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Identification and Characterization of the 5'-flanking Region of the Rat Galanin Gene

By

Hong Zhang

A Thesis

Submitted to the Faculty of Graduate Studies
in Partial Fulfillment of the Requirements for the Degree of

Master of Science

Department of Human Anatomy and Cell Sciences
University of Manitoba
Winnipeg, Manitoba

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IDENTIFICATION AND CHARACTERIZATION OF THE 5'-FLANKING REGION OF THE RAT GALANIN GENE

BY

HONG ZHANG

A Thesis submitted to the Faculty of Graduate Studies of The University of Manitoba in partial fulfillment of the requirements of the degree

of

MASTER OF SCIENCE

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To My Parents, My Wife And My Son....

ABSTRACT

Galanin, a neuropeptide widely distributed throughout the central and peripheral nervous systems, is a biologically active peptide which is strongly upregulated by nerve injury, estrogen and during development by mechanisms largely unknown. Here I present the initial molecular and functional characterization of the promoter region of the rat galanin gene. A rat genomic clone containing at least 4.5 kb 5'-flanking sequence has been isolated by PCR method. Partial sequence analysis of the clone revealed that the structure of the rat galanin gene is very conserved among species. Similar to the bovine, human and mouse genes, the rat gene has several transcriptional factor binding site consensus sequences such as SP1, AP-1, $ERE_{1/2}$, CREB. To locate functionally active regulatory elements of the rat galanin gene we have constructed plasmids containing various lengths of the rat galanin 5'-flanking sequence and the first exon, fused into a promoterless luciferase reporter plasmid. These hybrid genes were transfected into the human neuroblastoma (SK-N-MC) cell line, the rat pheochromocytoma (PC_{12}) cell line, the rat pituitary tumour cell line (GH_3), and the Hela cell line, by transient transfection. Galanin gene was transcriptionally active in both neuronal and endocrine cell lines while no activity was detected in Hela cells.

The first 200 bp of the galanin promoter contains strong neuronal and endocrine tissue specific elements and responsive elements to TPA and NGF. Strong negative regulatory elements are located upstream of the -657 bp for neuronal tissue and upstream of -486 for

endocrine tissue. Mobility gel shift assay identified a strong suppressor binding protein and a strong positive binding protein of the 5'-flanking region of the rat galanin gene in neuronal cells that is further induced by TPA. We conclude that functional neuronal tissue specific elements are resided within -200 bp of the proximal region of the rat galanin gene and that regulation of the rat galanin gene transcription in neuronal and endocrine cells appear to be mediated by both negative and positive regulatory element.

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TABLE OF CONTENTS

	Page
Abstract	i
Acknowledgments	iii
Table of Contents	v
List of Figures	x
List of Tables	xii
List of Abbreviations	xiii
Introduction	1
I. Chemistry and Molecular Structure of Galanin	1
I.1 Structure of galanin	1
I.2 The structure of galanin gene	7
I.3 Regulation of galanin mRNA	9
I.3.1 Regulation by sex steroid hormones	9
I.3.2 Regulation by Nerve Growth Factor (NGF)	10
I.3.3 Regulation by nerve injury	11
I.3.4 Regulation by TPA	11
II. Distribution of Galanin and Galanin Receptors in the Central Nervous System (CNS) and Peripheral Nervous System (PNS)	12

III. The Molecular Mechanisms of Galanin Action 14

IV. Physiology of Galanin 16

IV.1 The effects of galanin on growth hormone (GH), prolactin (PRL), and other hormones 16

IV.2 The effect of galanin on insulin secretion 18

IV.3 The effect of galanin on feeding behaviour 19

IV.4 Galanin and Alzheimer's disease 20

IV.5 The effect of galanin on learning and memory processes 22

IV.6 The effect of galanin on smooth muscle 23

IV.7 Other effects of galanin 25

V. Objectives 26

Materials and Methods 28

1. DNA and RNA Preparation 28

1.1 Isolation of total and messenger RNA 28

1.2 DNA Preparation 29

1.2.1 Maxi preparation of plasmid 29

1.2.2 Isolation and purification of genomic DNA from tissue 30

1.2.3 Isolation and purification of bacteriophage DNA 30

2. Restriction Endonuclease Analysis and Agarose Gel Electrophoresis 32

3. Recovery of DNA Fragments from Agarose Gel 32

3.1 Recovery of DNA fragments from agarose gel by Advantage™ PCR-Pure Kit 32

3.2 *In situ* electroelution of DNA from agarose gel 33

4. Subcloning of DNA Fragments 33

4.1 Mapping of lambda phage inserts 33

4.2 Subcloning of the A_{22} lambda phage inserts 34

4.3 Mapping and construction of luciferase reporter plasmids 34

5. Transformation of Competent *E. coli* Cells 37

5.1 Preparation of TG1λ competent cells 37

5.2 Transformation of TG1λ cells 37

6. DNA Labelling 38

6.1 Random priming 38

6.2 End labelling 38

6.2.1 Phosphate labelling with T_4 polynucleotide kinase 38

6.2.2 End-labelling with reverse transcriptase 39

7. Southern Blot 39

7.1 Southern transfer of DNA 39

7.2 Southern hybridization 40

8. Screening Genomic DNA Library by PCR 42

8.1 Titering the frequency of the original library for the rat galanin gene 42

8.2 Amplification of phage 42

8.3 Pooling of phage 44

8.4 Design of primers and PCR reactions 45

8.5 Analysis of PCR products 46

8.6 Plaque screening 46

9. DNA Sequencing and Sequence Structure Analysis 47

9.1 End labelling of primer 47

9.2 Sequencing reactions 47

9.3 Acrylamide gel electrophoresis 48

10. Primer Extension Reaction 49

10.1 Labelling of primers 49

10.2 Primer extension 49

11. Cell Culture 50

12. Transfection 51

13. Luciferase and β-Galactosidase Assays 52

13.1 Luciferase assays 52

13.2 β-Galactosidase assays 52

13.3 Transfection efficiency and modification of luciferase activity 53

14. Preparation of Nuclear Extract 53

15. Gel Mobility Shift Assay 54

Results 56

1. Isolation and Cloning of the Rat Galanin Gene 56

2. Determination of the Transcriptional Start Site 64

3. Reporter Gene Constructs 64

4. Basal Rat Galanin Promoter Activity in Neuronal and Endocrine Cell Lines 67

5. Induction of Galanin Promoter Activity by TPA 69

6. Induction of Galanin Promoter Activity by NGF 73

7. Induction of Galanin Promoter Activity by Estrogen 73

8. Identification of Transcription Factor-binding Sites in the 5'-flanking region 74

Discussion 78

1. Cloning and Characterization of the 5'-Flanking Region of the Rat Galanin Gene 78

2. Identification of Tanscriptional Activating Elements in the 5'-Flanking Region of the Rat Galanin Gene 81

2.1 Basal activity of the rat galanin promoter 81

2.2 TPA 84

2.3 NGF 87

2.4 Estrogen 87

References 90

LIST OF FIGURES

		Page
Figure 1.	Schematic representation of the rat, porcine, bovine, and human preprogalanin.	4
Figure 2.	Organization of the human preprogalanin gene.	8
Figure 3.	Deletion constructs and their locations in the rat galanin gene.	36
Figure 4.	Schematic diagram of the PCR screening procedure and diagrammatic representation of the PCR product detectable by hybridization.	43
Figure 5.	PCR screening of a rat genomic library for the galanin gene.	57
Figure 6.	Isolation of the rat genomic clone.	58
Figure 7.	Restriction mapping of the genomic clone A_{22}.	61
Figure 8.	Restriction map of the 5'-flanking region of the rat galanin gene and sequence strategy of the 8 kb-*Hind* III fragment.	63
Figure 9.	Nucleotide sequence of the proximal promoter region of the rat galanin gene and exon 1, exon 2, and the first intron of the gene.	65
Figure 10.	Determination of the transcriptional start site of the rat galanin gene by primer extension analysis.	66
Figure 11.	Rat galanin gene promoter activity in the Hela, SK-N-MC, PC_{12}, and GH_3 cell lines.	68
Figure 12.	Rat galanin gene promoter activity in the Hela and SK-N-MC cells.	70
Figure 13.	Rat galanin gene promoter activity in the GH_3 cell line.	71
Figure 14.	Stimulation of the rat galanin gene promoter activity by NGF in the SK-N-MC, PC_{12}, and GH_3 cells	72

Figure 15. Gel mobility shift and competition analysis of the fragment -208 to +13 5'-flanking region of the rat galanin gene. 75

