann; specific activity 6.0 Ci/mM; 5 μCi/g body wt) to insure comprehensive cell labeling. The prenatal developmental series contained 10 groups, the offspring of pregnant females given two successive daily injections progressively delayed by one day between groups (E12–E13, E13–E14, . . . E21–E22). Two or more pregnant females were injected for each group; 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). The postnatal developmental series had three groups of rat pups, each group containing males from at least two litters. The pups were given four (P0–P3, P2–P5, P4–P7) consecutive daily injections.

All animals were perfused through the heart with 10% neutral formalin on P6. The brains were kept for 24 h in Bouin’s fixative, then were transferred to 10% neutral formalin until they were embedded in paraffin. The brains of at least six animals from each group were blocked coronally according to the stereotaxic angle of the Pellegrino et al. atlas. Every 15th section (6 μm) through the olfactory tubercle and islands of Calleja was saved. Slides were dipped in Kodak NTB-3 emulsion, exposed for 12 weeks, developed in Kodak DIg, and post-stained with hematoxylin and eosin.

Anatomically matched sections were selected for quantitative analysis at five levels throughout the anteroposterior extent of the olfactory tubercle and islands of Calleja (drawings, Figs 3 and 7). For quantification, all cells within a designated area were counted and were further subdivided into two classes, labeled and nonlabeled. Cells with reduced silver grains overlying the nucleus in densities above background levels were considered labeled; obvious endothelial and glial cells were excluded. The proportion of labeled cells (% labeled cells/total cells) was then calculated from these data. Medium-sized cells in layer II were counted microscopically at × 312.5 in three lateral-to-medial strips (0.29 mm wide) between levels A10.8 and A9.0. Small to medium-sized cells both in layer III (labeled ‘small cells’ in Fig. 3B, C) and in the lateral and medial striatal bridges were counted microscopically at × 312.5 in unit areas set off by an ocular grid (approximately 0.084 mm²) at levels A10.4 and A9.4. The scattered large cells in layer III were counted in medial and lateral halves of the olfactory tubercle (all large cells in the section were counted) at levels A10.4 and A9.4. Granule cells in the islands of Calleja were counted microscopically at × 625 in unit areas set off by an ocular grid (0.034 mm²) at five levels between A10.8 and A8.6.

The determination of the proportion of cells arising (ceasing to divide) on a particular day utilized a modification of the progressively delayed comprehensive labeling procedure and is described in detail elsewhere. 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 nonmitotic 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 1 shows the data and calculations for the medial part of layer II at all levels throughout the rostrocaudal extent of the olfactory tubercle. To calculate the proportion of cells originating on day E17, for example, the proportion of cells labeled after the morning of E18 (60% in the E18–E19 injection group) is subtracted from the proportion of cells labeled after the morning of E17 (85% in the E17–E18 injection group) to give 25% as the proportion of cells originating

<table>
<thead>
<tr>
<th>Injection group</th>
<th>N</th>
<th>% Labeled cells*</th>
<th>Day of origin</th>
<th>% Cells originating†</th>
</tr>
</thead>
<tbody>
<tr>
<td>E15–E16</td>
<td>6 (A) 99 ± 0.6</td>
<td>E15</td>
<td>1 (A–B)</td>
<td></td>
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<tr>
<td>E16–E17</td>
<td>12 (B) 98 ± 1.7</td>
<td>E16</td>
<td>1 (B–C)</td>
<td></td>
</tr>
<tr>
<td>E17–E18</td>
<td>9 (C) 85 ± 5</td>
<td>E17</td>
<td>25 (C–D)</td>
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<tr>
<td>E18–E19</td>
<td>6 (D) 60 ± 9</td>
<td>E18</td>
<td>23 (D–E)</td>
<td></td>
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<tr>
<td>E19–E20</td>
<td>6 (E) 37 ± 5</td>
<td>E19</td>
<td>24 (E–F)</td>
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<tr>
<td>E20–E21</td>
<td>7 (F) 13 ± 4</td>
<td>E20</td>
<td>7 (F–G)</td>
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<tr>
<td>E21–E22</td>
<td>6 (G) 6 ± 1</td>
<td>E21–E22</td>
<td>5 (G–H)</td>
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<tr>
<td>P0–P3</td>
<td>6 (H) 1 ± 0.6</td>
<td>P0–P2</td>
<td>1</td>
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* X ± S.D.
† Graphed in Fig. 3A.
between the onset of injections on E17 and E18. Trends in cell labeling were analyzed in individual animals with the sign test; the rationale for the use of this statistic is provided elsewhere.2,3

