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TARGETING OF GONADOTROPIN RELEASING HORMONE TO THE MEDIAN EMINENCE: EVIDENCE OF THE ACTION OF CHEMOTROPIC FACTORS.

by

MARIE-CHRISTINE LAPOSTOLLE-ROGERS

A dissertation submitted to the Graduate Faculty in Biomedical Sciences in partial fulfillment of the requirements for the degree of Doctor of Philosophy, The City University of New York.

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Abstract

TARGETING OF GONADOTROPIN RELEASING HORMONE TO THE MEDIAN EMINENCE: EVIDENCE OF THE ACTION OF CHEMOTROPIC FACTORS.

By

MARIE-CHRISTINE LAPOSTOLLE-ROGERS

Adviser: Professor Marie J. Gibson

The projection of gonadotropin-releasing hormone (GnRH) neurons to the median eminence of the medial basal hypothalamus (MBH), where the hormone is secreted into the portal circulation to stimulate pituitary gonadotropins, is essential to reproductive function. This pathway, established early in development, is also seen when preoptic area (POA)-derived GnRH cell-containing grafts are placed in the third ventricle of hypogonadal mice (hpg). Evidence, derived from experiments on hpg mice receiving GnRH neurons containing grafts, suggested the existence of diffusible factors directing the GnRH axonal projection to the median eminence. The present work includes two parts: In an *in vitro* study, I elucidate some of the mechanisms responsible for the projection of GnRH axons, using organotypic cultures. *In vivo*, I established that GnRH axons from embryonic POA grafts placed in the mammillary bodies projected to the median eminence.

Using organotypic cultures in insert chambers, we showed that GnRH axons specifically grew in higher number and extended farther from the POA explant towards the MBH co-explant than other tissues. This effect was significant after 4 days in culture and maintained for 10 days in culture. Staining for growth associated protein 43 (GAP-43) labels a general population of neurons elongating their axons. In contrast to its effect on GnRH axons, the MBH did not induce a differential outgrowth of those axons labeled with GAP-43. The importance of contact-mediated guidance with glial elements was also assessed. Only erratic associations were seen between GnRH and glial processes extending on the membrane. However, GnRH axons consistently traveled in the company of GAP-43-labeled axons. We suggest that while employing an axonal substrate, GnRH axons follow diffusible chemoattractive signal(s) secreted by the MBH. *In vivo*, we implanted POA grafts in the mammillary bodies region, which never contains GnRH cell bodies in mice. One animal with grafts exclusively in the mammillary bodies had gonadal development and his median eminence was innervated. Other grafts innervated the median eminence from mammillary bodies and lateral hypothalamic locations. These findings suggest the existence of a gradient of diffusible factor chemotropic for GnRH axons released by the median eminence, capable of directing GnRH outgrowth *in vitro* and also *in vivo* from ectopic caudal locations.

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ABBREVIATIONS

CE. Cerebellum

E15. Embryonic day 15.

GAP-43. Growth associated protein 43.

GFAP. Glial fibrillary acidic protein

Hpg. Hypogonadal.

GnRH. Gonadotropin releasing hormone

MBH. Mediobasal hypothalamus

OVLT. Organum vasculosum of the lamina terminalis.

P1. Post natal day 1

POA. Preoptic area

SC. Spinal cord.

Chapter 1

GENERAL INTRODUCTION

Gonadotropin-releasing hormone (GnRH), the key element in the control of the reproductive axis, acts on the anterior pituitary to stimulate gonadotropin production and release (Burgus *et al.*, 1972; Schally *et al.*, 1971). The formation of the correct projection of GnRH axons to the median eminence, where the peptide is released into the hypophyseal portal circulation for transport to the anterior pituitary, is critical in the perpetuation of the species. Located on the ventral aspect of the mediobasal hypothalamus (MBH), the median eminence constitutes one of the specific targets for GnRH neurons.

GnRH neurons form a small population of approximately 800 neurons in the basal forebrain (Schwanzel-Fukuda and Pfaff, 1989; Wray *et al.*, 1989). They are not clustered into a discrete nucleus but instead are scattered in the basal forebrain primarily in the regions of the preoptic area and the hypothalamus (Silverman *et al.*, 1994). Despite their low number and their disseminated distribution, 60 to 70% of GnRH neurons succeed in targeting the median eminence (Silverman *et al.*, 1987) or circumventricular organs including the organum vasculosum of the lamina terminalis (OVLT) (Silverman *et al.*, 1989).

During normal development in the mouse, GnRH cells are first detected by in situ hybridization histochemistry and immunocytochemistry at E11.5 in the olfactory pit and the vomeronasal organ (Schwanzel-Fukuda and Pfaff, 1989; Wray *et al.*, 1989). They migrate into the brain via the nasal septum. By E14.5, the majority of GnRH cells have reached the basal forebrain including the septal/preoptic area, and GnRH terminals have arrived at the

median eminence (Livne *et al.*, 1993a; Wu *et al.*, 1994). Several pathways of GnRH axonal projections from the septal/preoptic area to the MBH have been described: along the ventral surface of the brain, in the ventral aspect of the III ventricle, or running parallel to the walls of the III ventricle and arching through the arcuate nucleus. The major termination is the lateral aspect of the palisade zone of the median eminence (Hoffman and Gibbs, 1982; Hoffman *et al.*, 1978; Hoffman *et al.*, 1992; King *et al.*, 1982; Silverman *et al.*, 1994). GnRH cells within embryonic or neonatal septal/preoptic area (POA) grafts, placed in the third ventricle of adult hypogonadal mice (hpg), also innervate the median eminence. The hpg mouse has a deletion of two exons in the GnRH gene and does not synthesize the peptide (Mason *et al.*, 1986). Its reproductive organs, therefore, remain undeveloped and the mice are infertile. Only when GnRH cells in the grafts innervate the host median eminence, do the mice undergo reproductive development (Krieger *et al.*, 1982), including ovulation and pregnancy (Gibson *et al.*, 1984a; 1984b).

GnRH neurons within grafts in the third ventricle consistently extend axons in the host recipient along the walls of the third ventricle to arch through the mediobasal hypothalamus and project to the lateral sides of the median eminence. The formation of functional contacts between GnRH terminals and the capillaries of the portal vasculature of the median eminence is associated with reproductive development in the POA grafted hpg mice (Silverman *et al.*, 1985). It is the specificity of this targeting to the median eminence, following some of the projection pathways observed during normal development (Hoffman

et al., 1992), which has suggested that the mediobasal hypothalamus and median eminence region are providing directional guidance cues to GnRH axons.

Several lines of evidence using the model of a normal POA graft into various sites within the central nervous system of the adult hpg mouse have suggested that the MBH or the median eminence itself plays a critical role in GnRH axonal targeting to the hypophyseal portal capillaries. When the graft is placed within the third ventricle, even in a very rostral location, GnRH axons ramify within the graft and exit into the central nervous system of the host when they reach the MBH or the median eminence (Silverman *et al.*, 1985). Similarly, GnRH neurons from E13 nasal septum grafts (taken during the migration of GnRH neurons from the olfactory placode to the POA), placed in the anterior hypothalamus or the host medial POA, project to the lateral borders of the median eminence, suggesting that one important element is not so much the rostral extent of the graft but its localization at the base of the brain close to the mediobasal hypothalamus and to the hypothalamic regions utilized by GnRH axons on their normal projection pathways (Livne *et al.*, 1992). If grafts are placed into the lateral ventricle, GnRH neurons survive but the axons, which enter the host brain, follow nearby fiber tracts such as the fornix, the corpus callosum, or the stria terminalis, but do not reach the median eminence (Kokoris *et al.*, 1987). Other GnRH axons even grew in the host lateral septum or the anterior hypothalamus, but never reached the median eminence. The location of the graft in the proximity of the MBH seems to be critical for proper targeting. GnRH neurons from accessory olfactory bulb tissue, which usually do not project to the median eminence

(Merchenthaler *et al.*, 1984), can be induced to do so by placement of the accessory olfactory bulb in the third ventricle (Perlow *et al.*, 1987). The importance of the MBH region in providing cues to GnRH axons was emphasized by experiments in which embryonic MBH was co-grafted with POA in the third ventricle. GnRH terminals robustly innervated the median eminence within the co-graft as well as the adult host median eminence (Saitoh *et al.*, 1992). These latter experiments also suggested that the cues for inducing axonal targeting were either similar in the adult and embryonic median eminence or had overlapping functions.

One possibility for this precise targeting is that a cellular compartment within the MBH forms an attractive surface for GnRH axons. Many of the GnRH axons exit from the graft, arching through the arcuate nucleus to terminate in the lateral aspect of the palisade zone of the median eminence, mimicking one of the normal pathways (Silverman *et al.*, 1985). The potential role of the neuronal and glial components of the region has been analyzed. Selective destruction of the majority of arcuate nucleus neurons of the hpg host by neonatal treatment with monosodium glutamate did not prevent grafted GnRH neurons from forming functional connections with the host median eminence, suggesting a limited role for this neuronal population (Silverman *et al.*, 1990). Graft-derived GnRH axons were found in association with glial channels and/or glial processes in this region, but the degree of reactive gliosis induced by the graft procedure might make this association fortuitous rather than instructive (Silverman *et al.*, 1991). Furthermore, since the increase in glial fibrillary acidic protein (GFAP), indicative of the reactive gliosis, was not confined to the

MBH, it seemed unlikely that the presence of these glia could explain the precise targeting that occurred. Whether the tanycytes, that are present in this region, play a role in this phenomenon could not be unequivocally determined.

The experiments described above suggest that direct surface interactions of GnRH axons with cells in the MBH are unlikely to be the only factor involved in the targeting. Another alternative is that the MBH and/or the median eminence produce a diffusible substance that attracts or sustains GnRH axonal growth. In recent years it has become clear that chemoattractants participate in axonal guidance in many systems (Goodman, 1996).

Therefore in this thesis, I attempted to elucidate some of the mechanisms involved in the guidance of GnRH axons to the median eminence, using organotypic cultures in insert chambers and ectopic POA grafts in the mammillary bodies of hpg mice. Axonal guidance to a specific target seems to be the result of coordinated action of several mechanisms directing axons: long range chemoattractive or chemorepellent forces acting at a distance on outgrowing axons through the release of diffusible factors; short range contact-mediated cues, either permissive or inhibitory, directing axons on tracts to extend towards their target (Tessier-Lavigne and Goodman, 1996). In this work, I address the following questions:

Are GnRH axons following a concentration gradient of diffusible chemoattractant factors released from the mediobasal hypothalamus, guiding them to the median eminence?

Is this effect due to some trophic action of the mediobasal hypothalamus or is it a real chemotropic effect, capable of orienting the GnRH axonal outgrowth?

Is this attraction specific to GnRH axonal outgrowth or does it affect the general population of axons elongating from the preoptic area?

Do GnRH axons require the presence of contact-mediated guidance, originating from glial cells or tanycytes, to extend in the direction of the mediobasal hypothalamus?

Do GnRH axons utilize any other substrate to grow in the direction of the mediobasal hypothalamus?

Can GnRH axons follow diffusible chemoattractant signals in the adult brain such that they extend to the median eminence from a caudal ectopic location?

Chapter 2

GONADOTROPIN-RELEASING HORMONE (GNRH) AXONS TARGET THE MEDIAN EMINENCE: *IN VITRO* EVIDENCE FOR DIFFUSIBLE CHEMOATTRACTIVE SIGNALS FROM THE MEDIOBASAL HYPOTHALAMUS, AS DEMONSTRATED WITH ORGANOTYPIC CULTURES.

To test the hypothesis that GnRH axonal outgrowth to the median eminence is regulated by diffusible substances from the target region, we established co-cultures of embryonic POA, containing GnRH neurons, with embryonic MBH, containing the median eminence. The explants were grown on the surface of a porous membrane of insert chambers and fed by capillary action by the underlying defined medium. This culture remained static for the time in culture from 1 to 10 days. This organotypic culture system has been used for studies of axonal targeting in other systems and is suitable for the study of chemotropic diffusible factors (Erzurumlu and Jhaveri, 1995; Erzurumlu *et al.*, 1993; Muller *et al.*, 1993; Yamamoto *et al.*, 1989, 1992; Yamamoto and Toyama, 1995).

MATERIALS AND METHODS.

Organotypic culture in insert chamber preparation.

Tissue explants were cultivated on the surface of the porous membranes of insert culture chambers (0.4μm pore diameter and 12mm diameter, cyclopore polyethylene terephthalate (P.E.T) transparent membrane with $0.8X10^6$ pores/cm^2, Falcon/Becton Dickinson Labware, Franklin Lakes, NJ). In preliminary experiments, membranes were coated with rat tail collagen (type I, 3mg/ml, Boehringer-Mannheim, Indianapolis, IN) in 0.2% acetic acid (Rogers *et al.*, 1994). Since laminin has been demonstrated to promote the extension of growth cones and axonal elongation of developing neurons (Lander, 1987), laminin (100μg/ml, Gibco BRL, Gaithersburg, MD) was added to the collagen substrate coating the insert chamber membranes. Under these conditions, GnRH axonal outgrowth

exiting the POA explant was enhanced (Rogers *et al.*, 1995) (see Appendix A). Therefore, in this chapter, all data are derived from the experiments with collagen/laminin coated membranes.

The tissue explants were fed through the porous membrane by the underlying medium. In all experiments the medium used was: Dulbecco's modified Eagle medium (DME) and Ham's nutrient mix F12 with L-Glutamine and 15mM HEPES without phenol red, supplemented with putrescine (10^{-4} M), sodium selenite (0.02 μg/ml), and apotransferrin (100 μg/ml, all medium ingredients from Sigma, St. Louis, MO). The coated insert chamber membrane was washed with 200μl of medium prior to use.

Dissections.

Embryos of gestational age E15 or newborn pups were used in these experiments as indicated in the text. For embryos, time-mated normal female mice (C3H/HeHx101H) were sacrificed by cervical dislocation on gestational day 15. The day the vaginal plug was found was counted as E0. The uterus was removed and placed immediately into a sterile petri dish on ice. Embryo staging was confirmed by comparison with Schambra (Schambra and Lauder, 1992). Newborn pups were collected less than 24 hours after delivery (this stage was defined as postnatal day 1, or P1) and placed on ice for anesthesia. The embryo (or the pup) was decapitated and the head was placed on a sterile stage under a binocular dissection microscope. When P1 pups were used, the head was rinsed with a solution of 70% ethanol before dissection to assure sterility. The skull was removed and the brain inverted on the

stage so that the ventral surface of the brain faced up. The POA was dissected under sterile conditions as described previously (Krieger *et al.*, 1982). Using a separate set of instruments, the MBH, including the median eminence, was dissected as a midline strip of tissue from the ventral surface of the brain caudal to the POA. Sections of cervical spinal cord (SC) or of cerebellum (CE) were also dissected in some experiments. Tissue explants were placed immediately on the collagen and laminin-coated surface of the insert chamber membrane (Figure 2.1 A). In the case of the POA explant, the block of tissue was sectioned at the midline after placement in the chamber and applied flat on the surface of the membrane. All explants were kept moist by the addition of 10-20μl of medium above the membrane. The chambers were then placed in wells of 12-well plates containing 1 ml of the same medium. In all experiments, the medium was not changed and care was taken to leave the cultures undisturbed until the time of fixation.

Experimental design of single POA cultures and co-cultures.

The POA explant was either cultivated alone or with other brain regions placed on the membrane adjacent to the POA. Using E15 tissue, the following explant partners were established: POA alone, or POA with MBH, SC, or CE. In another experiment the POA was separated in two halves during the dissection and both halves of POA were explanted separately on the same membrane. In experiments using P1 tissue, the POA explant was cultivated only with MBH. In the majority of experiments, explants were cultured for 7 days at 37°C, 5% CO_2, 100% humidity and then fixed by immersion in 4% paraformaldehyde for 3 hours. For a time course analysis, E15 co-explants of POA and

MBH were fixed after 1, 4, 7 or 10 days of culture. All fixed cultures were kept at 4°C in phosphate buffer (PB, 0.1M, pH 7.3) with 0.1% sodium azide (Sigma, St. Louis, MO) until they were processed by immunocytochemistry.

Some cultures were counterstained with a solution of 1% of cresyl violet in 20% ethanol solution. The staining was differentiated with a rinse in water, followed by a brief rinse in a solution of acidified 90% ethanol.

Immunocytochemistry.

Immunocytochemistry was performed directly on membranes after cutting them out of the chamber. To detect GnRH neurons and processes, SW1 antiserum (Rabbit polyclonal antiserum, gift from Susan Wray (Wray *et al.*, 1988)) was used at a dilution of 1:2500 in PB containing 0.1% Triton X100 (TX100, Sigma, St. Louis, MO), 1% normal goat serum (Gibco BRL, Gaithersburg, MD) or 3% normal donkey serum (Jackson Immunoresearch Labs., West Grove, PA) for 4 days at 4°C. The biotinylated anti-rabbit secondary antiserum (1:200, made in goat, Vector Labs., Burlingame, CA, or 1:200 made in donkey, Jackson Immunoresearch Labs., West Grove, PA) in the same diluent was applied overnight for increased penetration of the explants. Explants were then incubated in an avidin-biotin solution (Vector Labs., Burlingame, CA) conjugated with horseradish peroxidase for 2 hours. The chromogen was 1mg/ml 3,3'diaminobenzidine (DAB, Sigma, St. Louis, MO) and the H_2O_2 was generated by the oxidation of glucose by glucose oxidase (Sigma, St.

Louis, MO). There were no differences in GnRH labeling or background staining with the two secondary antibodies.

In a group of cultures, derived from E15 and P1 tissue, double label immunocytochemistry was performed to visualize GnRH and growth associated protein-43 (GAP-43). The visualization of GnRH in this case was done using SW1 with a secondary anti-rabbit antiserum directly conjugated with FITC (1:200, made in donkey, Jackson Immunoresearch Labs., West Grove, PA). The cultures were then rinsed overnight, treated with 3% normal donkey serum in PB with 0.1% TX100, incubated in the GAP43 antibody at a dilution of 1:1000 (mouse monoclonal antibody, clone 91E12, Boehringer-Mannheim, Indianapolis, IN) in PB containing 3% normal donkey serum and 0.1% TX100 for 4 to 6 days. The cultures were incubated subsequently in anti-mouse biotinylated secondary antiserum (1:200, made in donkey, Jackson Immunoresearch Labs., West Grove, PA) and the sites of antibody binding were visualized with Texas red conjugated avidin D (Vector Labs., Burlingame, CA).

Double labeling was also performed to characterize the possible interactions between GnRH and glial processes or cells. Glial cells were identified by using antisera against GFAP, vimentin or S-100. GnRH was visualized with the anti-GnRH mouse monoclonal antibody (#19304, QED Advanced Research Technologies, San Diego, CA) at a dilution of 1:5000 in 0.2% TX100 and 3% normal donkey serum in PB for 4 days at 4°C. The cultures were incubated overnight in biotinylated anti-mouse secondary antiserum (1:200, made in donkey, Jackson Immunoresearch Labs., West Grove, PA) and the avidin-

biotin reaction was carried out as described above with visualization using Texas red-streptavidin. The labeling of glial markers was performed with rabbit anti-GFAP (Dahl *et al.*, 1984) at a dilution of 1:1000, rabbit anti-vimentin (gift of Dr. Wang) at a dilution of 1:1000, or rabbit anti-S100 (DAKO, Carpinteria, CA) at a dilution of 1:2000. All antisera were prepared in 0.1% TX100 and 3% normal donkey serum. Visualization of the glial markers labeling was carried out with the use of an anti-rabbit antiserum conjugated with FITC.

After immunocytochemistry, the tissue was washed, mounted directly onto glass slides and immediately coverslipped with Gelmount (Biomedia, Foster City, CA). The cultures were observed and photographed under bright field or fluorescent optics as appropriate. In certain cases, observation of double labeled cultures was performed first with epifluorescence, then with a Leica inverted confocal laser scanning microscope, using an argon/krypton laser as its light source. For these studies the 40X objective was used with a pinhole of 40 which allowed confocal sections of 0.5 μm.

Quantitative analysis of the cultures.

GnRH axonal outgrowth was assessed in the co-cultures of POA-MBH, POA-SC, POA-CE and in cultures of POA alone. Cultures were not considered for quantitative analysis if the explants were fused or torn, if the location of the MBH or control tissue could not be determined, or if there were less than 50 GnRH immunoreactive cell bodies in the POA. Only GnRH immunoreactive fibers terminating in a growth cone were considered.

GnRH axons extending on the surface of the membrane from the POA explant were counted in two sectors: Sector I is the region of membrane facing the co-cultivated explant and sector II is the region of membrane away from the co-cultivated explant (Figure 2.1 B). A few GnRH cell bodies were detected within the MBH co-explants, similar to previous findings *in vivo* (T.J. Wu and A.-J. Silverman, unpublished observations). The occasional GnRH axons which extended out of the MBH were not included in the quantitative analyses. In cultures of POA alone, GnRH axons extending from the entire circumference of the POA were counted. In cultures where two halves of POA were placed separated from each other, GnRH axonal outgrowth was quantified in the region between the two pieces of POA and in the regions away from the co-cultivated half POA.

All values were expressed as mean ± standard error of the mean. When homogeneity of variances was not achieved, the appropriate transformation of the data was applied. If the means were proportional to the variances, the square roots of the data were used. Similarly if the means were proportional to the standard deviations, logarithmic transformation was then applied, according to the statistical protocols described by Kirk (Kirk, 1968). The number of GnRH axons in sectors I and II was then compared between POA-MBH co-cultures and control cultures, and similarly at each time point of the time course, with a two-way analysis of variance (ANOVA) and Newman-Keuls t-test with Duncan's correction for post-hoc comparisons, if $p<0.05$.

The maximum extent reached by GnRH axonal outgrowth from the POA explant was also assessed: The three longest extending GnRH axons from the POA explant were

measured in each sector with the help of a micrometer grid inserted in the eye piece of the light microscope as described by Lumsden and Davies (Lumsden and Davies, 1983). The measurements were defined as follows: the zero reference bar of the grid was placed tangentially to the interface between POA explant and membrane and the maximum distance reached onto the membrane by the growth cone of the farthest extending GnRH axons was measured, regardless of the winding path of the axon. These three lengths were averaged for sector I and sector II. Due to the nature of the data, the maximum axonal extent was analyzed using non-parametric tests: Kruskal-Wallis ANOVA was used to compare multiple groups. Comparisons between sector I and II were analyzed using Wilcoxon signed rank test.