Figure 16. Gel mobility shift analysis of the fragment -208 to -126 (lane 5-8) and -126 to +13 (lane 1-4) 5'-flanking regions of the rat galanin gene. 77

Figure 17. Organization of the rat preprogalanin gene. 79

LIST OF TABLES

		Page
Table 1.	Amino acid sequences of galanin in different species.	2
Table 2.	Primary structure of rat, porcine, bovine and human preprogalanin.	5
Table 3.	Analysis of the 5'-flanking and the first two exons and introns of the rat galanin gene for potential binding sites of known transcription factors.	82

LIST OF ABBREVIATIONS

AC	adenylate cyclase
ACh	acetylcholine
ACTH	adrenocorticotrophic hormone
AD	Alzheimer's disease
Ala	alanine
Arg	arginine
Asn	asparagine
Asp	aspartate
ATP	adenosine triphosphate
bGAL	bovine galanin
bp	base pair
BSA	bovine serum albumin
cAMP	cyclic adenosine monophosphate
CCK	acetylcholine
cDNA	complementary DNA
cGMP	cyclic guanosine monophosphate
ChAT	acetyltransferase
CIP	calf intestinal phosphatase
CMF	Calcium-magnesium-free
CNS	central nervous system
CORT	corticosterone
CRF	corticotrophin-releasing factor
DAG	1,2-diacylglycerol
dATP	dexyoadenosine triphosphate
dCTP	dexyocytidine triphosphate
ddH_2O	distilled and deionized water
dGTP	deoxyguanosine triphosphate
DMEM	Dulbecco's modification of Eagle's medium
DMSO	dimethyl sulfoxide
dsDNA	double strand DNA
DTT	dithiothreitol
dTTP	deoxythymidine triphosphate
DRG	dorsal root gangion
EDTA	ethylenediaminetetraacetic acid
E_2	17β-estradiol
GAD	glutamic acid decarboxylase
GAL	galanin

GAL-LI	galanin-like immunoreactivity
GH	growth hormone
GH_1	rat adenomatous cell line
GH_3	rat pituitary cell line
GHRH	GH-releasing hormone
Glu	glutamate
Gly	glycine
GMAP	galanin message-associated peptide
GRF	growth hormone releasing factor
FCS	fetal calf serum
Fig.	Figure
5-HT	5-hydroxytryptophan
hGAL	human galanin
His	histidine
IAS	opossum internal and sphincter
IP_3	inositol-1,4,5,-triphosphate
kb	kilobase pair
LB	L broth medium
LC	locus coeruleus
LES	lower esophageal sphincter
Leu	leucine
LH	luteinizing hormone
LHRH	luteinizing hormone-releasing hormone
LIF	leukemia inhibitory factor
Lys	lysine
MEN 1	multiple endocrine neoplasia type 1 disease
mGAL	mouse galanin
mRNA	messenger RNA
NaOAc	Sodium acetate
NGF	nerve growth factor
NMR	nuclear magnetic resonance
NPY	neuropeptide tyrosine
OD	optic density value
PBS	phosphate buffered saline
PCR	polymerase chain reaction
PC_{12}	rat pheochromocytoma cell line
pfu	plaque forming unit
PI	phosphoinositide
PKA	protein kinase A
PKC	protein kinase C
PLC	phospholipase C
PMSF	phenylmethylsulphonyl fluoride
PNS	peripheral nervous system

poly [dI-dC]	polydeoxyinosinic-deoxycytidylic acid
PRL	prolactin
Pro	proline
PTX	pertussis toxin
PVN	paraventricular nucleus
rGAL	rat galanin
RT-PCR	reverse transcriptase polymerase chain reaction
Ser	serine
SDS	sodium dodecyl sulfate
SK-N-MC	human neuroblastoma cell line
TFE	trifluoroethanol
TPA	12-O-tetradecanoylphorbol-13-acetate
TRH	thyrotropin releasing hormone
Trp	tryptophan
TSH	thyroid-stimulating hormone
Tyr	tyrosine
UV	ultraviolet
VDCC	voltage-dependent calcium channels
VIP	vasoactive intestinal polypeptide

INTRODUCTION

I. Chemistry and Molecular Structure of Galanin

I.1 Structure of galanin

The neuropeptide galanin was first isolated from porcine small intestine in 1983 and named for its amino-terminal glycine and amidated carboxy-terminal alanine residues (Tatemoto *et al.* 1983). The amino acid sequence of the rat galanin (Vrontakis *et al.* 1987 and Kaplan *et al.* 1988a), mouse galanin (Kofler *et al.* 1996), bovine galanin (Rokaeus *et al.* 1988), porcine galanin (Rokaeus *et al.* 1986), ovine galanin (Sillard *et al.* 1991), chicken galanin (Norberg *et al.* 1991), canine galanin (Boyle *et al.* 1994), alligator galanin (Wang *et al.* 1995), tuna fish galanin (Habu *et al.* 1994), and human galanin (Evans *et al.* 1991) have been determined (Table 1).

Galanin amino acid sequences from several species show that most mammalian galanin molecules are 29 amino acids long and are amidated at their C-terminus. The human galanin is an exception with its 30 amino acids and with a C-terminal free carboxylic acid. There is an absolute conservation of the N-terminal 15 amino acids in all species known, whereas there are several amino acid substitutions in the C-terminal portion of the molecule (Table 1).

Galanin is derived, through proteolytic processing, from a larger precursor protein known

Table 1. Amino acid sequences of galanin in different species.
Comparison of the amino-acid sequence of the rat, mouse, bovine, porcine, ovine, chick, human, canine, alligator, and tuna fish galanin. Nonconserved residues are bold.

					5					10					15					20					25				29	30
RAT	G	W	T	L	N	S	A	G	Y	L	L	G	P	H	A	I	D	N	H	R	S	F	S	D	K	H	G	L	T	
MOUSE	G	W	T	L	N	S	A	G	Y	L	L	G	P	H	A	I	D	N	H	R	S	F	S	D	K	H	G	L	T	
BOVINE	G	W	T	L	N	S	A	G	Y	L	L	G	P	H	A	**L**	D	**S**	H	R	S	F	**Q**	D	K	H	G	L	**A**	
PORCINE	G	W	T	L	N	S	A	G	Y	L	L	G	P	H	A	I	D	N	H	R	S	F	**H**	D	K	**Y**	G	L	**A**	
OVINE	G	W	T	L	N	S	A	G	Y	L	L	G	P	H	A	I	D	N	H	R	S	F	**H**	D	K	H	G	L	**A**	
CHICK	G	W	T	L	N	S	A	G	Y	L	L	G	P	H	A	**V**	D	N	H	R	S	F	**N**	D	K	H	G	**F**	T	
HUMAN	G	W	T	L	N	S	A	G	Y	L	L	G	P	H	A	**V**	**G**	N	H	R	S	F	S	D	K	**N**	G	L	T	**S**
CANINE	G	W	T	L	N	S	A	G	Y	L	L	G	P	H	A	I	D	N	H	R	S	F	**H**	**E**	K	**P**	G	L	T	
ALLIGATOR	G	W	T	L	N	S	A	G	Y	L	L	G	P	H	A	I	D	N	H	R	S	F	**N**	**E**	K	H	G	**I**	**A**	
TUNA FISH	G	W	T	L	N	**A**	A	G	Y	L	L	G	P	H	**G**	I	D	**G**	H	R	**T**	**L**	**G**	D	K	**P**	G	L	**A**	

as preprogalanin. Southern and Northern blot analysis of several tissues revealed that galanin is encoded by a single-copy gene and that its mRNA migrates as a single band of approximately 900 nucleotides (Vrontakis *et al.* 1987 and Kaplan *et al.* 1988a). No homologies have been found between galanin and any other known families of peptides.

In all species studied, preprogalanin mRNA encodes a 123-124 amino acid precursor protein. It contains a hydrophobic sequence of approximately 20 amino acids of signal peptide, 29-30 amino acids of galanin and a carboxy-terminal 59-60 amino acids galanin message-associated peptide, named as GMAP (Vrontakis *et al.* 1987, Kaplan *et al.* 1988a, Kofler *et al.* 1996, Rokaeus *et al.* 1986 and 1988, Sillard *et al.* 1991, Norberg *et al.* 1991, Boyle *et al.* 1994, Wang *et al.* 1995, Habu *et al.* 1994, and Evans *et al.* 1991). As shown in Figure 1, the galanin sequence consists of amino acids 33-62 in the precursor and is flanked by two pairs of basic amino acids (Lys-Arg) which are cleavage sites (Figure 1 and Table 2).

