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DEVELOPMENT OF HYPOTHALAMIC OPIOID NEURONS: A COMBINED IMMUNOCYTOCHEMICAL AND [^{3}H]THYMIDINE AUTORADIOGRAPHIC STUDY

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ABSTRACT

Using a combined technique of immunocytochemistry and [^{3}H]thymidine autoradiography, we have determined the "birth-date" of opioid peptide containing neurons in three hypothalamic nuclei. These include proopiomelanocortin neurons (indicated by ACTH immunoreactivity) in the arcuate nucleus, dynorphin A neurons in the supraoptic nucleus, and [Leu]enkephalin neurons in the periventricular nucleus. Arcuate proopiomelanocortin neurons were born very early in embryonic development, with peak heavy [^{3}H]thymidine nuclear labelling occurring on embryonic day E12. Supraoptic dynorphin A neurons were also labelled relatively early (peak at E13). By contrast,[Leu]enkephalin neurons in the periventricular nucleus exhibited peak heavy nuclear labelling on day E14. The results indicate a differential genesis of these three opioid peptide containing neuronal groups in three different hypothalamic nuclei.

INTRODUCTION

Most studies of ontogenetic development of opioid peptide neuronal systems have to date relied upon radioimmunoassay and immunohistochemical techniques (1-4). While each of these techniques provide a useful measure of peptide content or cell body and fiber distribution in the brain, both methods nevertheless suffer from obvious limitations of the technique used for the detection of minute quantities of peptide during early embryogenesis. Thus, neither method can accurately pinpoint the onset of neurogenesis. Neurogenesis, or the time of origin of neurons, can be determined by the prenatal exposure of embryonic neuroblasts to [^{3}H]thymidine and subsequent postnatal autoradiography to quantitate thymidine incorporation by the dividing neuroblast (e.g., 5). Yet the latter technique alone cannot account for the chemical identity of the neurons which are being "birth-dated." Thus, in the present study, we have utilized a combined [^{3}H]thymidine autoradiographic and immunocytochemical technique (6) to investigate the time of genesis of three distinct opioid peptide neuronal groups in the rat hypothalamus.

MATERIALS AND METHODS

Adult male and female Sprague-Dawley rats were obtained from Charles River Labs and were bred according to the procedure described previously (7). At least three pregnant rats each on embryonic days E12, E13, E14, E15, or E16 were injected with approximately 7 uCi/gm body weight of [^{3}H]thymidine (1 mCi/ml, specific activity 6.7 Ci/mmol) (New England Nuclear). The male progeny were collected for the experiment. Three or more adult males from each injection group (i.e., embryonic days E12-E16) were injected ICV with 50-200 ug/10ul colchicine 24-48 hours prior to sacrifice. Animals were anesthetized and perfused with 4% formaldehyde, and the brains were processed for peroxidase-antiperoxidase immunocytochemistry as described before (8). Primary rabbit antisera used were raised against ACTH (to localize proopiomelanocortin neurons in the arcuate nucleus), dynorphin A (magnocellular supraoptic nucleus) and [Leu]enkephalin (periventricular nucleus). After the completion of immunocytochemistry, the sections were processed for [^{3}H]thymidine autoradiography (see 9). After development and fixation, the sections were dehydrated through ethanols and xylenes and coverslipped. With the aid of a microscope, immunoreactive perikarya belonging to each specific peptide group, and with an identifiable nucleus, were counted in several sections. The cell nuclei were designated as heavy/medium labelled (H/M) or light/un-labelled (L/U) depending on the density of autoradiographic grains (Fig. 1). The results for each peptide group on each embryonic day were expressed as the percent H/M over the total number of immunoreactive perikarya (i.e., H/M : (H/M + L/U) x 100). "Blind" counts were made by independent observers to reduce experimental bias. Analysis of variance was performed for each neuronal population.

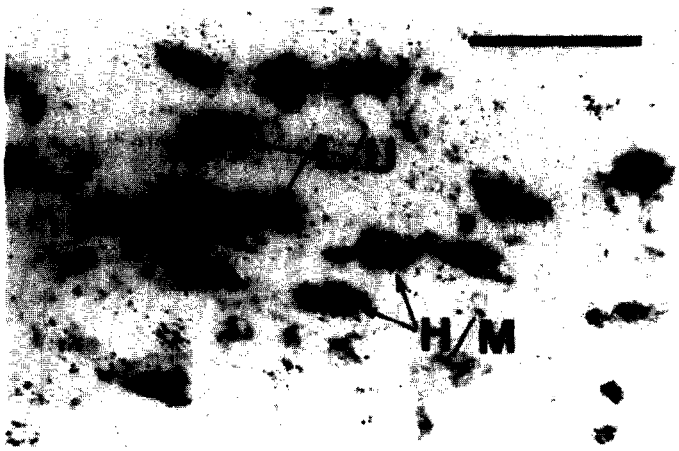

Fig. 1. ACTH immunoreactive perikarya in the arcuate nucleus exhibiting various densities of [^{3}H]thymidine autoradiographic grains over the cell nucleus: heavy/medium (H/M), and light/unlabeled (L/U). Bar = 50 um.