RESULTS

Neurogenesis in the olfactory tubercle

The olfactory tubercle has been traditionally divided into three layers.7,14,16,23,24 The superficial plexiform layer I is usually thicker laterally than medially. Layer II is the most well-defined morphologically and is probably responsible for having the olfactory tubercle classified as a cortex. It contains medium-to-small neurons packed 6–8 deep. Layer II forms loops and gives a corrugated appearance anteriorly, while it becomes parallel to the deep surface of the brain posteriorly (drawings, Fig. 3A). Layer II is nearly absent in the medial interhemispheric wall of the tubercle (pars medialis) and is most prominent in the large intermediate section of the tubercle along the base of the telencephalon (pars intermedia); it is quite irregular in a narrow lateral transition zone between the tubercle and the piriform cortex (pars lateralis).14 The neurons in layer II have traditionally been called pyramidal cells, but Golgi impregnations of these cells show an atypical and irregular structure distinguishing them from pyramidal cells in other cortical areas (Millhouse and Heimer, personal communication). Layer III contains scattered medium-to-small sized cells resembling the neurons in the striatum, and sparse large cells (arrows, Fig. 1), which Golgi impregnations show to resemble neurons in the globus pallidus and substantia inominata (Millhouse and Heimer, personal communication). Layer III is often spanned by ‘bridges’ of cells (SB, Fig. 1), forming a continuity with the striatum above and the layer II cells below.21

Figure 2 shows autoradiograms of layer II cells in the brain of a rat exposed to [3H]thymidine on E16+E17. There are fewer labeled cells in the pars lateralis (A) than in the medial part of the pars intermedia (B), indicating a lateral-to-medial gradient. To quantify the time of origin of neurons in layer II, cells were counted in the pars lateralis, and in lateral and medial areas of the pars intermedia at four levels between A10.8 and A9.0. The sign test showed that there were no significant differences in homologous areas along the rostrocaudal plane and the data were combined. The bottom graph in Fig. 3A is combined data from all transition areas adjacent to the piriform cortex at each level, while the top graph combines all medial areas. Neurogenesis shows a strong lateral-to-medial gradient (P<0.0001, all levels and comparisons), peaking between E14 and E16 in the transition area, between E16 and E18 laterally, and between E17 and E19 medially. There are a few scattered smaller neurons originating postnatally in all areas.

Small-to-medium sized cells were counted in layer III at lateral and medial areas, and in the lateral and medial striatal bridges at levels A10.4 and A9.4. The sign test showed no differences between homologous locations along the rostrocaudal plane and the data were combined. Figure 3B shows that the strong lateral-to-medial gradient seen in layer II is also found in layer III (P<0.0001) and in the striatal bridges (P<0.0001). The top graphs in Fig. 3B show histograms of cell origin in medial and lateral layer II cells (repeated from Fig. 3A) so that differences in time of origin along the superficial-deep plane can be more easily discerned. When the sign test is applied to these data, neurogenetic differences along the superficial-deep plane are not significant. Figure 1 is an autoradiogram from the brain of a rat exposed to [3H]thymidine on E16–E17. Notice that the small-to-medium sized neurons in both layers II and III and in the striatal bridge contain about the same proportion of labeled and unlabeled cells.

Large neurons are scattered throughout layer III (arrows, Fig. 1). The time of origin of these cells was separately determined and the results are compared with neurogenetic patterns of the small-to-medium sized cells in layer III (Fig. 3C, the histograms of the ‘small cells’ time of origin are repeated from Fig. 3B). The large cells have a much earlier time of origin than the small cells (P<0.0001), and, like the smaller cells of the olfactory tubercle, they show a strong lateral-to-medial gradient (P<0.0001), peaking on E14–E15 laterally and E15–E16 medially.

