Ontogeny of Galanin in the Mouse:

Galanin-Like Immunoreactivity in the Developing Mouse From Embryonic Day 10 to 15

By
Melissa Theresa Jones

A Thesis
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Department of Human Anatomy and Cell Science
University of Manitoba
Winnipeg, Manitoba

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I dedicate this thesis to my family for their endless

support and love.

Abstract

The Ontogeny of Galanin in the Mouse Embryo: Galanin-like immunoreactivity in the developing mouse embryo from Embryonic Day 10 to 15

Galanin is a highly conserved, 29-30 amino acid long putative neuropeptide widely distributed throughout the central and peripheral nervous systems of several adult species. This protein has been shown to affect the secretion of several hormones and the proliferation of some small cell lung cancer and lactotroph cell lines. Galanin is also expressed by certain types of neuroendocrine tumors. These findings suggest that galanin could be a mitogen. Mitogens cause cell proliferation in the normal development in the embryo for growth of tissues. Therefore, galanin may be expressed during development of the embryo. The purpose of this study was to determine the ontogeny of galanin in the mouse embryo from embryonic day 10 (E10) to embryonic day 15 (E15). We hypothesize that galanin-like immunoreactivity (GAL-LI) is present during this stage of development, when organogenesis is occurring. Using immunohistochemistry, GAL-LI was detected on sections of paraffin-embedded CD mouse embryos. At E10, GAL-LI was observed very abundantly in the outflow tract and endocardial cushions of the heart as well as in the mesenchyme surrounding the neural tube. From E12, GAL-LI could also be detected in the tissue associated with several bones, including the vertebrae, nasal capsule, and basioccipital and basisphenoid bones. It is of interest that in the adult bone, there is no expression of galanin, indicating the importance of galanin to the developing bone. GAL-IR cells are also located in the gastrointestinal tract, including the esophagus and midgut, by E13. From E13, the genitourinary system, including the ovary and

metanephros (definitive kidney), also contain GAL-LI. These results suggest that galanin is expressed in tissues derived from mesoderm and neural crest sources and is involved in the development of the heart, skeleton, gut, and genitourinary system of the mouse. These data, along with the high degree of conservation of galanin among species, imply that galanin is an important modulator of embryonic development.

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GAL-LI	-galanin-like immunoreactivity
GAL-IR	-galanin-immunoreactive
GMAP	-galanin message associated peptide
GAL-R#	-galanin receptor subtype number #
GALP	-galanin-like peptide
TPA	-10-O-tetradecanoylphorbol-13-acetate
DRG	-dorsal root ganglia
GH	-growth hormone
LH	-luteinizing hormone
PRL	-prolactin
ACTH	-adrenocorticotropic hormone
E#	-embryonic day #
P#	-postnatal day #
Mas-MIP	-myoinhibitory peptide from *Manduca sexta*
PLC	-phospholipase C
5-HT	- 5-hydroxytryptamine
BMP	-bone morphogenic protein
FGF	-fibroblastic growth factor
IGF	-insulin-like growth factor
Shh	-sonic hedge hog protein
H&E	-hematoxylin and eosin
VIP	-vasoactive intestinal peptide

1.0 Introduction

1.1 Galanin and its properties

Galanin was discovered in 1983 by the Swedish group of Dr. V. Mutt while isolating a broad spectrum of proteins from porcine intestine that had the common attribute of C-terminal amidation. It was named for its N-terminal glycine and its C-terminal alanine (Tatemoto *et al.* 1983). Only the 30 amino acid long human galanin protein is not amidated at its C-terminal end (Bersani et al 1991a, Evans et al 1991, Schmidt et al 1991).

The rat galanin gene has 6 exons and 5 introns and encodes a 123-124 amino acid preprohormone containing the galanin protein (Kaplan et al 1991). The mRNA of this precursor is arranged in a similar manner among species such as the cow, rat, human, and pig, such that the preprogalanin protein begins with a hydrophobic sequence of about 20 amino acids that is likely a signal sequence used to direct transport of the precursor to the endoplasmic reticulum. Amino acids 33 through 62 of preprogalanin correspond to galanin, which is flanked by lysine-arginine residues. The last 59 amino acids make up the galanin message-associated peptide (GMAP) (Vrontakis et al 1987, Rökaeus et al 1988, Kaplan et al 1988a, Rökaeus et al 1986). Galanin and GMAP are therefore theoretically produced in a 1:1 ratio.

Thus far, the galanin amino acid sequences of nine species have been determined (Bedecs *et al* 1995). The first 15 amino acids are fully conserved and the rest of the protein has up to six amino acid substitutions. Though 24 out of 36 amino acids of GMAP are conserved, overall, it is less conserved than galanin. However, the overall acidic nature of this protein is conserved, suggesting that its function is conserved as well

(Bedecs *et al.* 1995). For example, the amino acid sequence of the galanin protein (and its signal sequence) of the rat is completely homologous to that of the mouse, and the nucleotide sequence of the galanin gene of the mouse has only one substitution when compared to the sequence of the rat. The mouse GMAP has only 5 amino acid substitutions when compared to the rat GMAP protein (Lundkvist et al 1995).

Because the first 15 amino acids of the mature galanin protein are conserved in all the species studied so far, it is possible that this part may interact with a receptor that is also conserved. In many cases, the first 16 amino acids have been shown to be sufficient for full agonist activity, binding to the galanin receptor with an affinity comparable to that of full-length galanin (Amiranoff et al 1989, Crawley 1990, Fisone et al 1989, Land et al 1991, Xu et al 1990). Even shorter fragments of galanin can be sufficient to mimic the effect of the peptide. For example, the first 10 amino acids of galanin have been shown to cause contractions in rat smooth muscle preparations (Ekblad et al 1985). Any small fragments of galanin would probably be less stable than the full-length molecule since the full-length peptide adopts a horseshoe-like conformation, forming a β-turn around the proline[13]. This arrangement protects the N-terminal portion from proteolytic degradation (Bedecs et al 1995, Kulinski et al 1992, Rigler et al 1991).

In addition to fragments of full-length galanin, variant forms of galanin have been discovered that are also possible ligands for the galanin receptors (Bauer et al 1986a,b, Michener et al 1990, Bersani et al 1991a,b, McDonald et al 1992, Sillard et al 1992). These variant forms include elongated forms, such as gal(-9-29) and gal(-7-29) that were found in the adrenal medulla of the pig (Bersani et al 1991b). In the porcine brain, gal(-9-29) was found together with a truncated form of the protein, gal(5-29) (Bedecs *et al.*

1994). Thus, alternate forms of galanin do exist that can potentially mimic (an agonist) or block (an antagonist) the known effects of galanin.

Proteins with partial homology to galanin may also interact with the receptors of galanin and have similar biological effects. Galanin has been considered a unique peptide, showing no homology to other peptides. However, recently, Mas-MIP I and II (nine amino acids long) were discovered in the ventral nerve cord of the tobacco hornworm, *Manducta sexta*. Their amino acid sequences resemble the sequence of the first seven amino acids of galanin (see below). The function of these peptides may be similar to galanin since they affect ileal peristalsis in *Manducta sexta*, whereas in vertebrates galanin affects smooth muscle contractions in various ways, depending on the tissue being studied (Blackburn *et al.* 1995).

Mas-MIP I A W Q D L N S A W-amide

Mas-MIP II G W Q D L N S A W-amide

Galanin G W T L N S A...

Another protein that may be related to galanin was extracted from a pool of porcine hypothalamic tissue. Amino acids 9 to 21 of galanin-like peptide (GALP) match the first 13 amino acids of the galanin protein. The C-terminus of this protein is not amidated. GALP proteins were also deduced for the human and rat. Interestingly, this new protein binds to the second subtype of the galanin receptor, GAL-R2 (Ohtaki *et al.* 1999). Since this protein, as well as the synthetic galanin antagonists, have the first 13 amino acids of galanin, it is possible that they also adopt a horseshoe-like conformation and bind to the galanin receptors in a manner similar to galanin. It may be that galanin, including truncated and elongated forms, GALP, and Mas I and II are all members of the

same family of proteins. It is also possible that each member of this family binds to the various subtypes of the galanin receptor to exert effects that have, so far, only been attributed to galanin.

1.2 Galanin receptors

The galanin receptors characterized so far are coupled to G-proteins from a G_i / G_o-subfamily of transducing G proteins. The G-proteins mediate the effects of the effectors coupled to the galanin receptor, such as adenylate cyclase, phospholipase C, and potassium and calcium channels. Binding affinity of galanin is influenced by the presence of GTP and pertussis toxin. However, the mitogenic effect of galanin on small cell lung cancer (SCLC) cells, and the effect of galanin on intracellular levels in these cells, is insensitive to pertussis toxin and therefore may be mediated by a galanin receptor independent of pertussis toxin sensitive G proteins (Sethi *et al.* 1991, Seufferlein *et al.* 1996).

Through functional binding studies, three subtypes of the galanin receptor have been found in the adult rat and man. Each subtype is highly conserved across species, and yet, within each species, the receptor subtypes are less homologous. The distribution throughout the central and peripheral nervous system has *not* been found to perfectly overlap between the species (Kask *et al.* 1997, Wang *et al.* 1997, Kolakowski *et al.* 1998). Also, galanin receptors have also been detected in the embryo. For example, Xu et al found GAL-R1 receptors in the rat embryo from as early as E17 (Xu et al 1996).

1.3 Distribution of galanin in the adult

Galanin is present in the central and peripheral nervous systems of all of the species studied so far. Galanin expression is particularly strong in the arcuate, paraventricular, and supraoptic nuclei of the hypothalamus, in the median eminence, and in the posterior pituitary. The medulla, pons, and midbrain also express galanin, as do the superficial laminae of the spinal cord. The protein has also been localized to the nerve fibers of the respiratory tract, gastrointestinal tract, and genitourinary tract. Galanin is also present in non-neural tissues, such as in the anterior pituitary when under the influence of estrogen. The chromaffin cells of the adrenal gland also contain galanin (Vrontakis et al 1991), although the level of expression varies considerably between species. For example, most of the chromaffin cells of the adrenal gland of the chicken express galanin, but it is difficult to detect it in the rat adrenal gland (Zentel et al 1990). (For a detailed description of galanin distribution in the adult, please see table 1.)

Galanin distribution in the adult						Species				
organ/area	rabbit	sheep	pigeon	man	non-human primates	dog	cat	cow	pig	guinea pig
conceptus/ placenta				IH						
anterior pituitary				IH					RIA	
brain		O		IH, RIA	IH				IH,RIA, ISHH	
spinal cord				IH, RIA					IH,RIA, ISHH	
dorsal root ganglia				RIA					RIA	
sympathetic ganglia					ISHH,RIA	ISHH, RIA	IH-			
respiratory tract						RIA, IH	IH		IH,RIA	RIA,IH
thyroid		IH-					IH-	IH-	IH-	IH-
adrenal glands	O		IH	IH, RIA		RIA, O	RIA, O	IH, RIA, ISHH, NB	IH,RIA, ISHH, NB,O	IH,O
urinary bladder				IH, RIA					RIA-	
genital tract	IH, RIA							IH	RIA-	
pancreas				IH	IH,RIA	IH, RIA			RIA-	
GI tract				IH, RIA		RIA		O	IH, ISHH, RIA	IH-,RIA
liver						IH			RIA-	
kidneys									RIA-	
heart	RIA					RIA	RIA		RIA-	RIA
spleen										

Galanin distribution in the adult (cont'd)						
organ/area	rat	mouse	duck	chicken	hamster	References
conceptus/ placenta	NB, ISHH					Vrontakis *et al.* (1992), Graf *et al.* (1996)
anterior pituitary	IH					Bretherton-Watt *et al.* (1990), Hokfelt *et al.* (1992), Verchere *et al.* (1994), Vrontakis *et al.* (1990), Steel *et al.* (1989)
brain	IH,RIA, ISHH					Kaplan *et al.* (1988b), Michener *et al.* (1990), Skofitsch *et al.* (1986), Hokfelt *et al.* (1992), Rokaeus (1987), Sillard *et al.* (1991), Kasa *et al.* (1996)
spinal cord	IH,RIA					Michener *et al.* (1990),Skofitsch *et al.* (1986), Rokaeus (1987)
dorsal root ganglia	RIA,IH, ISHH					Michener *et al.* (1990), Skofitsch *et al.* (1985), Hao *et al.* (1999), Hokfelt *et al.* (1987), Kasiba *et al.* (1992), Villar *et al.* (1989)
sympathetic ganglia	IH-, ISHH, RIA,IH	IH-				Krummer (1987), Verchere *et al.* (1994), Longley *et al.* (1993), Mohney *et al.* (1994)
respiratory tract	IH,RIA, NB-			IH		Kaplan *et al.* (1988b), Cheung *et al.* (1985), Rokaeus (1987), Bretherton-Watt *et al.* (1990), Luts *et al.* (1989), Dey *et al.* (1993)
thyroid	IH	IH		IH-		Grunditz *et al.* (1987)
adrenal glands	IH,NB-, ISHH-		IH	IH-	IH	Kaplan *et al.* (1988b), Zentel *et al.* (1990), Rokaeus (1987), Fried *et al.* (1991), Bauer *et al.* (1986b), Bretherton-Watt *et al.* (1990), Rokaeus *et al.* (1988), Torsello *et al.* (1992)
urinary bladder	IH,RIA					Bretherton-Watt et al.(1990), Rokaeus (1987), Dixon et al.(1998)
genital tract	IH,RIA, NB, ISHH					Bretherton-Watt *et al.* (1990), Stjernquist *et al.* (1988), Rokaeus (1987), Lakomy *et al.* (1995), Torsello *et al.* (1992)
pancreas	IH, RIA, NB-, ISHH-					Kaplan *et al.* (1988b), Bretherton-Watt *et al.* (1990), Hokfelt *et al.* (1992), Rokaeus (1987), Taborsky *et al.* (1999), Ahren *et al.* (1991), Verchere *et al.* (1996), Torsello *et al.* (1992)
GI tract	IH,RIA, NB, ISHH	IH		O		Kaplan *et al.* (1988b), Bauer *et al.* (1986a), Hokfelt *et al.* (1992), Bishop *et al.* (1986), Rokaeus (1987), Norberg *et al.* (1991), Rokaeus *et al.* (1988), Torsello *et al.* (1992)
liver	NB					Kaplan *et al.* (1988b),Bretherton-Watt *et al.* (1990), Taborsky *et al.* (1999)
kidneys	NB					Kaplan *et al.* (1988b),Bretherton-Watt *et al.* (1990)
heart	RIA,IH	RIA				Bretherton-Watt *et al.* (1990), Xu *et al.* (1995)
spleen	NB					Kaplan *et al.* (1988b)

Table 1: Distribution of galanin in the adult.

The presence of galanin mRNA and/or GAL-LI in the adult of several species using various methods of detection, i.e. RIA= radioimmunoassay, IH= immunohistochemistry, NB= Northern blot, ISHH= in situ hybridization histochemistry, O= other. A (-) next to the method of detection denotes negative results for the presence of galanin.