For cell counts the POA was divided into two analogous regions, so that counts could be compared between the half facing the co-explant (side I) and the half facing away (side II) (Figure 2.1 B).

Analysis for trophic effects on GnRH cell survival was performed by counting the number of GnRH cell bodies in the POA in the presence or absence of the MBH co-explant and in the presence of control tissue co-explants. Possible trophic effects on GnRH axonal outgrowth were assessed by adding the GnRH axonal outgrowth in sector I and in sector II (which corresponds to the total outgrowth) in the presence and absence of the co-explants. Comparisons among groups were performed with a one-way ANOVA or t-test as appropriate.

RESULTS

Observations of the organotypic cultures in insert chambers.

The explants derived from either E15 or P1 tissue survived well in culture; the tissue showed no signs of necrosis or floating cells. Immunocytochemistry with the SW1 antiserum provided good labeling of GnRH cells despite the thickness of the whole mount preparation (Figure 2.2 A), and GnRH fibers, occasionally extending over very long distances (up to 1800μm), could be followed on the surface of the chamber membrane as they exited the explants (Figure 2.2 B). Most of these terminated in growth cones (Figure 2.2 B and C). Preabsorption of the antiserum with 10μg/ml of the GnRH peptide eliminated reaction product in cells and fibers. All the quantitative analyses were performed using the SW1 antibody.

The orientation and morphology of the GnRH cells in cultures appeared similar to what is seen *in vivo* at the same stage of development. GnRH cell bodies within the POA explants were often oriented similar to that seen *in vivo* (Figure 2.2 A). GnRH processes ramified extensively within the entire POA explant and numerous growth cones were visible therein.

Survival of the GnRH neurons in culture.

After one day of culture, the E15-derived POA region contained an average of 281 GnRH cells. During the first 7 days *in vitro* this number declined moderately and then

stabilized after an additional 3 days (the longest time point examined) (Figure 2.3). However in some cultures, a maximal number of approximately 410 GnRH neurons could still be detected after 7 days in culture. The normal mouse brain contains approximately 800 GnRH neurons scattered in the basal forebrain primarily in the region of the diagonal band, the preoptic area and the hypothalamus (Wray *et al.*, 1989).

For the 7 day cultures used in most experiments, the presence of a co-graft did not alter the number of surviving GnRH neurons (see Table 2.1) In addition, there was no difference among the cultures in the number of GnRH cell bodies within the POA in the region facing toward or away from any of the co-cultures, under any conditions tested.

Effect of the co-explants on GnRH axonal outgrowth.

The effect of co-explants on GnRH axonal outgrowth was assessed by counting axons in the sectors of membrane facing and opposite the co-explant. For E15 cultures, the presence of an MBH co-explant significantly altered the location of GnRH outgrowth such that there were more GnRH axons in sector I, facing the MBH, than in sector II, away from the MBH ($p<0.01$; Figure 2.4). This effect was not seen with SC or CE co-explants. GnRH axonal outgrowth towards the MBH was significantly greater than that towards the CE ($p<0.05$; Figure 2.4).

Furthermore, the GnRH axons extended significantly farther from the POA on the membrane in the sector facing the MBH ($p<0.001$; Figure 2.5) than opposite from it. This

effect was not observed with the control tissues. Indeed the GnRH axons extended longer in the sector away from the CE ($p<0.02$; Figure 2.5)

For P1 tissue, the number of GnRH axons growing in sector I was also significantly higher than in sector II in POA-MBH co-cultures ($p<0.01$; Figure 2.6). The total GnRH axonal outgrowth was also higher at P1 ($p<0.01$).

As the distance separating the co-explants might influence the GnRH axonal outgrowth, a correlation analysis was performed between the inter-explant distance and the number and extent of GnRH axons in sector I. The amount and the extent of GnRH axonal outgrowth into sector I were not correlated with the distance separating the POA and MBH explants ($p>0.5$).

To assess whether the MBH was providing trophic support for GnRH axons, we counted the total number of GnRH axons extending out of the POA explants grown alone or with the MBH in E15 cultures. The total number of GnRH axons on the membrane did not differ between the groups (POA alone: 42.2 ± 8.9, n=12; POA-MBH: 50.4 ± 4.5, n=48).

Effect of time on GnRH axonal outgrowth.

One possible explanation for differential GnRH axonal outgrowth towards the MBH after 7 day cultures is that the target provides support for the axons which project nearby. Under such conditions, GnRH axons would initially grow equally in all directions but only those extending towards the target would be maintained. To assess this possibility, we

performed a time course analysis of GnRH axonal outgrowth in the presence of a MBH co-explant, using E15 tissue.

The total number of GnRH fibers increased steadily between day 1 and day 10 of culture (Figure 2.7). The preferential location of GnRH fibers in sector I reached significance at day 4 ($p<0.01$) and was maintained until day 10 ($p<0.05$), consistent with the results described above (Figure 2.4).

The extent of GnRH axonal outgrowth on the membrane from the POA explant was significantly longer facing the MBH explant than opposite from it at day 4 and at every time point there after ($p<0.001$; Figure 2.8). This result is consistent with the observation that GnRH axons extended farther from the POA explant in the sector facing the MBH after 7 days in culture as described previously (Figure 2.5)

General axonal outgrowth.

Growth associated protein 43 (GAP-43), expressed in elongating axons, is used as a marker for general axonal elongation in dynamic systems (Goslin *et al.*, 1988). Therefore labeling for GAP-43 was used to assess general axonal outgrowth in POA-MBH co-cultures ($n=60$). Overall axonal outgrowth was robust and qualitatively did not show any preferential distribution (Figure 2.9 A and B).

Associations between GnRH axons and GAP-43 immunoreactive (-ir) axons.

The GAP-43-ir axons extended onto the membrane over long distances from both the POA and the co-explants, and formed a dense network (Figure 2.9 C), with numerous growth cones. The GnRH axonal outgrowth, which grew over comparatively shorter distances from the POA onto the membrane, was invariably found in company of GAP-43 axons. Observations using confocal microscopy confirmed that when associations between GnRH and GAP-43 axons were observed, GnRH axons traveled on top of GAP-43 axons (Figure 2.10 A) such that some GnRH fibers followed a bundle of GAP-43 axons (Figure 2.10 B), and others traveled on the surface of a network of GAP-43 fibers (Figure 2.10 C). Every GnRH axon observed with confocal microscopy was consistently found in the same confocal plane (estimated to be 0.5μm) as GAP-43 axons.

Co-localization of GAP-43 in GnRH axons.

Co-localization of GAP-43 in GnRH axons was noticed in some POA-MBH co-cultures as observed with confocal microscopy (Figure 2.11). The distribution of such double labeled axons did not seem to be influenced by the presence of a co-explant. In addition, not all GnRH axons were also GAP-43 immunoreactive.

Lack of association between GnRH axons and glial elements.

GnRH axons are seen close to tanycyte processes spanning the MBH *in vivo* and also appear to travel within glial channels to the median eminence (Kozlowski and Coates, 1985). For these reasons Kozlowski and Coates suggested that glial processes may guide

GnRH fibers toward the median eminence. To determine if GnRH axons required a glia surface for extension *in vitro*, we performed double labeling immunocytochemistry for GnRH and one of three markers of astroglia, vimentin (n=11), GFAP (n=3) and S-100 (n=5) in co-cultures of POA and MBH using P1 tissue.

As observed with confocal microscopy, no consistent association was found regardless of the sector examined. GFAP-ir cells and processes remained confined to the borders of the explants (Figure 2.12 A), such that GnRH axons extending onto the membrane were not found in close proximity to GFAP-ir cellular elements (Figure 2.12 B). Vimentin-ir cells and processes extended further onto the membrane (Figure 2.12 C), but GnRH axons were still not associated with them and did not align along vimentin-ir elements. Occasionally, a GnRH axon would cross over a vimentin-ir process or cell, but such occurrence seemed fortuitous. Most GnRH axons grew past the extent of the vimentin processes on the membrane and were then found unaccompanied (Figure 2.12 D). S-100 antibody labeled small astrocytes sparsely scattered on the membrane around the explants, with processes confined to the immediate surroundings of the cell bodies. GnRH axons did not contact S-100 labeled cells (Figure 2.12 E and F).

Presence of cellular elements on the membrane surrounding the explants.

A cresyl violet counterstain as well as direct observation with phase contrast microscopy revealed the presence of cells of globular or spindle-like shape on the membrane. These cells did not extend on the entire surface of the membrane but remained

confined to the region of membrane surrounding the explants. Only erratic associations of GnRH axons with such elements were observed (result not shown).

Presence of the median eminence within the MBH explant.

The median eminence was visible in most of the MBH explants dissected from P1-derived tissue after 7 days in culture. In single labeled experiments, GnRH axons concentrated in one region within the MBH which might be the median eminence (Figure 2.13 A), as suggested by its organization in a palisade structure. The origin of these axons probably included cells both in the POA and MBH explants. The presence of the median eminence within the MBH explant was confirmed in cultures double labeled for GnRH and glia. Vimentin and GFAP were present in tanycytes, the specialized ependymal cells of the MBH. They formed the characteristic arched palisade arrangement seen *in vivo* and GnRH axons terminated amongst them (Figure 2.13 B). Occasionally, a GnRH axon could be followed from the POA explant, across the membrane, to the MBH explant, ending in a growth cone close to the presumptive median eminence (Figure 2.14).

Intrinsic effects of the POA region on GnRH axonal outgrowth.

Within the majority of POA explants regardless of the co-explant, GnRH fibers tended to form dense, highly branched plexi. The ependymal lining derived from the III ventricle was often visible and GnRH fibers were abundant in this region (Figure 2.13 C). The morphological appearance of this area resembled the organum vasculosum of the lamina terminalis (OVLT), a circumventricular organ located at the anterior tip of the III

ventricle, which receives an abundant GnRH innervation in the normal animal (King *et al.*, 1982). Within the POA explant, tanycytes, immunoreactive for vimentin or GFAP, were detected (Figure 2.13 D) and GnRH axons were seen closely associated with these processes.

Occasionally some POA explants separated into two pieces of tissue. When this happened numerous GnRH terminals were seen crossing between the two segments. It is possible that the POA explant itself possesses some attractive properties, influencing GnRH outgrowth. To assess this hypothesis, cultures were performed in which the POA was intentionally dissected into two halves and placed separated on the surface of the membrane. The number of GnRH axons extending between the two pieces of POA was significantly higher than the number of GnRH terminals extending away from the pieces of POA (49.7 ± 5.8 and 31.8 ± 4.3, $n=24$, $p<0.05$).

DISCUSSION.

Innervation of the median eminence by GnRH axons, an essential condition for normal reproductive function, occurs early in development. As summarized in the introduction, grafted embryonic GnRH neurons send processes to the median eminence of an adult host brain. It is conceivable that several mechanisms act in concert to assure the correct projection of GnRH axons, so critical in the reproduction of species. These may include factors associated with the neuronal and/or glial substrate as well as diffusible substances that exert chemotaxic or chemotrophic effects. The present studies, using

stringent *in vitro* conditions, support the hypothesis that a major factor involved in the targeting during development and after transplantation is a diffusible substance originating from the region of the median eminence. In the organotypic cultures used, direct cell to cell interactions with glia did not seem to be required for an increased directional outgrowth of GnRH axons, while observations with GAP-43 double-labeling suggested the importance of an axonal substrate.

The MBH, which was dissected so that it contained the median eminence, exerted an action on GnRH axons that resulted in a longer GnRH axonal extent, as well as in higher numbers of such axons growing on the membrane region facing the MBH in the insert chambers. This result was not the consequence of an uneven distribution of GnRH cell bodies within the explant, nor was it a nonspecific effect exerted on all axonal outgrowth, as axons labeled with GAP-43 did not show such directionality. This preference for GnRH outgrowth toward an explant was specific to the MBH (and POA, see below) in that neither spinal cord nor cerebellum were attractive to GnRH axons.

Diffusible factors have been previously described to have chemotropic actions involved in the guidance of axons (Davies, 1994; Goodman, 1996). For example cortical axons, which form collaterals that grow towards the basilar pons, do so by following a diffusible signal originating from their target, as demonstrated with organotypic cultures in three dimensional collagen gel matrices (O'Leary *et al.*, 1991; Sato *et al.*, 1994). Similarly visual cortical axons project, directly, in culture to their appropriate thalamic target (Bolz *et al.*, 1990). Trigeminal neurons project to peripheral targets using a similar mechanism

though the molecular nature of this signal is not yet known (Lumsden and Davies, 1983; Lumsden and Davies, 1986). Recent studies performed in co-cultures at the surface of insert chambers suggest that factors originating from target tissue are responsible for some aspects of axonal projection and arborization of trigeminal neurons (Erzurumlu *et al.*, 1993), implying that the culture conditions in insert chambers are suitable to the study of such mechanisms. The floor plate of the spinal cord secretes diffusible factors, capable of orienting the outgrowth of commissural axons within collagen gel matrices (Placzek *et al.*, 1990a; 1990b; Tessier-Lavigne and Placzek, 1991; Tessier-Lavigne *et al.*, 1988). This observation has lead to the identification and isolation of netrins, expressed by floor plate cells, which form a family of factors involved in the guidance of commissural axons of the spinal cord (Kennedy *et al.*, 1994). They are homologous to a protein related to laminin involved in similar action in the nematode, *Caenorhabditis elegans* (Serafini *et al.*, 1994). The study of UNC-6 structure, the nematode netrin, showed that it is closely related to laminin, and might interact with it to promote the establishment of diffusible gradients involved in axonal guidance (Ishii *et al.*, 1992). Different domains of the netrin protein mediate tissue specificity and guidance direction (Wadsworth *et al.*, 1996).

The extent of GnRH outgrowth from the POA explant was longer on the side away from the cerebellum explant. These findings suggested that the cerebellum may have some repulsive activity on GnRH outgrowth. Chemorepulsion has been described previously in the central nervous system. Olfactory axons originating from the olfactory bulb grew away from the septum in organotypic cultures in a pattern suggesting the existence of inhibitory

diffusible signals (Pini, 1993; 1994). As noted above, although diffusible substances such as netrins have been shown to have some chemoattractive actions on developing commissural axons in the embryonic spinal cord (Kennedy *et al.*, 1994), it was recently demonstrated that the same proteins can have some chemorepellent activity towards other sets of axons, such as the trochlear motor axons (Colamarino and Tessier-Lavigne, 1995a). Connectin expressed by muscles has been demonstrated to have a repellent activity on motoneuron growth cones (Nose *et al.*, 1992; 1994). Chemoattraction and chemorepulsion seem to act in concert to assure the proper guidance of axons to their target over long distances by creating inhibiting and attractive territories along axonal projection pathways .

The persistence of an MBH effect on GnRH outgrowth after 10 days of culture suggests that the factors involved were synthesized by the MBH rather than simply associated with the tissue at the time of explantation. Blood borne factors present at the time of dissection would likely be destroyed over this extensive time course. Which cellular compartment manufactures this signal and the nature of the signal are unknown (see introduction). The fact that more and longer axons were present in sector I versus sector II from day 4 until day 10 suggests that a concentration gradient between the MBH and the POA was established early and was maintained (Molnar and Blakemore, 1995). In turn this supports our hypothesis that the diffusible signal is at least partly acting via a chemotaxic mechanism. One would expect that a neurotrophic substance diffusing throughout the undisturbed culture dish over 10 days would lead to a decline in the ratio of sector I versus

sector II and disappearance of significant differences between the two sectors. On the contrary, this significance was maintained at 10 days.

In the cited studies, the chemotropic activity of the targets (both final and intermediate) is differentiated from a purely localized trophic effect by the ability to rapidly orient or change the orientation of growing axons. In our study the GnRH axonal outgrowth is predominantly in the sector facing the MBH. Examples of GnRH axons extending straight in the direction of the MBH or exiting the POA laterally and turning towards the MBH were observed. However, other GnRH axons exiting in the sector facing the MBH followed diverse and tortuous paths and in some cases branched in multiple directions. Thus, the attractive properties of the MBH are less strongly chemotropic than, for example, the floor plate is for commissural axons. Furthermore, a pure trophic action also does not seem to be the only element involved in this targeting, since GnRH axons extended over longer distances from the POA explant on the sector of membrane facing the MBH after as early as 4 days in culture, which implies strong and rapid effects on the GnRH axonal outgrowth. MBH-derived trophic factors were implicated in studies by Daikoku using both organ cultures and transplantation *in vivo* (Daikoku *et al.*, 1991). In this study the number of GnRH neurons derived from rat early embryonic olfactory placode or vomeronasal organ was higher in the presence of the MBH in culture or in animals with transplants. In contrast, in our study the presence of the co-cultivated MBH explant did not influence the survival of GnRH cell bodies in the POA. Similarly, the distribution of GnRH cell bodies within the POA explant was not influenced by the presence of the MBH, suggesting there are no

sustaining trophic effects on GnRH neurons projecting in the direction of the target. The total number of GnRH axons growing out on the membrane was not influenced by the presence of the MBH. Rather, these GnRH axons were growing in greater numbers and over a longer extent on the membrane sector facing the MBH, instead of extending randomly all around the explant. Further, the progressive increase in GnRH outgrowth and extent facing the MBH in time course experiments, with the outgrowth in the opposite sector always remaining lower, as well as the absence of influence of the MBH on the total number of axons exiting the POA, suggests that other mechanisms than preferential maintenance of axons close to the target are involved.

In studies attempting to demonstrate that outgrowth follows a concentration gradient established by the release of a diffusible target-derived substance, one consideration is the distance separating the target from the innervating source. In our culture conditions, the distance separating the POA and the MBH explants did not affect GnRH axonal outgrowth. In a study of sensory fiber targeting, Lumsden and Davies showed that trigeminal ganglion explants could be placed one behind the other in collagen gel cushions and that both would still extend axons in the direction of the target, suggesting that the diffusible factor gradient was maintained past the boundaries of the first ganglion and could direct axonal outgrowth from the most distal ganglion regardless of the distance (Lumsden and Davies, 1983). Similar to Lumsden's experiment, GnRH axons in the present study adopted their pattern of preferential outgrowth whether they were close to or distant from the MBH explant, suggesting that a gradient of concentration was the important factor influencing the GnRH

outgrowth and challenging the purely trophic hypothesis, where the more distant the target the less effective the outgrowth.

Unfortunately, we cannot perform an experiment similar to the one by Lumsden and Davies described above as the POA itself has attractive properties. *In vivo*, there are abundant GnRH axonal projections to the organum vasculosum of the lamina terminalis (OVLT), which is located close to the majority of the GnRH cell bodies (Jennes and Stumpf, 1986; King *et al.*, 1982). The presence of an intrinsic target such as the OVLT would account for the abundant projection of GnRH terminals within the explant and between two separated pieces of POA. Since the POA possesses a target for GnRH projections in culture, it is less surprising that so few GnRH axons succeeded in extending out of the explant in the direction of the MBH. This observation emphasizes the power of the median eminence as a target, since axons exiting the POA may have to overcome an intrinsic attraction.

Although there appear to be chemoattractant factors for GnRH axons from the median eminence/MBH, the cellular sites of secretion of such factors, the chemical characteristics of the molecules involved, as well as the regulation of secretion and interactions between the site of secretion within the target and the GnRH axons, are unknown. Circumventricular organs, such as the median eminence or the OVLT may be attractive for GnRH axons because of their special nature. They lack a blood brain barrier and possess endothelial cells which do not form tight junctions, resulting in a fenestrated capillary structure (Broadwell *et al.*, 1983; Goldstein, 1988). The secretion of these

endothelial cells might reach the nervous tissue to direct axonal outgrowth. Endothelial cells have been shown to release many substances, including endothelins, which seem to have an important physiological role in the regulation of secretion of neuropeptides (Stojilkovic and Catt, 1992). Examples of blood borne molecules penetrating the brain tissue at the sites lacking blood brain barrier have been documented as well (Broadwell *et al.*, 1983), however this element is unlikely to be responsible for the directed targeting of GnRH axons *in vitro*, since the blood circulation is disrupted.

The tanycytes present in the MBH also might be involved in the release of diffusible trophic or tropic factors. Hypothalamic astrocytes produce factors that stimulate the differentiation of a GnRH secreting, immortalized tumorigenic cell line, GT1-7, with a reciprocal effect of these GnRH "neurons" on astroglial proliferation in culture (Gallo *et al.*, 1995). Astroglial conditioned medium, harvested from glial cultures treated with TGF-α, stimulated the release of GnRH from GT1-7 cells. The nature of these secreted substances remains unknown (Voigt *et al.*, 1996). GnRH axons in the region of the median eminence are also associated with glial elements immunoreactive for S-100 (Cummings and Brunjes, 1995), a protein expressed in glia (Yang *et al.*, 1996) and in certain sets of neurons (Rickmann and Wolff, 1995; Yang *et al.*, 1995a, 1995b), also known to have some trophic properties (Van Eldik *et al.*, 1991). Numerous other factors are present or secreted by astroglial cells and tanycytes of the arcuate nucleus and the median eminence region including insulin-like growth factor-I (Duenas *et al.*, 1994) and TGF-α (Ma *et al.*, 1992; 1994).

In addition to following a concentration gradient of diffusible signals, GnRH axons might need a specific substrate for proper guidance. The absence of association between GnRH axons extending on the membrane with glial elements suggests that the signals guiding GnRH axonal outgrowth towards the MBH are not provided by a direct cell to cell interaction with the surrounding glial environment. The interactions of neurons with glial elements during development has been widely documented to provide substrate-bound guidance to specific axonal projections (Marcus *et al.*, 1995; Silver, 1993; Silver *et al.*, 1993; Silver *et al.*, 1987; Silver and Rutishauser, 1984; Wu *et al.*, 1995). In our study, despite the presence of tanycytes in the preparation as visualized with vimentin and GFAP immunoreactivity, we did not find consistent interactions of GnRH projections towards the MBH (or in any other direction) with glial elements onto the membrane. Other mechanisms are likely to be involved in directing the GnRH projections from the POA to the median eminence, since their outgrowth in the sector facing the MBH does not require cell to cell associations with glia. Although the presence of unknown cellular elements extending onto the membrane of the insert chambers might provide some substrate for axonal outgrowth, these cellular elements were found on all sides of the explants. Thus it is unlikely that they provide a specific substrate. Moreover only erratic associations of GnRH axons with such elements were observed.