Neurogenesis in the islands of Calleja

The islands of Calleja are dense clusters of granule cells scattered throughout superficial and deep parts of the olfactory tubercle and along the dorsomedial border of the nucleus accumbens.
Fig. 3. Neurogenesis of the large and small-to-medium sized cells in the olfactory tubercle. All bar graphs are the proportion of cells originating on the indicated days (dashed line designates the time of birth). Table 1 indicates how the data were calculated. All arrows in drawings indicate gradients of neurogenesis. (A) Development of layer II neurons in the pars lateralis (PC transition area), and in lateral and medial parts of the pars intermedia. There is a prominent lateral-to-medial gradient. (B) Development of the small-to-medium sized cells throughout layers II and III, and in the striatal bridges at lateral and medial locations in the pars intermedia. There is no gradient in the superficial-deep plane, but a prominent lateral-to-medial gradient. (C) Development of the large and small-to-medium sized cells in layer III at lateral and medial locations. The large cells originate much earlier than the small cells, while both populations show a lateral-to-medial gradient.

Often, large cells are found in cores of neuropil enclosed by the granule cells;\textsuperscript{30} (arrows, Fig. 5D, C). All of the data presented here are based on counts of granule cells only. At all levels and locations, the neurons in the islands of Calleja originate significantly later ($P<0.0001$) than the small-to-medium sized neurons in layers II and III of the olfactory tubercle.

The superficial islands were counted in the transition area near the piriform cortex (pars lateralis), in lateral and medial parts of the main body of the olfactory tubercle (pars intermedia).
Fig. 1. The olfactory tubercle at a mid-rostrocaudal level from an animal exposed to [3H]thymidine on E16-E17 and killed on P60 (6 μm, paraffin, hematoxylin and eosin, bar = 0.2 mm). Layers I, II, and III are indicated with Roman numerals. Arrows point out some large cells in layer III. IC, island of Calleja; SB, a portion of a striatal bridge.
Fig. 2. Autoradiograms of layer II neurons from an animal exposed to $[^3]H$thymidine on E16-E17 and killed on P60 (paraffin, 6 μm, hematoxylin and eosin, bar = 25 μm). (A) Pars lateralis; (B) medial part of pars intermedia. Note the decrease in the number of labeled cells between (A) and (B).
Fig. 5. Autoradiograms of the islands of Calleja in animals exposed to [3H]thymidine on E20-E21 (A, C) and E19-E20 (B, D) and killed on P60 (6 μm paraffin section, hematoxylin and eosin, bar = 50 μm). (A) Medial superficial island; (B) lateral superficial island; (C) medial deep island; (D) lateral deep island. Both deep islands frequently associate with large pallidal-type neurons (arrows). The proportion of labeled neurons increases in both superficial-to-deep and lateral-to-medial directions.
Fig. 8. Autoradiograms of the large island of Calleja at anterior (A) and posterior (B) levels in an animal exposed to $[^3]$Hthy midine on E20–E21 (6 μm paraffin section, hematoxylin and eosin, bar = 50 μm). The proportion of labeled cells dramatically increases in the caudal level.
and along the medial wall of the hemisphere (pars medialis) at four levels between A10.8 and A9.0 (drawings, Fig. 4A). Superficial islands in the pars medialis are absent at levels A10.8 and A9.0, while those in the pars lateralis are absent at A9.0. The sign test showed no differences along the rostrocaudal plane so the data from homologous locations at each level were combined (Fig. 4A). Similar to the other cells in the olfactory tubercle, there is a very strong lateral-to-medial gradient \( P<0.0001 \), all levels and comparisons; arrows, Fig. 4A). The major part of neurogenesis in the pars lateralis occurs between E15 and E17, between E18 and E20 in the pars medialis.

Deep islands were quantified from A10.4 to A9.0. There are no deep islands in the pars lateralis. The lateral and intermediate deep islands are in the pars intermedia, while the medial deep island is in the pars medialis. The sign test showed no differences between levels along the anterior-posterior plane and data from homologous rostrocaudal locations were combined (Fig. 4B). Again, there is a strong lateral (peak on E18–E19) to medial (peak on E19–E22) neurogenetic gradient \( P<0.0001 \); arrows in Fig. 4B).