1.4 Galanin expression

The most distinctive feature of galanin gene expression is its dramatic upregulation by estrogen, primarily in the rat anterior pituitary. Although galanin is expressed in somatotrophs and thyrotrophs of the rat pituitary, estrogen treatment dramatically increases galanin protein and mRNA levels in lactotrophs (Steel et al 1990, Vrontakis et al 1989). Conversely, the removal of the ovaries in female rats decreases galanin levels in the pituitary (Kaplan et al 1988a, O'Halloran et al 1990). Also, during the 4 day long estrus cycle of the rat, galanin expression in the pituitary changes with fluctuating estrogen levels in the blood (Kaplan et al 1988a, 1991). Galanin expression in the hypothalamus of the rat is less dramatically affected by estradiol, showing that the magnitude of changes in galanin expression in response to the same steroid can differ depending on the tissue being considered (Kaplan et al 1991). The effect of a steroid can also depend on the species since galanin is only expressed in the corticotrophs of the human pituitary, with or without estrogen treatment (Vrontakis *et al.* 1990).

The glucocorticoid dexamethasone also causes an increase in galanin mRNA levels within the anterior pituitary of the rat, but its effects are otherwise different from those of estrogen. Its effects are more sustained in the pituitary than those of estrogen and are not accompanied by an increase in galanin-like immunoreactivity (GAL-LI). Dexamethasone also causes an increase in galanin mRNA levels in the uterus (Vrontakis *et al.* 1996). Therefore, estrogen and dexamethasone likely exert similar effects on galanin expression through different mechanisms.

Thyroid hormone influences galanin expression as well. Hypothyroidism in the rat results in decreased galanin mRNA levels in the pituitary and a decreased ability of estrogen to induce galanin expression (Hooi *et al.* 1997).

In the adrenal gland, forskolin and 12-O-tetradecanoylphorbol-13-acetate (TPA) both cause increased mRNA levels in bovine chromaffin cells (Rökaeus et al 1990), and a TPA responsive element was found associated with the galanin gene (Anouar *et al.* 1994).

Axotomy of sympathetic, sensory, and motor neurons alters expression of galanin dramatically. For example, although the small cells of the DRG of the rat normally express low levels of galanin, axotomy or crushing of the sciatic nerve leads to strong expression in the medium and large cells within the DRG as well (Hökfelt *et al.* 1987, Nahin *et al.* 1994). It was determined that, of those DRG cells that upregulate galanin expression after axotomy, most have unmyelinated axons, while only a small subpopulation have myelinated axons (Noguchi *et al.* 1993). Axotomy of the sciatic nerve also leads to increased galanin expression in the dorsal and ventral horns of the spinal cord on the affected side, at the level corresponding to the sciatic nerve (Villar et al 1989, Zhang *et al.* 1993). Transection of the vagus nerve results in increases in galanin mRNA levels in the corresponding motor neurons (Rutherfurd *et al.* 1992). A similar phenomenon is observed when certain central processes are damaged. For example, increased galanin levels are seen in the paraventricular and supraoptic nuclei of the hypothalamus after hypophysectomy (Merchenthaler et al 1993).

Normally, galanin is expressed only in low levels in the chromaffin cells of the rat adrenal medulla. However, after administration of insulin, the resulting splanchnic nerve

stimulation and hypoglycemic condition lead to strong galanin expression in these cells without affecting expression in the hypothalamus or pituitary (Anouar *et al.* 1995).

1.5 Functions and effects of galanin

The physiological significance of galanin remains to be determined, but clues are given from its biological effects and its widespread distribution. (More detailed discussions of the possible functions of galanin are given in reviews (Kask et al 1997, Bedecs et al 1995, Vrontakis 1991, Merchenthalter et al 1993, Bartfai et al 1993.)

In the pituitary, galanin increases secretion of GH, PRL, and LH and decreased secretion of TSH and ACTH (Merchenthalter et al 1993). Galanin also inhibits the release of insulin from ß-cells of the pancreas (Bartfai et al 1993). Galanin injection into the medial hypothalamus of rats causes an increase in food consumption, with a switch to preference for fat over carbohydrates (Bedecs et al 1995). Galanin impairs cognitive performance, and so may be important for memory and learning. Also, in the basal forebrain, those acetylcholine-containing neurons that degenerate during the progression of Alzheimer's disease have increased levels of GAL-LI (Merchenthalter et al 1993, Kask et al 1997). Its up-regulation during nerve injury (Merchenthalter et al 1993) suggests that the protein is also involved in nerve regeneration (Xu et al 1996). There is some evidence that galanin increases the pain threshold as well (Kask et al 1997).

1.6 Galanin and pregnancy

During the early stages of pregnancy in the rat, high levels of galanin are detected in the serum starting at about day 7 and peaking at day 11-12. Galanin protein and

mRNA are also detected in the decidual cells of the conceptus from day 7 and to E17 (Vrontakis *et al.* 1992). During this time period, these cells are both dividing and differentiating. In contrast to the conceptus and serum, the level of galanin expression in the pituitary is high from day 3 of pregnancy, and continues to remain high, particularly throughout the latter half of pregnancy. Decidual cells of the full term human placenta also contain GAL-LI (Graf *et al.* 1996). Thus, the function of galanin during pregnancy may be conserved among species, and may be related to growth of both the placenta as well as the embryo.

The function of galanin during pregnancy is to regulate the secretion of placental hormones. Alternatively, decidual cells may use the galanin they express as a growth or differentiation factor. Galanin may also be secreted into the serum to act on distant tissues (Vrontakis *et al.* 1992).

In the rat hypothalamus, the degree of co-localization of galanin in a subset of LHRH neurons depends on estrogen. However, during pregnancy and lactation, when progesterone levels are high, the pattern of expression in these neurons resembles that of the male rat. These differences might be due to the presence of progesterone. It could also explain why the galanin mRNA levels in the anterior pituitaries of intact cycling rats is higher than in those of pregnant rats (Merchenthaler *et al.* 1993, Vrontakis *et al.* 1992).

1.7 Galanin and cancer: galanin as a possible mitogen

In humans, galanin expression has been detected in pituitary tumors involving somatotrophs and lactotrophs, but especially in those tumors involving corticotrophs (Sano *et al.* 1991, Hsu *et al.* 1991) which normally express the peptide (Vrontakis et al

1990, Hsu et al 1991). Adrenal pheochromocytomas were one of the few types of tumors outside the pituitary that often expressed galanin (Sano *et al.* 1991).

In the rat, where galanin is normally expressed in somatotrophs, estrogen treatment can not only lead to increased galanin levels in lactotrophs (Vrontakis et al 1989, Steel et al 1990), but also to the formation of prolactin-secreting tumors (Vrontakis et al 1987). Lactotroph numbers increased in the pituitaries of female transgenic mice in which over-expression of the galanin gene was directed to these cells (Cai *et al.* 1999). Also, galanin increases the proliferation rate of the rat clonal lactotroph cell line, 235-1, while antibodies against galanin have the opposite effect (Wynick *et al.* 1993). Galanin may therefore act as a growth factor for lactotrophs.

Galanin appears to be a growth factor for two small cell lung cancer (SCLC) cell lines. It was also determined that galanin causes the kind of transient increase in intracellular calcium in these cells that is associated with early mitogenic signaling (Sethi *et al.* 1991). These effects are probably mediated through the activation of a pertussis toxin-insensitive receptor. This, in turn, leads to the activation of a mitogen-activated protein kinase (MAPK), which activates enzymes and transcription factors that affect cell proliferation (Seufferlein *et al.* 1996). Galanin did not, however, affect the proliferation rate of the insulin-producing cell line RINm5F (Sjöholm *et al.* 1995). Some human breast cancer tumors and cell lines contain more than two copies of the galanin gene, but there was only limited expression of the gene in these cells (Ormandy *et al.* 1998).

1.8 Summary

Galanin is a 29-30 amino acid long putative neuropepetide. Very few proteins are homologous to galanin. Those few that do have some homology to galanin resemble the N-terminal portion of the protein. The first 16 amino acids of galanin are fully conserved among the species studied so far. In the adult, galanin expression is found mainly in nervous tissue, particularly the hypothalamus, median eminence, and posterior pituitary. A distinctive feature of galanin is its dramatic up-regulation by estrogen and following nerve injury. Nerves of the gastrointestinal tract, genitourinary tract, and respiratory system also contain GAL-LI. Galanin has been shown to influence the secretion of pituitary hormones and insulin. It also affects food consumption, cognitive functions, and pain thresholds. Galanin is also expressed in many pituitary tumors and is a mitogen for lactotroph and two small cell lung cancer cell lines.

1.9 Rationale

During development, mitogens are required to facilitate the proliferation of cells needed for growth. Galanin is expressed in some tumors, particularly those of the anterior pituitary. Galanin is also expressed in and/or affect the proliferation of certain cell lines. In such cases, galanin may be considered a mitogen. Therefore, this protein may also act as a mitogen during development of the embryo.

Galanin is up-regulated during nerve injury, suggesting that it is involved in nerve regeneration. It is possible that this up-regulation represents the reactivation of an embryonic program used in the development of the corresponding nerves.

Galanin is highly conserved among the species studied so far. It is expressed throughout the central and peripheral nervous systems, and is also found in the pituitary and the adrenal gland. Proteins that are widely expressed and well conserved often have a universal function (or functions) and are therefore termed housekeeping genes. Many housekeeping genes are involved in embryonic development. Therefore, galanin may also be involved in development.

Galanin expression is plastic. It changes with alterations in steroid and hormone levels. It is up-regulated in injured nerves. It is possible that galanin expression is also plastic during development. That is, it is up-regulated during growth of the embryo, and down-regulated once it has served its purpose.

The mouse was chosen for this study because of its conveniently short gestation period of 17-19 days. Also many of the studies involving galanin expression are done in mice (or rats), and this study could be easily compared with those others. To assure that this study would be comparable to those using human embryos, the time period of mouse development between E10 and E15 was chosen. This is a period of rapid organogenesis, requiring rapid proliferation of cells. Galanin might be one of the mitogens expressed during this time. The period before E10 must also require rapid cell proliferation. However, the mouse embryo is in the process of turning at this stage. Therefore, mitogen expression in the mouse before E10 is not as comparable in the human.

Since this project involves galanin and its potential role in development of the embryo, a brief summary of embryonic development of the mouse is included below. The description given below combines those given by Rugh (1968), Kaufman (1999), and Hogan et al (1994).

1.10 Early development of the mouse embryo

After the egg is fertilized, it divides repeatedly. The blastocyst cavity forms within one end of the ball of cells. The outer cell layer of the blastocyst facilitates implantation of the embryo on the latter half of the fourth day of pregnancy. In the mouse, each blastocyst implants from the end containing the blastocyst cavity, in contrast to the human.

The remaining cells will become the embryo proper. As implantation proceeds, the primitive endoderm forms on the surface of the inner cell mass, now termed the primitive ectoderm, facing the blastocyst cavity, so that the embryo is bilaminar. On day 6 ½, the primitive streak forms in a posterior region of the ectoderm and eventually elongates to the tip of the egg cylinder. From the primitive streak, the mesoderm grows between the ectoderm and endoderm. Therefore, by day 7, the embryo is considered to be trilaminar. Please refer to figure 1 for a list of those tissues derived from each of the germ layers.

Starting at day 7, the embryo folds to assume the shape of an embryo. This causes the gut to become tubular and the heart, developing in the mesoderm cranial to the future brain region, to lie in the region of the future chest. The tissue that will form the notochord and the definitive lining of the gut appear at this time as well.

Also starting at day 7, the ectoderm above the developing notochord thickens. Neural folds appear at day 8 and fuse, beginning at day 9. Cellular differentiation of the central nervous system begins at about the time the neural folds fuse, at E8-8.5. From a population of proliferating cells lining the neural canal, first neuroblasts, and then glial cells, migrate outward, forming the future gray (mantle) and white (marginal) layers, respectively. The remaining cells remain in place to line the canal, becoming the ependymal layer. At about the time the neural folds fuse, the neural crest cells separate from the neuroepithelium and migrate to form, among other things, the cranial, dorsal root, and sympathetic ganglia of the peripheral nervous system. The future dorsal horns of the gray matter form connections with the nearby DRG. By day 9, all sense primordia and some cranial ganglia are already formed. By day 9½, the mesencephalic and prosencephalic vesicles and the metencephalic and myelencephalic areas of the brain are all recognizable. The cranial neuropore closes by about day 9 and the caudal neuropore closes by day 10-10.5 (Hogan et al 1994).

By day 7¾, the first pair of somites form, and each new pair is added caudally. By E15-16, 65 pairs of somites will have developed in the mouse embryo. Starting at day 9 ½ and continuing in a craniocaudal sequence, each somite differentiates into myotome, dermatome, and sclerotome, each part contributing to the formation of various muscular and skeletal elements. The precartilage primordium of each vertebra later forms when the caudal (dense) part of each sclerotome fuses with the cranial (loose) part of the sclerotome caudal to it. Sclerotomal cells remaining between the vertebrae form the annulus fibrosus of the intervertebral discs.

Beginning at day 8, as the embryo is folding, the mouse embryo rotates, or "turns." Thus, by day 9½, the endoderm initially located on the outside of the conceptus is inside, as in other vertebrates, and the embryo becomes surrounded by its extraembryonic membranes.

By day 9, the pituitary and forelimb buds are forming and the heart is beating regularly. The forelimbs begin to develop at this time, with the hindlimbs appearing slightly later, at day 9 ½, which are therefore slightly behind in their development compared to the forelimbs. By this time, the pronephric ducts and nephric primordium appear in intermediate mesoderm and the primitive streak has degenerated. Also at this time, the caudal-most portion of the spinal cord and its associated neural crest cells form from the tail bud, a remnant of the regressing primitive streak.

1.11 Summary of development from E10 to E15

1.11.1 Day 10 (30-39 pairs of somites)

On this day of development, Rathke's pouch and the infundibulum, the future anterior and posterior parts of the pituitary, respectively, begin to form. The horseshoe-shaped nasal processes that appear on the frontonasal prominence begin to approach each other. The medial nasal processes will fuse and become the philtrum of the lip, the premaxillary area of the maxilla and the associated gums, and the primary palate. The lateral processes of the nasal placodes will develop into the sides of the nose. By day 10 of embryonic development, the fourth pair of branchial arches develops in the future neck region. In the gut, the thyroid and cystic primordia are recognizable, the stomach region becomes identifiable as it dilates, and the umbilical hernia is observed as the midgut

elongates so much as to extend outside the body. The caudal neuropore closes and the neural crest-derived ganglia (cranial, dorsal root, sympathetic) are present, but very small, at this stage. Mesonephric vesicles and tubules are present in the intermediate mesoderm, but, in the mouse, they will never develop into glomeruli. In the heart, the spiral aorticopulmonary septum (that will divide the outflow tract into the aorta and pulmonary trunk) and the endocardial cushions (that will divide the atrioventricular canal into right and left channels) begin to develop.

1.11.2 Day 11 (40-44 pairs of somites)

The second branchial arch overgrows the third and fourth as the neck continues to develop. The very first evidence of palatal shelves is detected along the inner surface of the maxillary prominence of the first arch. The first and second arch cartilages are present in the form of precartilage. The forelimb develops distinct regions, including girdle, arm, and handplate, during this stage. The stomach has rotated so that it is to the left of the midline and horizontally situated. The tongue begins to form. The central nervous system begins to differentiate as the ependymal, mantle, and marginal layers are recognizable. The first indications of a definitive kidney are seen as the uteric bud grows from the mesonephric duct, dilates, and is surrounded by the metanephric blastemal tissue. The urorectal septum begins to divide the cloaca into anterior bladder and posterior hindgut regions. The gonad primordia are seen near the mesonephros.