These observations do not preclude that tanycytes are involved in the guidance of GnRH axons to their termination site close to the capillaries of the median eminence. The cell bodies of tanycytes are located close to the ventricle and their processes extend into the

circumventricular organs. (Bouchaud and Bosler, 1986; Eurenius and Jarskar, 1971; Kobayashi and Matsui, 1969). Since tanycytic processes have been shown to surround and make close contact with GnRH fibers (Kozlowski and Coates, 1985), their involvement in the guidance of GnRH projections to the median eminence has been suggested. In the hpg mouse, association between glial processes, expressing vimentin or GFAP, and GnRH axons extending from POA grafts in the third ventricle were observed, but provided inconclusive evidence because of the amount of gliosis induced in the host brain (Silverman *et al.*, 1991). However, within the MBH explant, GnRH axons were found in close proximity to tanycytes, as labeled with vimentin antiserum, forming a median eminence-like palisade structure. Similarly, within the POA explant, GnRH axons were found in association with tanycytes in a structure similar to the organum vasculosum of the lamina terminalis (OVLT), another region where GnRH axons project *in vivo*. It is possible that the tanycytes of the median eminence exert some role in the fine guidance of the GnRH terminals when they are close to the capillaries of the portal circulation, compatible with the role of tanycytes in the regulation of secretion of GnRH by dynamic plastic changes in their association with GnRH terminals (Garcia-Segura *et al.*, 1996a; 1996b; King and Rubin, 1994; 1995). However, our *in vitro* experiments show that association with tanycytes is certainly not required for GnRH outgrowth towards the MBH.

In contrast, a close association existed between GnRH and GAP-43 axons extending on the membrane. GAP-43-ir axons are a general population of axons evenly extending around the explants. Since GnRH axons were always observed within the same plane as

GAP-43-ir neurite and since the latter always extended farther, GAP-43-ir axons might provide a facilitative substrate. One other alternative is that GnRH axons might be guided by another subset of axons, included in the population of GAP-43-ir axons and extending predominantly towards the MBH. Therefore such a subset of axons would be the one following MBH derived-attraction. Our experiments, which require fixation and immunocytochemistry, do not allow us to study whether GnRH axons dynamically follow some other elongating axons. The present studies did not evaluate other neurosecretory cells known to innervate the median eminence.

The apparent co-localization of GAP-43 within certain GnRH axons is indicative that the culture conditions might be responsible for re-expression of GAP-43 in GnRH cells. Some GnRH neurons have been found to express GAP-43 only for a brief window of time during their early migration in the mouse embryo (Livne *et al.*, 1993a). The GAP-43 expression in GnRH neurons therefore does not coincide with the period of axonal elongation and targeting of GnRH axons to the median eminence. Consequently, expression of GAP-43 in GnRH neurons issued from P1 tissue is unusual. GAP-43 has been shown to be re-expressed in regenerating axons following transections in culture (Goslin and Banker, 1990). It is likely that our culture conditions enabling regeneration of GnRH axons following the transection of the projection to the median eminence at P1, allowed re-expression of GAP-43.

Alternative possibilities have been suggested to explain the specificity of an axonal projection to its target. On-going communication between axons and their target was

hypothesized to regulate the secretion of chemotropic factors in the developing spinal cord. For example, Substance P, secreted by some commissural terminals, increases the release of chemoattractants from the floor plate (De Felipe *et al.*, 1995). However, the secretion of the GnRH peptide by the GnRH axons is not necessary for proper targeting to the median eminence: axons originating from cell bodies containing the defective GnRH gene of hypogonadal mice, unable to synthesize the peptide, nevertheless reach the median eminence (Livne *et al.*, 1993b).

The precise nature of the guidance mechanism of the GnRH axons is still unknown. Present experiments were designed to test the hypothesis that the MBH provides signals to guide the GnRH axons to their target, the median eminence. These *in vitro* studies as well as the prior *in vivo* transplantation experiments clearly show that the MBH produces a diffusible factor whose most potent effect is to act as a chemoattractant to GnRH axons.

TABLE 2.1.

Distribution of GnRH cells in POA explants after 7 days in culture.

The number of GnRH cell bodies (Mean ± S.E.M.) was counted in the region of POA explant facing the co-explant (sector I) or opposite the co-explant (sector II).

Co-explant	Age of tissue	N	GnRH cell number in sector I	GnRH cell number in sector II	Total number of GnRH cells
no co-explant	E15	36			232.4 ± 14.6
MBH	E15	48	117.8 ± 7.9	121.0 ± 8.3	241.3 ± 9.7
SC	E15	17	99.7 ± 11.7	130.2 ± 12.9	231.2 ± 13.4
CE	E15	13	138.8 ± 16.9	110.5 ± 11.2	250.1 ± 16.7
MBH	P1	28	104.2 ± 8.3	108.5 ± 10.3	212.7 ± 10.2

FIGURE LEGENDS.

Figure 2.1: (**A**) Schematic drawing of an insert chamber, showing the co-explants placement. The POA explant is placed on the surface of a collagen and laminin coated membrane. The MBH explant (or control tissue) is placed similarly on the surface of the membrane. (**B**) Drawing of a POA-MBH co-culture showing the different sectors where the GnRH axons and the cell bodies are counted. The GnRH cell bodies and fibers are represented in black. The sectors are delineated by a vertical line. The GnRH axons were counted if they extended onto the membrane in sector I facing the co-cultivated explant or in sector II away from it. The GnRH cell bodies were counted within the POA explant in the corresponding sectors. Scale bar represents 400 μm.

Figure 2.2: (**A**) High power micrograph showing GnRH immunoreactive cell bodies within the POA explant in a 7-day POA-MBH co-culture. The orientation of the GnRH cell bodies depicted here is very similar to that seen *in vivo*. (**B**) GnRH axons with growth cones (arrows) exit the POA and extend onto the surface of the membrane in the direction of the MBH (empty arrow). The MBH itself is not shown. Scale bar represents 100 μm in **A** and **B**. (**C**) Three examples of GnRH axons terminated by growth cones extending onto the membrane. Scale bar represents 50 μm.

Figure 2.3: GnRH cell number in POA explants between day 1 and day 10 in culture with MBH. The number was maximal on the day of explantation, declined significantly by day 7 (• = $p<0.05$) and was stable thereafter.

Figure 2.4: GnRH axonal outgrowth on the membrane from E15 co-cultures after 7 days in culture. For the POA/MBH pairs only, the number of GnRH axons was greater in sector I (towards the explant) (•• = $p<0.01$). In addition, the number of immunoreactive axons on the sector I of the membrane was greater for this combination then for the POA/CE pair (**a** = $p<0.05$).

Figure 2.5: GnRH axonal extent on the membrane from the POA explant from E15 co-cultures after 7 days in culture. GnRH axons extended over longer distances from the POA on the membrane sector facing the MBH co-explant (•• = $p<0.001$). The other co-explants had no effect on the extent of GnRH outgrowth in sector I (facing SC) or increased the length of the GnRH extent in sector II (opposite to CE, • = $p<0.02$).

Figure 2.6: Comparison of GnRH axonal outgrowth in P1 and E15 cultures. In both cases, GnRH axon number is significantly greater in sector I than in sector II (•• = $p<0.01$). **a** represents a significant difference ($p<0.01$) between E15 and P1 cultures.

Figure 2.7: Time-course of GnRH axonal outgrowth by sectors. The number of GnRH axons in sector I increased over time, with those at day 7 more numerous than at day 1 and 4 (++ = $p<0.01$) and at day 10 greater than at days 7, 4 and 1 (◊ = $p<0.05$). The number of axons in sector I was significantly greater than in sector II at days 4 (•• = $p<0.01$), 7 (• = $p<0.05$) and 10 (• = $p<0.05$).

Figure 2.8: Time course of GnRH axonal extent from the POA explant in POA/MBH co-cultures. The extent of GnRH axonal outgrowth from the POA on the

membrane was longer in sector I, facing the MBH, than in sector II, away from the MBH at day 4, 7 and 10 of culture (•• = $p<0.001$)

Figure 2.9: (**A**) Low power micrograph showing GAP43 immuno-labeled axons extending on the membrane (Texas Red) in POA and MBH co-cultures in the direction of the MBH co-explant, and (**B**) away from the MBH co-explant. (**C**) High magnification of GAP-43 labeled axons extending on the membrane. In **A** and **B** scale bar represents 200 μm and in **C**, 100 μm.

Figure 2.10: Three confocal micrographs of double labeled POA and MBH co-cultures, showing GAP-43 in red and GnRH in green. GnRH axons consistently extended on the membrane along GAP-43-ir axons. (**A**) A GnRH axon (arrows) is associated with GAP-43-ir axons when extending on the membrane. (**B**) GnRH axons (arrows) traveled within a bundle of GAP-43-ir axons or (**C**) extended on the surface of a network of GAP-43-ir axons. Scale bar represents 20 μm.

Figure 2.11: Co-localization of GAP-43 in GnRH axons, as visualized with confocal microscopy. **A** and **C** represent GnRH axons, double labeled with GAP-43 in **B** and **D**. Scale bar represents 20 μm.

Figure 2.12: Confocal micrographs of POA and MBH co-cultures double labeled for glial elements (green) and GnRH axons (red). (**A**) Notice the absence of alignment of a short GnRH axon (arrow) with GFAP-ir processes (arrowhead) extending on the membrane along the borders of the POA explant (star). (**B**) GnRH axons (arrow) extending further onto the membrane, grew without associations with any GFAP labeled elements. (**C**)

Similarly, GnRH axons (arrows) did not align on vimentin-ir glial cells (arrowhead). (**D**) Vimentin-ir elements were more scarce on the membrane in regions further away from the POA explant. GnRH axons (arrows) were not associated with glial elements (arrowhead). (**E**) A GnRH axon (arrow), extending from the POA explant (star), grew in close proximity to a S-100-ir astrocyte (arrowhead) without making contact, while another GnRH axon (arrow) extended onto the membrane without the presence of S-100-ir astrocytes (**F**). Scale bar represents 20 μm.

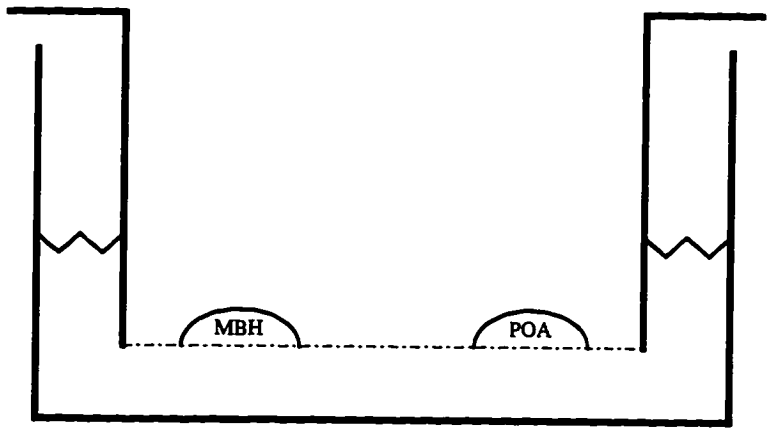
Figure 2.13: (**A**) GnRH axons (arrows) project to a region of the MBH co-explant, which resembles the median eminence (arrowhead) as seen in this micrograph. Notice the presence of a few GnRH cell bodies (asterisks) within the MBH explant. (**B**) The median eminence region within the MBH explant can be defined by the palisade arrangement of vimentin-ir tanycytes (green), showed in this confocal micrograph. GnRH terminals (red) (arrows) are found in this zone. (**C**) GnRH neurons (arrows) in the POA explant project to a region of the explant resembling the organum vasculosum of the lamina terminalis (OVLT) (arrowhead). (**D**) Confocal micrograph of GnRH axons (red) (arrows) coursing along GFAP-ir processes (green) within a structure resembling of the OVLT within the POA explant. Scale bar represents 100μm in **A** and **C** and 20μm in **B** and **D**.

Figure 2.14: A GnRH axon extended on the surface of the membrane from the POA explant to the MBH explant as visualized in this montage of 42 confocal micrographs. Notice the presence of a growth cone (arrow and shown magnified in insert) terminating the GnRH axon within the MBH explant. This GnRH axon projected close to the presumptive

median eminence (not shown). Scale bar represents 40μm and scale bar in insert represents 20 μm.