Figure 4C is a rearrangement of the histograms in Figs 4A, B so that neurogenetic gradients along the superficial-deep plane can be more easily discerned. Note that in the top group of histograms in Fig 4C ‘lateral superficial’ refers to pars intermedia lateral, ‘intermediate superficial’ refers to pars intermedia medial, and ‘medial superficial’ refers to pars medialis. Beneath these graphs are the histograms for the lateral, intermediate, and deep islands, respectively, shown in

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**Fig. 4. Neurogenesis in the islands of Calleja.** All bar graphs represent the proportion of cells originating on the indicated days. Arrows in drawings represent gradients of neurogenesis. (A) Superficial islands in four locations from the pars lateralis to the pars medialis have a lateral-to-medial gradient. (B) Deep islands at lateral and intermediate locations in the pars intermedia and deep islands in the pars medialis (medial location) also show the lateral-to-medial gradient. (C) A comparison of neurogenetic patterns in superficial and deep islands along the lateral-medial plane indicate strong superficial-to-deep gradients.
Fig. 4B. In all cases, neurogenesis begins significantly earlier in the superficial islands ($P < 0.0001$, lateral and intermediate; $P < 0.001$, medial). The arrows in the drawings in Fig. 4C indicate the superficial-to-deep neurogenetic gradient at some of the rostrocaudal levels examined.

The autoradiograms of the islands of Calleja shown in Fig. 5 illustrate qualitatively the two gradients described quantitatively above. Parts A and C are from superficial and deep islands, respectively, in the pars medialis of a rat exposed to $[^3]$H]thymidine on E19–E20. There are more labeled cells as one goes from the superficial islands to the deep islands, and from the lateral islands to the medial islands.

The deep islands in the pars intermedia extend rostrally to level A10.8 and have a neurogenetic gradient along the rostrocaudal plane (Fig. 6). Deep islands in the pars medialis do not extend this far rostrally. The intermediate deep island at level A10.8 begins to originate significantly earlier ($P < 0.029$) than the intermediate deep islands at levels A10.4–A9.0 (two top graphs, Fig. 6). Similarly, the lateral deep islands at level A10.8 begin to originate significantly earlier ($P < 0.0001$) than the lateral deep islands at levels A10.4–A9.0 (two bottom graphs, Fig. 6). The arrows in Fig. 6 indicate both the lateral-to-medial and rostral-to-caudal gradients seen in the deep islands.

![Fig. 6](image)

Fig. 6. Neurogenesis of granule cells in the deep islands of Calleja at level A10.8 (first and third graphs) and combined levels A10.4–A9.0 (second and fourth graphs) at lateral and intermediate locations. Bar graphs represent the proportion of neurons originating on the days indicated. Caudal levels originate later than the rostral level (arrows to left of drawings).

The large island of Calleja occupies part of the dorsomedial border of the nucleus accumbens and represents the most caudal extension of the islands (possibly a continuation of the deep islands in the pars medialis). Cells were counted in dorsal and ventral unit areas at levels A8.6, A9.0, and A9.4 (drawings, Fig. 7). The sign test showed no dorsoventral differences and the data were combined for each level (Fig. 7). Granule cells at level A9.4 begin to be generated significantly earlier than those at A9.0 ($P < 0.005$), those at level A9.0 are significantly earlier than granule cells at A8.6 ($P < 0.001$). Figure 8 shows the strong rostral-to-caudal gradient in the brain of an animal exposed to $[^3]$H]thymidine on E20+E21. Nearly all cells are labeled at the caudal level (B) while there are several unlabeled cells anteriorly (A). It is interesting to note that the deep medial island at level A9.4 begins to originate significantly before the even deeper large island at this same level ($P < 0.01$). Thus, the larger island participates in both the superficial-deep (Fig. 4C) and rostral-caudal gradients (Fig. 6) seen in the other islands.

**DISCUSSION**

The relationship of the olfactory tubercle to basal telencephalic structures

Neurogenetic gradients in both the striatum and pallidum continue into the olfactory tubercle. There is a predominant lateral-to-medial gradient in the caudoputamen complex in the nucleus
Neurogenesis in rat olfactory tubercle

Fig. 7. Neurogenesis of granule cells in the large island of Calleja between levels A9.4 (bottom) and A8.6 (top). Bar graphs indicate the proportion of cells originating on the days indicated. There is a prominent rostral-to-caudal gradient (arrow).