1.11.3 Day 12 (48-52 pairs of somites)

At this stage, the otic capsule surrounding the developing inner ear is precartilage. Rathke's pouch detaches from the oropharaynx, and it and the infundibulum proliferate. In the heart, the ridges of spiral septum fuse, the septum primum (dividing the primitive atrium of the heart into left and right sides) fuses with endocardial cushions, and the ostium secundum can be seen in the septum primum. The stomach has a distinct thick, glandular part and the liver is well differentiated. The forebrain, especially thalamus and hypothalamus, expands and differentiates and the ependymal layer in the spinal cord is less pronounced. The mesonephros begins to regress, but the metanephros has developed vesicles and tubules. The mammary gland primordia are seen at this stage as well.

1.11.4 Day 13 (52-56 pairs of somites)

The future olfactory lobe of the brain begins to expand forward to overlie the nasal cavity and olfactory (I) nerves pierce the precartilage ethmoid bone to approach this part of the brain. The primordia of hair follicles begin to form on the face. In the heart, all valves can be recognized and the ridges of the spiral septum of the outflow tract are completely fused. The muscular part of interventricular septum begins to form at this stage as well. Most of the esophagus is not patent due to the proliferation of the endodermal lining of gut. The mesenchyme surrounding the gastrointestinal and respiratory systems begins to condense. The formation of the diaphragm is complete. The pancreatic primordia begin to differentiate. In the spinal cord, the ependymal layer thins as the marginal and mantle layers thicken. The dorsal and ventral horns of spinal cord are recognized. The mesonephros has regressed and the cloaca is divided into

anterior and posterior parts. The gonads have begun to differentiate so the ovary appears spotty and the testis appears striped. Also at this stage, the metanephros enlarges and differentiates further and begins to "ascend" as the lower body elongates. The splenic primordium forms in dorsal mesentery of stomach region. The primordium of the cortex of the adrenal gland, derived from the ceolomic mesoderm, begins to develop at this stage as well. The first arch cartilage is observed in the mandible, and tooth primordia are recognizable. Some bones are condensations (the girdles), some are precartilage (petrous temporal bone, exoccipital bone, basioccipital bone), and others are cartilage late in this stage (vertebrae and long bones of limbs).

1.11.5 Day 14 (60 pairs of somites)

The palatal shelved that have formed on the internal surface of the maxillary prominence have gradually elongated, and, at this stage, begin to elevate, so that their edges approach each other at the midline. The nasal septum, which grows downward from the frontonasal prominence, fuses with the primary palate. In the heart, the caudal part of the fused ridges of the outflow tract fuses with the muscular part of the interventricular septum. Also, the septum secundum forms between the left and right atria. The umbilical hernia reaches its maximum size. The esophagus begins to canalize. The adrenal gland is more defined, and, by this stage, sympathetic fibers pass from the medulla (derived from the sympathetic nerve cells) to the sympathetic trunks. The kidney has glomeruli and collecting tubules. The testes and their drainage systems develop. The middle parts of the long bones are cartilage, as are the base of the skull, the nasal capsule and septum, the arch cartilage derivatives, the vertebrae and ribs, and clavicle and pelvis.

The distal regions of the limbs are precartilage, as are the sternebrae and the skeletal elements surrounding the upper respiratory tract. The webbing between the forelimb digits has disappeared by the end of this stage, but is present to some degree in the hindlimb at E15.

1.11.6Day 15 (60-65 pairs of somites)

At this stage, the eyelids grow. The pituitary differentiates. The skin becomes wrinkled and the development of hair follicles becomes more widespread. The palatal shelves fuse at the midline, but the primary palate and nasal septum do not fuse with them until next day or so. The heart and circulatory system are now well formed and will not develop much further until after birth. The esophagus is surrounded by muscle, and the duodenum and the midgut of the umbilical hernia develop villi and glands.
The splenic primordium becomes enlarged and elongated. The cerebellum and olfactory lobe differentiates and the marginal layer in spinal cord thickens. The gonads increase size. The first ossification centers are found in cartilage of the base of the skull, long bones of limbs, girdles, ribs, dorsal arches of vertebrae, as well as in the membrane bones of lateral palatal shelves, periorbtial region of face, mandible, frontal and parietal bones. The skeletal elements of respiratory system, most of pelvic girdle, and the sternum are cartilage. Intervertebral discs are also recognized at this stage.

1.12 Molecular control of embryonic development

During embryonic development of an organism, proliferation, differentiation, and programmed cell death are occurring to create new tissues and remodel and increase the

size of existing tissues. These events require cells of the developing embryo and the mother to interact appropriately with each other and the extracellular matrix. At each stage of development, each cell expresses a particular combination of genes in order to be able to live, produce the proper matrix components and signals, and respond to the environment. Therefore, gene expression affects the proliferation rate, histology, migration ability and path, and time of death of a population of cells. Theoretically, signals from other cells could change any of the above mentioned properties of a cell by signaling it to change which genes are turned on or off. Cells may affect each other, for example, through direct interaction of their surfaces, by secreting signal proteins, or by the extracellular matrix components they secrete. Furthermore, as one population of cells is induced to change, those changes can induce other cells to change which genes they express, thereby causing a chain reaction. One population of progenitor cells could differentiate into several different cell types, depending on which signals they were exposed to. It is not always clear when exactly a cell has been committed to a particular fate. Understanding how and when cells are induced to differentiate is a big part of understanding embryonic development.

The timing of gene expression within a cell is critical to the development of the embryo. Pleuripotent cells of the early embryo become increasingly restricted in what kind of tissue they can become. Also, the window of time in which a cell is able to respond to particular environmental signals is often small. Therefore, the timing of gene expression must be synchronized to lead to proper development. It is probably partly for this reason that, for one cell type to affect another, they must be in close proximity to

each other. Thus, the ability of one tissue to induce expression in another is limited by time and space.

The HOX gene family is an example of a group of genes being expressed in a particular combination to give rise to a particular effect. This family of genes is present in some form in mammals and in *Drosophila*. The various members of the family are turned on or off depending on the segmental level, as defined by the positions of the somites. These combinations of gene expression are thought to affect how segmentally arranged structures develop, such as spinal nerves, certain striated musculature, blood supply, and the initial sclerotomal vertebrae. The expression of certain members of this family may, in turn, be sensitive to other signals, such as retinoic acid (Larsen 1998).

Signals, or combinations of signals are used to designate which tissues will become dorsal structures versus ventral ones and which will become proximal rather than distal. For example, the sonic hedgehog (Shh) protein causes tissue to differentiate into a type of cell more typical for the ventral part of the neural tube. Similar dorsalizing signals help induce the ventromedial aspect of each somite to disperse as a sclerotome. Also, via extracellular matrix proteins as from the Wnt gene family, dorsalizing signals cause the dorsolateral somite to form a dermomyotome.

Many of the factors involved in development also have functions in the adult. For example, insulin and IGF-I from the somites and other midline structures signal the lateral plate mesodermal core of a developing limb bud to establish a proximodistal axis. Some members of the fibroblastic growth factor family help to maintain the outgrowth of the limb bud, so that the wrong combination of expression of FGF family members can

lead to the growth of extra limbs. Also, BMPs (bone morphogenic proteins), including BMP-2, are involved in mesenchymal condensation, chondrogenesis, and osteogenesis.

In summary, development progresses by different cell types inducing each other to differentiate in a particular way. This induction signal is limited in the distance it can travel and by the small window of time the responding tissue is receptive to such a signal. Each tissue present in the embryo is a result of a complex sequence of these signals, some of which are in the form of factors that also have functions in adult tissues.

Galanin could act as a signal, e.g. for proliferation of certain cell types, during development. The function and distribution of the protein may be different in the embryo as compared to the adult.

1.13 Hypothesis

Our hypothesis is that galanin is expressed during development of the mouse embryo, between E10 and E15.

1.14 Objective

The objective of this study is to determine the ontogeny of galanin-like immunoreactivity (GAL-LI) in the developing mouse embryo, from E10 to E15.

2.0 Methods

2.1 Mouse genetic background, housing, and breeding

Mice of the CD strain supplied by Central Animal Care at the University of Manitoba were used for this study. Animals were kept according to the University of

Manitoba animal protocol # . Temperature and the timing of light: dark cycles in the facility in which the mice were housed were kept constant.

Timed pregnancies were achieved by placing females and males in the same cage overnight. The following morning, if a mucous plug or other evidence of breeding was apparent, the female was separated and her future offspring considered to be at embryonic day 1, E1. Since the actual time of conception could have theoretically been any time during the dark cycle, and embryos were not necessarily collected at a particular time of day, the possible error in staging the embryos in this way was ±12 hours.

2.2 Tissue collection

At least two litters were collected at E10, E11, E12, E13, E14, and E15. Ether was used to anesthetize the pregnant mouse. A cut was made through the abdomen and chest to expose the uterus and heart. Using a 23G1 needle inserted into the heart, blood was collected. It was allowed to clot at room temperature for at least 30 minutes and then was centrifuged at 6000g for 10 minutes. The plasma was collected and stored at -20°C.

The right and left horns of the uterus were cut out and rinsed in cold saline (0.9% NaCl (w/v)). The uterus was removed from the saline and the embryos were dissected out. A dissecting microscope was useful for helping to recover the smaller embryos, i.e. E10 and E11, intact.

2.3 Tissue fixation and handling

Embryos were fixed in 4% paraformaldehyde (w/v) in PBS, pH7 (2X= 0.021M $Na_2HPO_4 \cdot 7H_2O$ (F.W.=268.07), 6.23mM $NaH_2PO_4 \cdot H_2O$ (F.W.=137.99), 0.246M NaCl (F.W.=58.44)) for 3 to 16 hours. Embryos were then rinsed overnight or longer in PBS.

Embryos were dehydrated through a graded series of ethanol solutions, cleared with two changes of chloroform and infiltrated and embedded in paraffin. Duration of fixation and dehydration varied with tissue size. Paraffin blocks were stored at room temperature.

2.4 Crown-Rump measurements

After fixation, the crown-rump length of each embryo was measured using a ruler marked with millimeter divisions. The length of each embryo was measured to the nearest tenth or quarter of a millimeter. Accuracy of measurements were assisted by the use of a dissecting microscope when measuring the embryos.

2.5 Paraffin sectioning

Paraffin sectioning was performed using a "feather" stainless steel microtome blade (Fischer Scientific cat. No. 12-631R, no.R35). Thickness for sagittal sections was 4μm for E10 embryos, 5μm for E12 embryos and 7μm for E13-15 embryos. Cross-sections were cut at 7μm for E12 and E13 and 10μm for E14 and E15 embryos.

Ribbons of sections were placed on a 40-42°C waterbath. The sections were separated and picked up onto "Fisherbrand superfrost/plus" slides (Fischer Scientific cat.

No. 12-550-15). Slides were drained and dried overnight on a slidewarmer at ~ 42°C. Slides were stored at room temperature in slide boxes.

2.6 H&E staining

Some slides were stained using a routine H&E procedure to show general histology. Slides were covered with 60mmX20mm coverslips (Fisher Scientific, cat. No. 12-545M) using Permount mounting medium (Fisher Scientific cat. No. SP15-500).

2.7 Immunohistochemistry

All steps for immunohistochemistry were performed at room temperature unless otherwise stated. Except for steps in tris buffer, ethanols, or xylol, all steps were done in a humid chamber on a benchtop orbital shaker. All solutions used were stored at 4°C until just before use. Paraffin sections were first deparaffinized (2X5 minutes xylol) and rehydrated (100% ethanol (2X5 minutes), 95% ethanol (5minutes), ddH_2O (5 minutes)).

Slides were then placed in 0.1M tris buffer (10X= 1M Tris-HCl, pH7.6) for 10 minutes. Each slide was then removed from the buffer and rings drawn around two sections using a hydrophobic PAP pen (Ted Pella, Inc., cat. No. 22303 and 22304). The sections were then covered with tris/triton solution (0.3% (v/v) triton X-100 in 0.1M tris buffer, pH7.6) for 10 minutes. Next, a solution of tris/triton/BSA solution (0.1% BSA (w/v) in 0.3% (v/v) triton X-100 in 0.1M tris buffer, pH7.6) covered the sections for 15 minutes. A universal blocker (DAKO diagnostics Canada, Inc., cat. No. CD310082) was added to the sections for 30 minutes, and then normal goat serum for 15 minutes. (Note:

The goat serum, anti-rabbit biotinylated antibody, and streptavidin-beta-galactosidase (used below) are from a commercial kit, i.e. Kirkegaard & Perry Laboratories, cat. No. 71-00-58.)

The anti-galanin antibody used in this study was raised in rabbit against the rat galanin protein and the IgG fraction was isolated from whole serum using a protein A agarose column (Peninsula Laboratories, Inc., code RGG-7141). The testing by manufacturer showed that the antibody has about a 6% cross-reactivity with porcine galanin, and a 0% cross-reactivity with porcine sectretin, human, porcine, and rat VIP, the porcine amide form of GMAP(1-41), the human and rat forms of NPY, substance P, and human PHM-27. The primary antibody was used at a 1:300 dilution using a commercial antibody dilutent (DAKO Diagnostics Canada Inc., cat. No. D2000S-250ml).

On each slide, the goat serum was removed from one section and the primary antibody solution added. The goat serum remained on the second section as a negative control. Previous experiments in our laboratory established that preabsorption of the antibody with galanin peptide abolished staining. The slides were then placed in the humid chamber at 4°C for two days.

The slides were then rinsed with tris/triton/BSA solution using a plastic pipette, and the sections covered with the same solution for 30 minutes. At this point, slides were gently rinsed with tris buffer and immersed in a fresh change of tris for 5 minutes. The goat anti-rabbit biotinylated antibody was then added to cover each section 1 hour. Afterwards, the slides were rinsed in tris buffer again and the sections were covered with streptavidin-beta-galactosidase for 1 hour. The slides were then rinsed in tris buffer.

The beta-galactosidase substrate solution was made from a kit (Kirkegaard & Perry Laboratories, cat. No. 54-13-00.) The solution covered each section and the slides placed in a humid chamber, protected from the light, at 37°C for 30 minutes.

The slides were then rinsed in ddH_2O, quickly dehydrated, passed through two changes of xylol, and coverslipped.

2.8 Pictures

Immunostained and H&E stained sections were scanned. Brightness and contrast were altered to show staining to the best possible effect using Abode Photoshop 4.0. Images were inserted into a word '97 and printed on glossy photo quality paper using a photo700 printer.

3.0 Results

3.1 Crown-Rump Measurements of CD Mouse Embryos

The average crown-rump length of each fixed embryo was measured. Table 2 compares the average lengths of embryos from time-impregnated CD mice from each stage as well as those listed in Rugh (1968) for the inbred CFI-S strain. (This strain is considered representative of most mice.) For each stage studied except E15, the average crown-rump length of the CFI-S strain falls within plus or minus two standard deviations of the average crown-rump length for our CD mouse embryos. The average crown-rump length of E14.5 embryos of the CFI-S strain falls within plus or minus two standard deviations of the average crown-rump length of our "E15" embryos.

Average Crown-Rump Lengths			
gestation age (days)	average length (mm) CD mice	CFI-S mice	n
9.50		2.80	
10.00	3.80 (SD=0.37)	3.50	6
10.50		4.34	
11.00	4.56 (SD=0.42)	5.10	12
11.50		6.10	
12.00	6.93 (SD=0.30)	7.00	10
12.50		8.03	
13.00	9.05 (SD=0.24)	9.09	11
13.50		9.31	
14.00	10.08 (SD=0.26)	10.40	10
14.50		10.70	
15.00	11.43 (SD=0.39)	12.51	12
15.50		13.31	
16.00		15.18	

Table 2: Average crown-rump lengths.

