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Adrenal steroid receptor immunoreactivity in cells born in the adult rat dentate gyrus

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Several lines of evidence indicate that cell birth in the adult rat dentate gyrus is regulated by adrenal steroids. The expression of adrenal steroid receptors by mitotic cells in the dentate gyrus would support the hypothesis that these hormones act directly on granule cell progenitors. We performed a survival time course of in vivo [^{3}H]thymidine autoradiography combined with immunohistochemistry for mineralocorticoid receptors (MR) and glucocorticoid receptors (GR) and found that very few [^{3}H]thymidine labeled mitotic cells express these receptors. By 4 weeks following [^{3}H]thymidine administration, the vast majority of [^{3}H]thymidine labeled cells were immunoreactive for MR and GR. These results suggest that adrenal steroids do not act directly on granule cell progenitors in the adult rat dentate gyrus.

In most mammalian brain regions, neurogenesis occurs only during development. In the rat dentate gyrus, however, neurons continue to be born in adulthood[5,16]. Granule neurons are born in the hilus, subgranular zone and granule cell layer of the adult dentate gyrus; neurons that are born in the hilus migrate to the granule cell layer[7]. These newly born cells receive synaptic input[15], survive for several months following mitosis[3] and express a neuron specific marker[7]. The unusual persistence of neurogenesis in this brain region makes the dentate gyrus a useful model in which to study the cues that regulate this process in the absence of similar changes occurring in related structures.

Several lines of evidence suggest that neurogenesis in the adult rat dentate gyrus is inhibited by adrenal steroids. First, developmental neurogenesis occurs in 2 distinct phases both of which are inversely correlated with the levels of circulating adrenal steroids[6] (see also ref. 23 and compare with 22). Second, the administration of glucocorticoids or mineralocorticoids to developing rat pups results in a decrease in the birth of granule cells[6,12]. Third, removal of circulating adrenal steroids by adrenalectomy results in an increase in the rate of cell birth in the adult dentate gyrus; many of these cells appear to be neurons[13].

Despite the evidence that adrenal steroids regulate granule cell genesis, it is presently unknown whether these hormones act directly on granule cell progenitors. The expression of adrenal steroid receptors by adult dentate gyrus neuroblasts would support this hypothesis. Granule cells of the adult rat dentate gyrus express both types of adrenal steroid receptors, mineralocorticoid or Type 1 receptors (MR)[1,21] and glucocorticoid or Type 2 receptors (GR)[10,21]. However, no previous study has addressed the possibility that granule cell progenitors in the adult dentate gyrus express these receptors. In order to determine whether this is the case, we performed a survival time course of [^{3}H]thymidine autoradiography, combined with Nissl staining and immunohistochemistry for GR and MR in adult rats.

Adult (4 months old) male Sprague–Dawley rats were used for this study. Groups of rats were injected with [^{3}H]thymidine either 1 h, 24 h, 1 week, 2 weeks, 3 weeks or 4 weeks prior to perfusion. The 1 h time point was chosen to label mitotic cells as this amount of time is sufficient for uptake of [^{3}H]thymidine but not for

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completion of mitosis or migration[7,18,20]. The 24 h time point was chosen to identify cells that have divided once; the cell cycle of dentate gyrus granule cells is approximately 16 h[19]. At the time of perfusion, the rats were deeply anesthetized with Metofane and transcardially perfused with 4.0% paraformaldehyde in 0.1 M phosphate buffer with 1.5% (v/v) picric acid. The brains were dissected from the skulls and postfixed in a solution having the same composition as the perfusate for 24 h. The brains were then cut (40 μm) on a Vibratome into a bath of PBS and processed for immunohistochemistry combined with autoradiography as previously described[13]. Briefly, the sections were incubated overnight in one of the following primary antibody solutions: (1) polyclonal antisera against the mineralocorticoid receptor (Affinity Bioreagents, hMRsN, for characterization see ref. 2) diluted to 10 μg/ml in PBS, or (2) monoclonal antibody against the glucocorticoid receptor (Affinity Bioreagents, Clone BuGR 2, for characterization see ref. 11) diluted to 2.5 μg/ml in PBS. The sections were then rinsed in PBS and placed in biotinylated secondary antisera directed against the appropriate species for 2 h. The sections were rinsed again and incubated in avidin-biotin-HRP for 2 h. After a final rinse in PBS, the sections were reacted with diaminobenzidine and hydrogen peroxide for 15 min. Control sections were processed as described above with omission of the primary antibody and showed no nonspecific staining. The sections were mounted onto gelatinized slides, rinsed in distilled water, dipped in photographic emulsion (NTB-2, Kodak) and stored in the dark at 4°C for 4 weeks. The slides were then developed in Dektol, rinsed in water, fixed in Ektaflo, rinsed again and counterstained for Nissl using Cresyl violet.