Rodent, mouse, porcine and bovine preprogalanins are all composed of a signal sequence, followed by a Lys-Arg cleavage site, then the 29 amino acids galanin peptide, and finally, the Gly-Lys-Arg at the C-terminus that contains the amide donor glycine and the cleavage site Lys-Arg. Interestingly, in the human preprogalanin, a serine residue replaces the glycine. Due to this change, the carboxy-terminal amidation does not occur, i.e. human galanin is not amidated and contains one additional amino acid (Evans *et al.* 1991). The entire human galanin amino sequence, including the signal peptide exhibits 85% identity with the rat, porcine and bovine galanin amino acid sequence (Table 2).

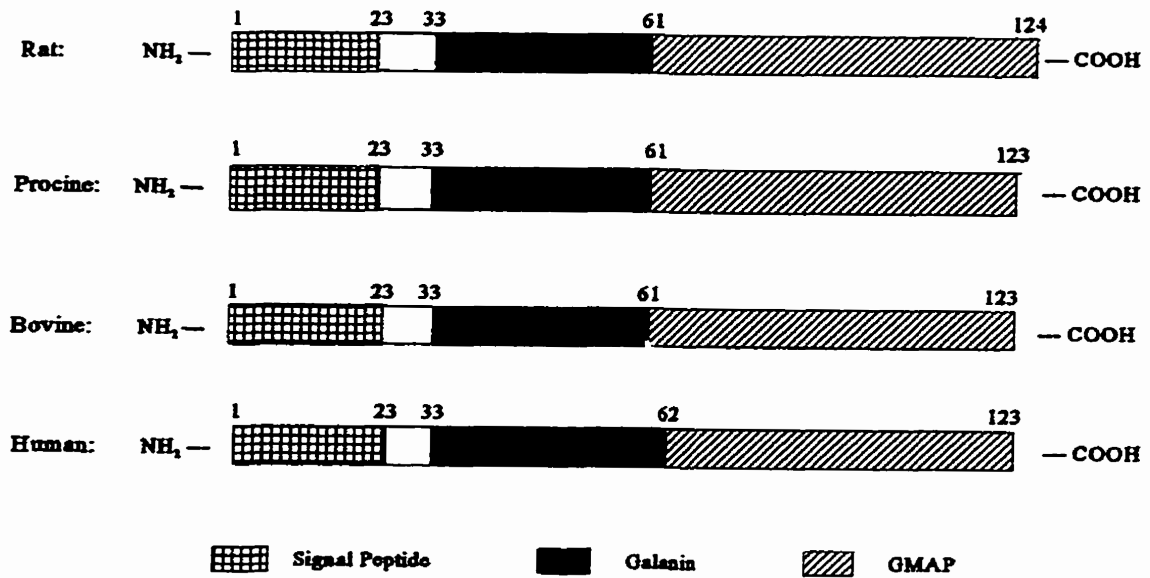


Figure 1. Schematic representation of the rat, porcine, bovine, and human preprogalanin.

Table 2. Primary structure of rat, porcine, bovine and human preprogalanin.
The conserved areas are bold

	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19
Rodent	**Met**	**Ala**	**Arg**	**Gly**	**Ser**	**Val**	Ile	**Leu**	**Leu**	**Ala**	Trp	**Leu**	**Leu**	**Leu**	Val	**Ala**	Thr	**Leu**	**Ser**
Porcine	**Met**	Pro	**Arg**	**Gly**	Cys	Ala	**Leu**	**Leu**	**Leu**	**Ala**	**Ser**	**Leu**	**Leu**	**Leu**	**Ala**	Ser	**Ala**	**Leu**	**Ser**
Bovine	**Met**	Pro	**Arg**	**Gly**	**Ser**	**Val**	**Leu**	**Leu**	**Leu**	**Ala**	**Ser**	**Leu**	**Leu**	**Leu**	**Ala**	**Ala**	**Ala**	**Leu**	**Ser**
Human	**Met**	**Ala**	**Arg**	**Gly**	**Ser**	Ala	**Leu**	**Leu**	**Leu**	**Ala**	**Ser**	**Leu**	**Leu**	**Leu**	**Ala**	**Ala**	**Ala**	**Leu**	**Ser**
	20	21	22	23	24	25	26	27	28	29	30	31	32	33	34	35	36	37	38
Rodent	**Ala**	**Thr**	**Leu**	**Gly**	**Leu**	**Gly**	Met	**Pro**	Thr	**Lys**	**Glu**	**Lys**	**Arg**	**Gly**	**Trp**	**Thr**	**Leu**	**Asn**	**Ser**
Porcine	**Ala**	**Thr**	**Leu**	**Gly**	**Leu**	**Gly**	**Ser**	**Pro**	**Val**	**Lyr**	**Glu**	**Lys**	**Arg**	**Gly**	**Trp**	**Thr**	**Leu**	**Asn**	**Ser**
Bovine	**Ala**	**Thr**	**Leu**	**Gly**	**Leu**	**Gly**	**Ser**	**Pro**	**Val**	**Lyr**	**Glu**	**Lys**	**Arg**	**Gly**	**Trp**	**Thr**	**Leu**	**Asn**	**Ser**
Human	**Ala**	Ser	Ala	**Gly**	**Leu**	Trp	**Ser**	**Pro**	Ala	**Lyr**	**Glu**	**Lys**	**Arg**	**Gly**	**Trp**	**Thr**	**Leu**	**Asn**	**Ser**
	39	40	41	42	43	44	45	46	47	48	49	50	51	52	53	54	55	56	57
Rodent	**Ala**	**Gly**	**Tyr**	**Leu**	**Leu**	**Gly**	**Pro**	**His**	**Ala**	**Ile**	**Asp**	**Asn**	**His**	**Arg**	**Ser**	**Phe**	**Ser**	**Asp**	**Lys**
Porcine	**Ala**	**Gly**	**Tyr**	**Leu**	**Leu**	**Gly**	**Pro**	**His**	**Ala**	**Ile**	**Asp**	**Asn**	**His**	**Arg**	**Ser**	**Phe**	His	**Asp**	**Lys**
Bovine	**Ala**	**Gly**	**Tyr**	**Leu**	**Leu**	**Gly**	**Pro**	**His**	**Ala**	Leu	**Asp**	Ser	**His**	**Arg**	**Ser**	**Phe**	Gln	**Asp**	**Lys**
Human	**Ala**	**Gly**	**Tyr**	**Leu**	**Leu**	**Gly**	**Pro**	**His**	**Ala**	Val	Gly	**Asn**	**His**	**Arg**	**Ser**	**Phe**	**Ser**	**Asp**	**Lys**
	58	59	60	61	62	63	64	65	66	67	68	69	70	71	72	73	74	75	76
Rodent	**His**	**Gly**	**Leu**	**Thr**	**Gly**	**Lys**	**Arg**	**Glu**	**Leu**	Pro	Leu	**Glu**	Val	**Glu**	Glu	Gly	**Arg**	Leu	**Gly**
Porcine	Tyr	**Gly**	**Leu**	Ala	**Gly**	**Lys**	**Arg**	**Glu**	**Leu**	**Glu**	**Pro**	**Glu**	**Asp**	**Glu**	**Ala**	-	**Arg**	**Pro**	**Gly**
Bovine	**His**	**Gly**	**Leu**	Ala	**Gly**	**Lys**	**Arg**	**Glu**	**Leu**	**Glu**	**Pro**	**Glu**	**Asp**	**Glu**	**Ala**	-	**Arg**	**Pro**	**Gly**
Human	Asn	**Gly**	**Leu**	**Thr**	Ser	**Lys**	**Arg**	**Glu**	**Leu**	Arg	**Pro**	**Glu**	**Asp**	Asp	Met	-	Lys	Pro	**Gly**
	77	78	79	80	81	82	83	84	85	86	87	88	89	90	91	92	93	94	95
Rodent	**Ser**	Val	Ala	Val	**Pro**	**Leu**	**Pro**	**Glu**	Ser	**Asn**	**Ile**	**Val**	**Arg**	**Thr**	**Ile**	Met	**Glu**	**Phe**	**Leu**
Porcine	Gly	**Phe**	**Asp**	**Arg**	Leu	Gln	Ser	**Glu**	**Asp**	Lys	Ala	Ile	**Arg**	**Thr**	**Ile**	Met	**Glu**	**Phe**	**Leu**
Bovine	**Ser**	**Phe**	**Asp**	**Arg**	**Pro**	**Leu**	Ala	**Gln**	**Asp**	**Asn**	Val	**Val**	**Arg**	**Thr**	**Ile**	**Ile**	**Glu**	**Phe**	**Leu**
Human	**Ser**	**Phe**	**Asp**	**Arg**	Ser	Ile	**Pro**	**Glu**	**Asn**	**Asn**	**Ile**	Met	**Arg**	**Thr**	**Ile**	**Ile**	**Glu**	**Phe**	**Leu**
	96	97	98	99	100	101	102	103	104	105	106	107	108	109	110	111	112	113	114
Rodent	**Ser**	**Phe**	**Leu**	**His**	**Leu**	**Lys**	**Glu**	**Ala**	**Gly**	**Ala**	**Leu**	**Asp**	Ser	**Leu**	**Pro**	**Gly**	Ile	**Pro**	Leu
Porcine	Ala	**Phe**	**Leu**	**His**	**Leu**	**Lys**	**Glu**	**Ala**	**Gly**	**Ala**	**Leu**	Gly	**Arg**	**Leu**	**Pro**	**Gly**	**Leu**	**Pro**	Ser
Bovine	Tyr	**Phe**	**Leu**	**His**	**Leu**	**Lys**	Asp	**Ala**	**Gly**	**Ala**	**Leu**	Glu	**Arg**	**Leu**	**Pro**	Ser	**Leu**	**Pro**	Thr
Human	**Ser**	**Phe**	**Leu**	**His**	**Leu**	**Lys**	**Glu**	**Ala**	**Gly**	**Ala**	**Leu**	**Asp**	**Arg**	**Leu**	Leu	Asp	**Leu**	**Pro**	Ala
	115	116	117	118	119	120	121	122	123	124									
Rodent	**Ala**	Thr	**Ser**	**Ser**	**Glu**	**Asp**	Leu	**Glu**	Glu	**Ser**									
Porcine	**Ala**	**Ala**	**Ser**	**Ser**	**Glu**	**Asp**	**Ala**	Gly	Gln	**Ser**									
Bovine	**Ala**	Glu	**Ser**	Ala	**Glu**	**Asp**	**Ala**	**Glu**	**Arg**	**Ser**									
Human	**Ala**	**Ala**	**Ser**	**Ser**	**Glu**	**Asp**	**Ala**	**Glu**	**Arg**	**Ser**									