Figure 2.1

A



B

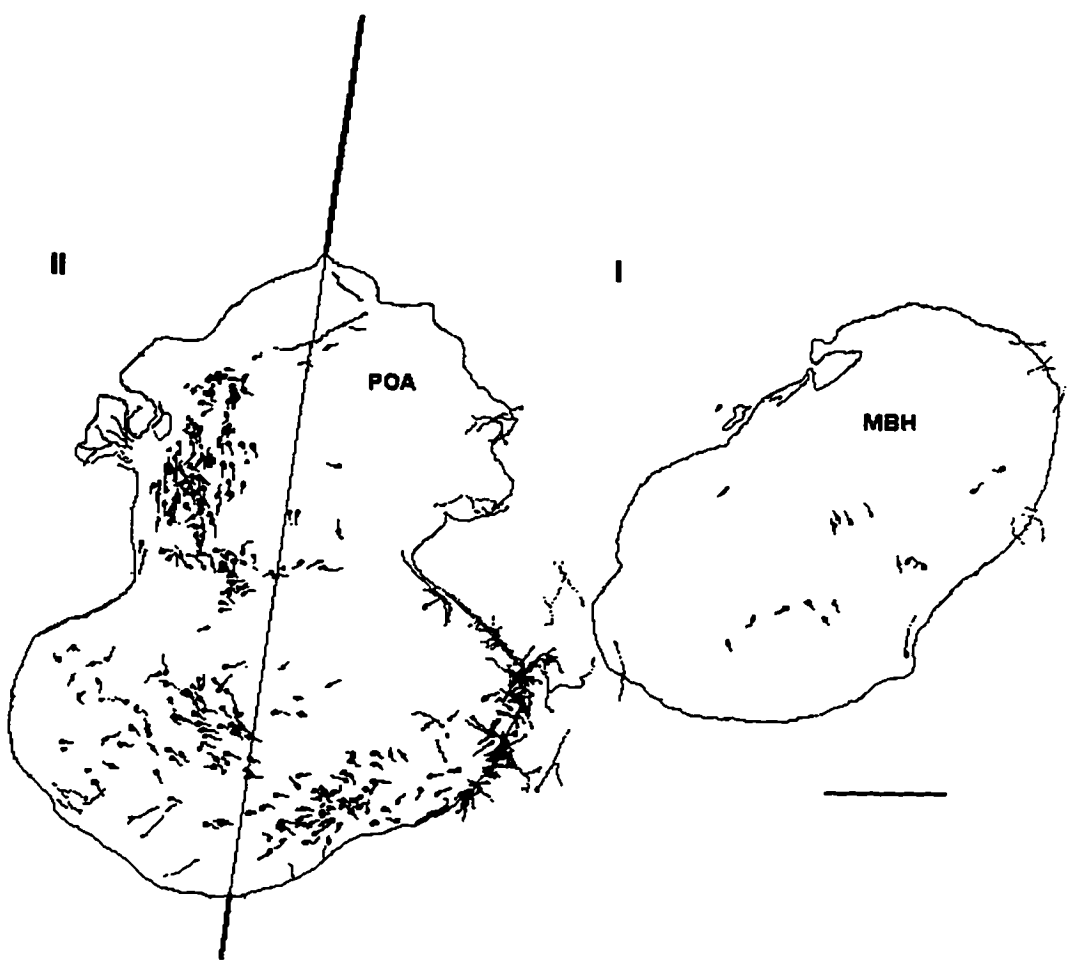


Figure 2.2

Figure 2.3

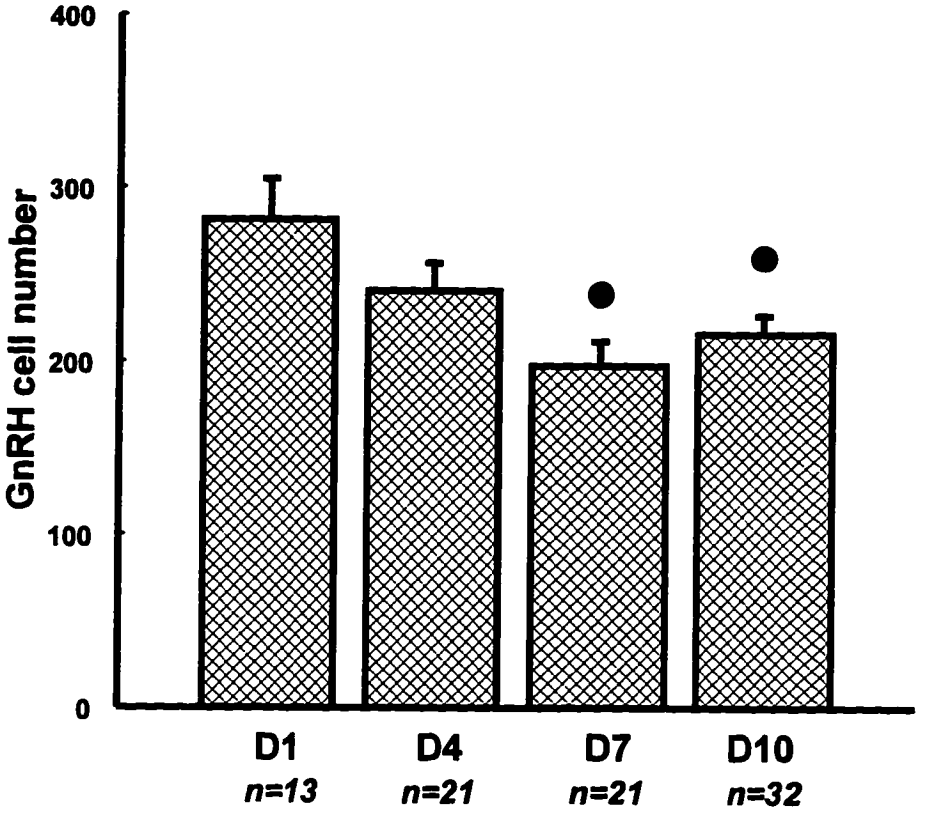


Figure 2.4

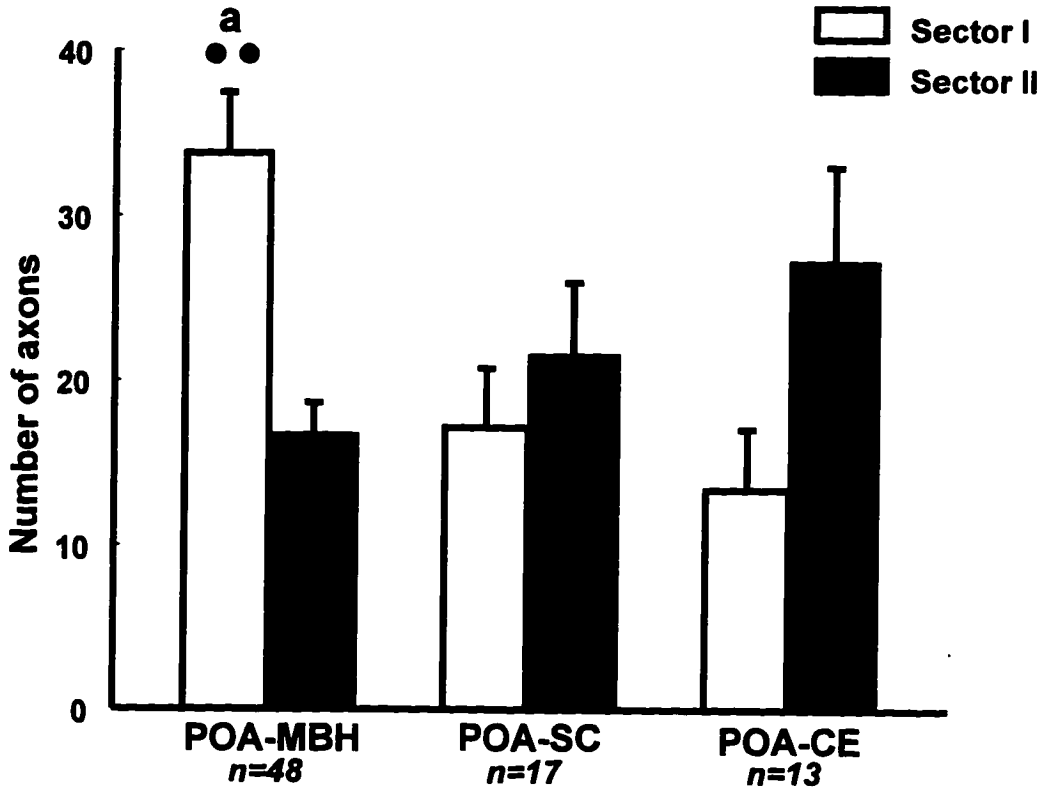


Figure 2.5

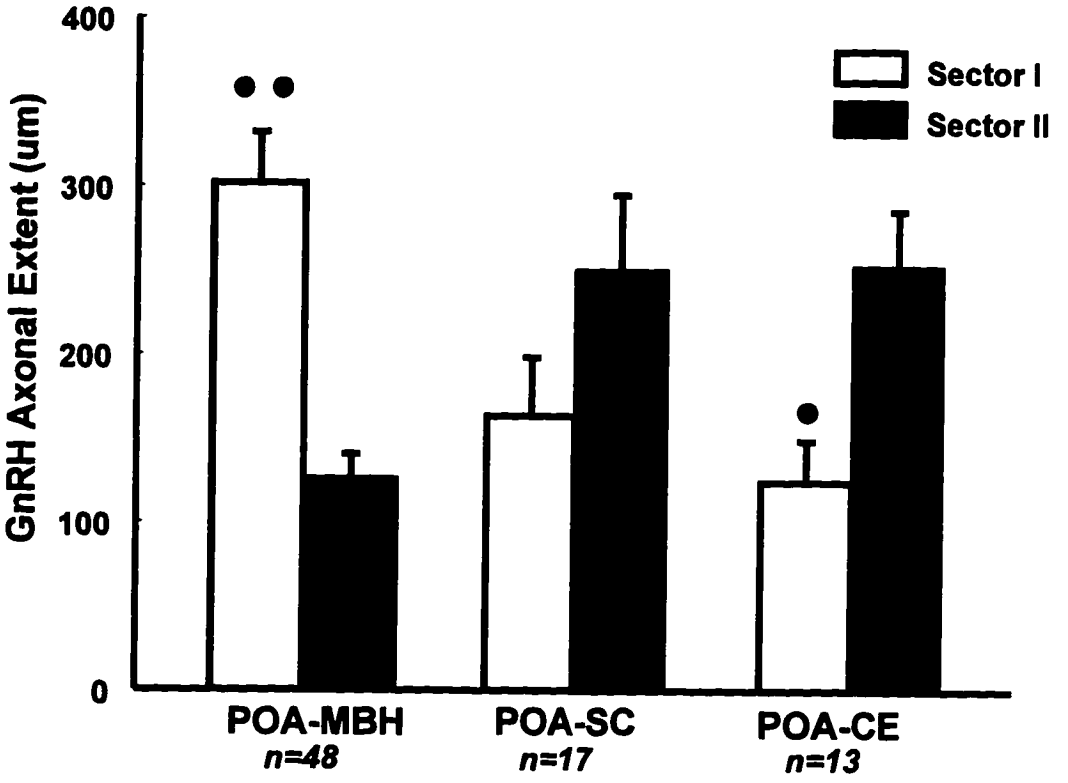


Figure 2.6

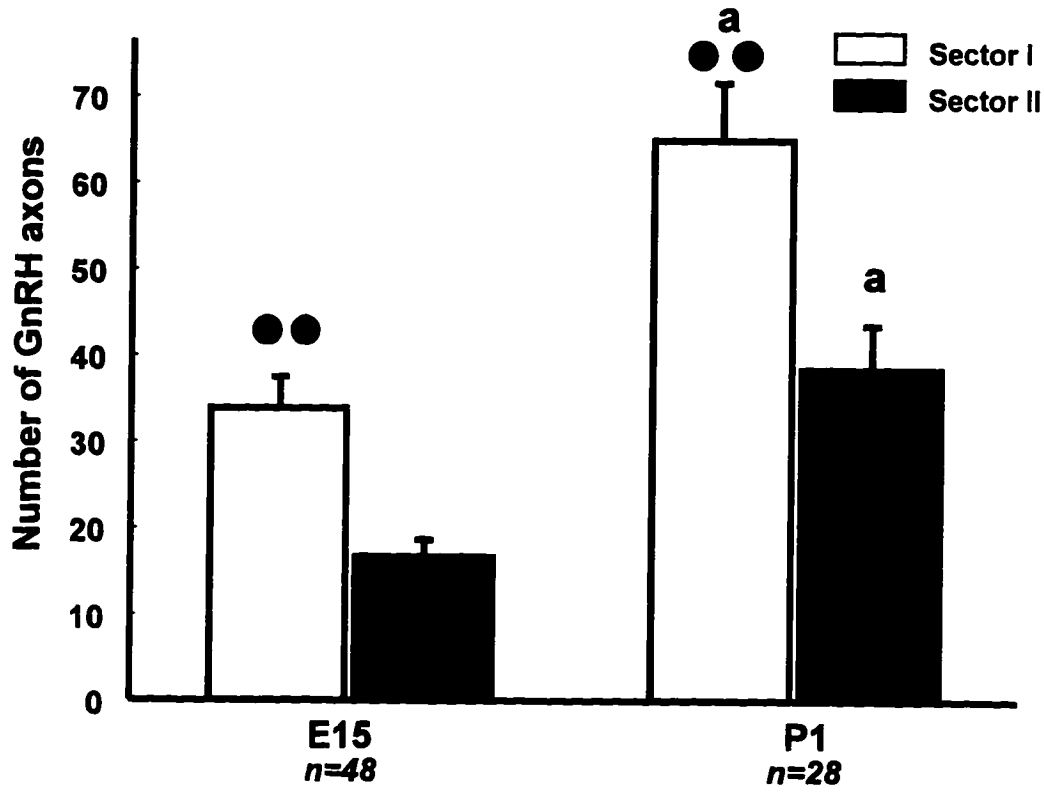
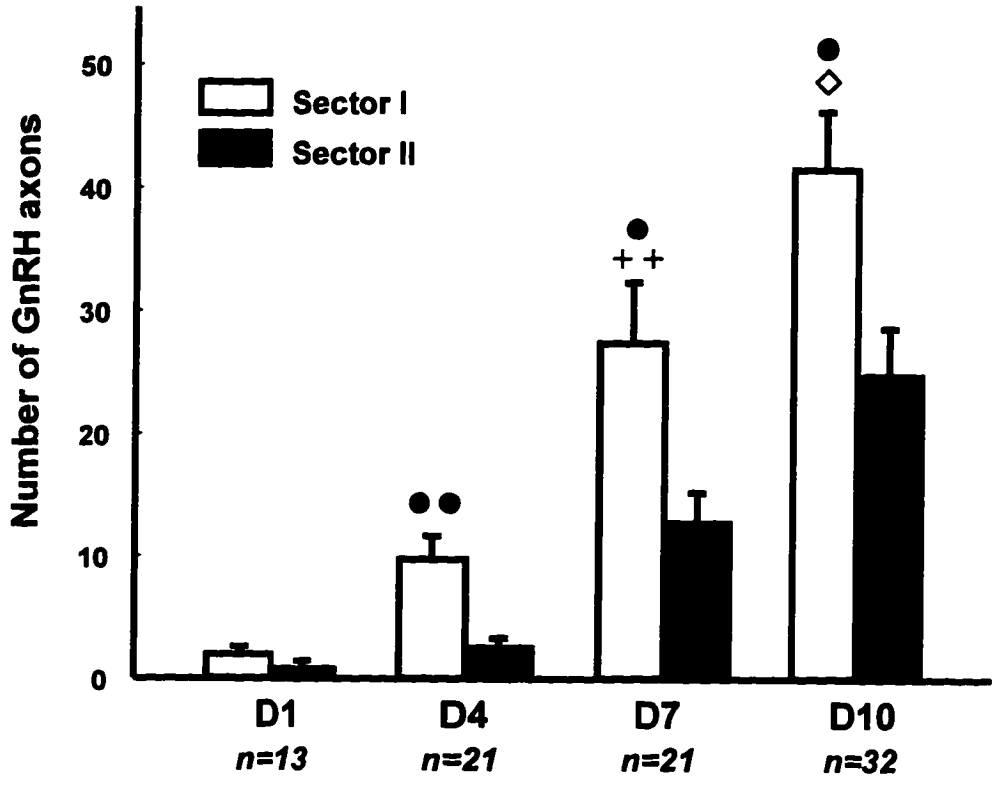


Figure 2.7



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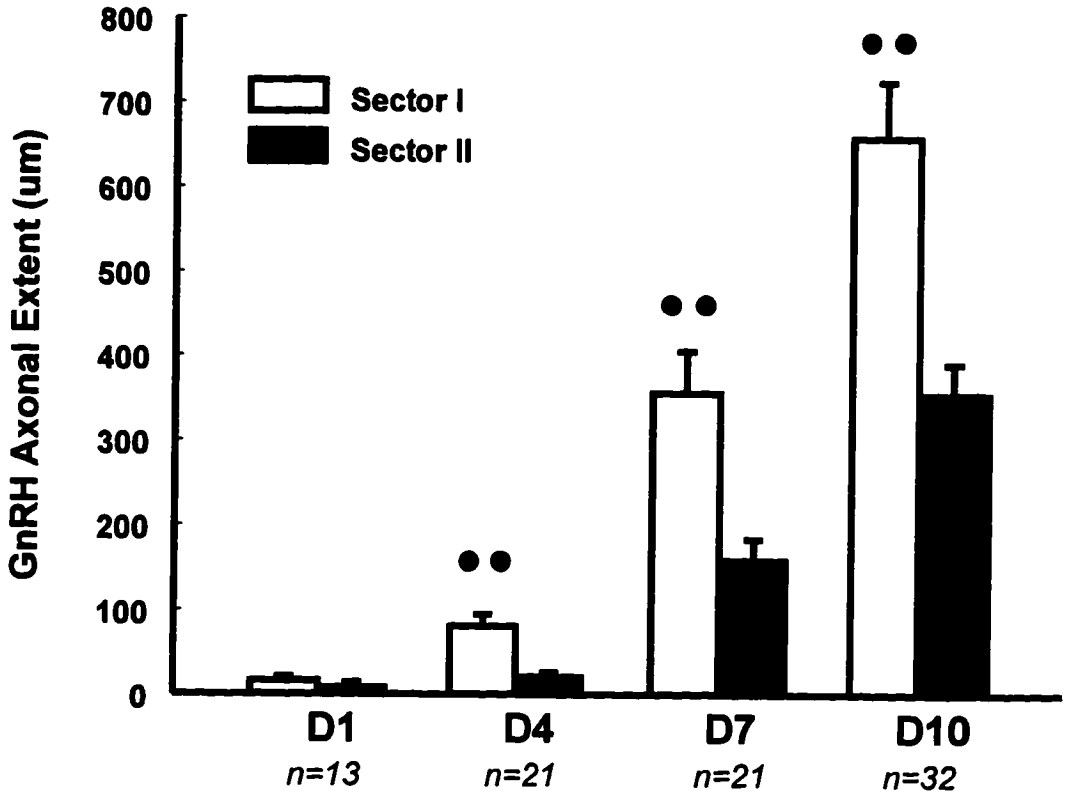

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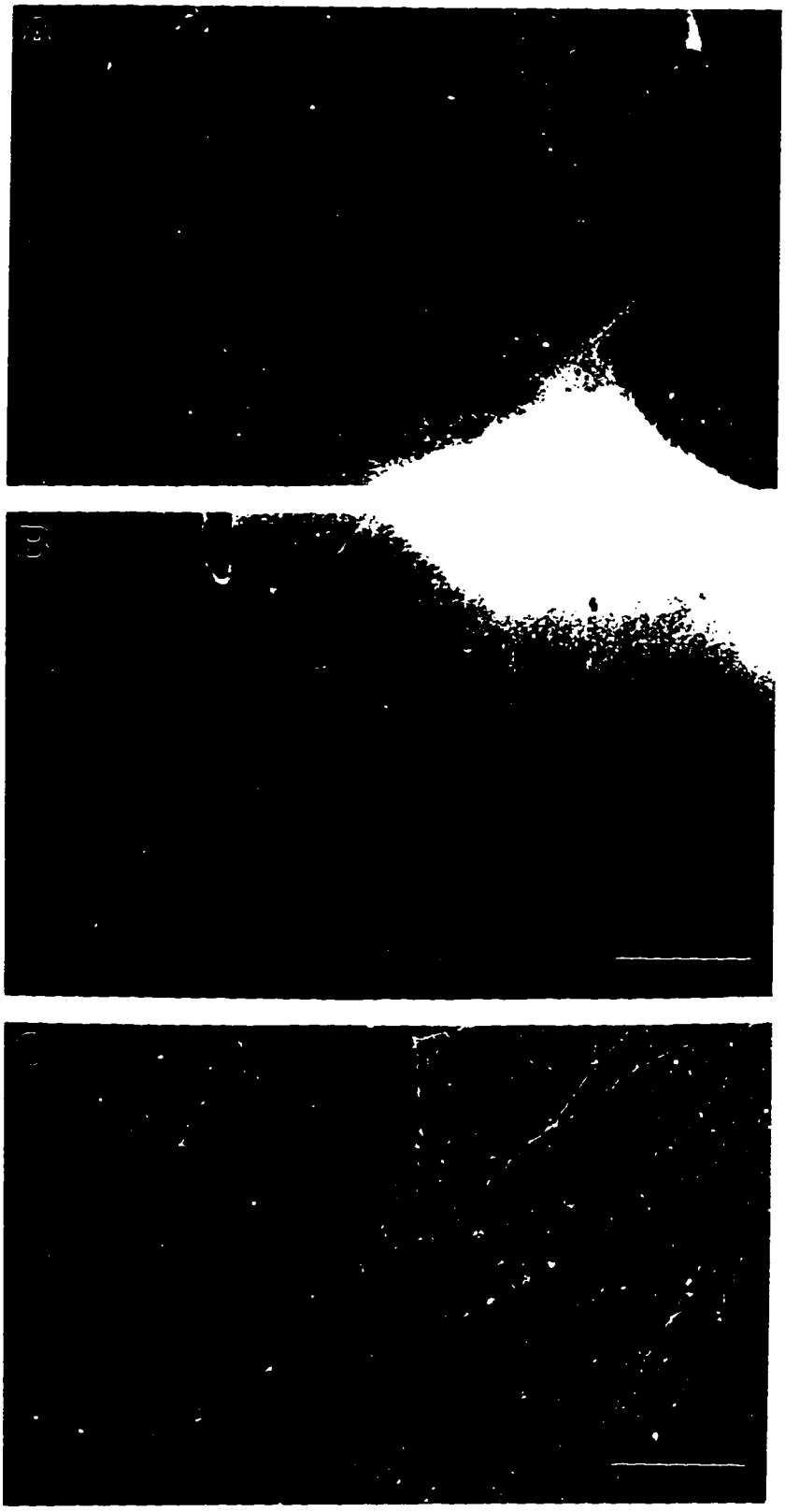

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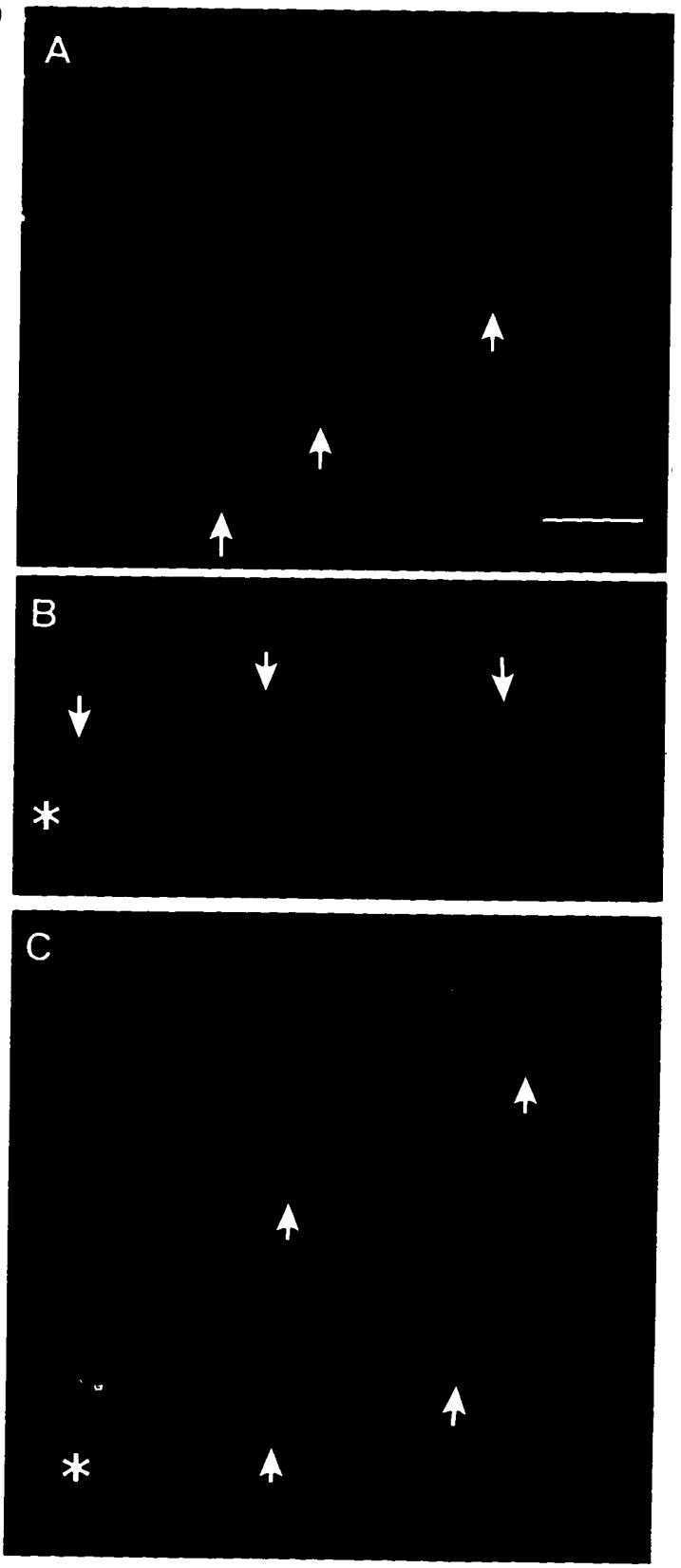

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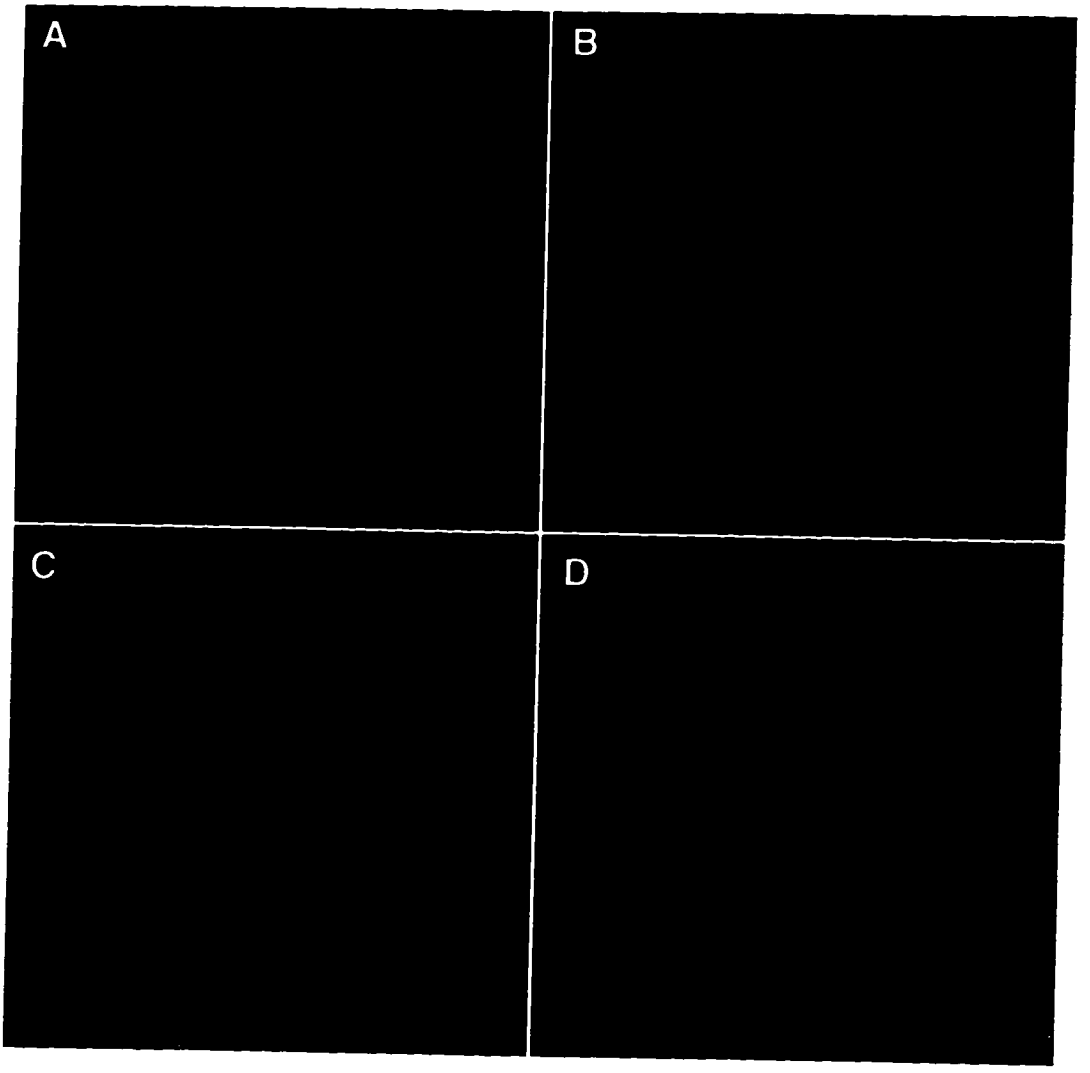

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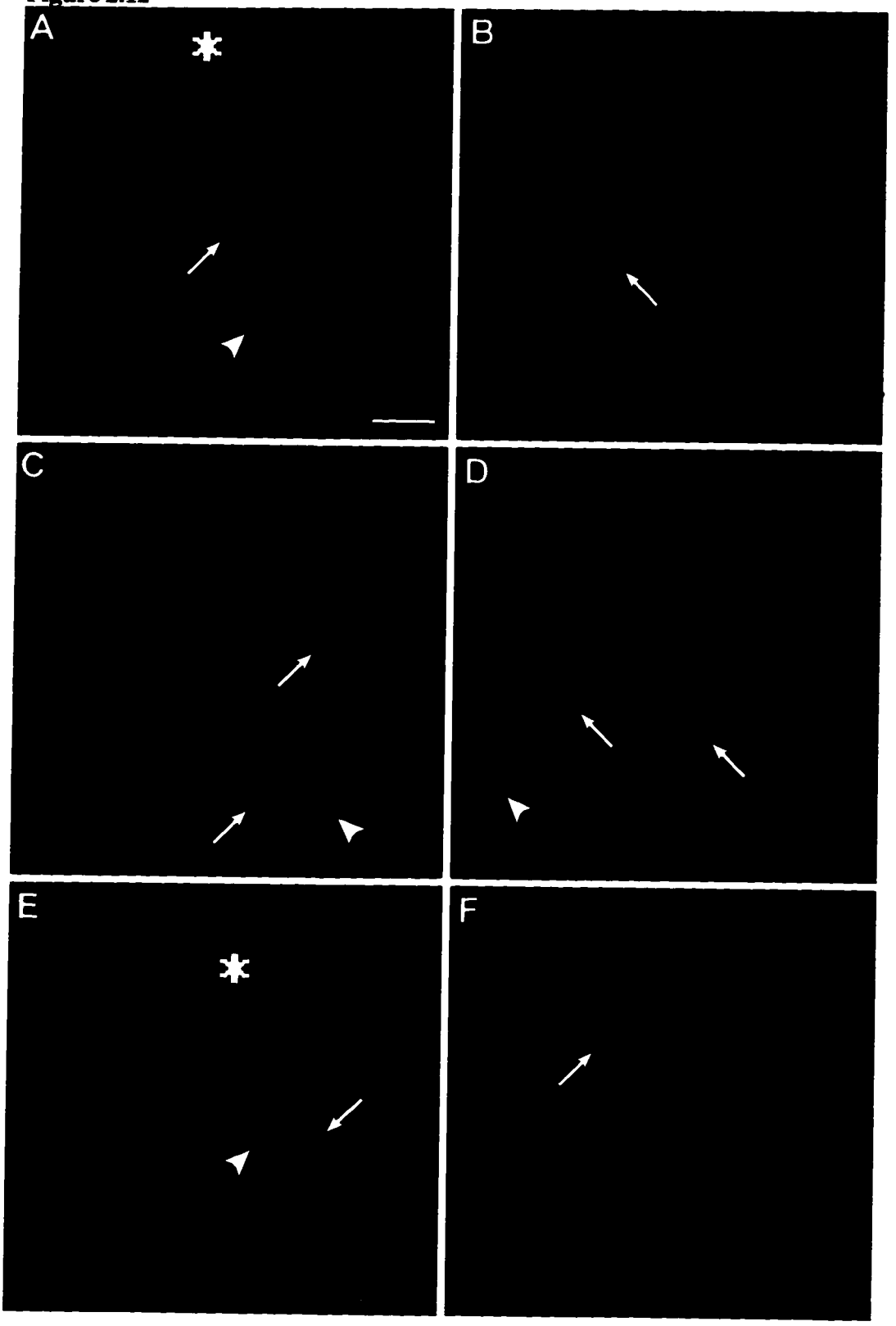