The numbers of [^{3}H]thymidine labeled cells that were immunoreactive and those that were non-immunoreactive in the hilus and granule cell layer were counted in sections stained for MR or GR. The cross-sectional areas of the granule cell layer and hilus were determined for each section using a camera lucida drawing tube and a Zeiss Interactive Digitizing Analysis System. The data were expressed as densities (number of cells per 10^6 μm^2) and percentages of [^{3}H]thymidine labeled cells that were immunoreactive. An average of 4 sections (8 sides) were counted for each brain. Means were determined for each animal and the data were subjected to one-way analysis of variance with Tukey HSD post hoc comparisons.

Light microscopic examination of MR-immunoreactive tissue revealed moderate staining of cells in the granule cell layer as well as in the hilus. The MR staining in the granule cell layer appeared to be neuronal. Likewise, in the hilus MR staining was localized to neurons, e.g. mossy cells and modified pyramidal cells. Quantitative analysis of MR immunoreactive tissue showed significant overall differences in densities and percentages of [^{3}H]thymidine labeled cells that were MR immunoreactive with time ($P < 0.01$; Fig. 1). At 1 h following injection, the percentage of [^{3}H]thymidine labeled cells that were MR immunoreactive was low (12%, Fig. 1). By 2 weeks following injection the percentage of [^{3}H]thymidine labeled cells that were MR immunoreactive was significantly higher ($P < 0.05$, Fig. 1). This percentage remained high through the latest time point examined (81.8% at 4 weeks; Fig. 1). Many [^{3}H]thymidine labeled cells in the adult dentate gyrus showed morphological characteristics of neurons, i.e. medium sized round or oval cell bodies (Fig. 2). All [^{3}H]thymidine labeled MR immunoreactive cells had these features (Fig. 2). At 4 weeks following injection all [^{3}H]thymidine labeled MR immunoreactive cells were located in the granule cell layer and most [^{3}H]thymidine labeled non-immunoreactive cells were located in the hilus.

Light microscopic examination of GR-immunoreactive tissue revealed moderate to intense staining in the granule cell layer as well as in the hilus. Quantitative analysis of GR-immunoreactive [^{3}H]thymidine labeled tissue showed significant overall differences in densities and percentages of [^{3}H]thymidine labeled cells that were immunoreactive for GR with time ($P < 0.01$, Fig. 3). Very few (10%) [^{3}H]thymidine labeled cells were immunoreactive at the earliest time point examined, 1 h following [^{3}H]thymidine injection (Fig. 3). Twenty four hours following [^{3}H]thymidine injection, a

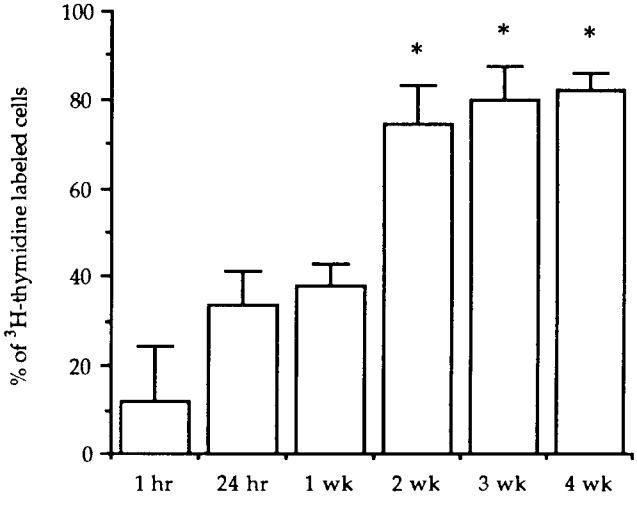

Fig. 1. The percentage of [^{3}H]thymidine labeled cells in the dentate gyrus that are immunoreactive for MR at 1 h, 24 h, 1 week, 2 weeks, 3 weeks and 4 weeks following [^{3}H]thymidine administration. Values represent mean + S.E.M. obtained from 3 brains. Asterisks represent significant difference from 1 h and 24 h; $P < 0.05$.