The sequence of the amino-terminus of galanin (1-15) is completely conserved. The carboxy- terminal region of galanin exhibits substantial species variability (Table 1) which might be responsible for the species specific effects of galanin on several endocrine systems, e.g. on insulin and glucagon secretion (Gilbey *et al.* 1989 and Miralle *et al.* 1990). The conserved amino-terminus might also be responsible for the receptor interaction. For instance, residues 1-16 of galanin have full agonistic properties in inhibiting muscarinic agonist-mediated stimulation of phosphatidyl inositol turnover in slices of rat ventral hippocampus (Fisone *et al.* 1989a and 1989b) and residues 1-15 can alter forskolin-stimulated cAMP production and inhibit insulin release from RIN m5F cells (Aminaroff *et al.* 1987).

Within the porcine, bovine and human preprogalanin, the sequence of galanin is followed by a 59 amino acid peptide called galanin message-associated peptide (GMAP) which extends from amino acid 65 to 123 and includes the carboxy-terminus of the predicted primary translation product. In the rat, the GMAP sequence contains an extra amino acid absent in other species (Table 2). At the carboxy-terminus of GMAP, a 36 amino acid portion is highly conserved among species with 24 invariant amino acids. Although the amino-terminal 23 amino acids of GMAP show little homology in amino acid sequence among species, the overall acidic nature of the peptide C-terminal portion is conserved, suggesting a role for the region in interactions with polycovalent cations and/or of involvement in transport processes.

Preprogalanin is processed to yield two proteins, galanin and GMAP, and their distributions in different tissue are also overlapping in most regions. However a heterogeneous distribution of the two gene products has been shown by immunohistochemical methods in a few areas, such as the pancreas and the eye, which could reflect tissue-specific post-translational processing (Hokfelt *et al.* 1992). The differential distribution together with the finding of well conserved regions of GMAP, imply a physiological role for this peptide.

I.2 The structure of galanin gene

Previous studies have shown that in human the galanin gene is composed of six exons spanning 6.5 kb from the start of exon 1 to the site of polyadenylation (Figure 2). The first exon (about 190 bp) encodes only the 5' untranslated region of the preprogalanin mRNA. The intron between exon 1 and exon 2 is about 190 bp. Exon 2 starts with the methionine codon of the signal peptide and terminates just before the tryptic cleavage site preceding galanin. The tryptic cleavage site and the first 13 amino acids of galanin are encoded on the third exon. The remaining portion of galanin and the first 10 amino acids of GMAP form the fourth exon. The exon 5 encoding the next 26 amino acids of GMAP. The exon 6 contains the remaining portion of GMAP and the 3' untranslated region.

Evans has shown that there is a single gene for human preprogalanin located in the q13.3-q13.5 region of chromosome 11. This region is of interest since it is the breakpoint region for translocation t (11;14) (q13;q32), an important abnormality associated with chronic lymphocytic leukemia and diffuse β-cell lymphoma (Evans *et al.* 1993).

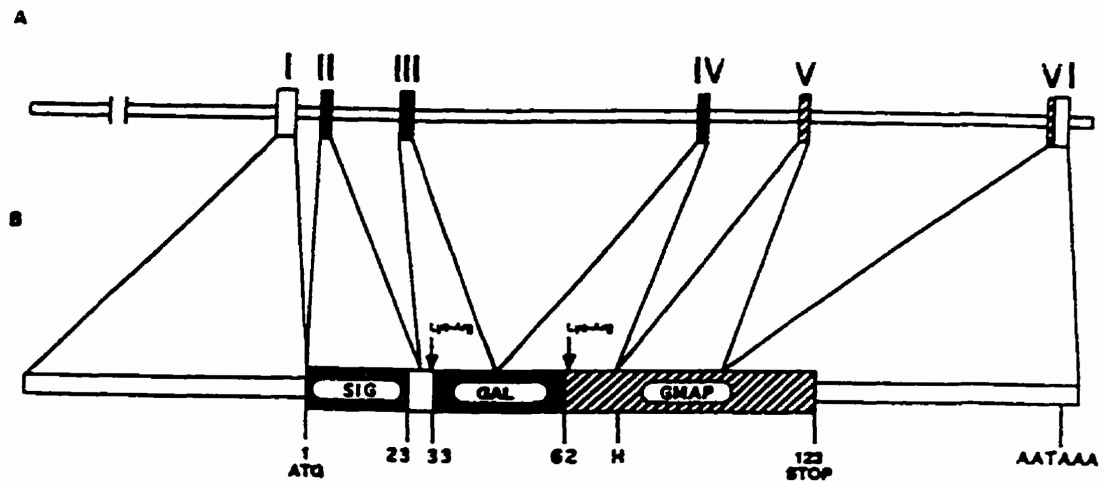


Figure 2. Organization of the human preprogalanin gene. (**A**) Schematic representation of the human preprogalanin gene. (**B**) the positions of the six exons within the galanin cDNA are shown. Numbers refer to the amino acid position of preprogalanin. The position of the translation initiation site (ATG), the poly (A) site, and the termination codon are indicated. GAL, galanin; SIG, signal peptide; GMAP, galanin message-associated peptide (Evans *et al.* 1993).

I.3 Regulation of galanin mRNA

I.3.1 Regulation by sex steroid hormones

Transcription of the galanin gene in the rat pituitary is strongly induced by estrogen in a dose and time dependent manner. The induction by estrogen is rapid and reversible. In estrogen induced prolactinoma, a 1000 to 4000 fold increase of galanin mRNA has been detected (Vrontakis *et al.* 1987, 1989a and 1992, Kaplan *et al.* 1988b, and O'Halloran *et al.* 1990). Ovariectomy in rats results in decreased levels of galanin mRNA as well as galanin-like immunoreactivity (GAL-LI) in the pituitary. Interestingly, the galanin content is high in rat posterior pituitary. Normal male and ovariectomized female rats contain nearly undetectable levels of galanin mRNA in the anterior pituitary. Estrogen treatment, however, dramatically upregulated galanin gene expression and peptide synthesis in the rat anterior pituitary (Vrontakis *et al.* 1987 and 1989b, Kaplan *et al.* 1988b). The fact that anterior pituitaries of normal female rats contain more galanin peptide than that of normal males is most likely the result of increased levels of circulating estrogen in the female rat (Gabriel *et al.* 1990). In contrast to its strong effect on pituitary galanin expression, estrogen regulates hypothalamic galanin expression to a considerably more limited extent.