Figure 2.13

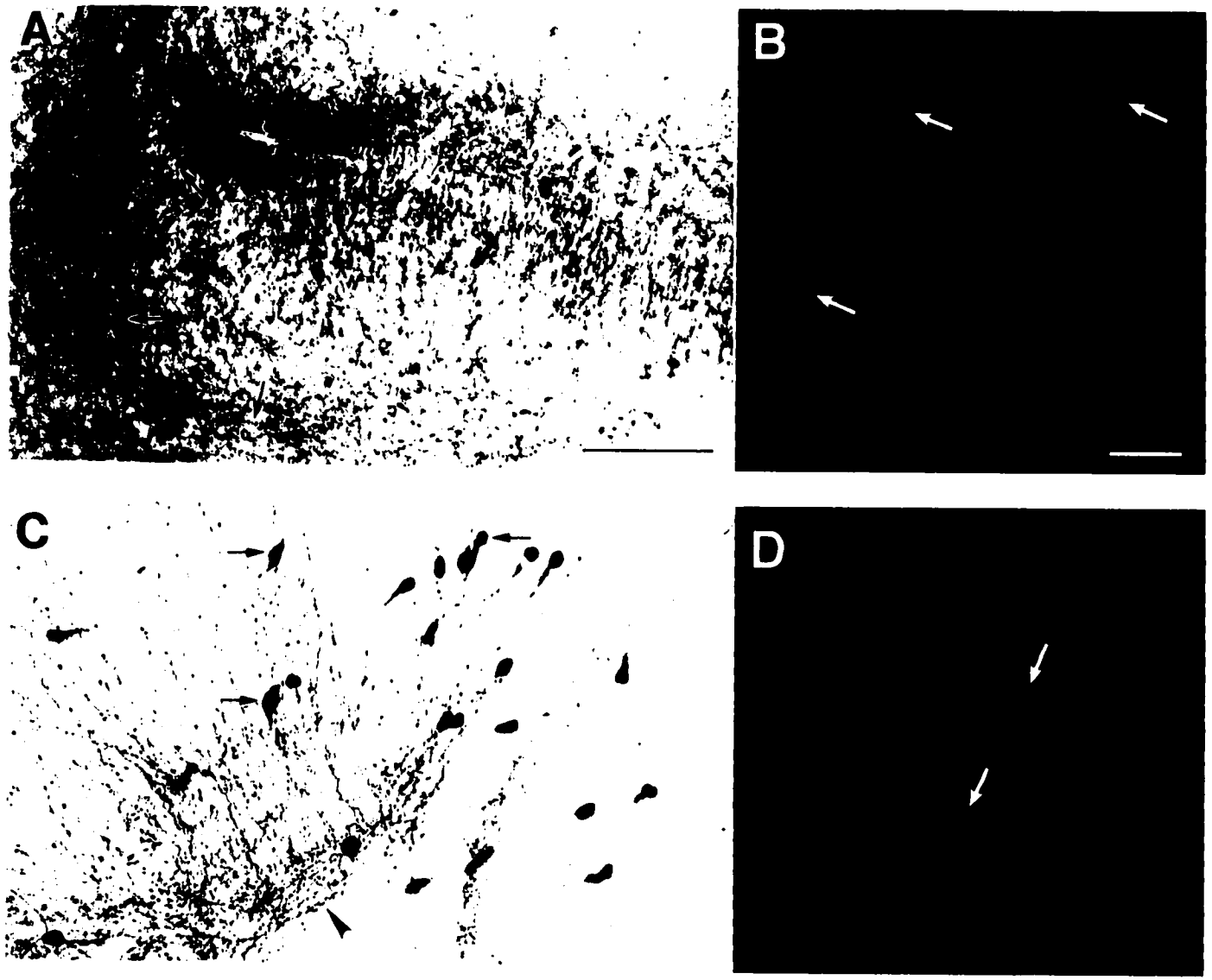
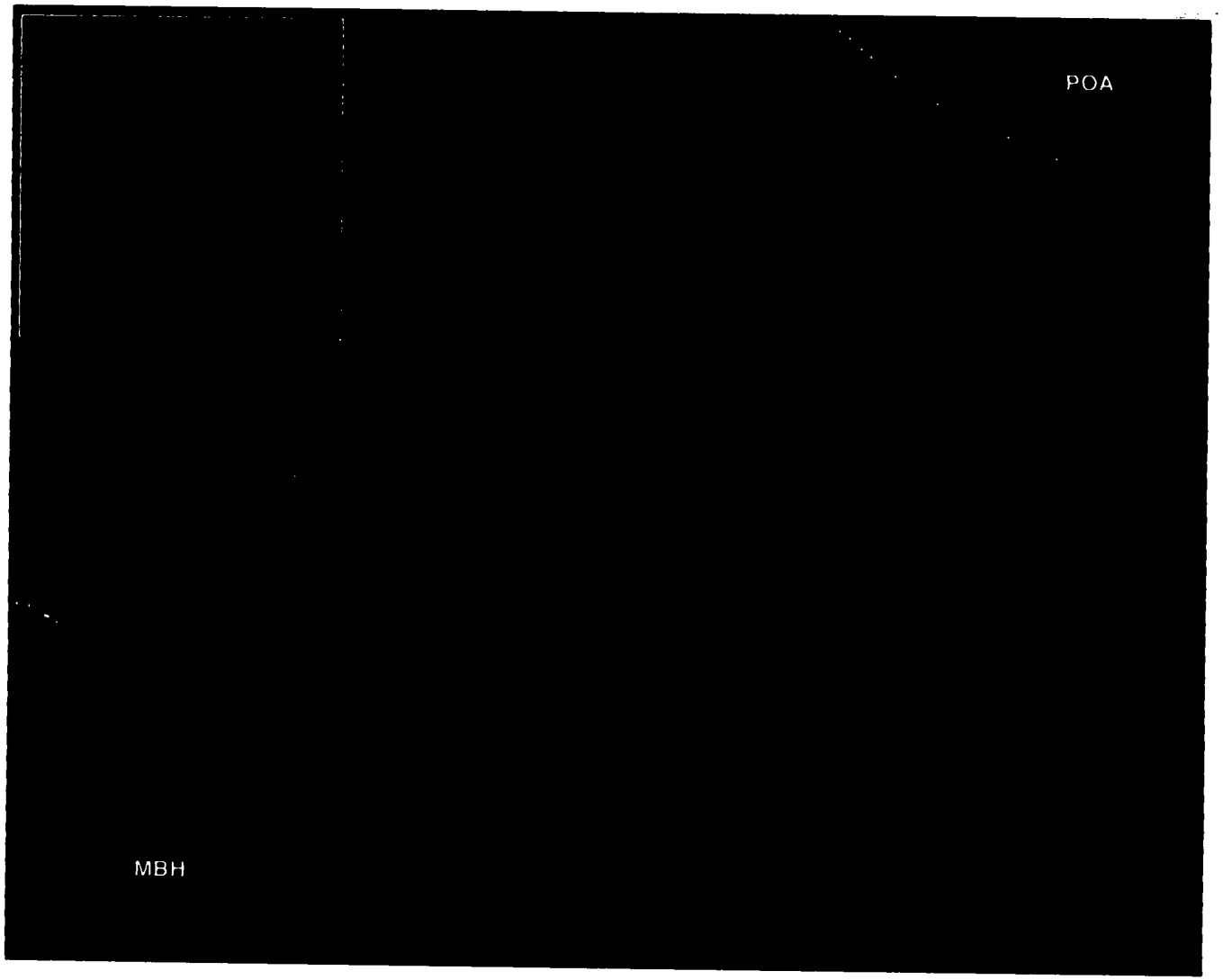


Figure 2.14



Chapter 3

IDENTIFYING THE NATURE OF THE SIGNAL FROM THE MBH: EXPERIMENTS IN "INVERTED CHAMBERS".

The studies described in Chapter 2, as well as previous studies using transplantation in hpg mice, support the hypothesis that the MBH appears to release a signal attractive for GnRH axons, such that they grow longer and in higher numbers onto the membrane sector facing the MBH. The nature of this signal is still unknown. However, the factors attracting GnRH outgrowth are likely to be diffusible chemoattractants, originating from the region of the median eminence. Interactions with glia did not seem to be required for the proper targeting of GnRH axons in culture. However, the possibility of some interactions with a substrate has not been eliminated, since GnRH axons extended on the membrane in the company of GAP-43-ir axons. To confirm the diffusible chemoattractant hypothesis, I designed an organotypic culture (named "inverted chamber") where the co-explants were separated by the membrane of the insert chamber with pore size of 0.4 μm, creating a physical barrier that allowed the diffusion of large molecules (Figure 3.1).

METHODS

Dissection of E15 mouse preoptic area (POA) and mediobasal hypothalamus (MBH) was performed as described in Chapter 2. The POA was kept in a drop of sterile defined medium until further use. Insert chambers coated, as previously described, with a mixture of rat tail collagen (type I, 3mg/ml, Boehringer-Mannheim, Indianapolis, IN) in 0.2% acetic acid and laminin (100μg/ml, Gibco BRL, Gaithersburg, MD) were turned upside down on a sterile petri dish to expose the under-surface (uncoated) of the membrane. The MBH explant was placed slightly off-center onto the under-surface of the membrane. A

drop (10-20 μl) of rat tail collagen (type I, 3mg/ml in 0.2% acetic acid), mixed in a 9/1 ratio with 10 times concentrated Dulbecco's modified Eagle medium (DME) and Ham's nutrient mix F12 with L-Glutamine and 15mM HEPES without phenol red (Sigma, St. Louis, MO) and 5 μl 0.8M sodium bicarbonate, was applied to the MBH explant and allowed to polymerize for 15-30 minutes. Upon polymerization the insert chamber was turned and placed in a well containing defined medium (described in Chapter 2). The POA explant was then placed on the surface of the membrane separated by a short distance from the MBH explant. I modified these cultures by adding laminin (100μg/ml) within the cushion, hoping that this would allow better adhesion of the cushion onto the membrane. The presence of laminin did not affect the physical properties of the cushion. The cultures were kept 7 days at 37 °C and fixed by immersion in 4% paraformaldehyde. Immunocytochemistry for GnRH was performed as described in Chapter 2.

GnRH axons were counted and the maximum extent reached by GnRH axons from the POA explant was measured and analyzed as described in Chapter 2.

RESULTS and DISCUSSION

The preparation of the inverted chambers allowed good survival of the POA explants after 7 days of culture, since the cells appeared healthy and no discernible difference was observed with routine organotypic cultures in which both explants were placed above the membrane (Chapter 2). Immunocytochemistry revealed a few GnRH cell bodies and axons within the MBH, indicating that embedding within the collagen (or

collagen and laminin) gel was supporting survival of the tissue. The preparation had an increased thickness when compared to the regular cultures in insert chambers. However this did not interfere with the ability to identify GnRH cell bodies within the MBH explant, which was applied directly under the surface of the chamber.

The collagen (or collagen and laminin) gel became brown while remaining translucent as the DAB reaction produced a precipitate within the gel. This reaction produced a high background, which, in conjunction with the increased thickness of the preparation, made visualization of GnRH axons difficult although not impossible in the sector I facing the MBH co-explant. Moreover, the collagen gel formed a cushion of diameter far larger than the MBH explant, which in certain cases extended under the space between POA and MBH co-explants, and occasionally under the POA explant. Upon mounting on a glass slide, the gel cushion distorted the membrane such that GnRH axons extending on the surface of the membrane had to be followed over different planes of focus.

GnRH axons were counted in sector I facing the MBH explant embedded in a gel and in sector II on the opposite side. There was no significant difference between the numbers of GnRH axons when the MBH was embedded in collagen gel (n=32) or in collagen and laminin gel (n=53), so the two groups were pooled. The number of GnRH axons in sector I was not significantly different than in sector II (respectively 23.8 ± 2.2 and 20.2 ± 1.8, n=85).

The maximum distance reached by GnRH axons was measured also as previously described. However, due to the difficulty of visualization of GnRH axons in sector I, special care was taken to identify the longest distances reached by the GnRH axons: High magnification was used (Objective 100X, with oil immersion) which reduced the depth of focus and allowed better identification of GnRH axons against the background formed by the underlying cushion. The membrane was easily identified by the presence of pores. In only one instance a GnRH axon seemed to enter a pore. All other GnRH axons remained above the membrane, and could not be confused with some of the few GnRH axons extending in the collagen gel cushion from the MBH explant, since these extended in a different plane of focus. The maximum distance reached by GnRH axons was measured only in the cultures where the MBH was embedded in collagen only. Three cultures were eliminated because of the inability to focus on most GnRH axons extending in sector I, due to an extensive protrusion of the collagen gel cushion underneath the POA explant. The maximum extent (μm) reached by GnRH axons was significantly higher in sector I than in sector II (311.8 ± 30.2 and 235.9 ± 22.6 respectively, $n=29$, $p<0.05$).

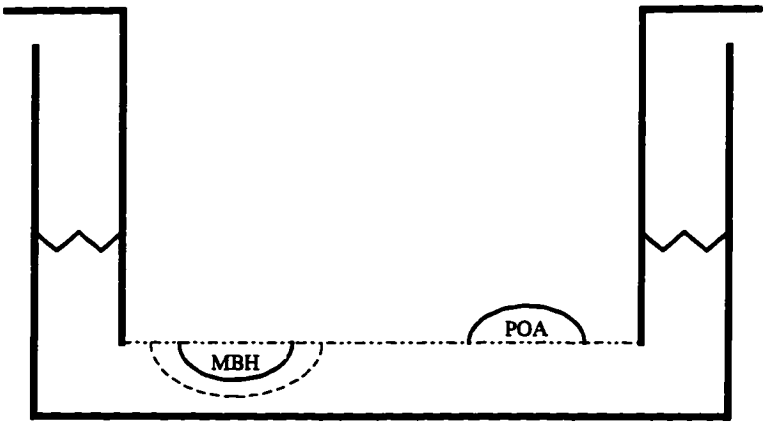
Comparison of the extent (μm) of axonal outgrowth in inverted insert chamber with the routine insert chambers, where both explants are above the membrane, reveals that the extent of GnRH outgrowth in sector I is unchanged (311.8 ± 30.2, $n=29$ and 301.0 ± 30.8, $n=48$, respectively, $p>0.5$), while the extent of outgrowth in sector II is significantly increased when the chambers are inverted (235.9 ± 22.6, $n=29$ and 125.2 ± 15.5, $n=48$, respectively, $p<0.01$).

The MBH exerted an action on the maximum extent reached by GnRH axons from the POA explant on sector I of the membrane, since this extent was higher in sector I than II. Unlike routine insert chambers, where the POA and the MBH explants were placed side by side on the surface of the membrane, the inverted insert chambers did not allow differences in GnRH axon number between sector I and II to be discernible. It is possible that the conditions of visualization of the GnRH axons against a high background, in only sector I, lead to an under-representation of the number of GnRH axons growing on the membrane in this sector, since many axons are extremely fine and pale and can easily be missed. However the measurement of the GnRH axonal extent from the POA explant was made possible by the extensive care taken to avoid missing the longest axons and was less subject to errors than counting the absolute number of axons. This measurement, therefore, constituted a more sensitive test of the action of the MBH, since, observed in routine insert chamber cultures (Chapter 2), the difference of GnRH axonal extent between sector I facing the MBH and sector II was over twofold.

The significant difference in the GnRH axonal length between sector I and II indicated that the MBH action on GnRH axonal outgrowth did not require extensive contact between tissue extending from the MBH and GnRH axons. This result supported the diffusible factor hypothesis, since the MBH placed underneath the membrane could influence the GnRH axonal outgrowth only by way of diffusion of substances through the membrane pores.

Figure 3.1: Schematic drawing of an inverted insert chamber showing placement of the co-explants. The MBH explant, included in a polymerized collagen gel cushion, is maintained on the under surface of the membrane. The POA explant is placed on the membrane.

Figure 3.1



Chapter 4

GONADOTROPIN-RELEASING HORMONE (GNRH) NEURONS IN POA GRAFTS IMPLANTED INTO THE MAMMILLARY BODIES/POSTERIOR HYPOTHALAMUS OF HYPOGONADAL MICE PROJECT TO THE MEDIAN EMINENCE DESPITE THEIR CAUDAL LOCATION.

Earlier reports on grafting of GnRH-containing tissue in hpg mice suggest that the environment of the mediobasal hypothalamus provides clues necessary for proper targeting to the median eminence. When POA grafts were placed in the third ventricle, even in a very rostral location, GnRH axons followed the walls of the third ventricle until they reached the mediobasal hypothalamus, where they arched to their target (Silverman *et al.*, 1985). Similarly, GnRH neurons from E13 nasal septum grafts (taken during the migration of GnRH neurons from the olfactory placode to the POA), when placed in the anterior hypothalamus or host medial POA, occasionally sent axons toward the third ventricle which then turned ventral to project to the lateral borders of the median eminence. It has also been shown that the rostral extent of the graft is less important than its localization at the base of the brain close to the mediobasal hypothalamus and to hypothalamus regions utilized by GnRH axons on their normal projection pathways (Livne *et al.*, 1992). The median eminence was never innervated by GnRH fibers, when grafts were positioned in the lateral ventricle, despite survival of many GnRH cells within the grafts. Some GnRH axons grew in the nearby host tissue, following host bundles of fibers, such as the fornix, the corpus callosum, or the stria terminalis (Kokoris *et al.*, 1987). Other GnRH axons even grew into the host lateral septum or the anterior hypothalamus, but never reached the median eminence.

In the previous experiments POA grafts were always placed rostral to the median eminence. The paths taken by GnRH axons on their way to the median eminence would be like the ones observed in the development of the normal animal. One hypothesis is that the

GnRH axons exiting the graft would find a facilitative environment to grow in the host tissue, borrowing already established pathways to the median eminence. However, studies using co-grafts of POA and embryonic mediobasal hypothalamus in hpg mice, where both host and co-grafted median eminence were innervated by GnRH neurons, suggested that the guidance to the median eminence requires more than just positioning along organized structures of the basal section of the third ventricle (Saitoh *et al.*, 1992).

In the present study, we placed POA grafts caudal to the median eminence at the level of the mammillary bodies. The mammillary bodies do not contain GnRH cell bodies in the normal mouse (Jennes and Stumpf, 1986). We hypothesized that, if the median eminence region releases diffusible substances of guiding GnRH axons, these would be capable of guiding GnRH axons from such a location.

MATERIALS AND METHODS

Animals

Normal (C3H/HeHx101H) and hpg mice were housed in a colony room with lights on at 2300h and lights off at 1300h daily. They were maintained under constant temperature with food and water at libitum. All protocols for animal treatments and surgeries were reviewed and approved by the Mount Sinai School of Medicine Institutional Animal Care and Use Committee.

Preparation of donor tissue.

Normal embryos of gestational age E15 to 19 were used as tissue donor for the grafts. The day the vaginal plug was found was counted as gestational age E0. Time-mated females were sacrificed by cervical dislocation after brief anesthesia with CO_2. The uterus was removed immediately and placed in a sterile petri dish on ice until dissection. Embryos staging was confirmed by comparison with the external morphological criteria defined by Schambra (Schambra and Lauder, 1992). The POA was dissected from the ventral surface of the brain as previously described (Krieger *et al.*, 1982). The tissue was kept in a drop of sterile saline on ice until implantation.

Surgeries

Hpg host male mice used were at least 1.5 months old and no older than 4 months at the time of the surgery. The animals were anesthetized with chloral hydrate (360mg/kg, i.p., Sigma, St. Louis, MO) and Isoflurane vapors (used as a supplement when needed). Animals were placed in a stereotaxic apparatus (David Kopf Instruments, Tujunga, CA) adapted for the mice. Grafts of embryonic POA tissue were implanted bilaterally with a 20-gauge needle into the mammillary bodies (n=10) or the anterior hypothalamus (n=2) of the hpg hosts. For the mammillary bodies grafts, the stereotaxic coordinates were -2.6 mm caudal from bregma, ±0.6mm laterally from the midline, and -5.2mm from the surface of the brain. For anterior hypothalamus grafts all lateral and depth coordinates were similar, but the graft was placed -1.0mm caudal from bregma (Slotnick and Leonard, 1975). Animals were allowed to recover 4 to 7 months before perfusion.

Perfusions.