The average crown-rump lengths, in millimeters, of CD embryos from each stage are compared to results from Rugh (1968) using the inbred CFI-S strain of mice. In making any comparisons, it should be kept in mind that rate of development can vary with the strain. SD= standard deviation, n= number of individual CD embryos used to calculate the average crown-rump length at each stage.

3.2 Immunohistochemistry

See table 3 for a summary of the results. More details are given below. At least two embryos from each of two litters were stained using immunohistochemistry for galanin except for E10, where only one embryo was examined per litter.

3.2.1 E10

At embryonic day 10, galanin-like immunoreactivity (GAL-LI) is present in the mesenchyme around the neural tube (figure 3). There is also clear staining indicated the mesenchymal spiral ridges of the outflow tract of the heart (figure 2b). Another immunostained section shows that the endocardial cushions stain for galanin as well (figure 2a).

3.2.2 E12

At embryonic day 12, GAL-LI continues to be detected in the mesenchyme (of varying cell density) surrounding the developing neural tube. GAL-IR tissue in this region includes the dense and diffuse portions of the mesenchymal sclerotomes, which are the precursors of the vertebrae, and mesenchyme surrounding the dorsal aorta and the cardinal and subcardinal veins (figure 4,5, and 6). The maxillary component of the first branchial arch (the precursor of the upper jaw) (figure 4) and the medial and lateral nasal processes (that will form the nose) contain GAL-LI as well (figure 6).

The spiral septum of the outflow tract and the endocardial cushions of the heart continue to stain as it did at E10 (figure 4 and 6). The trabeculae of the ventricle wall of the ventricle and the septum primum appear to stain as well (figure 6).

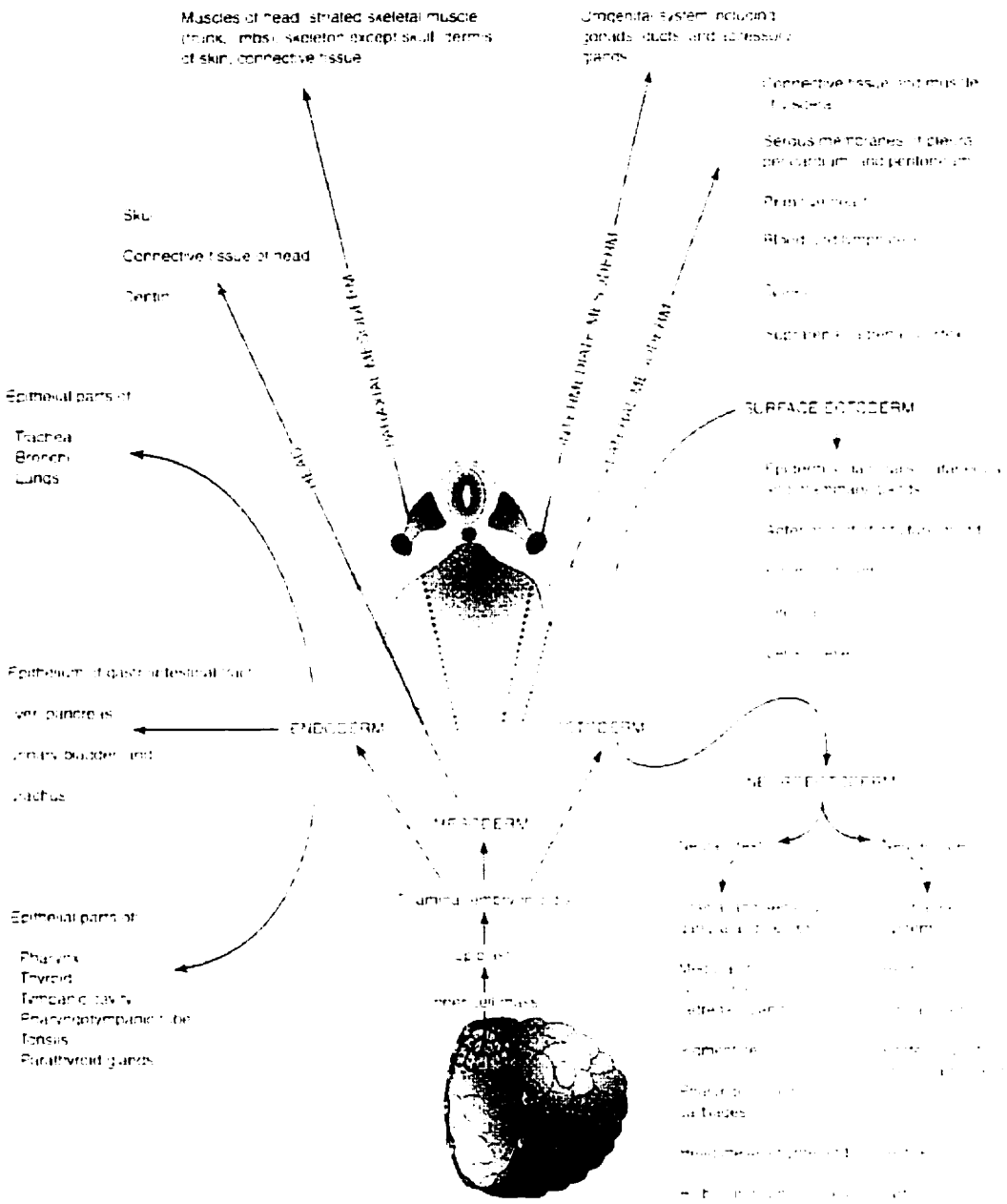

Figure 1 : Schematic drawing illustrating the derivatives of the three germ layers: ectoderm, endoderm, and mesoderm. Cells from these layers make contributions to the foundation of different tissues and organs; for example, the endoderm forms the epithelial lining of the gastrointestinal tract and the mesoderm gives rise to connective tissues and muscles (Moore and Persaud 1998).

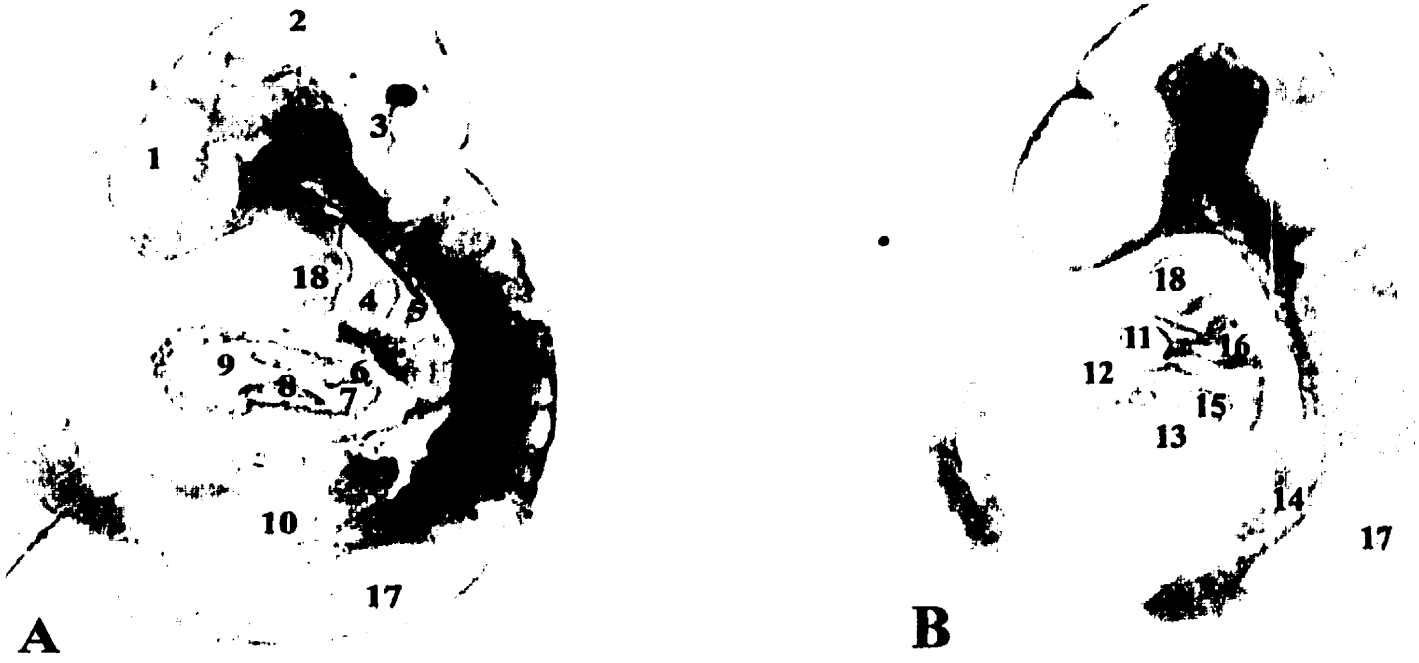

Figure 2 : Sections of mouse embryo at E10c, immunostained for galanin..
(A) Parasagittal section, (B) Mid-sagittal section

1. Telencephalic vesicle
2. Mesencephalic vesicle
3. Hindbrain
4. Second branchial artery
5. Third branchial artery
6. Septum primum
7. Atrium
8. Endocardial cushion
9. Ventricle
10. Peritoneal cavity
11. Truncus arteriosus
12. Bulbus cordis
13. Septum transversum
14. Dorsal aorta
15. Sinus venosus
16. Outflow Tract
17. Neural tube
18. Mandibular process
19. Mesenchyme

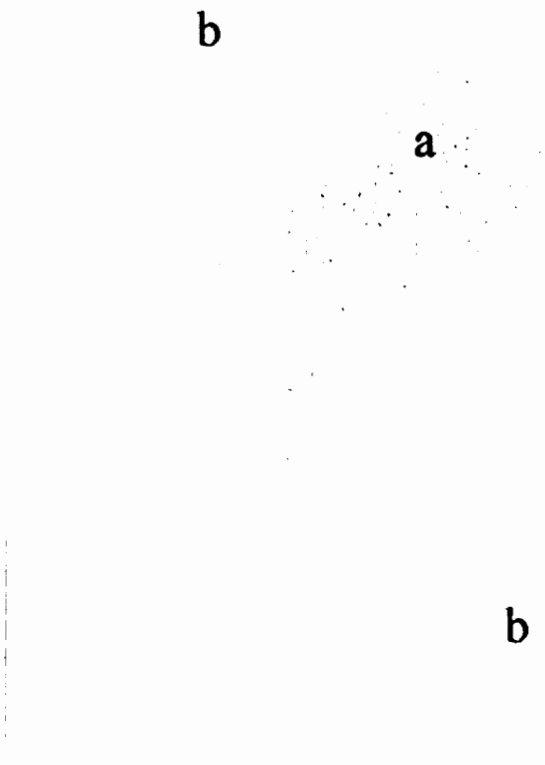

Figure 3 : High magnification of head of embryo at E10 immunostained for galanin, from figure 2B. Note that the cephalic mesenchyme (a) is strongly immunotreactive for galanin, while the neuroepithelium (b) has not stained.

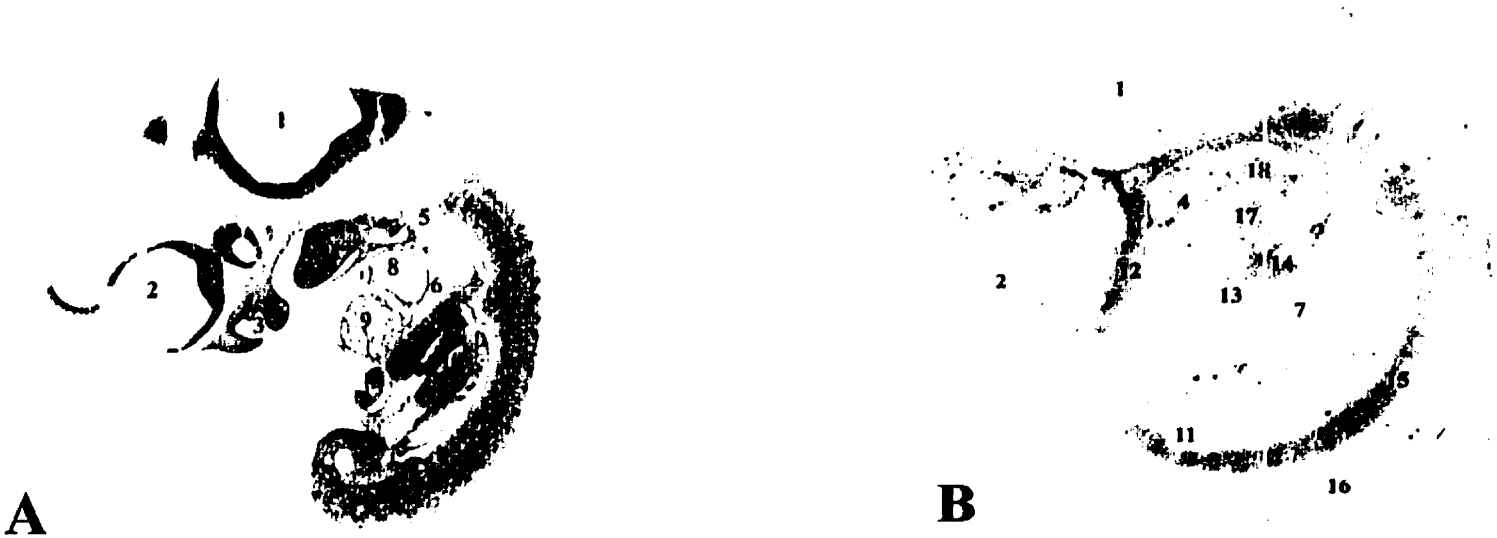

Figure 4 : Sections of mouse embryo at E12.
(A) H & E stained parasagittal section, (B) Mid-sagittal section immunostained for galanin.

1. Fourth ventricle
2. Lateral ventricle
3. Nasal pit
4. Mandibular process
5. Anterior cardinal vein
6. Common cardinal vein
7. Hepatic primordium
8. Atrium
9. Bulbus cordis
10. Duodenum
11. Subcardinal vein
12. Maxillary process
13. Ventricle
14. Endocardinal cushion
15. Sclerotomes
16. Neural tube
17. Outflow tract
18. Second branchial artery

Figure 5 : Caudal cross-sections of mouse embryo at E12.
(A) H & E stained, (B) Immunostained for galanin.

1. Dorsal aorta
2. Gonadal ridge
3. Peritoneal cavity
4. Inferior mesenteric artery
5. Hindgut
6. Neural tube
7. Dorsal root ganglion
8. Sclerotome
9. Subcardinal vein
10. Dorsal mesentery
11. Abdominal wall
12. Lower limb bud
13. Umbilical artery

Figure 6 : Cranial cross-sections of mouse embryo at E12.
(A) H & E stained, (B) Immunostained for galanin.

1. Neural Tube
2. Dorsal root ganglion
3. Trachea
4. Esophagus
5. Upper limb bud
6. Anterior cardinal vein
7. Pericardio-peritoneal canal
8. Septum Primim
9. Atrium
10. Ventricle
11. Nasal pit
12. Lateral nasal process
13. Medial nasal process
14. Lateral vesicle
15. Abdominal wall
16. Sclerotome
17. Endocardial tissue
18. Dorsal aorta

The dorsal mesentery is also GAL-IR (figure 5), as is the abdominal wall (figure 5 and 6). The tissue immediately surrounding the esophagus also stains for galanin (figure 6).