In the rat pituitary galanin is expressed in somatotrophs and thyrototrophs, however, following estrogen treatment galanin mRNA is also expressed in lactotrophs (O'Halloran *et al.* 1990, Kaplan *et al.* 1988b and 1991). In the human, galanin mRNA is expressed only in corticotrophs, and no galanin immunoreactivity is present in lactotrophs even in estrogen-treated subjects (Vrontakis *et al.* 1987 and Hsu *et al.* 1991). These observations indicate that

the cellular distribution and expression of galanin are strikingly different in the human and rat pituitary.

Recently, many findings indicate that steroid hormones participate in the control of galanin gene expression in a tissue- and hormone-specific fashion in rat. It has been reported (Vrontakis *et al.* 1993 and Torsello *et al.* 1992) that galanin is expressed in the male and female rat reproductive system. In the vas deferens and seminal vesicles, galanin mRNA has been localized at a cellular level in fibroblast-like cells. In the uterus, galanin mRNA is expressed in stromal cells of the endometrium. The regulation of galanin mRNA expression by estrogen in the uterus and pituitary is markedly different. Estrogen dramatically increases galanin gene expression and protein synthesis in the rat anterior pituitary, while the expression in the uterus of the same animals is transient Vrontakis *et al.* 1993). Also, testosterone up regulates galanin mRNA in the stria terminalis (Miller *et al.* 1993).

I.3.2 Regulation by Nerve Growth Factor (NGF)

NGF upregulates galanin gene expression in a dose- and time-dependent fashion in cultured rat pheochrmocytoma cells (PC_{12}) which contain little or no galanin mRNA in the absence of NGF (Kaplan *et al.* 1991). NGF also strongly induces the galanin gene expression in rat basal forebrain cholinergic neurons, but there is no effect on galanin gene expression in other noncholinergic forebrain regions. In rats, NGF prevents age- and lesion-induced degeneration of basal forebrain cholinergic neurons which coexpress neuropeptide galanin. These data indicate that the concurrent induction of galanin in the forebrain could limit the

ameliorating actions of NGF on cholinergic dysfunction (Planas *et al.* 1997).

I.3.3 Regulation by nerve injury

Autonomic, sensory and motor neurons are capable of regenerating in adult mammals following axonal damage. Axonal injury induces dramatic changes in neuropeptides expression in these neurons. One characteristic of axotomized neurons is that there is a increase of the galanin expression in these neurons. Nerve injury increases galanin levels dramatically in sensory neurons, trigeminal sensory neurons, cranial motoneurons, and adrenal ganglia cells, as well as in sympathetic ganglia (Hokfelt *et al.* 1987, Villar *et al.* 1989 and 1990, Cortes *et al.* 1990, Wiesenfeld-Hallin *et al.* 1992, Melander *et al.* 1985, 1986, and Fibiger 1982).

I.3.4 Regulation by TPA

Galanin gene expression is upregulated by phobol esters (such as TPA), and forskolin in chromaffin cells (Rokaeus *et al.* 1990). TPA (12-O-tetradecanoylphorbol-13-acetate), which directly activates protein kinase C (PKC), produces a 50-fold increase in galanin mRNA levels in chromaffin cells. The TPA stimulation of galanin mRNA level is desensitized completely with time by down-regulation or depletion of the PKC. TPA is known to activate PKC by affecting the abundance and activity of AP-1 complexes (Anouar *et al.* 1994). Forskolin stimulates galanin gene expression by activating the protein kinase A (PKA) pathway.

In summary, the galanin gene expression is dependent on several factors, including cell type, differentiation state and hormonal background. Lesions of primary sensory and motor neurons upregulated galanin gene expression. Within the anterior pituitary, galanin gene expression is dependent on cell type, and the observed cell lineage specificity appears different in rat and human.

II. Distribution of Galanin and Galanin Receptors in the Central Nervous System (CNS) and Peripheral Nervous System (PNS)

Numerous galanin immunoreactive cell bodies were observed in the rat telencephalon, the nucleus of the diagonal band continuing into the medial septum, and the medial aspects of the central amygdala (Merchenthaler *et al.* 1993). In the primate telencephalon, galanin-positive cell bodies apparently are more widespread than in the rat (Kordower *et al.* 1992). Thus, positive cell bodies in the primate were observed in the anterior olfactory nucleus, the basal forebrain, the endopiriform nucleus, hippocampus, and the bed nucleus of the stria terminalis.

Of particular interest is the distribution of galanin-like immunoreactivities (GAL-LI) in the basal forebrain, in part because of its possible relation in Alzheimer's disease and because of apparent species differences. Galanin in the rat is restricted to the septal diagonal band complex (Skofitsch and Jacobowitz 1985, and Lamour *et al.* 1988), but galanin has an apparently wider distribution in the monkey basal forebrain (Kordower *et al.* 1992).

In the diencephalon, the hypothalamus and median eminence are rich in GAL-LI and the pattern of distribution among mammalian species is similar. The distribution included the medial and lateral preoptic nuclei, the arcuate nucleus, the periventricular nucleus, the dorsomedial nucleus, the lateral hypothalamus/medial forebrain bundle area, the superoptic and paraventricular magnocellular nuclei, and the area lateral to the mamillary recess. In rat, the galanin-positive cell bodies in the thalamus were only seen in the anterior dorsal and periventricular nuclei. No cell bodies were observed so far in the primate thalamus, but small numbers of fibers could be seen in various nuclei including the ventral tegmental area (Merchenthaler *et al.* 1993).

Galanin is also widely distributed in the trigeminal, medulla, nucleus of the solitary tract, and dorsal horn of the spinal cord (Skofitsch and Jacobowitz 1985, Melander *et al.* 1986, and Kordower *et al.* 1992). In the peripheral nervous system galanin is present in the pituitary, terminal innervation of the gastrointestinal, respiratory, urogenital tracts, pancreas, adrenal medulla, and ganglia innervating the heart, kidney, liver and spleen (Bauer *et al.* 1986a, Dunning *et al.* 1986, Cheung *et al.* 1985, and Xu *et al.* 1995b).

One peculiarity of galanin is the fact that GAL-LI is difficult to visualise in some systems under normal circumstance. After certain types of stimulation, however, it is synthesized at high levels. This suggests that galanin is a highly plastic peptide in certain systems, and that under normal circumstances it may not be synthesized at all, or only at very low levels. The peptide, then, may only reach functional significance under certain conditions, such as

damage to neurons.

Human and rat galanin receptors have been cloned by high affinity binding of radiolabelled galanin. Three subtypes of galanin receptors have been identified and characterized so far in both species (Fathi et al. 1997, Wang *et al.* 1997a, and 1997b). Galanin receptors show a wide spread distribution in the CNS and PNS, and also in neurons innervating the gastrointestinal tract and the pancreas. The distribution of galanin binding sites closely matches the distribution of GAL-LI in rat (Skofitsch and Jacobowitz 1986, and Melander *et al.* 1988), monkey (Kohler *et al.* 1989), and human (Kohler *et al.* 1990).

III. The Molecular Mechanisms of Galanin Action

So far, all galanin signal transduction pathways have in common the coupling of galanin receptors to a pertussis toxin-sensitive, ADP-ribosylatable G_i/G_o protein as the transducing element (Bartfai *et al.* 1993). In the rat and monkey ventral hippocampus, acetylcholine, which acts through muscarinic receptors, inhibits adenylate cyclase (AC) activity, stimulates cGMP synthesis, and elicits phosphoinositide (PI) breakdown. PI is hydrolysed by phospholipase C (PLC) to inositol-1,4,5,-triphosphate (IP_3) and 1,2-diacylglycerol (DAG). Both of these end-products act as second messengers. Furthermore, IP_3 releases Ca^{2+} from intracellular, nonmitochondrial stores and DAG activates protein kinase C (PKC). The activation of the galanin receptor through the binding of its galanin ligand leads to lower the Ca^{2+} influx through "N-type", voltage-dependent calcium channels (VDCC), thereby to

inhibit the activity of PLC (Palazzi *et al.* 1991).