accumbens and in the olfactory tubercle (present study). The large cells of the olfactory tubercle fit into the caudal-to-rostral gradient found in the globus pallidus, substantia innominata and horizontal limb of the diagonal band (Bayer, in preparation). In addition, neurogenesis is nearly simultaneous in the three structures. Large cells in the olfactory tubercle are generated early and in patterns resembling neurogenesis in the magnocellular basal telencephalic nuclei (Bayer, in preparation). The small to medium-sized cells in layers II and III of the olfactory tubercle as well as the medium-sized cells of both nucleus accumbens and caudoputamen are mainly produced 4–6 days before birth with minimal neurogenesis in the early postnatal period. This 'striatal' pattern to neurogenesis in the olfactory tubercle has been noted in previous studies. There has been long-standing histochemical evidence of continuity between the olfactory tubercle and the striatum since these areas are among the structures most intensely stained for acetylcholinesterase and for the immunocytochemical localization of choline acetyltransferase. There is also ample histochemical evidence to include large cells of the olfactory tubercle in the ventral pallidum. Finally, there are similarities in anatomical connections: the globus pallidus is the chief target of caudoputamen projections, while the ventral pallidum (substantia innominata and horizontal limb of the diagonal band) is the chief target of projections from the medium-sized cells in the nucleus accumbens and olfactory tubercle. Thus, both developmentally and anatomically, the olfactory tubercle is more closely related to the striatum and pallidum than to other telencephalic structures.

Both the caudoputamen and nucleus accumbens are typical ganglionic structures. On the other hand, the olfactory tubercle resembles a cortex, although this is a point of controversy in the descriptive anatomical literature. True cortical structures in the telencephalon show a typical 'inside-out' neurogenetic gradient. In her analysis of olfactory tubercle cell birthdays, Creps concluded that the olfactory tubercle showed a characteristic cortical neurogenetic gradient. However, this is true only when the large neurons in layer III (the polymorph cells) are included in the analysis. The data presented in Fig. 3B show that the olfactory tubercle does not have an inside-out gradient when only small-to-medium sized cells are quantified. In a true cortex, the deeper neurons, even if they are small, are usually generated before more superficial neurons. For example, the smaller neurons in layer V of the entorhinal cortex are generated earlier than the larger stellate neurons in layer II. A similar situation to that found in the olfactory tubercle is present in the posterolateral part of the 'cortical' nucleus of the amygdala which also does not have the typical cortical neurogenetic gradient. Both the cortical nucleus of the amygdala and the olfactory tubercle get main olfactory bulb input to layer I which creates a cell-sparse plexiform layer. The presence of layer I makes the olfactory tubercle look like part of the olfactory cortex, when there is a strong probability that it is a ganglionic structure like the rest of the striatum. However, the presence of direct olfactory input makes the olfactory tubercle a unique component of the striatum.
Correlations between neurogenic gradients and anatomical patterns

The strong lateral-to-medial neurogenic gradient correlates with differential anatomical connections of the olfactory tubercle. The lateral part of the tubercle gets input from the anterior piriform cortex, while the medial part gets input from the posterior piriform and entorhinal cortices. Limbic neocortical areas project more heavily to medial rather than lateral parts of the tubercle. There are also differential mediolateral projections to the tubercle from the basolateral and basomedial nuclei of the amygdala. Fallon reported some evidence of a mediolateral topography between the thalamic and hypothalamic inputs to the tubercle. Finally, there are differences in the efferent projections of the tubercle; lateral parts project to the lateral ventral pallidum while medial parts project to the medial ventral pallidum. In an extensive study of the chemical neuroanatomy of the olfactory tubercle, Fallon et al. found many differences along the lateral-medial plane. For example, met-enkephalin, substance P, and leutinizing hormone releasing hormone are more concentrated in medial parts of the tubercle. More recently, Fallon et al. found epidermal growth factor, a gut peptide with mitogenic activity, to be preferentially located in fibers ramifying in pallidal structures, including the neuropil surrounding the large cells associated with the islands of Calleja. The peptide is more widespread in lateral rather than medial parts of the tubercle. Thus, medial and younger parts of the tubercle get a different complex of afferent, project to different targets, and have a unique set of neurochemical characteristics than do more lateral and older parts of the tubercle. Possibly, precise patterns of neurogenesis are important developmental events in bringing about the future expression of anatomical and neurochemical differences.

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Neurogenesis in rat olfactory tubercle