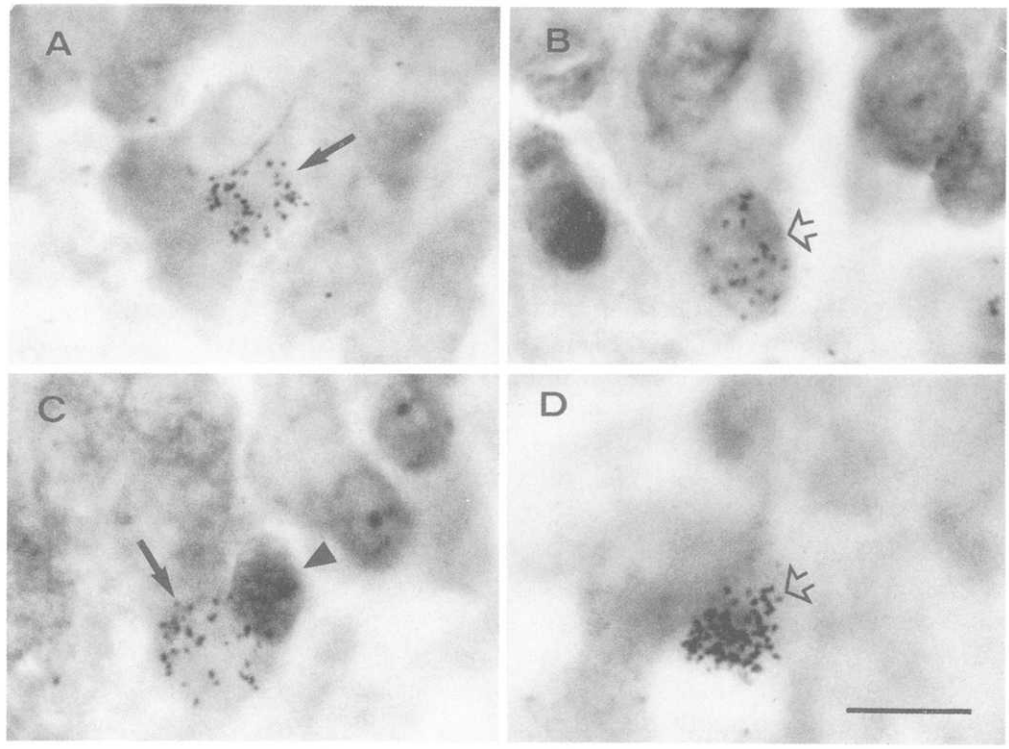

Fig. 2. Photomicrographs of [^{3}H]thymidine labeled cells in the dentate gyrus that are nonimmunoreactive (solid arrow) for MR (A), immunoreactive (open arrow) for MR (B), nonimmunoreactive for GR (C) and immunoreactive for GR (D). Observe the neuronal morphology of the labeled cells in A, B and C. Arrowhead in C indicates immunoreactive unlabeled cell. Bar = 30 μm and applies to all frames.

significant increase in the percentage of GR immunoreactive [^{3}H]thymidine labeled cells was observed ($P < 0.05$, Fig. 3). The percentage of [^{3}H]thymidine labeled cells that were GR-immunoreactive steadily increased with time; by 4 weeks following [^{3}H]thymidine administration, the vast majority (approximately 85%) of [^{3}H]thymidine labeled cells were GR immunoreactive (Fig. 3). Many [^{3}H]thymidine labeled cells

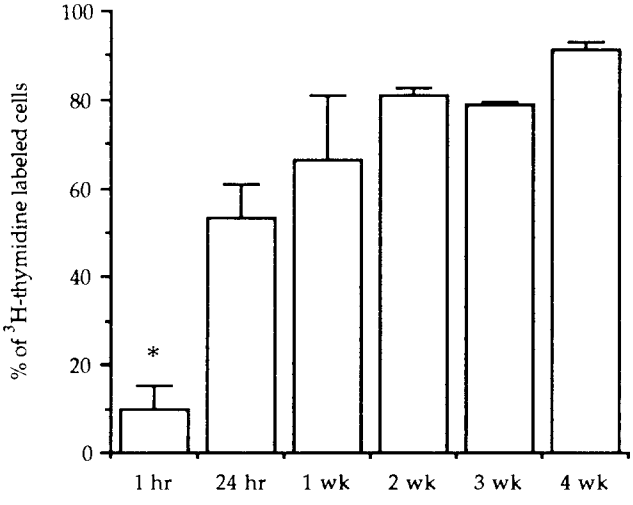

Fig. 3. The percentage of [^{3}H]thymidine labeled cells in the dentate gyrus that are immunoreactive for GR at 1 h, 24 h, 1 week, 2 weeks, 3 weeks and 4 weeks following [^{3}H]thymidine administration. Values represent mean + S.E.M. obtained from 3 brains. Asterisk represents significant difference from all other groups; $P < 0.05$.

observed in the adult dentate gyrus showed morphological characteristics of granule neurons, i.e. round or oval medium sized cell bodies (Fig. 2). However, not all GR immunoreactive [^{3}H]thymidine labeled cells could be identified as neurons based on their morphological characteristics. At the 4 week time point, the majority of [^{3}H]thymidine labeled cells in the granule cell layer were GR-immunoreactive. The hilus contained both GR-immunoreactive and nonimmunoreactive [^{3}H]thymidine labeled cells at this time.