3.2.3 E13

Diffuse mesenchyme associated with the developing brain and spinal cord, which is the future subarachnoid space, stains at E13 (figure 7, 8, and 10). The roof of the oropharynx, including the cartilage primordia of the basioccipital and basisphenoid bones, the otic capsule, the nasal capsule and septum, and the primary palate stain at this time as well (figure 9). Also in the facial area, tissue in the lower jaw and proximal tongue also contain GAL-LI (figure 7 and 8).

The ventricle and the atrioventricular bulbar and endocardial cushion tissue (figure 7, 8, and 9) of the heart continues to stain for galanin at this stage. The abdominal wall also continues to stain for galanin (figure 7 and 8). Tissue surrounding the developing ribs is GAL-IR at this stage. The cartilage vertebrae of the proximal tail region, as well as the precartilage of the vertebrae of the distal tail region and interzones between them stain for galanin (figure 7 and 8). Also in the tail, mesenchymal tissue surrounding the segmental nerves stains for galanin at this stage, as illustrated in figure 9 and 10. The centrum of the more rostal vertebrae contains GAL-LI (figure 10). The mesothelial lining of the pleural cavity and the layer of the midgut beneath the epithelia contain GAL-IR (figure 7). The mesenchyme at the tip of the genital tubercle stains for galanin at E13. The paramesonephros (the future female reproductive organs), not

Figure 7 : Parasagittal sections of mouse embryo at E13.
(A) H & E stained, (B) Immunostained for galanin.

1. Rathke's pouch
2. Fourth ventricle
3. Lateral ventricle
4. Tongue
5. Ventricle
6. Genital tubercle
7. Liver
8. Midgut
9. Dorsal root ganglia
10. Atrium
11. Origin of posthepatic part of inferior vena cava
12. Paramesonephros
13. Future subarachnoid space
14. Cartilage primordium of rib
15. Lining of pleural cavity
16. Abdominal wall
17. Membranous part of interventricular septum

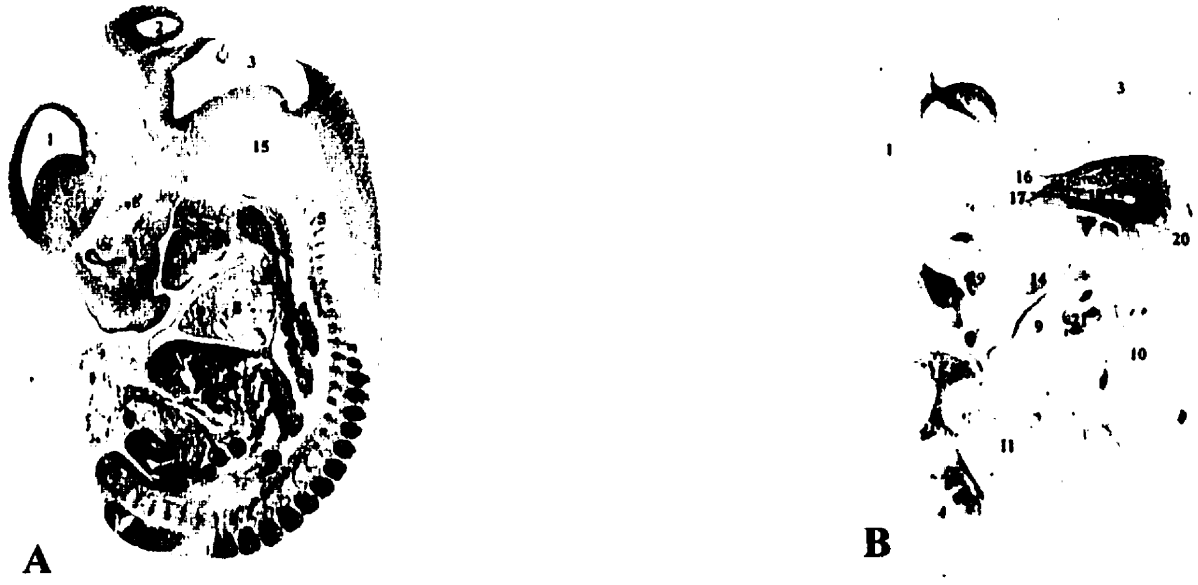

Figure 8 : Parasagittal section of mouse embryo at E13.
(A) H & E stained, (B) Immunostained for galanin.

1. Lateral ventricle
2. Mesencephalic vesicle
3. Fourth ventricle
4. Neural tube
5. Cartilage primordium of vertebra
6. Posthepatic part of inferior vena cava
7. Atrium
8. Membranous part of interventricular septum
9. Muscular part of interventricular septum
10. Lung
11. Vesicular part of urogenital sinus
12. Cartilage primordium of nasal septum
13. Dorsal root ganglion
14. Lower jaw
15. Future subarachnoid space
16. Rathke's pouch
17. Cartilage primordium of basisphenoid bone
18. Cartilage primordium of basioccipital bone
19. Primary palate
20. Cartilage primordium of rib
21. Atrioventricular septum and endocardial cushions

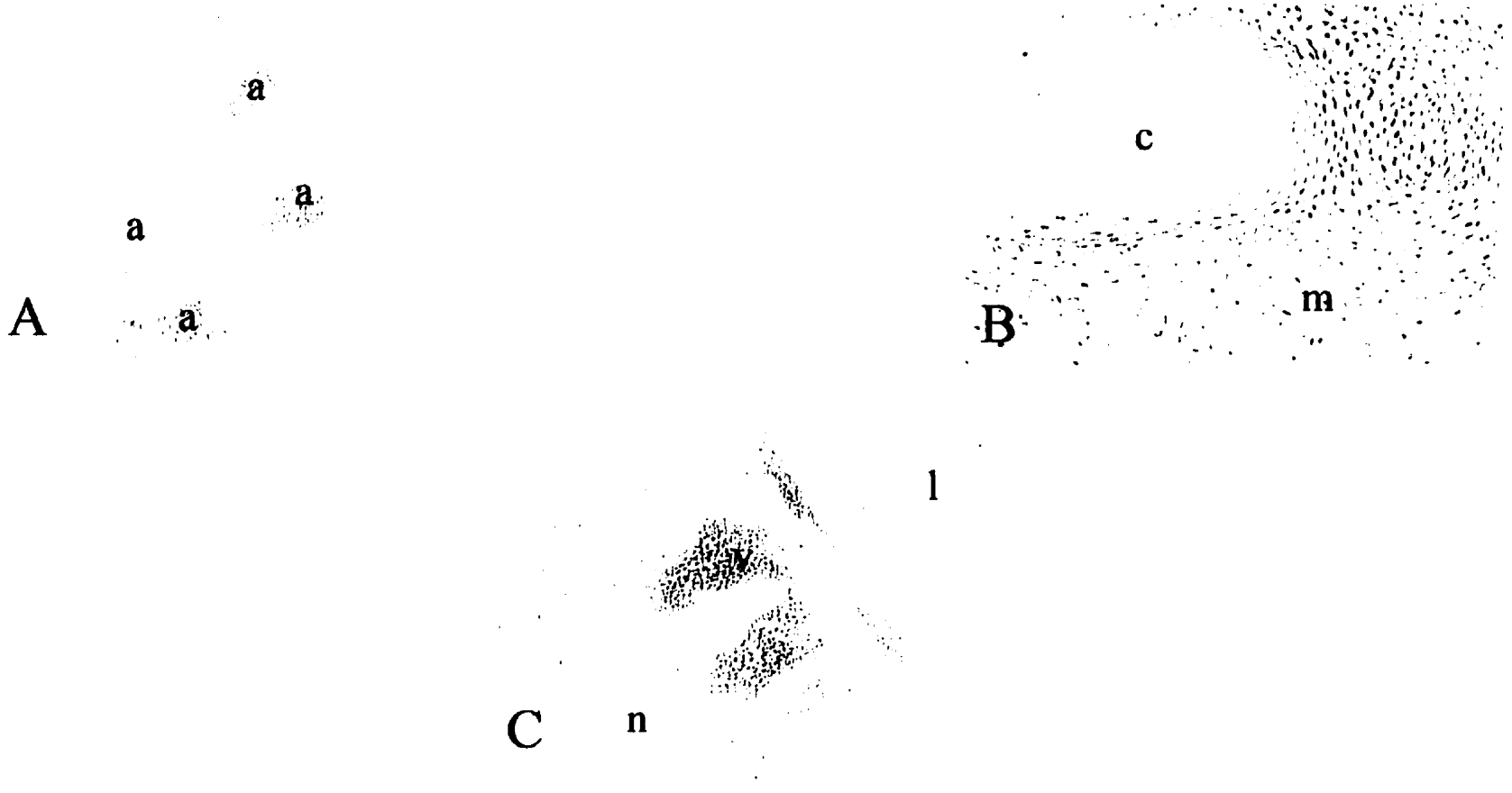

Figure 9 : High magnification of embryo at E13 immunostained for galanin, from figure 8. (A) Detect GAL-LI in the heart, (B) in developing bone, and in the (C) developing vertebrae of tail. (a) aorticopulmonary septum and endocardial cushions, (c) cartilage primordium of basisphenoid bone, (m) mesenchyme, , (v) primordium of vertebra, (n) neu ral tube, (l) liver.

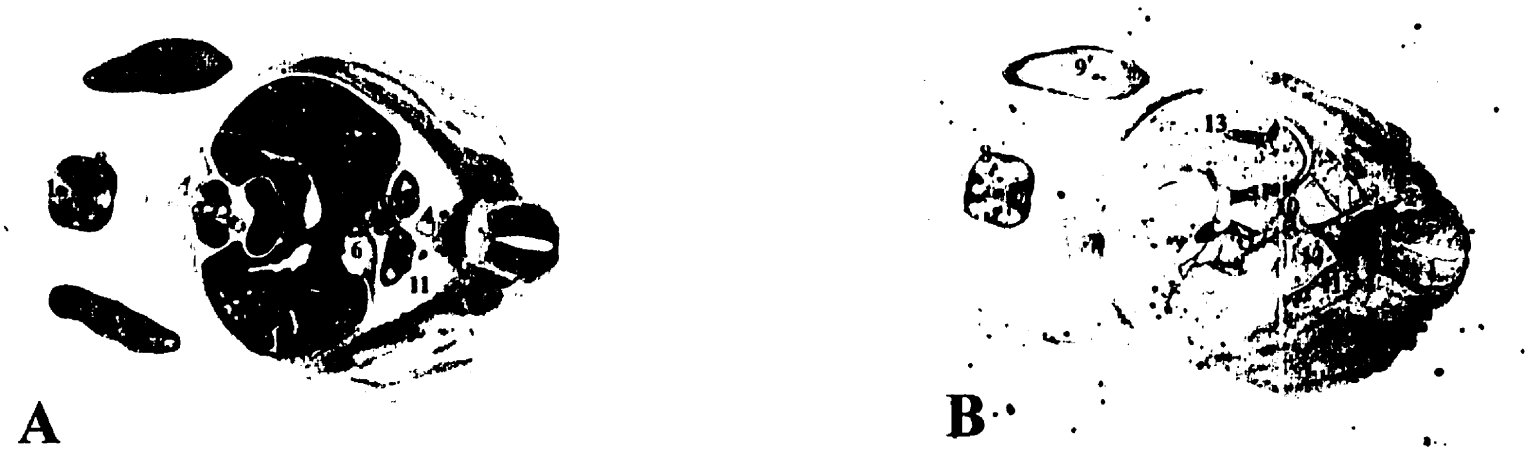

Figure 10 : Cross-sections of the mid-section of mouse embryo at E13.
(A) H & E stained, (B) Immunostained for galanin.

1. Neural tube
2. Dorsal root ganglion
3. Centrum of cartilage primordium of vertebra
4. Thoracic aorta
5. Proximal part of shaft of cartilage primordium of rib
6. Origin of posthepatic part of inferior vena cava
7. Duodenum
8. Segmental nerve in tail
9. Hindlimb
10. Esophagus
11. Wall of pericardio-peritoneal cavity
12. Lung tissue
13. Liver

including the paramesonephric duct that runs along its side, stains for galanin at this stage as well (figure 7).

The site between the entrances of the esophagus and larynx also stains for galanin.

In the lower body, the tissue immediately surrounding the head of the femur also appears to stain, similar to that seen at E14 (see also figure 13 and 18).

3.2.4 E14

The distribution of GAL-LI seen at E14 is very similar to the distribution observed at E13. Mesenchyme of the future subarachnoid space surrounding the central nervous system and the mesenchyme surrounding the cartilage primordia of the vertebrae, basioccipital bone, and basisphenoid bone stain for galanin. The cartilage of the nasal capsule (figure 11 and 12) and the mesenchyme surrounding the turbinate bones (supporting the conchae of the nasal cavities) (figure 13) also contain GAL-LI at this stage. The palate also stains for galanin (figure 11 and 13). Parts of the lower jaw and the gut, including the esophagus (figure 13) and midgut (figure 11), stain at E14 as well. The petrous region of the temporal bone contains GAL-LI (figure 11 and 14). The primordia of the middle ear ossicles do not stain for galanin, but are surrounded by GAL-IR mesenchyme (figure 14).

The bulbar ridges of the outflow tract forming the membranous part of the interventricular septum continue to stain (figure 13). The skin of the developing limbs clearly stains at this stage as well, as illustrated in the hindlimb in figure 13. The

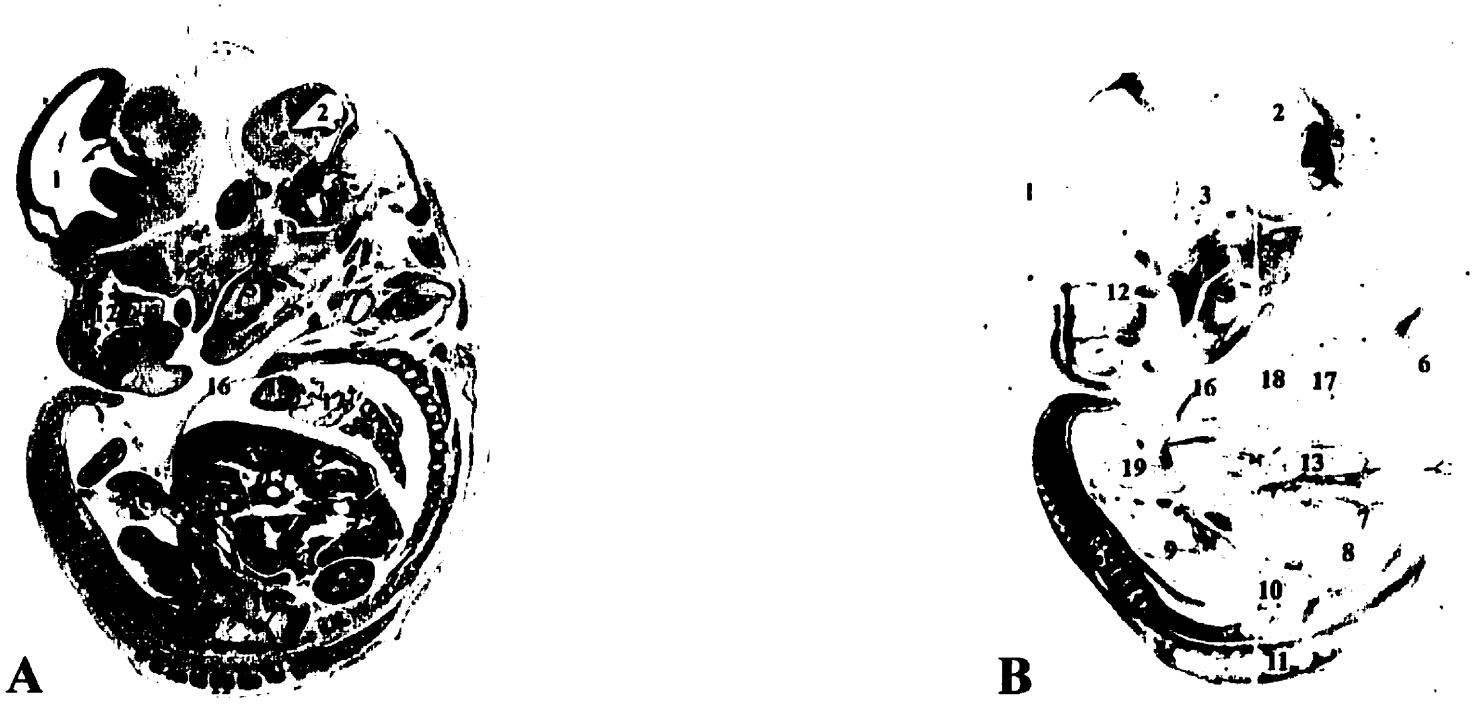

Figure 11 : Parasagittal sections of mouse embryo at E14.
(A) H & E stained, (B) Immunostained for galanin.