In the spinal cord, a reduction in K^+-stimulated cGMP production was observed after galanin application. Depolarisation of neuronal tissue leads to increase influx of Ca^{2+} by opening VDCCs, which in turn causes activation of cGMP synthesis (Tjornhammar *et al.* 1984). This may either be a direct inhibition of guanylate cyclase (GC) by the agonist occupied galanin receptor or result from the galanin mediated closure of voltage-dependent Ca^{2+} channels via a PTX-sensitive G protein, leading to a lowering of the free cytoplasmic Ca^{2+} concentration or a combination of both (Bedecs *et al.* 1992).

In rat brain, agonists binding to galanin receptors are coupled via inhibitory G-proteins to adenylate cyclase. As a result, a reduced 3,5,-cAMP level was observed after galanin application. In cultured cell line of the same tissue, galanin receptor activation leads to the opening of ATP-sensitive K^+ channels. The cells are then hyperpolarized leading to the closure of voltage-dependent Ca^{2+} channels and thus to a reduced intracellular Ca^{2+} concentration. In the dissociated normal mouse β-cells, however, galanin acts on a different type of K^+ channel that is sulphonylureas insensitive and has lower single-channel conductance (Wahlander *et al.* 1991). There is evidence indicating that this cation channel is the same K^+ channel that is activated by adrenaline, which also inhibits insulin release, e.g. during stress. Galanin coexists with noradrenaline and adrenaline in the sympathetic nerves innervating the pancreas. Adrenaline is known to mediate stress-induce inhibition of insulin secretion. This has raised the possibility of galanin taking part in this stress response.

In pheochromocytoma (PC_{12}) cells, galanin, like somatostatin, activates K^+-channels which has the effect of hyperpolarizing the membrane, thereby leading to a decreased probability of Ca^{2+} entry through Ca^{2+} channels (de Weille *et al.* 1989).

In summary, agonist stimulation of galanin receptors results in a reduced intracellular Ca^{2+} concentration in most cell types. This reduces transmitter release and inhibits other Ca^{2+}-dependent processes. The actual mechanism behind the reduction may be either:

- direct through closure of Ca^{2+} channels;
- indirect via opening of various kinds of K^+ channels which would hyperpolarize the membrane and thereby restrict Ca^{2+} entry via voltage-dependent Ca^{2+} channels (VDCC);
- indirect by an inhibition of adenylyl cyclase; or
- a combination of these mechanisms.

IV. Physiology of Galanin

IV.1 The effects of galanin on growth hormone (GH), prolactin (PRL) and other hormones

Galanin is present and active in the neuroendocrine system and has been suggested to have paracrine as well as endocrine effects in the hypothalamic-pituitary axis. Galanin plays a significant role in the regulation of GH secretion in some species. Human galanin is able to elicit GH secretion and is able to increase the GH response to GH-releasing hormone (GHRH) in normal man. Also recent data show that galanin has a small but significant direct

stimulatory effect on GH release from monolayer cultures of rat anterior pituitary cells (Bauer *et al.* 1986b, Gabriel *et al.* 1988, and Giustina *et al.* 1994). The GH-releasing action of galanin is far less potent than that of GHRH in normal somatotrophs. Interestingly, the same dose of galanin that is able to stimulate GH secretion in normal somatotrophs exerts quite a potent inhibitory effect on GH release from a cultured rat adenomatous cell line (GH_1). The same dose of galanin that results in stimulation of GH secretion in normal men is, on the contrary, able to induce a significant inhibition of GH secretion in acromegalic patients (Giustina *et al.* 1992). Pretreatment with GHRH antibodies suppresses the plasma GH increase, induced by intravenous and intracerebroventricular infusion of galanin, which suggests that as a final common pathway in the rat, the stimulatory effect of galanin on GH secretion is mediated by hypothalamic GHRH. The mechanism of this stimulating action of galanin is unclear. Experimental studies suggest that the action of galanin may be mediated either by a decrease in hypothalamic somatostatin, which has an inhibitory effect on GH secretion, or by an increase in endogenous GHRH secretion which has a stimulating effect on GH secretion (Loche *et al.* 1990 and Kitajima *et al.* 1990).

In rats, galanin has a stimulating effect on PRL secretion (Koshiyama *et al.* 1987). However, its PRL-releasing activity is low and does not seem to have physiological relevance. In women infused with galanin, there was a slight, but not statistically significant, increase in PRL level. However, galanin is able to amplify the lactotrope responsiveness to several stimuli such as TRH. In normal male volunteers, however, galanin failed to affect the PRL response to TRH (Arvat *et al.* 1995 and Giustina *et al.* 1994). All of these suggest that

galanin plays a greater role in the modulation of PRL in women than in men.

In rat, galanin shows an stimulating role in the control of luteinizing hormone (LH) secretion. Galanin stimulates LH-releasing hormone (LHRH) secretion from nerve terminals of the median eminence and, at the pituitary level, enhances both basal and LHRH-induced LH secretion (Lopez *et al.* 1990 and 1991). Also, adrenocorticotrophic hormone (ACTH) and thyroid-stimulating hormone (TSH) release seems to be influenced by galanin in some animals. In the rat, treatment with galanin antiserum induces a clear rise in ACTH levels, while either a decrease or an increase in TSH levels has been reported (Hooi *et al.* 1990 and Ottlecz *et al.* 1988). Moreover, galanin clearly inhibits the ACTH response to stress although its administration does not modify spontaneous ACTH secretion, while it has been reported to inhibit or not to affect TSH release. In *vitro* studies on rat pituitary cells have shown that galanin does not influence ACTH release but increases a TRH-induced TSH rise. Porcine, rat and human galanin have no significant effect in spontaneous secretion of FSH, LH, ACTH, and TSH in human so far.

IV.2 The effect of galanin on insulin secretion

The very first discovered galanin-inducing effect was that administration of porcine galanin into dog elevated plasma glucose levels in a dose-dependent manner and concomitantly lowered basal plasma insulin levels due to an inhibition of insulin release from pancreatic β-cells (Tatemoto *et al.* 1983 and McDonald *et al.* 1985). Galanin also blunted canine

insulin responses to a number of known insulin secretagogues (Hramiak *et al.* 1988 and McDonld *et al.* 1986). In contrast, it has been reported that the administration of synthetic replicates of porcine and human galanin into human does not affect either plasma glucose or insulin levels, but does elevate plasma human growth hormone levels (Ahren *et al.* 1990, Gilbery *et al.* 1989, McDonald *et al.* 1994, and Holst *et al.* 1993). In *vitro* studies on isolated human islets have shown a direct inhibitory effect of galanin secretion (Adeghate *et al.* 1996).

IV.3 The effect of galanin on feeding behaviour

Galanin has a potent stimulating effect on food ingestion, particularly high-fat diet. Microinjection of galanin directly into the PVN results in a 300% increase in food consumption in satiated rats (Leibowiz *et al.* 1991). The effect can be seen with intraventricular injection of native galanin (1-29), as well as with galanin (1-16), indicating the importance of the first 16 N-terminal amino acids in the biological action of the peptide. This response, in contrast, is not produced by other galanin fragments such as 1-9, 10-20, 12-29, 17-29, or 21-29, which, in equilibrium binding experiments, exhibits relatively low affinity for the hypothalamic galanin receptor (Land *et al.* 1991).

The importance of the PVN in the stimulating action of galanin in feeding is reflected with the finding that galanin has generally little impact in food consumption when microinjected into other hypothalamic nuclei, as well as certain extrahypothalamic areas (Kyrkouli *et al.*

1990). It is also relatively ineffective in animals that have received damage to the PVN by electrolytic lesions (Kyrkouli *et al.* 1990). These findings suggest that the PVN and its neurochemical innervation have a critical role in controlling the consumption of specific macronutrients. Interestingly, the PVN injection of galanin preferentially increases the ingestion of a fat diet, rather than carbohydrate or protein diets. The selectivity of this effect is underscored by the finding that it can occur in essentially all rats regardless of their natural preference for fat and other macronutrients (Tempel *et al.* 1990b).

Recent biochemical studies provide strong support for the proposal that endogenous galanin receptors, specifically those in the PVN, mediate the action of exogenous galanin in the PVN and are physiologically active in modulating fat ingestion in freely feeding animals. This is also a significant positive correlation between spontaneous daily fat intake and GAL-LI specifically in the PVN (Menendez *et al.* 1992). This further shows a possible contribution of endogenous galanin to body weight control and a propensity towards obesity.

IV.4 Galanin and Alzheimer's disease

Extensive studies related to coexistence of galanin with acetylcholine (ACh) in the basal forebrain have been undertaken (Melander *et al.* 1985). GAL-LI and galanin mRNA are found in cholinergic basal forebrain neurons of the rat and several species of monkeys. Although apes and humans do not show GAL-LI or galanin mRNA in these cholinergic neurons, small galanin immunoreactive interneurons are wide spread in the nucleus basalis

of Meynert and diagonal band areas and in the diagonal band cell bodies (Benzing *et al.* 1993 and Mufson *et al.* 1993).