Animals were sacrificed with an overdose of chloral hydrate. Intracardial perfusion was performed using saline followed by ice cold solution of 4% paraformaldehyde (Sigma, St. Louis, MO) in 0.1M phosphate buffer (PB, pH 7.3). Testes and seminal vesicles were removed and weighed. The brain was removed from the skull with special care to maintain the pituitary attached to the base of the brain and postfixed in 4% paraformaldehyde (Sigma) overnight at 4°C. The brains were embedded in a solution of 8% gelatin and 8% albumin (bovine serum albumin, Sigma) and allowed to polymerize for 48 hours in contact with 38% formalin fumes. The brains were sectioned in the coronal plane at 50 μm with a vibratome from the level of the septal nuclei to the most caudal pons. Floating sections were washed in 0.1M PB containing 0.1% sodium azide (Sigma) until immunocytochemistry was performed for GnRH.

Immunocytochemistry.

Floating sections were washed in 0.5% H_2O_2 solution to block endogenous peroxidase activity. For detection of GnRH neurons and processes, SW1 antiserum (Gift from Susan Wray (Wray *et al.*, 1988)) was used at a dilution of 1:2500 in PB containing 0.1% Triton X100 (TX100, Sigma, St. Louis, MO) and 3% normal donkey serum (Jackson Immunoresearch Labs., West Grove, PA) for 2-4 days at 4°C. The biotinylated anti-rabbit secondary antiserum (1:200 made in donkey, Jackson Immunoresearch Labs., West Grove, PA) in the same diluent was applied for 1 hour. The sections were then incubated in an avidin-biotin solution (Vector Labs., Burlingame, CA) conjugated with horseradish

peroxidase for 2 hours. The chromogen was 1mg/ml 3,3'diaminobenzidine (DAB, Sigma, St. Louis, MO) and the H_2O_2 was generated by the oxidation of glucose by glucose oxidase (Sigma, St. Louis, MO). Sections were mounted on gelatin-coated slides and allowed to dry. They were coverslipped with Permount (Fisher) or Gelmount (Biomedia, Fostercity, CA) and were examined and photographed using light microscopy.

RESULTS.

Graft survival and placement.

All animals receiving grafts remained healthy after the surgery and did not show any signs of neurological damage at the time of the perfusion. Immunocytochemical processing with SW1 antibody revealed GnRH labeling in all brains. All hosts were confirmed to be hpg, since no immunoreactive GnRH neurons were found in the septal/preoptic area, where GnRH cells are present in the normal mouse. Every graft (n=12) survived well with little necrosis and contained GnRH neurons (Table 4.1), whose morphological appearance did not differ from GnRH neurons in third ventricle grafts or in the normal mouse (Figure 4.1 A). GnRH neurons were present within the graft with the exception of a few GnRH cells which appeared to have migrated in the nearby host tissue in PP01 (Figure 4.1 B and C). The individual data for each animal is summarized in Table 4.1.

Hpg hosts receiving POA transplants bilaterally in the mammillary bodies as defined by the stereotaxic coordinates described above could be separated into categories upon the final localization of the grafts and GnRH cell bodies:

In only one animal, PP01, were grafts correctly positioned in the mammillary bodies and restricted to that main region. For PP20, LL30 and LL24, GnRH neurons were found principally in the region of the grafts which were located caudally in pons and nucleus interpeduncularis levels, within the mammillary bodies and posterior hypothalamus. Some were also in the more rostral lateral hypothalamus, in certain cases at the level of the median eminence. Some parts of the grafts (at rostral levels) were found also in dorsal and lateral hypothalamic locations for LL24 and LL30. A fragment of tissue very likely originating from the graft was found bulging into the third ventricle in PP20, but no GnRH neurons were detected in that fragment. In LL24 and LL30, a section of the graft extended ventrally and laterally close to the caudal median eminence. A tear in the tissue at the level of the caudal third ventricle and mammillary recess due to sectioning procedure did not allow us to eliminate the possibility of some graft merging within the ventricle at that level. However no evidence of graft fragments within the third ventricle was observed at more rostral levels in these animals. In the case of LL30 a small fragment of graft was also located above the infundibular stalk. It is possible that this piece merged within the infundibular recess. The large size of the grafts, merging medially in the mammillary bodies and bulging underneath the ventral surface of the brain to fill the meningeal space between the brain, the pituitary and the infundibulum, distended the host tissue making the identification of structures laborious.

In other animals, the grafts fragmented along a dorso-ventral axis following the needle tracts of the bilateral injections. In the case of PP44, QQ06, and PP52, large sections

of grafts, containing GnRH cell bodies, evidently filled the third ventricle and the mammillary bodies. Likewise, the grafts of hpg hosts receiving bilateral POA tissue injections in the anterior hypothalamus (LL33 and LL23) were found merging within the third ventricle. Fragments of these grafts were also found laterally in the anterior hypothalamus and in dorsal locations.

In PP42, QQ05, QQ07, GnRH neurons were exclusively located in the dorsal fragments of the grafts. No GnRH neurons were found in the mammillary bodies. Details of the individual graft placements can be found in Table 4.1.

Gonadal development of the POA grafted hpg hosts.

Untreated hpg mice have low testes and seminal vesicle weights, 7.5mg ± 2.5 (standard deviation) and 17.2mg ± 4.7 respectively (Perlow *et al.*, 1987). Development of the testes and the seminal vesicles served as a bioassay of the stimulation of the pituitary-gonadal axis by a successful and well integrated POA graft. An animal was considered a responder, when the testes weight was at least two standard deviations heavier than that of untreated hpg males. All hpg mice receiving grafts with GnRH cells placed in the mammillary bodies or the posterior hypothalamus (n=4) were responders. In particular, PP01 with cells located exclusively within the mammillary bodies was the one of the best responders. Three animals (PP01, PP20 and PP24) had a remarkable increase in seminal vesicle and testes weights (Table 4.1). Similarly, the testes development was evident in all animals in which grafts merged within the third ventricle, whether the graft was originally

aimed at the anterior hypothalamus (n=2) or to the mammillary bodies (n=3). However, when GnRH neurons were found exclusively in a dorsal location above the third ventricle, the pituitary-gonadal axis was not stimulated. Only one animal (QQ05) from three grafted hpg hosts, with GnRH neurons exclusively found in dorsal locations of the graft, had some slight increase in testicular weight, probably due to an individual variation (Table 4.1) as had been observed by Kokoris (Kokoris *et al.*, 1987).

GnRH projections to the median eminence of the host hpg mice.

When the graft containing GnRH neurons was located in a very caudal as well as ventral location, at the level of the mammillary bodies or the posterior hypothalamus, some GnRH fibers exited the grafts on their dorsal borders and turned to project medially towards the base of the brain at more rostral levels (PP01, LL24, PP20) (GnRH projections from grafts located in the mammillary bodies of PP01 were schematically drawn in Figure 4.2). These GnRH fibers were followed at the midline as they extended rostrally from a ventral location to innervate the caudalmost part of the median eminence (Figure 4.3 A, B, C, D, E). The most caudal fibers were seen turning medially towards the base of the brain to accumulate within the midline, indicating that they might orient perpendicularly to the coronal section towards the median eminence. Other GnRH fibers extended laterally from the graft away from the midline of the brain.

In PP01 a GnRH cell migrated rostrally out of the graft and was found located along the borders of the mammillary recess with axons oriented towards the mammillary

recess (Figure 4.1 D) . It is possible that this cell contributes to the innervation of the caudal median eminence observed in this animal.

GnRH axons extended from the ventral borders of graft sections (LL30), when these were localized in the dorsal aspects of the mammillary bodies, to project towards the base of the brain and abundantly innervate the mammillary bodies. Furthermore, GnRH fibers could be followed on more rostral sections, arching through the posterior hypothalamus towards the mammillary recess, as they extended in the direction of the caudal median eminence.

Regardless of the final position of the graft within the mammillary bodies or the posterior hypothalamus, whether it was located along the ventralmost borders of the brain, in dorsal mammillary bodies or more rostrally in the lateral posterior hypothalamus, some GnRH fibers exited the graft and oriented medially towards the mammillary recess and the median eminence. At median eminence levels, GnRH axons arched through the arcuate nucleus and mainly projected along the lateral sides of the median eminence (Figure 4.3 B, C and D) .

Remarkably, numerous GnRH fibers extending within the mammillary bodies (PP20) or within the grafts (at caudal levels placed in the meningeal space between pituitary and ventral surface of the brain for LL30 and LL24) were oriented along a dorso-ventral axis and seemed to accumulate at the base of the brain with growth cones directly facing the pia mater and the pituitary (LL30, PP20, LL24) (Figure 4.4 A, B, and C). In the case of

LL24, the grafts bulged underneath the ventral surface of the brain directly above the pituitary, because of the distention in the host tissue created by the large size of the grafts. GnRH fibers extended upward to the caudal median eminence from a ventral location, while other GnRH fibers oriented within the grafts downward with growth cones facing the pia mater and the pituitary. In one instance (LL23), GnRH axons descended along the infundibular stalk to the borders of the pituitary.

When some pieces of graft containing GnRH neurons fragmented in more rostral locations in the lateral hypothalamic area, or when grafts containing GnRH neurons merged within the third ventricle (PP44, QQ06, PP52, LL33, LL23), GnRH fibers markedly extended in the direction of the median eminence and arched through the arcuate nucleus to innervate the lateral sides of the median eminence (Figure 4.5 A). GnRH neurons within the graft often projected in the direction of blood vessels, their axons wrapping around the lumen (Figure 4.5 B) .

GnRH neurons located in dorsal grafts (in PP42, QQ05, QQ07) never projected to the median eminence.

GnRH projections in other regions of the host brain.

GnRH neurons in grafts located in the mammillary bodies or the posterior hypothalamus, and extending in certain cases caudally at pons levels, projected along the pons (Figure 4.6 A) and to the interpeduncularis nucleus (PP01, PP20, PP52) (Figure 4.6

B). GnRH axons, extended also within the fasciculus retroflexus, traveling rostrally and dorsally within the fiber bundle (PP01) (Figure 4.6 C). This region was also innervated by GnRH axons even when the graft was placed dorsally, close to the habenular nucleus or in the thalamus (PP42, QQ05). In that case, several unbranched GnRH fibers could be followed over long distances within the fasciculus retroflexus and terminated in the nucleus interpeduncularis or the mammillary bodies.

Other GnRH fibers originating from neurons in dorsal thalamic grafts were found in the corpus callosum (PP42) or entered the hippocampus (PP42, QQ05, QQ07) (Figure 4.6 D). These GnRH fibers however remained in low number. A few GnRH axons were also detected along the optic tract and in the amygdala (LL24, LL33). When grafts merged within the rostral aspect of the third ventricle (PP44, PP52), axons were seen innervating the organum vasculosum of the lamina terminalis (OVLT) (Figure 4.6 E), a circumventricular organ receiving abundant GnRH innervation in the normal animal (King *et al.*, 1982).

DISCUSSION.

Previous studies have consistently established that GnRH axons from grafts placed within the III ventricle (Gibson *et al.*, 1982; 1983; 1984a; Krieger *et al.*, 1982; Silverman *et al.*, 1985) or the anterior hypothalamic area (Livne *et al.*, 1992) successfully innervated the median eminence of host hpg mice, mimicking some of the pathways followed by GnRH axons projections from the septal/POA to the median eminence (Hoffman *et al.*, 1992). The

specificity of the GnRH axonal targeting suggested the existence of precise mechanisms directing the GnRH projections towards the median eminence. The release of diffusible attractive factors from the median eminence region, directing the GnRH axons, seemed to be the most compelling hypothesis to account for these results (Saitoh *et al.*, 1992).

In addition, GnRH projections from the graft might follow some substrate-bound guiding cues, specific to the mediobasal hypothalamus. It was shown for instance that GnRH neurons in POA grafts implanted in the lateral ventricle never projected to the median eminence (Kokoris *et al.*, 1987). Such grafts might be located outside the range of action of some median eminence-derived diffusible substances, in regions of central nervous system which might not provide an appropriate substrate for axonal guidance.

The present studies showed that POA grafts in the mammillary bodies or the posterior hypothalamus, regions of the brain which never contain GnRH cell bodies in the normal rodent (Jennes and Stumpf, 1986; Silverman *et al.*, 1979; 1994), successfully innervated the median eminence, leading to gonadal development in the hpg hosts. Such grafts were not adjacent to the pathways utilized in the normal mouse by GnRH axons to project from septal preoptic regions towards the median eminence (Hoffman *et al.*, 1992) nor pathways borrowed by GnRH axons exiting third ventricle grafts (Silverman *et al.*, 1985). The most unambiguous case was PP01, with all GnRH neurons located in the mammillary bodies. The pattern of innervation of the median eminence, as observed in this animal, proved unquestionably that GnRH neurons placed caudally to the median eminence can target the median eminence. The graft in PP01 was so well integrated within the host

brain, that its borders were indefinite and some GnRH cells migrated into the host tissue. The GnRH neuron closest to the median eminence had migrated close to the mammillary recess and may have extended projections from this caudal location. Although rare, migration of GnRH neurons into the host tissue has been observed when grafts derived from embryonic nasal septum were placed into the host anterior hypothalamic area (Livne *et al.*, 1992). Such pathfinding is consistent with the hypothesis that the axons are guided by an attractive substance released by the median eminence.

It was more difficult to determinate the location of the neurons giving origin to the GnRH terminals innervating the median eminence in the remaining grafts placed in the mammillary region as these grafts extended more rostrally in regions of the lateral hypothalamus at the level of the caudal portion of the median eminence. In those instances, the pattern of GnRH fiber outgrowth to the host tissue suggested that the innervation of the caudal part of the median eminence originated from graft tissue in both the mammillary bodies/posterior hypothalamic grafts and from some fragments in the lateral posterior hypothalamus. Regardless of the dorso-ventral, lateral or rostro-caudal position of the grafts, GnRH axons exited the graft in the direction of the median eminence, often turning medially in its direction. This observation strongly suggested that the GnRH outgrowth to the median eminence is directed by diffusible chemoattractants secreted by the target.

When GnRH neurons were placed at some distance from the median eminence region, in grafts in dorsal regions of the brain, including the dorsal thalamus, the habenula and the foramen of Monroe connecting the lateral and third ventricle, no innervation of the

median eminence by GnRH neurons or gonadal development was observed. In only one instance (QQ05), minor testis development was observed without seminal vesicle growth and without innervation of the median eminence. This absence of median eminence innervation by GnRH cells located dorsally was similar to the results described by Kokoris, in which POA grafts were placed in the lateral ventricle of hpg host mice (Kokoris *et al.*, 1987).

Despite the fact that neither dorsal brain regions nor posterior hypothalamic regions normally contain GnRH neurons, GnRH axons from grafted tissue in these two regions projected differently: axons from dorsally implanted GnRH neurons never reached the median eminence, while caudally implanted GnRH neurons innervated their target. GnRH axons extending from grafts placed in a dorsal location of the brain or within the lateral ventricle might be too far (over 3000 μm) from their target to orient in response to a diffusible chemoattractant, while GnRH neurons in mammillary bodies grafts are located approximately 150 to 250 μm from the median eminence (Franklin and Paxinos, 1997).

The region of the mammillary bodies receive GnRH projections in the rodent (Merchenthaler *et al.*, 1984; Silverman *et al.*, 1994; Witkin *et al.*, 1982). This region is also abundantly innervated by GnRH cells in the grafts placed in the mammillary bodies or the posterior hypothalamus. Certainly, the mammillary bodies and the posterior hypothalamus seems to provide some facilitative environment for GnRH outgrowth. Nevertheless some GnRH axons exiting the graft orient towards the median eminence suggesting that they might respond to diffusible signals. GnRH neurons within grafts located in the third

ventricle targeted the median eminence, inducing stimulation of the pituitary-gonadal axis as described in previous studies (Gibson *et al.*, 1982; 1983; Silverman *et al.*, 1985).

Moreover, the pattern of the GnRH projections from the caudal/ventral grafts suggested that GnRH fibers might orient towards the pia mater or towards the pituitary. In these cases, GnRH axons accumulated either at the base of the mammillary bodies or the graft, with GnRH axons markedly oriented downwards along the borders of the tissue. Growth cones directly accumulated along the ventral surface of the brain facing the pia mater and pituitary. Indeed, *in vitro* studies had inferred that the pituitary might be attractive for GnRH axons, since they innervated pituitary tissue in culture (Wray *et al.*, 1988). *In vivo*, GnRH axons likewise projected to pituitary tissue co-grafted with POA in third ventricle of hpg mice (Saitoh *et al.*, 1992), while others targeted the host median eminence. In lower vertebrate species, such as teleosts, the pituitary is directly innervated by GnRH axons, in the absence of a median eminence (Muske, 1993). However in our experiments, GnRH axons were seen within the pituitary stalk in only one instance. The accumulation of GnRH axons along the surface of the brain facing the pituitary does not strictly confirm an action of the pituitary. GnRH axons have been shown to extend close to the pia mater, containing numerous blood vessels (Witkin *et al.*, 1982). We have observed that GnRH axons seemed attracted by blood vessels as well. Furthermore in our study, this attraction for GnRH axons did not prevent targeting of the median eminence, since both projections occurred in the same brains and were observed often at the same coronal level. This observation is in accord with previous observations, in which GnRH axons in third

ventricular grafts would nevertheless target the median eminence of hypophysectomized hpg mice (Saitoh *et al.*, 1992).

The dramatic accumulation of GnRH terminals in the sub-arachnoid space directly above the pituitary in some animals suggested that the GnRH peptide might be released directly into the CSF to stimulate the pituitary gonadotropes. Nevertheless, GnRH fibers terminated in the median eminence in all animals presenting testicular development. This observation is in accordance with previous studies, in which testicular development was observed with the innervation of the median eminence by GnRH axons originating from third ventricular grafts (Krieger *et al.*, 1982; Silverman *et al.*, 1985). Moreover, PP01 presented a robust testicular development, with a moderate innervation of the median eminence from caudal grafts, but no evidence of axons projecting directly to the pituitary.

Studies using organotypic co-cultures of POA and mediobasal hypothalamus explants, containing the median eminence, demonstrated that GnRH axons' attraction to the mediobasal hypothalamus explant likely derived from chemotropic properties (Chapter 2). In these *in vitro* studies, we demonstrated that the mediobasal hypothalamic region is releasing some diffusible signal attractive for GnRH axons, likely to be of a chemotropic nature. Such a diffusible chemoattractant would likely direct the targeting of GnRH terminals to the median eminence from any site within its range including caudal ectopic locations.

However the same *in vitro* studies have shown that while following factors originating from mediobasal hypothalamic co-explants, GnRH axons grew in association with other axons, labeled with an antibody against growth associated protein 43 (GAP-43). Since this association was observed consistently, we hypothesized that GnRH axons used a population of GAP-43 labeled axons as a substrate for outgrowth.

However when grafts were placed dorsally, GnRH axons (in low numbers) followed bundles of fibers as they extended within the host brain. GnRH axons were then found within the hippocampus, the corpus callosum or the fasciculus retroflexus. The same observation was made by Kokoris with grafts placed in the lateral ventricle (Kokoris *et al.*, 1987). The presence of GnRH fibers within bundles of fibers is not surprising, since in the normal animal extrahypothalamic GnRH axons are found in fiber tracts (Baker *et al.*, 1975; Jennes, 1987; Merchenthaler *et al.*, 1984). GnRH axons extended within the fasciculus retroflexus as they projected from habenular grafts to the nucleus interpeduncularis. This projection has been described *in vivo*; GnRH neurons located in the septal area send axons to and through the habenula, down the fasciculus retroflexus to the interpeduncularis nucleus and the mammillary bodies (Jennes, 1987). Similarly, GnRH neurons within mammillary bodies grafts sent axons upstream in the fasciculus retroflexus, suggesting that this bundle of fibers might provide a facilitative substrate for GnRH outgrowth.

In the basal brain GnRH axons consistently extended and oriented towards the median eminence regardless of the position of the graft. In contrast, GnRH neurons in dorsal grafts were apparently unable to target the median eminence. These observations are

consistent with the hypothesis of a diffusible chemoattractant released by the median eminence to direct the GnRH projections. Furthermore, a facilitative environment within the ventral aspect of the diencephalon seems to play a role to promote GnRH outgrowth.

TABLE 4.1: Detail of GnRH projections to the host hpg brain receiving POA grafts in the mammillary bodies (MB) or the anterior hypothalamus (AH). Animals are separated based on graft locations. Italics indicate location of portions of grafts without detectable GnRH neurons. TW and SVW indicate testis weight and seminal vesicle weigh.

Animal	TW (mg)	SVW (mg)	Number of GnRH cells	Graft location	Projections
PP01	100	180	63	MB, PH	ME, p, rf, IPN
PP20	113	98	162	MB, PH, LH, *IIIV.*	ME, p, IPN, Pit[1]
LL30	14.7	18.5	259	p, MB, PH, LH.	ME, Pit[1]
LL24	35	75	130	p, MB, PH, Th, HB.	MB, Pit[1], ME, PH, Hip, Amy.
PP44	54.1	43.5	85	IIIV., LH, Th, MB	OVLT, AH, ME, MB
QQ06	27.7	20	38	*MB*, IIIV., Th, HB.	ME
PP52	15	14	83	IIIV., LH, MB, *p.*	OVLT, AH., ME, PH, MB, IPN
LL33	15	25	71	IIIV, AH, PH, Th	ME, Amy, Th, HB. AH
LL23	8	42	125	IIIV, TH, LH, *LV.*	AH, Th, ME, PH.
PP42	5	-	60	Th, HB, *LV*, Fm	fr, HB, MB, IPN, Hip.
QQ05	17	12	54	HB, Th , PH, *MB.*	fr, Hip, cc., MB
QQ07	7	-	11	Th, HB, Hip.,	Hip.

List of abbreviations: AH: Anterior Hypothalamic area. Amy: Amygdala. Fm: Foramen of Monroe. HB: Habenula. Hip: Hippocampus. IPN: Inter-peduncularis nucleus. LH: Lateral hypothalamus. LV: Lateral ventricle. ME: Median eminence. MB: Mammillary bodies. OVLT: Organum vasculosum of the lamina terminalis. PH: Posterior hypothalamus. Pit.: Pituitary. IIIV: Third ventricle. Th: Thalamus. cc : Corpus callosum. p : Pons. rf : Fasciculus retroflexus. [1]GnRH axons extend in the direction of the pia mater and the pituitary only.

FIGURE LEGENDS.