The results of this study show that most cells born in the adult rat dentate gyrus do not express adrenal steroid receptors during mitosis. At 1 h following [^{3}H]thymidine injection, approximately 10% of labeled cells were immunoreactive for MR and an equal percentage were immunoreactive for GR. However, cells born in the adult developed MR or GR immunoreactivity with time. A significant increase in the percentage of [^{3}H]thymidine labeled cells that were immunoreactive for GR occurred at 24 h following [^{3}H]thymidine injection whereas a significant increase in the percentage expressing MR did not occur until 2 weeks following DNA synthesis.

Since 1 h is enough time for the uptake of circulating [^{3}H]thymidine by cells in the S-phase of mitosis but not enough time for cell division or migration to occur[7,18,20], the [^{3}H]thymidine labeled cells observed

after a 1 h survival time are part of the dentate gyrus progenitor population. Granule cell mitosis is complete within hours of DNA synthesis[18,19], indicating that [^{3}H]thymidine labeled cells in the 24 h survival group represent a population that has divided once. The increase in GR-immunoreactive [^{3}H]thymidine labeled cells at 24 h suggests that these receptors are expressed shortly after mitosis is complete. In contrast, the percentage of [^{3}H]thymidine labeled cells that are MR-immunoreactive did not increase until 2 weeks after mitosis. Since many newly born granule cells migrate from the hilus to the granule cell layer before this time[7], it is possible that expression of mineralocorticoid receptors does not occur until both cell division and migration are completed.

These findings are consistent with those of a developmental study which showed a presumed lag time between the birth of cells and the appearance of corticosterone binding in the postnatal dentate gyrus[25]. In a previous study we found that at postnatal day 6, mitotic cells, i.e. those [^{3}H]thymidine labeled after a 1 h survival time, were not immunoreactive for MR and only a few such cells (10%) were immunoreactive for GR[14]. The present results indicate that approximately the same percentage of [^{3}H]thymidine labeled mitotic cells are GR immunoreactive in adulthood as during development. However, in contrast to the developmental profile, some [^{3}H]thymidine labeled cells are MR immunoreactive in adulthood. This difference raises the possibility that some of the cells that divide in the adult exist in a more differentiated state, i.e. they express MR immunoreactivity, than during development.

Although it is not possible to determine with certainty whether the [^{3}H]thymidine labeled cells in the present study are neurons or glia, most of these cells have the morphological characteristics of neurons. Moreover, most of the [^{3}H]thymidine labeled cells are located in the granule cell layer, a region that contains few glia[17]. However, the few [^{3}H]thymidine labeled cells located in the hilus probably represent a more mixed population of both cell types. Because this study did not involve double labeling for MR and GR on the same section, it is not known whether these two populations of immunoreactive [^{3}H]thymidine labeled cells are the same. Previous studies have shown that MR is expressed primarily by neurons (see ref. 1 for exceptions) whereas GR is expressed by both neurons and glia[10] suggesting that these two populations are not identical. The observation that no [^{3}H]thymidine labeled MR-immunoreactive cells are located in the hilus at 4 weeks suggests that these cells have migrated into the granule cell layer. In contrast, many [^{3}H]thymidine labeled GR-immunoreactive cells remain in the hilus at this time point. Since newly born neurons have been shown to migrate from this region[7], it is likely that these GR-immunoreactive hilar cells are primarily glia.

In adulthood, removal of circulating adrenal steroids results in an increase in dentate gyrus cell birth[13]. The present finding that very few mitotic cells express MR or GR makes it unlikely that adrenal steroids act directly on granule cell progenitors to regulate cell birth and indicates that an as yet unidentified factor is probably involved. Two lines of evidence indicate that excitatory afferents may control cell birth in this brain region. First, excitation and afferent input have been shown to regulate cell birth in many other systems[4,8,9,24]. Second, blockade of the *N*-methyl-D-aspartate subtype of glutamate receptors increases cell birth in the developing rat dentate gyrus (unpublished observations). Since the adult dentate gyrus retains many characteristics typically seen only during development, e.g. cell birth and migration[7,16], it is likely that factors which regulate these processes in the young animal continue to do so in adulthood. In this regard, it is noteworthy that many excitatory afferents to the dentate gyrus, e.g. hilar mossy cells and medial septal cells, show immunoreactivity for MR or GR[1,10]. Future studies in our laboratory will explore the possibility that adrenal steroids influence dentate gyrus cell birth in the adult by acting directly on an afferent population.

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