The most consistent marker for neuronal loss in Alzheimer's disease (AD) has been the decline in numbers of cholinergic neurons of the nucleus basalis of Meynert (Coyle *et al.* 1983). Dramatic reductions in choline acetyltransferase (ChAT), the synthetic enzyme for ACh, and acetylcholinesterase, the metabolic enzyme for ACh, are routinely seen in postmortem samples of basal forebrain and cortical samples from Alzheimer's victims, as compared to age matched controls. Cholinergic deficits are highly correlated with dementia severity, independent of age (Bierer *et al.* 1995).

Histological analyses show that galanin immunoreactive fibers and terminals are present in much greater densities in the basal forebrain in AD. The mechanism by which galanin immunoreactive terminals hyperinnervate cholinergic neurons in the basal forebrain in AD is not known. Since galanin is overexpressed after neuronal injury (Hokfelt *et al.* 1994), it may be speculated that the galanin overexpression in AD is the results of neuronal damage caused by the progressive accumulation of amyloid protein.

Recently, evidence shows that cholinergic transmission is inhibited by galanin both pre- and post-synaptically in the ventral hippocampus (Palazzi *et al.* 1991). In the ventral hippocampus, presynaptic inhibition by galanin results in an inhibition of potassium-evoked ACh release in *vitro* and scopolamine induced release in *vivo* (Fisone *et al.* 1987).

Furthermore, galanin inhibits muscarinic receptor mediated stimulation of phosphoinositide breakdown by closing ω-conotoxin sensitive (N-type) calcium channels (Palazzi *et al.* 1988).

In summary, galanin coexists with ACh in the nucleus basalis of Meynert. Galanin immunoreactive interneurons are greatly increased in the nucleus basalis of Meynert of the Alzheimer's patient. Galanin hyperinnervates the cholinergic neurons in the nucleus basalis of Meynert and via its hyperpolarizing action further reduces the release of ACh. Dramatic reductions in ChAT and acetylcholinesterase are routinely seen in the basal forebrain of Alzheimer's victims.

IV.5 The effect of galanin on learning and memory processes

As described earlier, galanin coexists with ACh in the basal forebrain cholinergic neurons of the basal frontal projecting to the frontal cortex and of the septum projecting to the hippocampus which is involved in cognitive functions.

Recent studies showed that galanin plays an important role in learning and memory processes (Ukai *et al.* 1995 and Ogren *et al.* 1992). Centrally administered galanin has inhibitory actions in several rodent learning and memory paradigms. Acquisition in the Morris water maze tank was slower in rats treated with daily intraventricular administration galanin receptor antagonist, M35, produced a small increase in the rate of acquisition in the Morris swim maze tank without increasing swim speed (Ogren *et al.* 1992). This suggested

that endogenous galanin may contribute an inhibitory effect on swim maze learning. Rats treated before training with an intraventricular infusion of 8 μg of galanin, showed significantly higher numbers of errors and longer latency to reach the food reward in a starburst radial maze tank on the retention trial the next day (Malin *et al.* 1992). Intraventricular administration of galanin (5 μg) immediately before the test session reduced choice accuracy on a delayed non-matching to position working memory tank (Robinson *et al.* 1993).

In the ventral hippocampus, the inhibitory effects of galanin were dose-dependent and delay-dependent. At lower dose, 0.1 nmole and 0.4 nmole, galanin produced a delay-dependent reduction of choice accuracy, indicating a specific mnemonic deficit. At the highest dose, 1.6 nmole, galanin produced a delay-independent reduction in choice accuracy, indicating that non-specific procedural components of the task were impaired. These findings indicate that higher doses of galanin may induce selective mnemonic actions, i.e. produce specific deficits on memory peers at the ventral hippocampus site (Ukai *et al.* 1995 and Ogren *et al.* 1992).

IV.6 The effect of galanin on smooth muscle

Galanin has been reported to have a number of effects on the gastrointestinal tract. Its actions on gastrointestinal motility are highly species- and locus-specific. Galanin has been shown to be i) an excitatory neurotransmitter that increases the motility of the gut and causes

contraction of the gastrointestinal smooth muscle in porcine (Brown *et al.* 1990); ii) an inhibitory factor for gastrointestinal motility in human (Bauer *et al.* 1989); and iii) a modulator regulating the motility of gastrointestinal smooth muscle in porcine (Brown *et al.* 1990) and dog (Fox-Threlkeld *et al.* 1991). In some cases the effects of galanin are neurally mediated or due to direct interaction with galanin receptors on the target tissue (Brown *et al.* 1990, Chakder *et al.* 1991, Ekblad *et al.* 1985, Fox *et al.* 1988, Haring *et al.* 1991, and Katsoulis *et al.* 1990).

The variability in action of galanin on different gastrointestinal muscle preparations is not only due to the possibility that galanin could have variable mechanism of action. Possible mechanisms include that galanin acts either directly with receptors on smooth muscle cells or directly on neural elements releasing various stimulating neurotransmitters. It is also due to interaction with different subtypes of galanin receptors. According to the evidence, Gu *et al.* (1994) suggest that at least two different subtypes of galanin receptors exist in gastrointestinal smooth muscle that can be differentiated by their relative affinities for N- and C-terminal galanin fragments.

Furthermore, Botella *et al.* (1994 and 1995) indicated that contraction and relaxation in smooth muscle are induced by different subtypes of galanin receptors. They also found that galanin triggers different intracellular pathways in smooth muscle cells when it interacts with each of the two subtypes receptors. In the longitudinal muscle layer of dog colon and the circular muscle layer from pig ileum, galanin induces a cell contraction via an activation

of a pertussis toxin-sensitive G protein and an influx of extracellular calcium by altering the activity of K^+ and Ca^{2+} channels. In the circular muscle layer from dog colon, galanin induces a cell relaxation by activating the adenylate cyclase complex and PKA.

IV.7 Other effects of galanin

Galanin has been found in neurons of the locus coeruleus (LC). It colocalizes with noradrenaline in the LC (Skofitsch *et al.* 1985, Holets *et al.* 1988). The functional consequences of this coexistence are unclear.

Experiments show galanin can depress the firing rate of LC neurons. Galanin causes hyperpolarization and a reduction in input resistance. The hyperpolarization and lower input resistance persists when the membrane potential is manually clamped to its pre-drug value, and increases a potassium permeability by opening a class of K_{ATP} channels (Sevcik *et al.* 1993).

Zini *et al.* (1993) demonstrated that galanin hyperpolarizes hippocampal neurons and inhibits the release of glutamate (Glu) and aspartate (Asp) during anoxia. It is known that Glu and Asp are the major excitatory neurotransmitters of the two principal intrinsic pathways of the hippocampus, i.e. the mossy fibers and the Schaffer collaterals (Corradetti *et al.* 1981). Galanin modulates the release of excitatory amino acids from mossy fiber terminals through a direct or indirect coupling to K^+ channels sensitive to glibenclamide

receptors. Therefore galanin can protect hippocampal neurons from the deleterious effects of anoxia (Zini *et al.* 1993).

Galanin is also of importance in the control of certain cardiac functions and/or of circulation (Xu *et al.* 1995b). GAL-LI is present in the heart nerve fibers of mouse and rat, as well as in the heart of dog, cat, rabbit, and guinea-pig. Moreover, the chromatographic analysis showed that GMAP is also produced in the heart. Galanin may exert some vascular action at the microcirculation level rather than directly on the main arteries or veins.

In addition, galanin also inhibits monoaminergic neurotransmissions such as dopaminergic neurons from the median eminence (Nordstrom *et al.* 1987 and de Weille *et al.* 1989) and histaminergic neurons from the hypothalamus and hippocampus (Arrang *et al.* 1991).

V. Objectives

Galanin is widely distributed throughout the CNS and PNS and in endocrine tissue. It has been shown to have a diversity of biological functions. The amino acid sequence of galanin is conserved across all species examined so far. Taken together these suggests that galanin is an important messenger for inter cellular communication in the nervous and endocrine system. The differential regulation of galanin expression in the nervous and endocrine systems suggests that galanin gene, a single gene, may be activated by multiple pathways. Therefore the molecular mechanism of galanin gene activation in different tissues are of

particular interest.