Figure 4.1: GnRH neurons are numerous in this graft (G) fragment localized within the mammillary bodies at the base of the brain in LL30. Notice the abundant GnRH innervation of the graft. Pit: pituitary (**A**). A GnRH neuron (arrowheads) migrated within the hpg host brain at caudal levels in PP01. The graft borders are indicated by a dashed line (**B**). In **C**, a GnRH neuron (arrowhead) has migrated close to the mammillary bodies recess (r) in PP01. Notice numerous GnRH axons extending towards the midline at the base of the brain (arrows). Such axons could be followed on adjacent sections as they innervated the caudal median eminence. The scale bar represents 100μm in **A** and **B**. The scale bar in **C** represents 50μm.

Figure 4.2: Schematic representations of successive coronal sections in PP01, illustrating the position of POA grafts (stripped area) located in the mammillary bodies (MB) and the axonal projections (dotted lines) of GnRH neurons. Coordinates relative to bregma are indicated for each section. Notice the innervation of the caudal part of the median eminence (ME) and the presence of the GnRH axons in the lateral hypothalamus. GnRH fibers were seen also in the fasciculus retroflexus (fr) and the nucleus interpeduncularis (IPN). Amy: Amygdala. Hip: Hippocampus. RN: Red nucleus. SN: Substantia nigra.

Figure 4.3: Examples of innervation of the median eminence by GnRH fibers, which issued from cells in grafts located in the mammillary bodies or the posterior

hypothalamus. In **A**, innervation of the caudal median eminence, in PP01, in a coronal section rostral to the micrograph in Figure 4.1 C. Notice how GnRH fibers (arrows) seen in Figure 4.1 C can be identified on the more rostral section as they project to the median eminence (*). V: third ventricle. Innervation of PP20 median eminence is shown in **B, C, D** and **E**. GnRH fibers (arrows) innervate the lateral part of the median eminence. Innervation at the most caudal levels of the median eminence (**B, C** and **E**) appear to originate from GnRH cells in grafts in the mammillary bodies, as they can be followed on adjacent sections. GnRH innervation at more rostral levels (**D**). Scale bar represents 50μm in **A, B, C, D** and **E**.

Figure 4.4: GnRH axons (arrows) accumulated along the ventral surface of the brain facing the pia mater and the pituitary in LL30 as seen in coronal sections. G: graft. Pit: pituitary. GnRH axons seemed to be oriented towards the pia mater and the pituitary in absence of tissue bridges between pituitary and the ventral surface of the brain. The space within the tissue in **A** might have been created by tearing a piece of graft at the time of sectioning. **B** represents a higher magnification of an adjacent section. In **C**, GnRH axons extending directly above the pia mater and the pituitary tissue and oriented towards it, are seen at higher magnification on an adjacent coronal section. Scale bar represents 100μm in **A**, and 50μm in **B** and **C**.

Figure 4.5: GnRH innervation (arrows) of the lateral parts of the median eminence (*) from third ventricular grafts containing GnRH neurons (**A**) in a coronal section of PP44. Notice the position of the graft (G) within the third ventricle containing a GnRH

neuron (arrowhead). High magnification micrograph of a GnRH neuron extending processes wrapping around a blood vessel in PP44 (**B**). Scale bar represents 100μm in **A** and 50μm in **B**.

Figure 4.6: GnRH fibers extended to other locations within the host brain. From caudal grafts GnRH axons often were seen at the level of the pons (**A,** in PP20) or in the nucleus interpeduncularis (**B,** in PP01). GnRH fibers (arrows) often utilized other bundles of fibers when they extended within the host brain. From the caudal grafts in PP01, they extended within the fasciculus retroflexus (**C**). Other projections were seen within the hippocampus from dorsal grafts (**D**) and from rostral third ventricle grafts to the organum vasculosum of the lamina terminalis (**E**). Scale bar represents 50μm in **A**, **C**, **D**, **E** and 200μm in **B**.

Figure 4.1

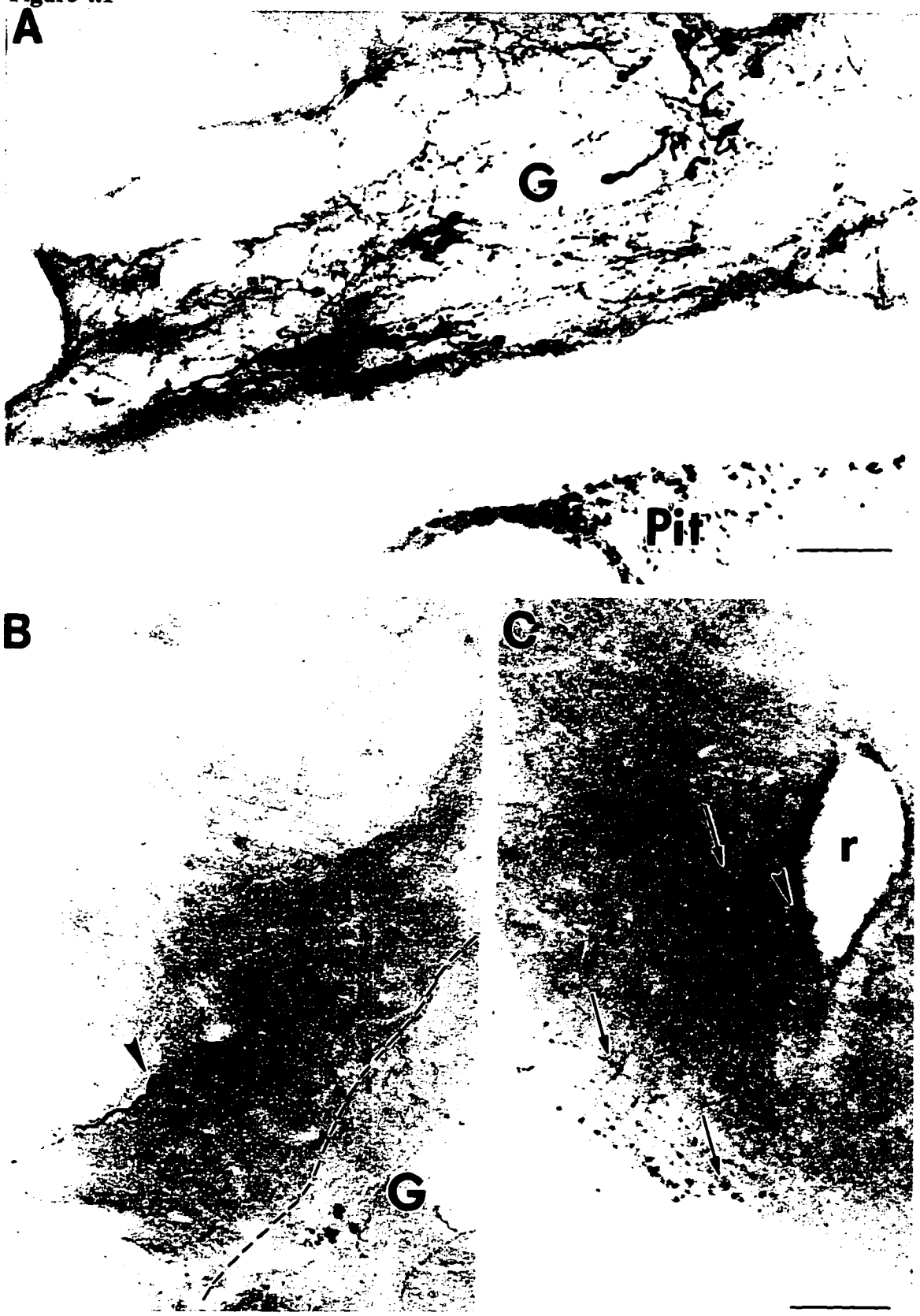


Figure 4.2

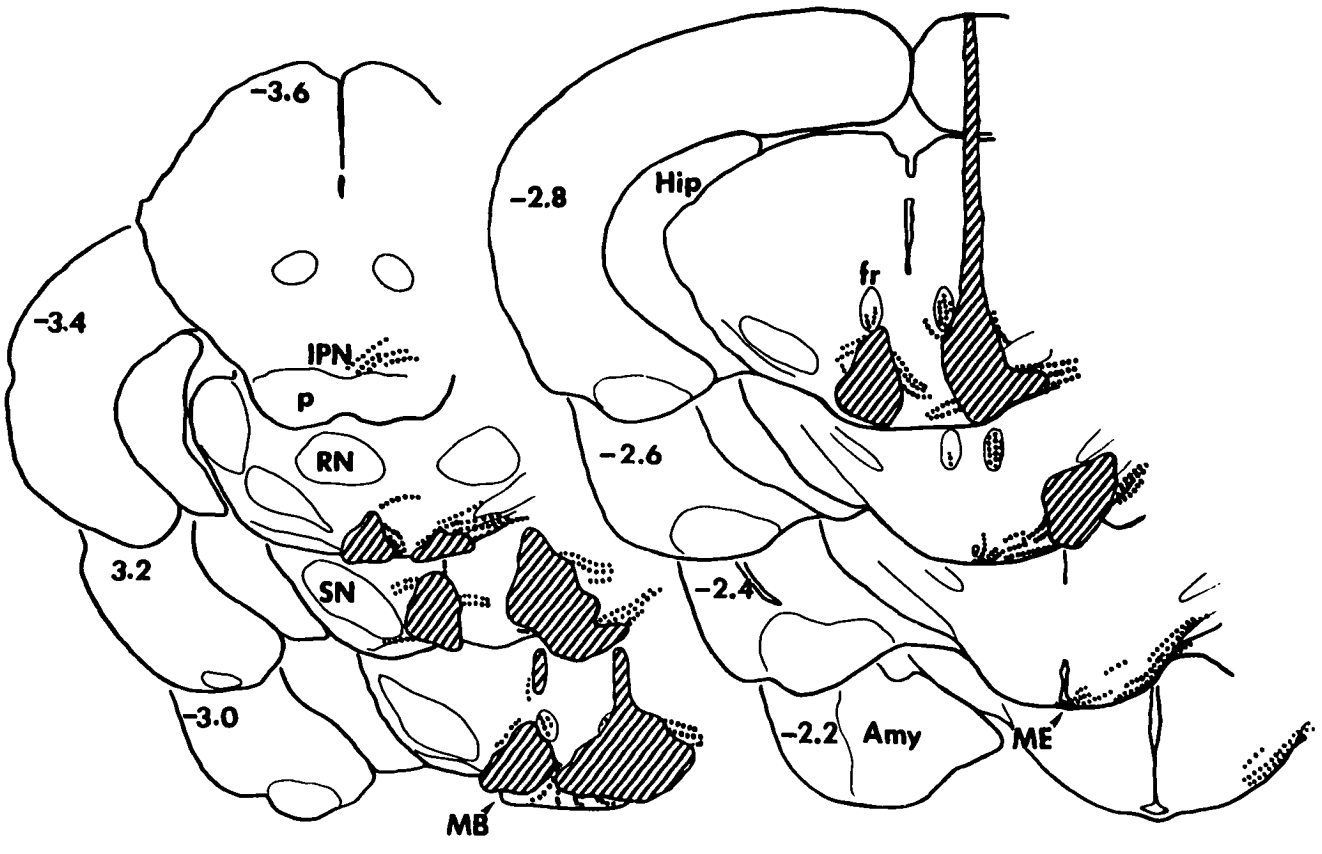


Figure 4.3

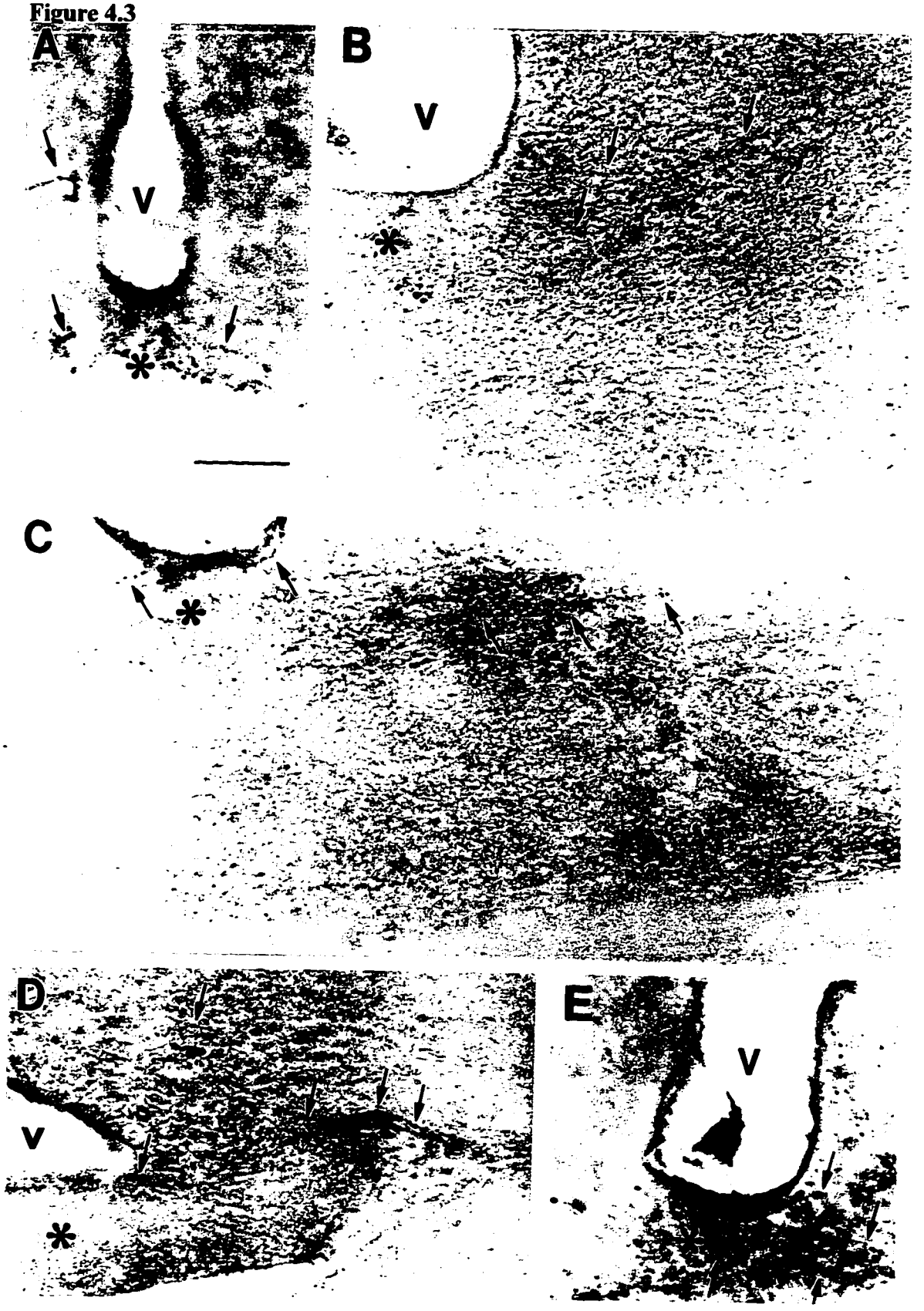


Figure 4.4

Figure 4.5

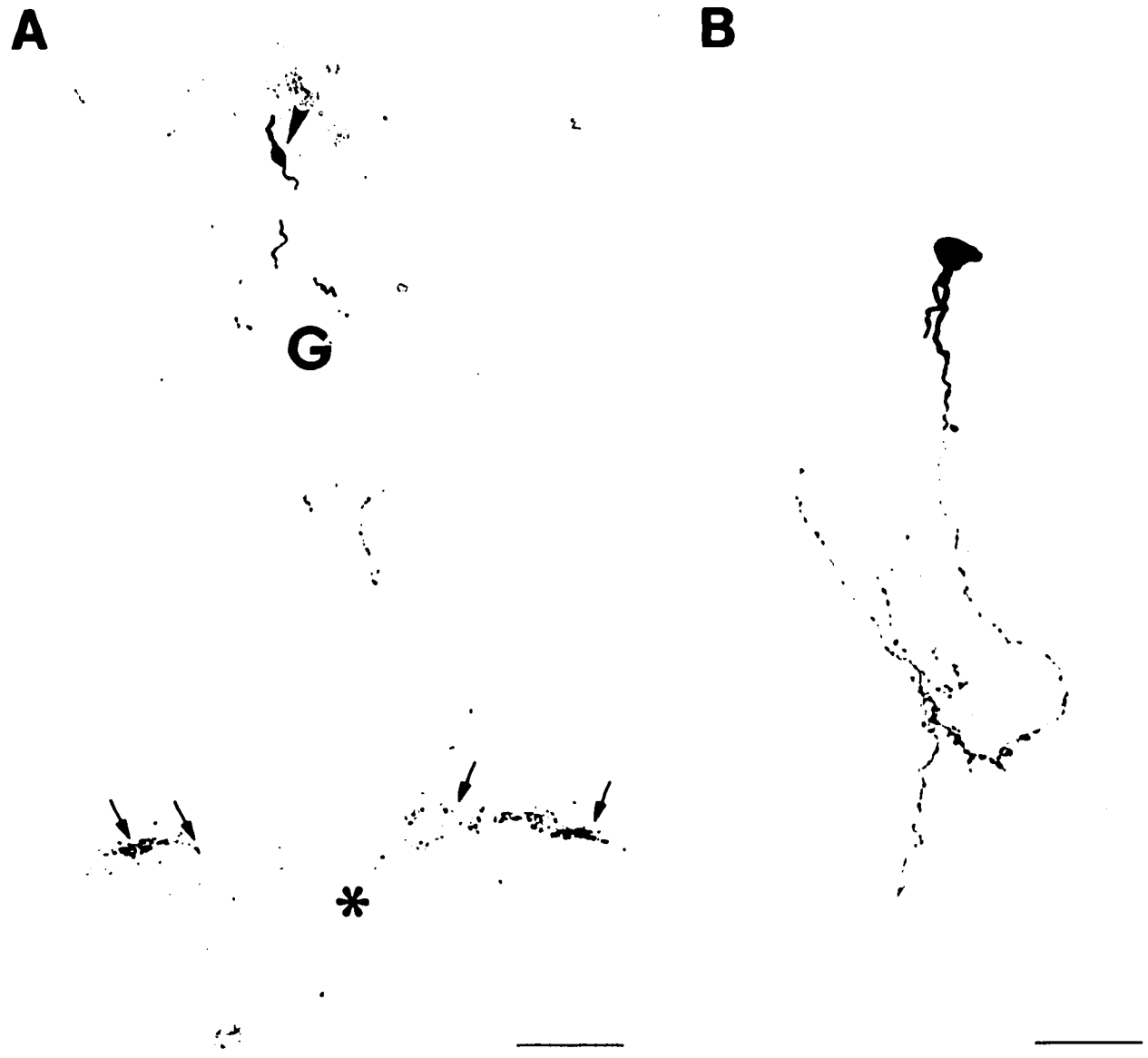
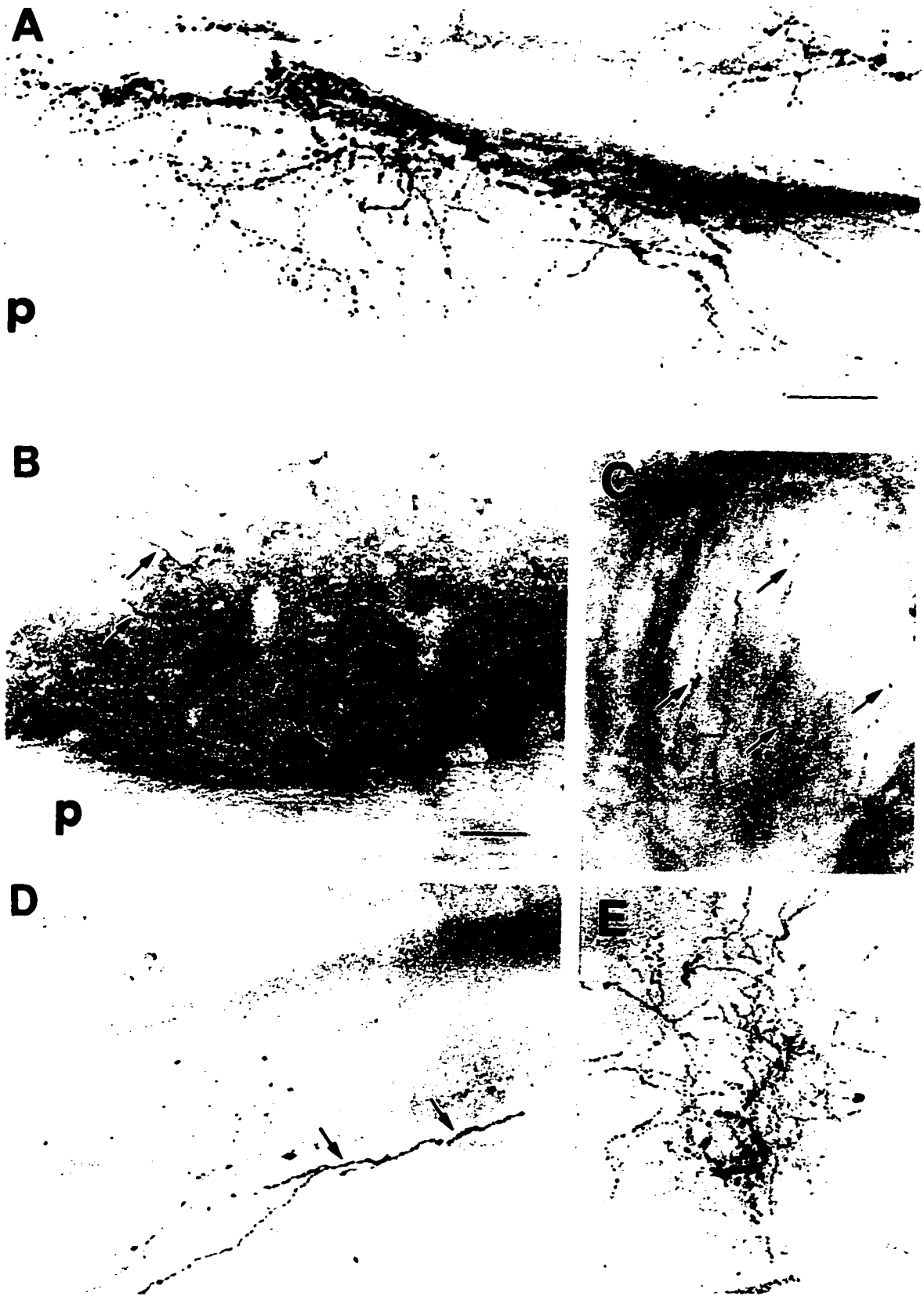


Figure 4.6



APPENDIX A: PRELIMINARY RESULTS USING CO-CULTURES ON COLLAGEN COATED MEMBRANES.