RESULTS

The ACTH-containing perikarya in the arcuate nucleus showed heavy nuclear-labelling patterns at early embryonic stages studied. On days E12 and E13 alone over 50% of immunoreactive cells exhibited [^{3}H]thymidine labelling. The labelling patterns declined gradually from E14 through E16 (only 2% of cells on the last day). The dynorphin A-containing magnocellular perikarya of the supraoptic nucleus exhibited peak heavy [^{3}H]thymidine labelling on day E13. Nevertheless, a major proportion (over 80%) of dynorphin A cells were "born" prior to E15, with only a minor population being labelled heavily on days E15 and E16 (approximately 4% on each day). Among [Leu]enkephalin-containing perikarya in the periventricular nucelus, a

relatively minor proportion were "born" on days E12 and E13 (12% and 13%, respectively), while peak heavy [^{3}H]thymidine labelling occurred on day E14 (25%). Labelling was negligible on days E15 and E16. These results are shown in Table 1 below.

TABLE 1

Nucleus/Ab	E12	E13	E14	E15	E16	
ARC/ACTH	29 ± 2.3	24 ± 2.8	23 ± 2.6	9 ± 1.8	2 ± 0.8	p<.0005
SON/DYN-A	28 ± 2.4	39 ± 6.1	19 ± 3.8	4 ± 2.7	4 ± 1.5	p<.001
PV/Leu-ENK	12 ± 5.3	13 ± 5.5	25 ± 3.2	.6 ± 0.4	2 ± 0.7	p<.01

TABLE 1. Percentage of cells born on embryonic days E12-E16 (± S.E.M.) in three hypothalamic nuclei, arcuate (ARC) immunostained with ACTH, supraoptic (SON) immunostained with dynorphin A (DYN-A), and periventricular (PV) immunostained with [Leu]enkephalin (Leu-ENK). Ab = antibody.

DISCUSSION

Some of the observed patterns of neurogenesis in the present study correspond well with previous developmental studies, while others do not. The present finding of the early origin of ACTH-containing neurons in the arcuate nucleus (29% at E12) corresponds well with immunocytochemical localization of ACTH and other proopiomelanocortin products in arcuate neurons as early as day E12 (2,4). On the other hand, the finding of peak labelling of supraoptic dynorphin A neurons on day E13, as well as peak labelling of periventricular [Leu]enkephalin neurons on day E14, does not agree with the observed immunoreactivity in developing embryos during late gestation, e.g., E17-E18 (3,4). Based on these observations, there appears to be no time lag between the onset of neurogenesis of arcuate proopiomelanocortin neurons and the initation of peptide synthesis. Conversely, a time lag of several days seems to exist between the onset of neuron differentiation and dynorphin A or [Leu]enkephalin synthesis. One simple explanation may be that the antisera used for the detection of the latter peptides in developmental immunocytochemical studies are not as sensitive as the anti-ACTH serum or that these peptides exist mainly in precursor form and/or in minute quantities during early gestation. Thus, the low quantities or precursor "masking" might render the latter peptides undetectable at such early gestational stages (i.e., E13-E14). Alternatively, these supraoptic and periventricular neurons might not begin synthesis of their respective opioid products until several days after they are born, or might express nonopioid products earlier (e.g., vasopressin in the case of supraoptic neurons).

Another interesting observation in the present study is that the patterns of neurogenesis of opioid peptide-containing neurons in any given region of the hypothalamus do not necessarily correspond to the patterns of cell birth in that region as a whole (see 5). For example, the arcuate nucleus is among the latest arising of nuclei in the hypothalamus (over 60% at E14-E16 according to Altman and Bayer, 6), while the ACTH cells which make up a minor

population among arcuate neurons arise very early (over 70% at E12-E14). However, these patterns correspond well for the dynorphin A neurons in the supraoptic nucleus and the [Leu]enkephalin neurons in the periventricular nucleus.

In conclusion, the present combined immunocytochemical and [^{3}H]thymidine autoradiographic technique offers a means for the precise determination of the time of origin of chemically identified opioid neurons, that cannot be inferred simply from the patterns of neurogenesis in an entire nucleus. It further demonstrates that there may exist a time lag between the time of neuron differentiation and the initation of peptide synthesis. More importantly, the present findings indicate a differential genesis of three distinct opioid peptide neuronal populations in three separate hypothalamic regions.

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