1. Lateral ventricle
2. Fourth ventricle
3. Trigeminal ganglion
4. Palatal shelf
5. Meckel's cartilage
6. Cartilage primordium of rib
7. Testis
8. Metanephros
9. Genital tubercle
10. Cartilage primordium of imnominate bone
11. Dorsal root ganglion
12. Nasal cavity
13. Liver
14. Nasal Capsule
15. Petrous part of temporal bone
16. Abdominal wall
17. Atrium
18. Ventricle
19. Midgut

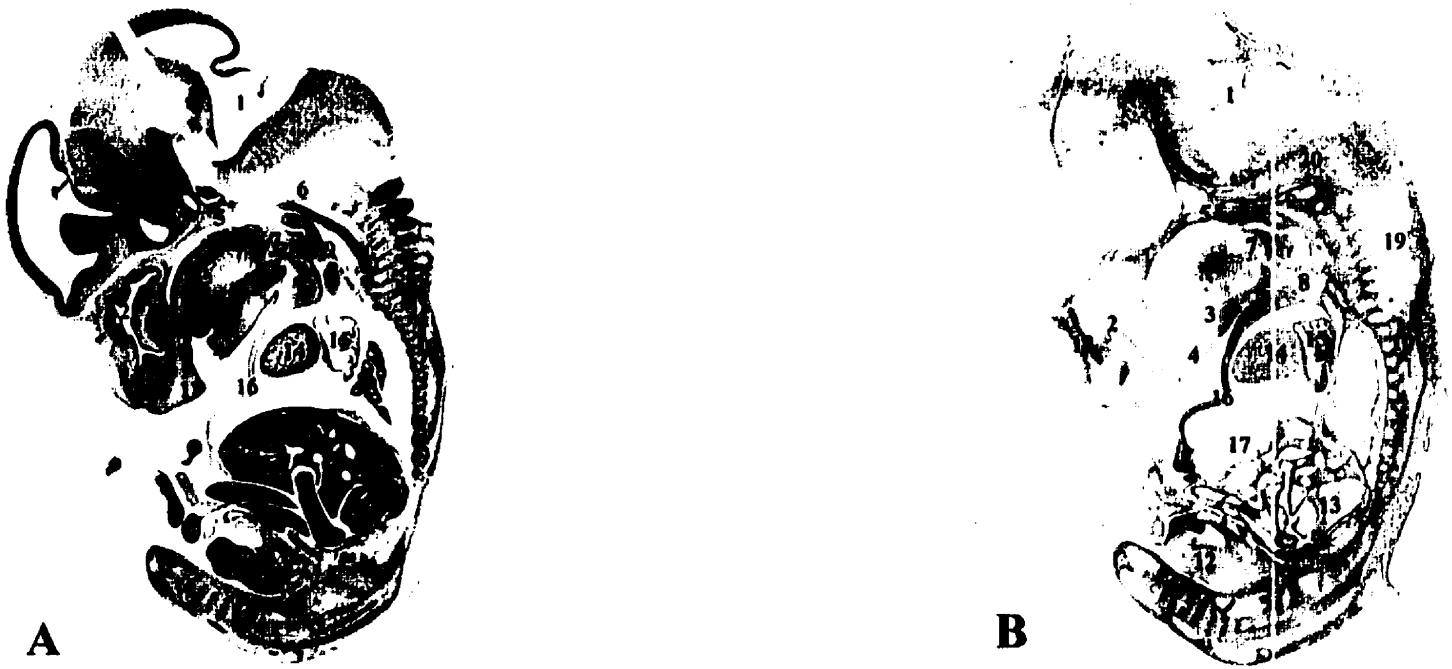

Figure 12 : Parasagittal sections of mouse embryo at E14.
(A) H & E stained, (B) Immunostained for galanin.

1. Fourth ventricle
2. Nasal cavity
3. Primordium of lower incisor
4. Lower lip
5. Cartilage primordium of basispheniod bone
6. Cartilage primordium of basioccipital bone
7. Cartilage primordium of hyoid bone
8. Thymus
9. Cricoid cartilage
10. Cartilage primordium of rib
11. Upper lip
12. Genital tubercle
13. Metanephros
14. Ventricle
15. Atrium
16. Abdominal wall
17. Liver
18. Nasal capsule
19. Dorsal root ganglion
20. Future subarachnoid space

Figure 13 : Mid-sagittal sections of mouse embryo at E14.
(A) H & E stained, (B) Immunostained for galanin.

1. Lateral ventricle
2. Fourth ventricle
3. Rathke's pouch
4. Meckel's cartilage
5. Cartilage primordium of basioccipital bone
6. Cartilage primordium of vertebra
7. Cartilage primordium of proximal shaft of humerus
8. Hindfoot
9. Stomach
10. Thymus
11. Aorta
12. Ventricle
13. Atrium
14. Metanephros
15. Adrenal gland
16. Head of cartilage primordium of femur
17. Palate
18. Cartilage primordium of basisphenoid bone
19. Esophagus
20. Neural tube
21. Future subarachnoid space
22. Dorsal root ganglion
23. Cartilage primordium of turbinate bones
24. Membranous part of interventricular septum
25. Upper lip
26. Lower jaw

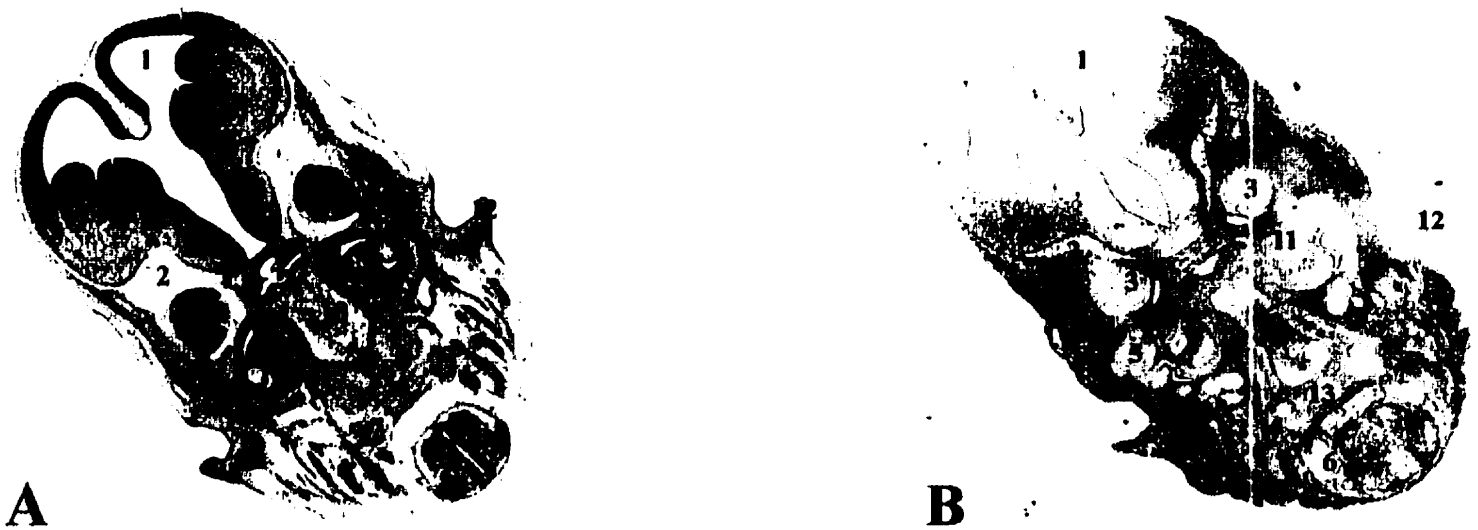

Figure 14 : Cranial cross-sections of mouse embryo at E14.
(A) H & E stained, (B) Immunostained for galanin.

1. Lateral ventricle
2. Future subarachnoid space
3. Trigeminal ganglion
4. Pituitary
5. Primordia of middle ear ossicles
6. Dorsal root ganglion
7. Cartilage primordium of exoccipital bone
8. Cartilage primordium of basioccipital bone
9. Neural tube
10. Ossification within parachordal plate
11. Otic capsule
12. Pinna
13. Neural arch of vertebra

Figure 15 : Cross-sections of the mid-section of mouse embryo at E14.
(A) H & E stained, (B) Immunostained for galanin.

1. Abdominal muscles
2. Stomach
3. Ovary
4. Paramesonphric duct
5. Mesonephric tubules (regressing)
6. Centrum of cartilage primordium of vertebra
7. Liver
8. Hind limb
9. Pancreas
10. Midgut
11. Neural tube
12. Dorsal root ganglion
13. Segmental nerve of tail

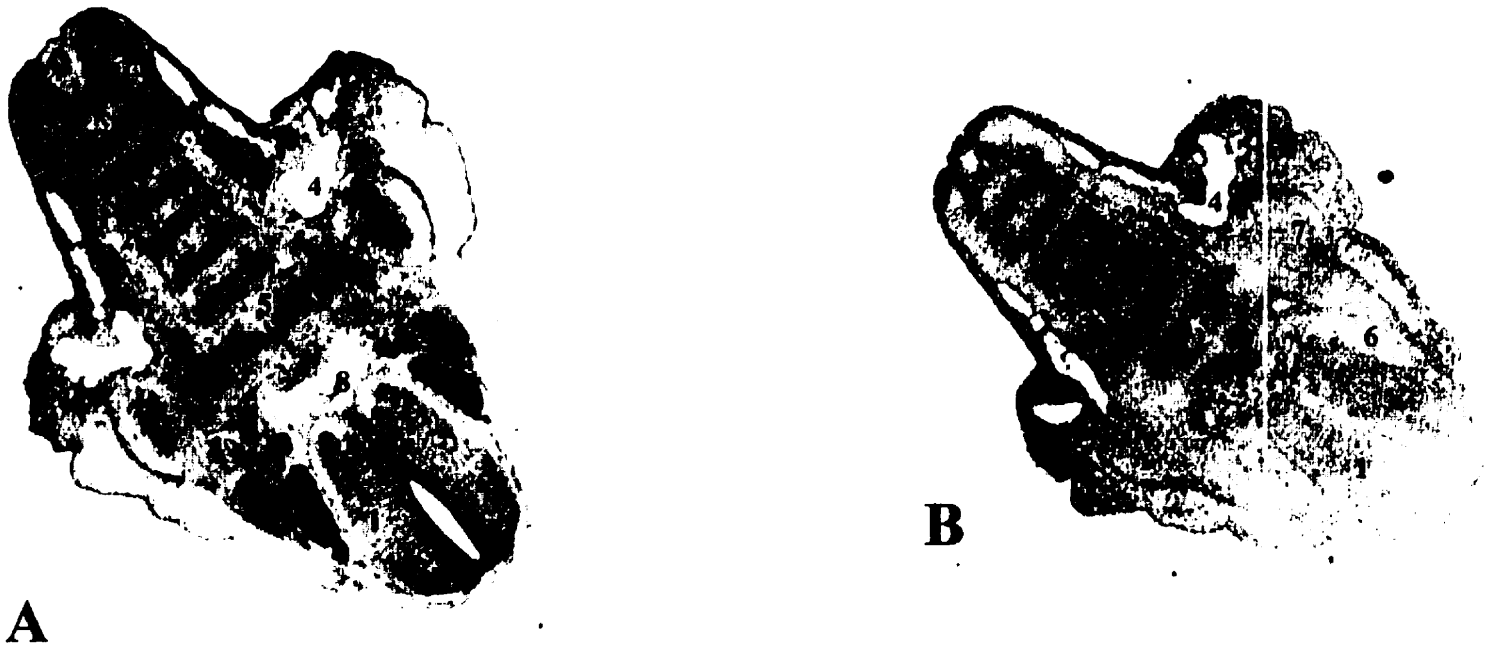

Figure 16 : Caudal cross-sections of mouse embryo at E14.
(A) H & E stained, (B) Immunostained for galanin.

1. Neural tube
2. Nerve trunk
3. Cartilage primordium of vertebra
4. Femoral vein
5. Notochord
6. Dorsal root ganglion
7. Muscle mass
8. Future subarachnoid space

mesenchyme surrounding the head of the cartilage primordia of the femur also stains for galanin (figure 13).

The vertebrae of the tail and the ribs stain for galanin in a similar manner as at E13 (figure 11, 12, and 16), though the interzones between the caudal vertebrae stain less intensely at this stage. However, the more rostal vertebrae contain much more GAL-LI at their borders, where intervertebral discs and connective tissue are developing, and near the notochord (figure 13 and 15). The tissue surrounding the segmental nerves in the tail also stains for galanin (figure 15 and 16).

Within the metanephros (definitive kidney), diffuse mesenchyme stains for galanin (figure 11 and 12). The abdominal wall, including developing muscle and skin, contains GAL-LI (figure 11, 12, 13, and 15). The ovary and dorsal mesentery (figure 15) stain at this stage, as does the stomach (figure 15) and the midgut (figure 11, 12, 13, and 15).

3.2.5 E15

The distribution of GAL-LI at embryonic day 15 is similar to the distribution at day 14 in the bones, head, gut, and heart (figure 17, 18, and 19). Staining is observed in the proximal part of the rib (figure 18) but not the in their distal tips (as they approach the sternum) in the chest wall (figure 17). The ventricle and atrium shown staining for galanin (figure 17) as in some embryos of previous stages (figure 4, 6, 5, and 12). Mesenchyme packed around the optic nerve and in the developing eyelid contains GAL-LI at E15 as well (figure 19).

Figure 17 : Parasagittal section of mouse embryo at E15, immunostained for galanin.