When the project was initially designed to study the targeting of GnRH axons to the median eminence, a technique of organotypic culture in collagen gel matrices as a support embedding the explants was initially chosen, based on the abundant literature describing the targeting of commissural axons towards the floor plate of the developing spinal cord (Placzek *et al.*, 1990a; Tessier-Lavigne *et al.*, 1988). The collagen gel matrices in that case allowed rapid axonal outgrowth as they followed the diffusion of chemoattractant molecules, which have been further identified as the netrin family of chemotropic factors (Kennedy *et al.*, 1994; Serafini *et al.*, 1994). Such techniques are valuable in studying very rapid growth (24 to 48 hours) of a homogenous and numerous population of axons labeled with the lipophilic dye DiI, which does not require immunocytochemical processing. We performed preliminary studies using collagen three dimensional matrices (Wu *et al.*, 1993).

The GnRH neuronal population in the mouse brain includes approximately 800 cells (Wray *et al.*, 1989) and in culture an average of 180 GnRH cell bodies remained in the POA explant. The GnRH axonal outgrowth is not comparable to the number of axons put out by a homogenous neuronal population. Identification of these cells within the explant and axons in the gel required immunocytochemical staining, which could not be performed on whole collagen gel matrix. Therefore sectioning was required, with the consequent loss of

positioning information of the explants as well as of the axonal outgrowth, making further analysis and quantification difficult.

I started to investigate other types of methodology for organotypic cultures which did not require sectioning and could be visualized directly on whole mount preparations. Organotypic cultures in insert chambers allowed good visualization of immunocytochemically stained material without sectioning. We pursued our experiments with the insert chambers and kept collagen as a basic coating substrate for the membrane. These cultures in insert chambers behaved in accordance with the previous studies using similar culture systems, (Buchs *et al.*, 1993; Stoppini *et al.*, 1991; 1993) and are suitable for the study of chemotropic or chemotrophic actions (Erzurumlu *et al.*, 1993; Yamamoto *et al.*, 1989; 1992) or axonal projection formation during development (Li *et al.*, 1993; 1995).

In this section, I will present some of the preliminary results obtained with collagen coated membranes. To improve the GnRH axonal outgrowth on the membrane, laminin was added to the collagen in further experiments as described in Chapter 2, since it had been described as a substrate favorable for axonal elongation (Lander, 1987). I will compare the results of experiments using only collagen to those using collagen and laminin. The experiments using collagen and laminin are extensively described in Chapter 2.

METHODS

Dissections and preparation of the cultures as well as the immunocytochemistry processing are described in Chapter 2. Insert culture chamber membranes (Falcon, 0.45μm pore diameter, P.E.T. transparent membrane) were coated with a rat tail collagen (Type I) solution (3mg/ml, Boehringer-Mannheim) in 0.2% acetic acid or, in other experiments, with a solution of collagen (3mg/ml) mixed with laminin (100μg /ml, Gibco).

RESULTS

Co-cultures on collagen coated membrane.

Local effects of co-cultivated explants on GnRH outgrowth exiting the POA were assessed in the sectors of membrane facing and opposite to the co-explant as described in Chapter 2. There were significantly more GnRH axons exiting the explant in the sector facing the MBH than in the sector away from the MBH ($p<0.01$). The spinal cord and cerebellum had no effect on GnRH axonal outgrowth (Figure 5.1). GnRH axonal outgrowth in the region facing the MBH was significantly greater than in the region facing the CE ($p<0.05$) and not statistically different from that in the region facing the SC.

Since the distribution of GnRH cell bodies within the POA may affect the direction of outgrowth, with more outgrowth seen where the higher number of cells resides, the number of GnRH cells were counted within the POA in the region facing the co-cultivated explant and in the region opposite from it. There was no difference among the cultures in

the number of GnRH cell bodies within the POA in the region facing or away from any of the co-cultures, whether MBH, SC or CE. (Table 5.1).

Comparison with co-cultures on collagen and laminin coated membranes.

As laminin has been shown to be a substrate promoting axonal elongation, POA and MBH or control tissue were cultivated for 7 days on a membrane coated with collagen and laminin as described in Chapter 2.

The total number of GnRH axons extending on the membrane in POA-MBH was significantly higher in the cultures on collagen plus laminin than in the cultures on collagen alone (51.4 ± 4.5; 36.3 ± 3.3 respectively $p<0.01$). The total number of GnRH axons extending on the membrane was not different when the membrane was coated with collagen or with collagen and laminin for the other co-cultures.

The extracellular matrix molecule laminin has been demonstrated to have effects on growth cones and axonal elongation of developing neurons, promoting outgrowth and possibly involving guidance mechanisms (Lander, 1987). Laminin has been shown to promote neurite extension and axonal regeneration in culture of E15 rat basal forebrain axotomized neurons (Schinstine and Cornbrooks, 1988; 1990). Growth cones of sympathetic neurons responded dramatically to the addition of laminin with ultrastructural changes reflecting an acceleration of their growth (Rivas *et al.*, 1992). Laminin coated artificial guideposts provide guidance to growth cones of dorsal root ganglion in culture, as well as increasing their velocity (Kuhn *et al.*, 1995). Other reports have shown that laminin

molecules might be involved in neuronal migration as well, promoting migration (Calof and Lander, 1991; Calof and Reichardt, 1985) or providing the cells with a differential guidance system by a "gated migration" mechanism involving s-laminin (Porter and Sanes, 1995).

In the hpg mouse with graft, laminin immuno-reactive staining was not confined specifically to the region of GnRH outgrowth although it was present on astrocytes in the region of the MBH (Silverman *et al.*, 1991). These findings suggest that while laminin is not the key element involved in the guidance of GnRH terminals, it might act as a substrate enhancing the outgrowth of GnRH terminals in POA-MBH co-cultures.

The presence of laminin had no effect on GnRH fiber outgrowth when spinal cord or cerebellum were the co-explants. When cultivating the explants on a surface of collagen and laminin, GnRH outgrowth in absence of any real chemotropic activity was not enhanced.

TABLE 5.1.

Distribution of GnRH cells in POA explants on collagen coated membranes after 7 days of culture.

The number of GnRH cell bodies (Mean ± S.E.M.) was counted in the region of POA explant facing the co-explant (sector I) or opposite the co-explant (sector II).

Co-explant	Age of tissue	N	GnRH cell number in sector I	GnRH cell number in sector II	Total number of GnRH cells
MBH	E15	56	95.4 ± 6.0	109.0 ± 7.4	203.3 ± 8.2
SC	E15	26	96.1 ± 11.7	100.2 ± 13.6	192.6 ± 13.9
CE	E15	28	106.3 ± 12.7	89.9 ± 10.5	196.2 ± 12.9

Figure 5.1: GnRH axonal outgrowth on collagen coated membranes in POA-MBH, POA-SC and POA-CE co-cultures after 7 days. For the POA/MBH co-cultures, the number of GnRH axons was greater in sector I (towards the explant) (•• = $p<0.01$). In addition, the number of immunoreactive axons on the sector I of the membrane was greater for POA/MBH then for POA/CE pair (**a** = $p<0.05$).

Figure 5.1

APPENDIX B. FURTHER CHARACTERIZATION OF POA-MBH CO-CULTURES.

As I designed organotypic co-cultures of POA with other tissues, the MBH, SC and CE, I performed some experiments designed to understand and further study the properties of these organotypic cultures. Among these experiments, I sectioned one POA-MBH co-culture perpendicularly to the plane of the membrane after this culture was processed for immunocytochemistry for GnRH and counted, to assess thickness of the preparation and efficiency of the antibody penetration within the thickness of the explants. Other experiments involved double labeling of POA-MBH co-cultures with GnRH antibody and with β-endorphin or tyrosine hydroxylase antisera in an attempt to identify the arcuate nucleus within the MBH co-explant. The purpose of labeling the arcuate nucleus was to visualize the morphology of the MBH explant after 7 days in culture.

METHODS

Sectioning an organotypic culture in insert chamber.

POA and MBH explants were cultivated for 7 days and processed for immunocytochemistry for GnRH using the SW1 antiserum as described in Chapter 2. Co-cultures were mounted and analyzed as described in Chapter 2. A culture representative of most POA-MBH co-cultures was chosen based on a good survival of GnRH neurons and

increased GnRH axonal outgrowth on the membrane sector facing the MBH. This POA-MBH culture was washed 72 hours in cold phosphate buffer (PB; 0.1M), to allow the coverslip to detach. The culture was washed three times in PB. The culture was then embedded in solution of 8% gelatin and 30% albumin (bovine serum albumin, Sigma) with care taken to mark the orientation of the POA and MBH explants and allowed to polymerize for 48 hours in contact with 38% formalin fumes. The block was sectioned perpendicularly to the surface of the membrane and in the plane containing both POA and MBH explant at 50 μm with a vibratome. Sections were immediately mounted in order of sectioning on gelatin coated glass slides and coverslipped with Gelmount (Biomedia, Foster City, CA).

Double labeling for GnRH and β-Endorphin or tyrosine hydroxylase.

Organotypic co-cultures of POA and MBH were double labeled for GnRH and β-endorphin or tyrosine hydroxylase to mark the arcuate nucleus within the MBH explant. GnRH was visualized with the anti-GnRH mouse monoclonal antibody (#19304, QED Advanced Research Technologies, San Diego, CA) at a dilution of 1:5000 in 0.2% TX100 and 3% normal donkey serum in PB for 4 days at 4°C. The cultures were incubated overnight in biotinylated anti-mouse secondary antiserum (1:200, made in donkey, Jackson Immunoresearch Labs, West Grove, PA) and the avidin-biotin reaction was carried out as described above with visualization using Texas-red-Streptavidin, as described in Chapter 2. The β-Endorphin was visualized using a rabbit-polyclonal antiserum (ICN) at a dilution of 1:2000 in 0.2% TX100 and 3% normal donkey serum for 7 days at 4°C, followed by an

anti-rabbit antiserum conjugated to FITC overnight (1:200, made in donkey, Jackson Immunoresearch Labs, West Grove, PA). Tyrosine hydroxylase was visualized using a rabbit-polyclonal antiserum (Chemicon) at a dilution of 1:5000 in 0.2% TX100 and 3% normal donkey serum for 7 days at 4°C, followed by an anti-rabbit antiserum conjugated to FITC overnight as described above.

RESULTS

Observation of sectioned organotypic culture in insert chamber

Sectioning preserved the integrity of the membrane and the tissue remained attached to the surface of the membrane. The thickness of the explants was approximately 150 to 200μm after 7 days in culture. Immunocytochemistry for GnRH revealed GnRH cell bodies within the entire thickness of the POA explant, indicating that the cells survived through the entire explant and that the antibody was penetrating the tissue. GnRH axons were seen spanning the entire extent of the explants. A few GnRH axons were seen also extending on the surface of the membrane mostly in the section of membrane between POA and MBH.

Immunocytochemistry for GnRH and β-endorphin

β-Endorphin cell bodies were detected within the MBH explant. They were grouped in two nuclei resembling the arcuate nucleus. No β-endorphin cell bodies were seen within the POA explant. β-Endorphin axons were abundant within the MBH explant. They also extended onto the membrane around the MBH explant.

Immunocytochemistry for GnRH and tyrosine hydroxylase.

Tyrosine hydroxylase immunoreactive cell bodies were abundant in POA and MBH explants. In the MBH explant the arcuate nucleus was easily recognizable, with the ventricle visible as a background autofluorescence. Within the POA explant, tyrosine hydroxylase neurons were also grouped in two nuclei, likely the zona incerta (A13) or A14 or part of the periventricular tyrosine hydroxylase cell bodies. Tyrosine hydroxylase axons were seen in the culture as they extended abundantly from the explants onto the membrane.

SUMMARY

During embryonic development, neurons extend axons ending in growth cones to specific targets where they establish synaptic contacts. Mechanisms directing these highly specific projections involve guidance cues derived from the environment of elongating axons, which influence pathfinding of the growth cones (Goodman, 1994; 1996; Goodman and Shatz, 1993; Goodman *et al.*, 1993; Tessier-Lavigne and Goodman, 1996). The guidance cues directing elongating axons to their target can be of differing natures, with several types of mechanisms acting in concert to assure the specificity of the targeting. The pioneer axons, the first axons to extend over long distances, might use intermediate targets to orient in the developing brain as described extensively in the grasshopper embryo (Bentley and Caudy, 1983; Bentley and Toroian Raymond, 1986; Caudy and Bentley, 1986). Their orientation depends on contact mediated guidance with guidepost cells, serving as intermediary targets (O'Connor *et al.*, 1990). Other axons might develop later, when other axonal pathways are already established, using these as tracts for their projection (Goodman, 1996; Grenningloh and Goodman, 1992). Such mechanisms of guidance may use contact-mediated interactions with the environment, involving non-diffusible molecules on the cell surface or extracellular molecules acting as substrates permissive for axonal outgrowth (Bixby and Jhabvala, 1990; Calof and Reichardt, 1985; Carbonetto *et al.*, 1987; Chang *et al.*, 1987; Hammarback *et al.*, 1988; Kuhn *et al.*, 1995; Lander, 1987; Lemmon *et al.*, 1989; Neugebauer *et al.*, 1988). In addition, cell surface

substrates or extracellular molecules might inhibit axonal outgrowth, therefore orienting elongating axons away from unwanted pathways (McKeon *et al.*, 1991; 1995; Pindzola *et al.*, 1993; Silver, 1994; Snow *et al.*, 1990; 1991; Tessier-Lavigne, 1995; Westerfield, 1987). This type of guidance acts over short distances. Other guiding mechanisms involve long-range chemoattraction, in which diffusible substances, secreted by the target, act at a distance to orient and attract the elongating axons (Colamarino and Tessier-Lavigne, 1995b; Davies, 1994; Tessier-Lavigne, 1992; Tessier-Lavigne *et al.*, 1988). Netrin was the first identified diffusible chemotropic factor, attractive for commissural axons, isolated from the ventral floor plate of the spinal cord (Kennedy *et al.*, 1994; Serafini *et al.*, 1994). Other long range guiding diffusible factors involve chemorepellents (Pini, 1993; 1994), such as netrin, which is repellent for another set of axons, the trocheal motor axons (Colamarino and Tessier-Lavigne, 1995a), or the semaphorins family (Kolodkin *et al.*, 1993; Matthes *et al.*, 1995; Messersmith *et al.*, 1995).

The experiments described in this dissertation were designed to understand some of the mechanisms underlying the projection of GnRH axons to the median eminence. GnRH projection to the median eminence is critical to the perpetuation of the species, since this neuroendocrine cell releases in the hypophyseal portal circulation the peptide controlling the secretion of luteinizing hormone and follicle stimulating hormone by the gonadotropes of the pituitary. It was previously demonstrated that the GnRH neurons required the guidance from the mediobasal hypothalamus environment, since GnRH neurons of the accessory olfactory bulb, which do not normally project to the median eminence, would do

so when placed in the third ventricle of hpg mice (Perlow *et al.*, 1987). Therefore, GnRH axons follow some guidance from the environment as they project to the median eminence. The specificity of the GnRH projection to the median eminence *in vivo* during normal embryonic development or from embryonic preoptic area (POA) grafts to the adult hpg host median eminence suggested the presence of diffusible substances released from the median eminence region directing the GnRH axonal outgrowth as described in Chapter 1 (Introduction). We have developed a system of organotypic cultures to test whether GnRH axons followed attractive cues from the median eminence region. Moreover, while allowing various manipulations, organotypic cultures permitted study of the nature of the mechanisms involved in the GnRH targeting to the median eminence: to test the hypothesis that GnRH axonal projection is guided by the long range action of diffusible signals released by the median eminence, to verify the specificity of these signals, and to assess the importance of contact-mediated interactions of elongating GnRH axons. These experiments were followed by additional *in vivo* experiments in which POA grafts, containing GnRH neurons were placed in an ectopic position within the mammillary bodies, to test the hypothesis that diffusible factors could direct GnRH axons to the median eminence from a caudal location in which there are no GnRH neurons normally.

The hypothesis of a diffusible guiding signal released from the median eminence implies that a concentration gradient of chemoattractive substances, followed by elongating GnRH axons, is established from the target. In the present studies, GnRH axons extended in higher numbers and over farther distances from the POA facing the mediobasal

hypothalamus (MBH) than away from it in organotypic cultures, when POA and MBH explants are co-cultivated on the surface of collagen (Appendix A) or of collagen and laminin coated membranes in the very stringent conditions of a defined medium (Chapter 2). Experiments in 'inverted' chambers (Chapter 3) supported the hypothesis that this attraction was due to the diffusion of factors from the MBH, since a porous physical barrier between POA and MBH did not prevent the establishment of some of this outgrowth pattern. Moreover time-course experiments (Chapter 2) showed that the GnRH preferential outgrowth to the MBH was established as early as 4 days in culture and maintained for up to 10 days in culture, suggesting that the mediobasal hypothalamus actively released attractive cues rather than carrying explanted blood borne factors from the embryo. Such a rapid GnRH axonal outgrowth towards the MBH co-explant lasting for 10 days suggested that the outgrowing axons followed attractive cues, rather than being maintained preferentially by trophic factors upon arrival to their target. As the distance between explants did not influence the GnRH outgrowth facing the MBH co-explant, we concluded that GnRH seemed to follow a gradient of concentration of attractive substances from the MBH, based on observations by Lumsden (Lumsden and Davies, 1983) that axons would grow upward of a chemotropic concentration gradient, regardless of their distance from the target.

The preferential GnRH outgrowth facing a co-explant was specific to the MBH, since neither spinal cord or cerebellum exerted such an effect. Moreover the cerebellum seemed to have some repulsive action on GnRH axons, since these extended farther from

the POA explant on the side facing away from the cerebellum (Chapter 2). The action of the MBH did not influence the general axonal outgrowth, as labeled by growth associated protein 43 (GAP-43), a protein expressed in most neurons in the course of elongating axons during development (Goslin and Banker, 1990; Goslin *et al.*, 1988; 1990). This demonstrated that the action of the MBH is at least specific for GnRH axons, but does not exclude other neuroendocrine cells' projecting to the median eminence.

As GnRH projections appeared to be directed by diffusible chemoattractant substances released by the mediobasal hypothalamus region, we asked whether GnRH axons might also require some contact-mediated guiding cues in addition to a long range chemoattractant activity. In chapter 2, I evaluated the role of cell-contact mediated guidance, including interactions with glial elements which seemed to be involved in the guidance of GnRH axons to the median eminence. It had been suggested that GnRH axons traveled within glia channels through the mediobasal hypothalamus and that these channels might be involved in the guiding of GnRH projections to the median eminence (Kozlowski and Coates, 1985). It is recognized that axonal outgrowth is guided by interaction with glial environment in many systems (Silver, 1993; Silver *et al.*, 1993). The observation that GnRH axons align on tanycytes processes, a specialized ependymal cell, within the median eminence, likely involved in functional regulation (King and Rubin, 1994), supported this hypothesis. However, when POA grafts derived GnRH axons were found associated with glial processes within the hpg host, it was difficult to infer that these glial processes were actually involved in the guidance, since the degree of reactive gliosis following the graft

implantation was substantial and not only confined to the mediobasal hypothalamus region (Silverman *et al.*, 1991). I have here demonstrated that associations of GnRH axons with glial elements are not necessary for the proper extension of GnRH projections towards the mediobasal hypothalamus. Indeed, GnRH axons travel over long distances on the membrane of insert chambers without the presence of tanycytes or glial cells.

In contrast, GnRH axons were associated with other axons immunoreactive for GAP-43. GAP-43 axons might provide a facilitative substrate in such stringent conditions of culture. Another alternative is that GnRH axons might follow a sub-population of GAP-43 axons itself attracted to the MBH. If this was the case, the MBH then would provide guiding clues to such a population of unknown axons.

The presence of the median eminence was evaluated within the mediobasal hypothalamus as dissected for the organotypic co-culture. The arcuate nucleus was identified within the MBH explant, indicating that the dissection of the MBH contained the region of the median eminence (Appendix B). Moreover labeling for glial markers indicated the presence of a palisade structure containing tanycytes, reminiscent of the median eminence structure, with numerous GnRH axons projecting to it and aligning along the tanycytes processes (Chapter 2). It is difficult to confirm that these GnRH axons are all originated from cells in the POA explant, since a few GnRH cells remain within the MBH explant. However, several GnRH axons were seen crossing onto the surface of the membrane from the POA to the MBH, indicating that a few GnRH axons might extend to the median eminence.

In Chapter 4, I demonstrated that GnRH axons can target the median eminence, when the embryonic POA graft is placed in an ectopic location, the mammillary bodies. However, when grafts are placed in locations dorsal to the third ventricle, including the lateral ventricle, GnRH neurons did not project to the median eminence as had also been described by Kokoris (Kokoris *et al.*, 1987). The guidance of GnRH axons from caudal locations such as the mammillary bodies supported the hypothesis that the signals originating from the median eminence are diffusible, since they can direct GnRH axonal outgrowth from a caudal but nevertheless close location.

The nature of these diffusible substances remains unknown. Further studies are under way to determine the cell type within the median eminence secreting such factors. The presence of fenestrated capillaries in circumventricular organs lacking blood brain barrier, such as the median eminence or the organum vasculosum of the lamina terminalis (OVLT), adds an interesting dimension to the GnRH projection. Indeed, GnRH axons have a tendency to be attracted by blood vessels in the brain, as I observed *in vivo* with grafts in the mammillary bodies or in the third ventricle of hpg hosts, where GnRH axons wrapped around some blood vessels or extended in the direction of the pia mater. A similar phenomenon was often observed in the normal animal (Witkin *et al.*, 1982). It is possible that endothelial cells, lining the blood vessels, release some attractive factors for GnRH axons. Such factors would be made available to guide GnRH axons in higher quantities in circumventricular organs due to the fenestrated nature of their capillaries, allowing easier diffusion of chemoattractive substances into the parenchyma.

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