1. Lateral Ventricle
2. Mesencephalic vesicle
3. Cartilage primordium of nasal capsule
4. Palatal shelf
5. Ossification within proximal shaft of rib
6. Ventricle
7. Atrium
8. Lung
9. Liver
10. Cartilage primordium of distal rib within abdominal wall
11. Cartilage primordium of vertebrae
12. Genital tubercle
13. Otic capsule
14. Lower jaw
15. Tongue

Figure 18 : High magnification of embryo at E15 immunostained for galanin. Detect GAL-LI in (A) developing ribs, and (B) femur of developing hindlimb, (r) rib. (s) skin, (p) pleural cavity, (f) cartilage primordium of femur.

Figure 19 : Cranial cross-sections of mouse embryo at E15.
(A) H & E stained, (B) Immunostained for galanin.

1. Lateral ventricle
2. Third ventricle
3. Hair follicle
4. Eye
5. Trigeminal ganglion
6. Rathke's pouch
7. Tubo-tympanic recess
8. First branchial cleft
9. Precartilage primordium of temporal bone
10. Premuscle masses
11. Precartilage primordium of exoccipital bone
12. Cranial tube / caudal part of medulla oblongata
13. Future subarachnoid space
14. Caudal part of medulla
15. Cartilage primordium of basioccipital bone
16. Eyelid
17. Otic capsule

Table 3 : Summary of results					
Day	Bones	Gut	Urogenital System	Heart	Central Nervous System
E10				Outflow tract, endocardial cushions	Mesenchyme surrounding neural tube
E12	Sclerotomes, lateral and medial nasal processes	Mesenchyme surrounding esophagus, dorsal mesentery, abdominal wall		Outflow tract, endocardial cushions, ventricle, septum primum	Mesenchyme surrounding neural tube
E13	Basioccipital bone, basisphenoid bone, otic capsule, nasal capsule and septum, primary palate, ribs and vertebrae, head of femur	Mesenchyme beneath epithelium of midgut	Paramesonephros, genital tubercle	Outflow tract, endocardial cushions, ventricle	Mesenchyme surrounding neural tube (future subarachnoid space)
E14	Basioccipital bone, basisphenoid bone, petrous temporal bone, nasal capsule and septum, palate, ribs and vertebrae, head of femur, turbinate bones	Mesenchyme beneath epithelium of midgut (including stomach) and esophagus, abdominal wall	Ovary, mesenchyme of metanephros	Outflow tract including membranous part of interventricular septum	Mesenchyme surrounding neural tube (future subarachnoid space)
E15	(Distribution similar to E14.)	(Distribution similar to E14.)		Distribution similar to E14, but includes atria and ventricle walls	Distribution similar to E14, except mesenchyme around optic nerve and eyelid

4.0 Discussion

4.1 Crown-Rump Lengths

Average crown-rump lengths of the CD embryos used in this study match those for the CFI-S stain of mice obtained by Rugh (1968) for E10 to E14. Assuming these two strains develop at comparable rates, these results support that the predicted embryonic ages are accurate. The average crown-rump length for the litter representing "E15" is more consistent with embryos at E14.5. When mice are mated overnight, there can be as much as a 16 hour variation in the gestational ages of the resulting offspring. In addition, the mice were not collected at a set time on the required day. For these reasons, we accepted a possible error of ±12 hours in our estimation of the gestation ages of the embryos. This is relevant because knowing at which stages galanin is expressed can help determine which processes in which it is involved, e.g. proliferation versus differentiation.

4.2 Immunohistochemistry

4.2.1 General considerations when interpreting immunohistochemistry

When interpreting results, it is important to remember that when using immunohistochemical procedures like the one used here, "galanin-like immunoreactivity" (GAL-LI) is detected. The polyclonal antibody will not only interact with rat galanin, but any other proteins that are sufficiently similar in structure. Though *very* few proteins have been discovered that are homologous to galanin, there may be more galanin-like proteins that have yet to be discovered. Thus, any GAL-LI detected in this study may be galanin or some other as yet unknown protein. A concentration of the

antibody has been chosen to maximize the chances of detecting galanin-like proteins while minimizing the chances of background due to non-specific interactions. Future studies using in situ hybridization will help confirm the presence of galanin by localizing the corresponding mRNA.

When staining is observed at E10, it is possible that staining first appeared at an even earlier stage. Similarly, distribution of the staining seen at E15 could continue beyond this stage. Future studies will be required to determine when galanin *first* appears and when it finally disappears.

In cases where previous studies do not appear to agree with the results obtained here, it is possible that this can be due to the differences in technique used. Fixative choice and timing of fixation, handling of tissue, and the methods used to make the tissue sections more permeable to antibodies and blocking agents are among those variations in procedure that can make the difference in sensitivity.

4.2.2 Heart

From the few E10 sagittal sections available, immunostaining localized GAL-LI in the endocardial cushions and the spirally arranged aorticopulmonary septum developing in the outflow tract of the heart. These tissues continued to stain on E12. On sections of E14 embryos, GAL-LI was detected in the membranous part of the interventricular septum, which is composed partly of the most caudal portion of the fused aorticopulmonary septum. The endocardial cushions begin to form at about E9.5, growing between the primitive atrium and primitive ventricle to divide this region into right and left atrioventricular canals. The ridges of the aorticopulmonary septum fuse, and

the two resulting channels separate to form the aorta and pulmonary trunk. By E15, the walls of the heart are GAL-IR as well.

No previous studies have dealt with the expression of galanin development of the embryonic heart, and only a few have addressed the role of galanin in the function of the adult heart. Using RIA, GAL-LI was found in the adult hearts of the rat, mouse, guinea pig, rabbit, cat, and dog, but not the pig (Bretherton-Watt *et al.* 1990). Immunohistochemistry of the rat heart indicated that there are scattered GAL-IR, presumably neuronal, fibers throughout the heart (Xu *et al.* 1995). In this study, the GAL-LI observed in the embryonic heart is not restricted to fibers. The histology of GAL-IR cells in the endocardial cushions and spiral septum is similar to mesenchymal cells, i.e. small cells with large intercellular spaces. The staining of the atrium and ventricle is less defined. The identity of these GAL-IR cells could be determined in double-staining techniques using antibodies against galanin and various cell markers. Since neural crest cells contribute greatly to the formation of the aorticopulmonary septum and other parts of the heart, it is possible that some of the GAL-IR cells of the heart are derived from the neural crest as well as the cardiac mesoderm. The spiral aorticopulmonary septum is actively growing at the stages GAL-LI was present, so galanin may be a mitogen for those cells.

4.2.3 Bone

This study showed that GAL-LI was detected from the earliest stages of bone formation. GAL-LI is present during the formation of the vertebrae, from E12, when the dense and diffuse potions of the sclerotomes are combining to form the precartilage

models of these bones. A similar distribution pattern is seen later in the more caudal vertebrae, and this is expected since they develop in a craniocaudal sequence. GAL-LI is also present in the region of the nose, from as early as E12 when the medial and lateral nasal processes are still approaching each other to become circular olfactory pits. The mesenchyme that will become the bones of the base of the skull start exhibiting GAL-LI at E12 as well. As the precartilage and cartilage models of each bone are formed, GAL-LI becomes more restricted to the mesenchyme surrounding it. This phenomenon is also observed in the development of the basioccipital and basisphenoid bones, the ribs, and the femur.

The bones mentioned above develop using endochondral ossification since they form from cartilage models. Thus, galanin may be involved in the early stages of endochondral ossification. In the ribs, by E15, GAL-LI is detected within the bone, not just in the tissue surrounding the cartilage, when ossification is occurring. Thus, galanin could be involved throughout this process, from when the mesenchyme condenses to when the bony matrix is laid down. Galanin may also be involved in membranous ossification since the petrous part of the temporal bone, or otic capsule, which develops by this process, also contains GAL-LI from E13.

The facial bones, those bones encasing the sensory organs, the palate, and the basiospenoid bone are thought to be at least partly derived from the neural crest (Couly 1993), while other bones are derived from the sclerotomes. Thus, galanin may be involved throughout both processes of bone formation. Because GAL-LI is present throughout these processes, it is not possible to determine, from the timing of its appearance and its distribution, exactly what the function of the protein is. Galanin could

be involved in, for example, the condensation of mesenchyme or the differentiation of mesenchyme cells into chondroblasts and/or osteoblasts. The source of the mesenchyme does not appear to be a predictor of which tissues will contain GAL-LI since the mesenchyme is derived from both the somites and the neural crest. Galanin may also have some function in the development, e.g. proliferation, differentiation, or condensation, of other tissues derived from mesenchymal progenitor cells. These tissues include fibroblasts, muscle cells, stromal cells of the bone marrow, and other connective tissue such as adipocytes (fat cells) and dermal cells of the skin.

As in the heart, very little work has been done to determine if galanin plays a role in bone development before birth or after birth. Therefore, there is little data with which we can compare our results. In one such study using oligonucleotides, galanin mRNA was first detected in large cells, presumably osteoclasts, of developing bone of E19 rat embryos (Xu *et al.* 1996). We detect GAL-LI relatively earlier in a pattern that suggests that galanin is not only involved in osteoclast function. If we had extended our study to stages later than E15 in mouse, we also may have detected GAL-LI in the osteoclasts of new bone. Another study found that exogenous galanin inhibited DNA synthesis in cultured ovine growth plate chondrocytes and reduced the ability of gastrin-releasing peptide (GRP) to increase DNA synthesis of these cells (Hill *et al.* 1992). Those findings would contradict the idea that galanin is a mitogen, but a protein could act differently in vivo versus in culture. Some growth factors require interactions with molecules in the extracellular matrix, including those not produced by chondrocytes, in order to produce the proper effect (Hall 1994). Also, those cells may already be producing the protein, and therefore react to exogenous sources of galanin in a way unlike the in vivo response.

Finally, subpopulations within a population of cultured chondrocytes may respond different to galanin, but only the overall response is the one being measured. Thus, galanin may be a mitogen for mesenchymal cells, chondrocytes, or osteoblasts at any particular stage in their development.

There has been no report of galanin expression in intact adult bone or the surrounding tissues, nor has it been implicated in the process of bone repair. Bone repair is thought to involve similar molecular and cellular events as bone development. The repair of an unstable and stable fractures are thought to involve processes similar to endochondral ossification and membranous ossification, respectively. Length-wise growth of bones also uses endochondral ossification. Thus, galanin may be involved in development, growth, and repair of bones.

In summary, GAL-LI is present throughout development of various bones that are constructed using mesenchyme from both neural crest and somitic sources. It could be involved in one or more aspects of bone development, including chondrocyte and/or osteoblast proliferation or differentiation, manipulation of the blood supply (i.e. repelling the blood supply from cartilage or attracting it at the beginning of bone formation), or osteoclast (and osteoclast-like) activity or proliferation. Future studies may show that galanin has roles in bone development, growth, and repair.

4.2.4 Gut

From E10 in the mouse, the layer of mesenchyme beneath and surrounding the neural tube contains GAL-LI. The dorsal mesentery and esophagus begin to express GAL-LI at E12, and the midgut also contains GAL-LI from E13. At this time, the gut is

elongating and the splanchnic mesoderm surrounding it is condensing and will eventually differentiate into the connective tissue and smooth muscle layers of the GI tract. During E13, as the gut continues to elongate, the endoderm proliferates to obliterate the lumen, and then recanalizes and differentiates. Galanin could be involved in any of these processes. Alternatively, galanin could influence the development of other tissue types within the gut, including nervous elements, cartilage, i.e. near the esophagus and trachea, and blood vessels.

It should also be noted that although the location and histology of this tissue suggest that it is indeed of mesodermal origin, double staining with the appropriate neuronal and mesenchymal cell markers will need to be performed to determine the identify of these GAL-IR cells. Several studies have been done on galanin in relation to the development of the gut, mostly in relation to the enteric nervous system. Day 14 of gestation is the earliest point in development in which galanin (and GAL-R1) was detected in the gut of the rat (Xu *et al.* 1996), confirming an earlier RIA study that found GAL-LI in the GI tract of the rat at E15 (Gabriel *et al.* 1989). Other studies found GAL-LI in the nervous elements of the human gut, including the esophagus, starting at the 13th week of gestation (Hitchcock *et al.* 1992, Larsson *et al.* 1987). The appearance of GAL-LI is therefore well after the 8th week when the neuroblasts first arrive from the neural crest. In the chicken, on the other hand, GAL-LI is found in these cells shortly after they arrive at the developing gut from the neural crest, starting at incubation day 4. Such early expression suggests that galanin is a growth or differentiation factor for these cells. By E15, the distribution of the peptide has become adult-like in this species, present in nervous elements of the muscular layers and the lamina propria, often right up to the

epithelium (Salvi *et al.* 1998, 1999, García-Arrarás *et al.* 1999). In the rabbit, GAL-LI is detected in the duodenum only in small amounts just before birth at E30 (Wikström *et al.* 1993), though such a difference in the appearance of GAL-LI in the gut may partly be due to the differences in antibody sensitivities or other technical factors. It could be that the GAL-IR cells of the gut in the mouse observed in this study are neurons. Again, double-labeling with the appropriate neuronal markers should distinguish between nervous and mesodermal tissue in this region.

4.2.5 Spinal Cord, Brain, Trigeminal and Dorsal Root Ganglia

With the techniques used in this study, we did not detect GAL-LI in the central nervous system of the mouse from E10 to E15. However, intense GAL-LI was found in the mesenchyme surrounding the developing neural tube at least from E10, the earliest time studied. This tissue is likely mostly derived from the mesoderm, but neural crest cells, which may also be present in this region as they migrate to their various destinations, may also be in this location. Double staining using the appropriate cell markers would help determine which cell types in this region contain GAL-LI. Galanin derived from this mesenchyme could therefore affect the development of the central nervous system. In addition, this protein could affect the development of structures in this region, including bones (e.g. vertebrae, skull), blood vessels, and ganglia. Some of this GAL-IR tissue closest to the central nervous system will develop into the subarachnoid space of the meninges, which are thought to be partly derived from the neural crest. Thus, galanin may be involved in the development of the meninges as well. Therefore, as with

the development of the bones, galanin may be involved in the development of tissue derived from both the mesoderm and neural crest.

Brain

We did not detect GAL-LI within the brain of the developing mouse, but others have studied the appearance of GAL-LI in the rat brain. Sizer *et al* first detected GAL-LI in the brain starting at P2, and it increased to adult levels and distribution in the first postnatal month. They concluded the appearance of galanin in the brain (and spinal cord) to be entirely postnatal (Sizer *et al.* 1989), but others used RIA to detect GAL-LI in the rat brain from E15, during a period of rapid neurogenesis (Gabriel *et al.* 1989). Using in situ hybridization, weak signals for galanin mRNA were found in the future hypothalamus at E17 (Giorgi *et al.* 1995). Another study found galanin (and GAL-R1) signals in the developing rat brain as early as E14 (Xu *et al.* 1996). Thus, even within the same species, results are contradictory. These discrepancies are probably due to the sensitivity of the methods employed, including proper tissue fixation and preventing protein (and the mRNA) degradation. It seems clear that galanin expression in the brain of the rat (and probably of the mouse) is not entirely postnatal and, with sensitive enough techniques, we would expect to detect GAL-LI by E13 in the mouse brain. Also, it is important to be aware that low levels of expression of galanin at one stage of development (prenatal or postnatal) does not necessarily mean that it expression is not up-regulated at other stages, i.e. that the protein is transiently expressed.

The central nervous systems of other species have also been investigated for galanin expression during development. In the chicken (incubation 20-21 days), GAL-LI

is detected in extrahypothalmic tissues at incubation day 4, and within the future hypothalamus by day 6. By day 16, the distribution and levels of GAL-LI approach that of the adult. This is in contrast to the rat, where detectable levels of GAL-LI are present in several regions of the brain during the late embryonic stages of development, which decrease up to birth and increase again postnatally (Józsa *et al.* 1994).

Galanin expression in the pig (gestation 114 days, cellular level gonadal differentiation E26) brain has also been detected at least as early as E30, but almost exclusively in hypothalamic structures. Generally, the number and distribution of GAL-IR cell bodies and fibers seem to increase prenatally, and then decrease in the early postnatal period to adult levels (Pearson *et al.* 1996).

In Bazilian opossum, sexual differentiation, differentiation, and neurogenesis progress more slowly, so that the brain of the newborn is quite immature compared to the brains of newborns of other species. Galanin already appears in the brain at P1 in this species, while neurogenesis is occurring and before many of the regions in the brain can be identified morphologically. The number and distribution of GAL-IR neuronal elements increases gradually to adult levels. No transient expression is observed with the exception of a small number of cells that contain GAL-LI in the hippocampus at P25, but not at P10,16, or 60 (Elmquist *et al.* 1992).

Since galanin expression has been observed in the brain during prenatal development of the rat, chicken, and pig, and during the early postnatal development of the Bazilian opossum, galanin probably is expressed in the developing brain of the mouse as well. Our failure to detect GAL-LI in the mouse brain at least from E13 when others detected it in the rat at an equivalent stages in development suggests that our techniques

(tissue handling, immunohistochemistry, etc.) were less sensitive than those of Xu et al. For example, it is possible that the primary antibody did not penetrate certain types of tissue in the sections, including the brain and spinal cord. The use of a different fixative, or microwaving or autoclaving the sections may allow us to detect GAL-LI in the brain of the developing mouse.

There are no other reports of GAL-LI in the mesenchyme associated with the nervous system or any other system. Since we obtained negative results where others had positive results, i.e. in the nervous system, it would appear that our techniques were *less* sensitive than those of other investigators. On the other hand, we obtained positive results with respect to mesenchymal tissues, suggesting our techniques were *more* sensitive. One possible explanation is that our results were not suited to detect GAL-LI in nervous tissue, but were appropriate for detection of the protein in mesenchymal tissues.

Spinal Cord

Other investigators have detected GAL-LI first in the spinal cord at E15 in the rat (ventral horn), fetal week 6 in the human (ventral horn), and incubation day 2 in the chicken (Marti *et al.* 1987, Józsa *et al.* 1994). GAL-LI was not detected in the relatively immature spinal cords of the newborn dog (Bonfanti *et al.* 1991), although it was not determined whether or not galanin was transiently expressed earlier in this species. GAL-IR fibers were also identified in the autonomic portion of the spinal cord, the intermediolateral columns, at E21 of the rat and from the 20th fetal week of the human (Marti *et al.* 1987).

GAL-R1 mRNA also appears during the prenatal development of the rat spinal cord. It is detected in the ventral horn beginning at E17 (where GAL-LI was detected in this region at E16 of the rat and in the 8th week in the development of the human fetus (Marti *et al.* 1987)) and in the dorsal horn at E19 (Xu *et al.* 1996).

Thus, it is reasonable that GAL-LI would be detected in the developing spinal cord during prenatal development. It may be that the reason we could not demonstrate GAL-LI in the mouse spinal cord is that it is not expressed in detectable amounts in this species until after E15, the latest stage investigated in this study.

Trigeminal Ganglia and Dorsal Root Ganglia

We did not detect the protein in either the trigeminal or dorsal root ganglia in this study. However, our preliminary studies showed that galanin mRNA is present in these ganglia. Also, in other studies, galanin mRNA and protein were detected in the rat trigeminal ganglia and DRG at E15 and GAL-R1 mRNA was detected in these ganglia at E17 and E21, respectively. The protein was detected in both central and peripheral processes, suggesting that it is transported both centrally and peripherally (Xu *et al.* 1996). GAL-LI was also detected in the DRG of the human relatively later in development, at the 14th week of gestation (Marti *et al.* 1987). In the case of nervous tissues, it may be that our method, including the choice of antibody, contributed to our difficulty in confirming the results of others. The protein may have been degraded or the antibody was unable to penetrate the nervous tissue. It seems clear from the above reports, however, that the mRNA is translated and that the protein is not transported out of the cell too quickly or masked in such a way that no antibody could possibly detect it.

Katz et al could not detect GAL-LI in the trigeminal and dorsal root ganglia of the rat at E11.5-13.5, a point in development when some of the neurons are still dividing (Katz *et al.* 1992), supporting the idea that the protein may not be expressed until later in development of these tissues in the mouse. If galanin is expressed after E15 in the mouse, this would suggest galanin is not a mitogen for these particular cells since most of them have exited mitosis by ~E15.5 (Katz *et al.* 1992).

If galanin is present in the neurons of these ganglia during embryonic development of the mouse, there are several possibilities as to its function. It could be a growth or differentiation factor for the cells that produce it. The protein could also influence the development of cells nearby the neuronal cell bodies or their processes, such as oligodendrocytes or Schwann cells or fibroblasts in nearby connective tissue. We did detect GAL-LI in the mesenchyme surrounding the DRG and trigeminal ganglia. Therefore, the protein may affect the development of the ganglia when it is surrounding the corresponding neurons.

4.2.6 Adrenal Gland

The developing adrenal gland of the mouse did not contain GAL-LI at the stages studied here. However, the timing, distribution, and the level of expression of galanin depend on the species being considered. In the chicken, all or most of the future medullary cells, derived from the neural crest, contain GAL-LI beginning when they penetrate the future adrenal cortex at incubation day 4 and continuing at least up to hatching. This expression is also present in the chromaffin cells of adult adrenal medulla (Sánchez-Montesinos *et al.* 1996). In contrast, in the rat, only insignificant numbers of

chromaffin cells stained for galanin in the postnatal period and in the nervous tissue of the adrenal at P2 (Holgert *et al.* 1994), when the adrenal nervous elements are nearly mature. In the rabbit, the level of galanin expression is low in the chromaffin cells at E28 but increases to adult levels during the postnatal period (Wikström *et al* 1993). Thus, although galanin does not appear to be expressed at E14 or E15 in the mouse, it may be expressed at later stages.

4.2.7 Other peripheral tissues

Galanin appears to be expressed in tissues associated with the developing eye. We found that GAL-LI is present in the mesenchyme surrounding the optic nerve and in the tissue immediately surrounding the vitreous body of the posterior chamber at E15. At this stage, the mesenchyme within the eyelids also contains GAL-LI as they begin to grow toward each other to eventually fuse. Although we detected galanin expression in mesenchymal cells, some of which may be neural crest derived, other studies found galanin expression only in the nervous tissues of the eye. Protein and mRNA corresponding to galanin, but not GAL-R1, have been detected in the developing eye of the rat from E15, particularly in the inner layers of the retina (Xu *et al.* 1996). In the Brazilian opossum, the ganglion cells of the retina and the axons of the optic nerve contained GAL-LI when the nervous system is still relatively immature, at P1-16 (Elmquist *et al.* 1992).

At every time point we studied, from E10 to E15, there was GAL-LI in the mesenchyme close to the surface of the skin in various regions of the body, including the limbs, face, back, and chest. In the face, galanin appeared to be expressed in the

mesenchyme surrounding the developing hair bulbs, but not the hair bulbs themselves, which are derived from the ectoderm. At E15-17 in the rat, galanin mRNA was found just below the surface of the epithelium of the skin (Xu *et al.* 1996). However, previously, Marti et al did not detect GAL-LI in the skin of the rat from E12 or of the human fetus from 6 weeks gestation (Marti *et al.* 1987).

The development of the gonads is a complex process that occurs in the intermediate mesoderm of the embryo. Briefly, the mesodermal epithelium of the urogenital ridge forms projections into the mesenchyme beneath it. (In the female, these primary sex cords eventually disappear.) Later, secondary cords derived from the mesothelium project into the mesenchyme and break up, and, together with germ cells originally from the yolk sac, form follicle primordia. Meanwhile, longitudinal invaginations of the mesothelium along each urogenital ridge form the paramesonephric ducts which project into the urogenital sinus. These paramesonephric ducts fuse with each other caudally and give rise to the uterus. The mesenchyme surrounding the paramesonephric ducts and the ovary stains for the protein and so galanin may be involved in the development of these structures. The timing of the appearance of the protein (E13) and the lack of staining in the testis suggest that galanin may be involved in sexual differentiation of the genital system.

The pituitary also lacks the presence of GAL-LI throughout this period studied. This suggests that galanin is not involved in the early development of this tissue, i.e. between E10 and E15, in the mouse.

4.3 Transient Expression

Others and we have found that GAL-LI and/or galanin mRNA to be transiently expressed during prenatal development (Marti *et al* 1987, Xu *et al* 1996). This may be due to a real temporary expression of the protein or could have more to do with technical problems, e.g. pour penetration of the antibody into nervous tissue or degradation of the protein or mRNA being detected. Also, the protein may be transported out of the cell that made it and into surrounding cells or the extracellular matrix during particular stages of development. In other cases, the immunoreactive cells have undergone programmed cell death or migrated to another location.

When determining the function of a protein that is transiently expressed transiently expressed during development, it is important to consider that it may be very different from the function in the adult. For example, future targets of 5-HT (5-hydroxytryptamine) innervation require intracellular and circulating 5-HT as a differentiating signal before they even receive that innervation (Lauder et al 1978). Alternatively, the protein may have a trophic effect on a population cells early in development that is unnecessary later on. In fact, this effect may be reactivated during injury of the tissue or during the formation of cancerous tumors involving that cell type. Thus, determining the reason for the apparent transient expression can help determine the function of the protein during the stage in which it is expressed.

4.4 Possible Functions of Galanin

In the adult, galanin expression changes in response to various factors such as the changing levels of estrogen and nerve damage (Bartfai *et al.* 1993), but the expression

and the changes in expression in response to various stimuli depend on the species being considered. When galanin is expressed in response to a stimulus, it may be the reactivation of the ontogenic program. When discussing the expression of galanin in development of the rat, Xu *et al* went so far as to suggest that "...the main role of GAL/GMAP is during development." (Xu *et al.* 1996). The function of the protein in the embryo may also be different from the function in the adult and may even act through different receptors (or combination of receptors), mechanisms, and pathways.

One possible function of galanin during development is as a mitogen. This action could be towards the cells that express it, i.e. mesenchyme derived from the mesoderm and neural crest, or the nearby cells, such as nervous tissue of the central or peripheral nervous system. Galanin acts as a mitogen in some cell culture situations, and so may also act in such a way during development of some tissues (in vivo). In the rat hypothalamus, GAL-R2 is coupled to phospholipase C (PLC) through G-proteins in such a way as to cause increases in intracellular calcium levels upon its activation. This response is known to be associated with mitogenic signaling and is similar to the response observed after activation of the G-coupled receptor of bombesin (Smith et al 1997, Rozengurt 1991). Thus, receptors like GAL-R2 may mediate any mitogenic effects of galanin. Using the proper conditions, including allowing enough time for galanin to elicit an effect or also adding the appropriate growth factors, future studies may show that galanin is a mitogen for individual cell types in the embryo using GAL-R2-like receptors. Galanin may only have a mitogenic effect for, e.g. mesenchymal, cells during a very short period in their differentiation. That is, the effect of galanin may be dependant on

their state of differentiation since the expression of certain receptors may be transient as well.

Timing of expression during development alone is insufficient to predict whether or not galanin is a mitogen. In general, differentiation and proliferation seem to oppose each other. However, in the embryo, these processes are not so well separated. In fact, it was suggested that the actions of growth factors in controlling differentiation are linked to their control on cell division (Mercola *et al.* 1988). Thus, it is too simplistic to state that on a particular day in embryonic development, a particular cell type is *either* proliferating *or* differentiating. They may be alternating between processes rapidly. Since mechanisms inhibiting proliferation may also have a role in stimulating differentiation. there may be expression during both processes. Since galanin is widespread throughout development between E10 and E15, when both proliferation and differentiation are occurring, the role galanin plays during development appears to be complex.

4.5 Galanin and Mesoderm

Galanin expression has never before been associated with the development of mesenchymal tissue. We find GAL-LI in the mesenchyme of the developing bones, gut. skin, heart, and (female) urogenital system, as well as the mesechymal tissue associated with the eye. However, much of the work concerning the pathways through which mitogens act is done with mesenchymal Swiss 3T3 cells or intact fibroblasts (Rozengurt 1991). That work links mitogenic pathways to enzymes which galanin receptors are coupled to, such as PLC (Smith et al 1997). However, there have been no reports documenting whether or not galanin has a mitogenic effect on Swiss 3T3 cells or

fibroblasts. In the future, galanin may be found to be a mitogen for these cells as well as other mesenchymal cells.

Another growth factor that is associated with mesenchymal development is TGF-ß. Its expression in the mouse embryo from E8-18 has some similarities to the expression of galanin. TGF- ß has been detected in mesenchyme, including the sclerotome, and tissues derived from mesenchyme, such as cartilage, bone, and connective tissue. These tissues include those derived from neural crest, including the palate, facial mesenchyme, nasal sinuses, and meninges. Regions in the heart and in developing hair follicles also appear to express TGF- ß (Heine et al 1987). Although TGF-ß distribution does not perfectly overlap the distribution of GAL-LI, their functions may be related in the regions that they do overlap. For example, TGF- ß is involved in chemotactic, proliferative, and differentiative actions in various mesenchymal cells. Therefore, it is useful to compare the expression of various proteins. It could be that TGF-ß is involved in turning on the galanin gene of certain cells.

5.0 Conclusions

GAL-LI is present in the mesenchyme of many regions of the mouse embryo from E10 to E15, including developing bone (skull, vertebrae, ribs, limbs), the skin, the heart (spiral septum, endocardial cushions), the gut, the meninges, and the urogenital system (ovary and uterus). In the case of bone development, GAL-LI is present throughout the process of both endochondral and membranous ossification. Galanin may therefore be involved in the development of these tissues. In situ studies need to be performed in order to confirm that galanin, and not another undiscovered peptide,

corresponds to the GAL-LI detected in this study. The bone and meninges, which both contain GAL-LI in the mouse embryo, are derived from both mesoderm and neural crest sources, and so galanin expression is likely not restricted by the source of the mesenchyme. The signaling for growth and differentiation in the embryo is complex and the two processes are often related. Thus, timing of the appearance of galanin is only one clue in determining the function of galanin. For these reasons, it is reasonable to conclude that galanin, or a galanin-like peptide, is involved in development, and that the signaling pathways that mediate the effects of this protein in the embryo are complex.

Studies on specific cell lines and tumors suggest that galanin is a mitogen and that this action is tissue specific. Therefore, galanin may be a mitogen in the embryo. More studies will have to be performed, e.g. on cultures of individual embryonic cell types, including the effect of galanin on intracellular calcium levels. It may be that galanin is influencing the development of tissues nearby those that contain GAL-LI, such as the central and peripheral nervous systems.

Since GAL-LI is present in certain mesenchymal tissues of the mouse embryo from at least E10 to 15, it is probably involved somehow in the development of those tissues. Galanin may act as a mitogen for certain cell types, but alternative or additional roles, such as in cell differentiation or condensation, are also possible.

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