The objectives of my thesis are:

i) to isolate the 5'-flanking region of the rat galanin gene;

ii) to explore the transcriptional activation of the rat galanin gene; and

iii) to locate functionally active (tissue and hormonal specific) regulatory elements.

MATERIALS AND METHODS

1. DNA and RNA Preparation

1.1 Isolation of total and messenger RNA

Total RNA was isolated by the guanidinium isothiocyanate method (Chomczynski and Sacchi 1987). Briefly, after harvesting from the tissue culture plates, cells were resuspended in 600 μl of Solution D (4 M guanidinium thiocyanate, 0.5% sarcosyl, 0.1 M 2-mercaptoethanol, 25 mM sodium citrate, pH 7.0) and drawn through a 23 gauge needle several times. The solution was added to a suspension containing 60 μl of 2 M sodium acetate, pH 4.0, 600 μl phenol, and 120 μl chloroform/isoamyl alcohol and incubated on ice for 20 minutes. The mixture was then centrifuged at 10,000 × g for 20 minutes at 4°C. The aqueous phase was precipitated with isopropanol at -20°C overnight. The pellet was redissolved in 180 μl of Solution D, and reprecipitated with isopropanol. The pellet was rinsed with 70% ethanol and finally resuspended in 20 μl of 0.5% SDS at 65°C. The concentration of RNA in the samples was determined by measuring the absorbance at 260 nm and applying the formula: 1 OD_{260} = 40 μg/ml RNA.

Messenger RNA was isolated using a Poly A Tract Isolation Systems III kit (Promega). Briefly, total RNA was incubated with 150 pmol biotinylated-oligo(dT) in 5 × SSC (1× SSC is 150 mM NaCl and 15 mM sodium citrate, pH 7.0), heated to 65°C and then cooled to

room temperature for annealing. After complete cooling, the annealing reaction was then mixed with washed SA-PMP magnetic particles (Promega) at room temperature for 10 minutes. The supernatant was removed after the SA-PMPs were captured by using a magnetic rack and the particles were washed four times with 0.1 × SSC. To elute the mRNA, the SA-PMP pellet was resuspended in 1 ml of RNase-free water. The SA-PMPs were magnetically captured and the aqueous phase containing the eluted mRNA was removed. The mRNA concentration was determined spectrophotometrically.

1.2 DNA preparation

1.2.1 Maxi preparation of plasmid

A single colony of *E. coli* TGIλ was inoculated into 5 ml of L broth (LB) and grown for 8 hours at 37°C with shaking. One ml of this culture was added to 200 ml LB culture and grown overnight at 37°C with shaking. The culture was centrifuged at 10,000 × g for 15 minutes at 4°C, the supernatant was removed, and the pellet was resuspended in 4 ml of solution I (50 mM Tris-HCl, pH 7.5, 10 mM EDTA, 100 μg/ml RNase A). The resuspended bacteria were mixed with 4 ml solution II (0.2 M NaOH and 1% SDS) by gently inverting the tube several times and incubated at room temperature for 15 minutes. Subsequently, 4 ml of solution III (1.32 M potassium acetate, pH 4.8) was added to the tube, following by gentle mixing. The resulting solution was centrifuged at 14,000 × g for 15 minutes at 4°C. The supernatant was carefully removed to a new tube and mixed with 10 ml Wizard™ DNA Purification Resin (Promega). The entire solution was passed through a Wizard™ Midicolumn. The column was then washed twice with 15 ml of Wash Solution (80 mM

potassium acetate; 8.3 mM Tris-HCl, pH 7.5; 40 μM EDTA; 55% ethanol). The DNA was eluted with 300 μl ddH_2O (distilled and deionized water). The absorbance at λ = 260 nm was measured to determine the DNA concentration.

1.2.2 Isolation and purification of genomic DNA from tissue

A rat kidney, which was cut into small pieces, was lysed with 1 ml 10% SDS and 1 ml of proteinase K (10 mg/ml) at 37°C for 2 hours. An additional 1 ml of proteinase K (10 mg/ml) was added before incubation overnight at 37°C. After overnight digestion, 1.3 ml of 5 M NaCl was mixed into the solution. The mixture was extracted three times with an equal volume of phenol/chloroform (1:1), once with an equal volume of chloroform, and centrifuged at 2500 × g for 10 minutes. The aqueous phase was precipitated with ethanol and rinsed with 80% ethanol. After precipitation, the pellet was redissolved in Tris/salt buffer (10 mM Tris-HCl, pH 7.4, 25 mM EDTA, and 0.5 M NaCl) and incubated with 20 μl DNase-free RNase A (10 mg/ml) at 37°C for 30 minutes. The solution was extracted again with phenol/chloroform, then chloroform, and finally the aqueous phase was precipitated with 2.5 volumes of 100% ethanol, then rinsed with 80% ethanol. The pellet was air-dried for 30 minutes and dissolved in TE buffer (10 mM Tris-HCl, pH 8.0 and 1 mM EDTA, pH 8.0). The DNA concentration was determined by measuring the OD_{260} and DNA aliquots were stored at -20°C.

1.2.3 Isolation and purification of bacteriophage DNA

A standard method for recovery of a large amount of bacteriophage DNA (Sambrook *et al.*

1989) was used with slight modifications. Five ml LB medium (5 g/l NaCl, 5 g/l Bacto-yeast extract, 10 g/l Bacto-tryptone, pH 7.5) containing 10 mM $MgSO_4$ and 0.2% maltose was inoculated with a single colony of *E. coli* XL1-Blue MRA (P2) and grown overnight at 37°C on a rotary shaker (200 rpm/minute). Four ml of this culture and 4 ml of 10^{10} pfu of bacteriophage were used to inoculate a 200 ml culture of LB/10 mM $MgSO_4$/0.2% maltose. The mixture was grown at 37°C with shaking until lysis of the bacteria occurred (6-8 hours). Four ml of chloroform was added to the flask and incubated a further 15 minutes with shaking. Cellular debris were separated by centrifugation at 8,000 × g for 10 minutes at 4°C. PEG 8000 (Fisher Scientific) and NaCl were dissolved in the recovered supernatant at a final concentration of 10% (w/v) and 0.5 M, respectively, and the solution was cooled on ice for 1 hour to allow bacteriophage particles to precipitate. The particles were recovered by centrifugation at 11,000 × g for 10 minutes at 4°C. The supernatant was removed and the pellet was resuspended in 4 ml TM buffer (50 mM Tris-HCl, pH 7.5 and 10 mM $MgSO_4$). The PEG 8000 was removed by a chloroform extraction and the aqueous phase was recovered. RNase A and DNase I were added to the solution in a final concentration of 100 μg/ml and incubated for 30 minutes at 37°C. The resulting crude phage solution was loaded onto a DEAE-cellulose (Whatman) column and eluted with 10 ml TM buffer. The eluate was collected and NaCl and ice-cold isopropanol were added to a final concentration of 400 mM and 40%, respectively, and then the mixture was placed at -80°C for 15 minutes. The phage were centrifuged at 8,000 × g for 10 minutes at 4°C and resuspended in 0.8 ml TE buffer. The phage solution was extracted once with phenol, twice with phenol/chloroform (1:1), and once with chloroform/isoamyl alcohol (24:1). The phage DNA was precipitated with 2

volumes of 100% ethanol and washed with 70% ethanol. The phage DNA pellet was resuspended in TE buffer and the concentration was determined spectrophotometrically.

2. Restriction Endonuclease Analysis and Agarose Gel Electrophoresis

The cloned phage and plasmid DNA were digested with restriction endonucleases to formulate a detailed restriction enzyme map prior to sequencing or subcloning. Briefly, 1 to 3 μg of phage or plasmid DNA was digested with a specific restriction endonuclease (1 to 3 U/μg) for one to four hours at the temperature recommended by the manufacturer. The reaction was then loaded onto a 0.6 - 1.8% agarose gel and separated by electrophoresis in 1× TBE buffer (89 mM Tris-borate and 2 mM EDTA, pH 8.0) at 10 - 130 volts. Ethidium bromide (0.5 μg/ml) was incorporated into the gel to allow visualization of the DNA bands under ultraviolet light. DNA fragments were sized by comparison to a marker, and by digesting the samples with combinations of restriction endonucleases, the positions of the enzyme recognition sequences were determined.

3. Recovery of DNA Fragments from Agarose Gel

3.1 Recovery of DNA fragment from agarose gel using Advantage™ PCR-Pure Kit

The DNA containing the fragment of interest was loaded on a conventional agarose gel and separated by electrophoresis in 1× TAE buffer (for 50 × : 242 g Tris base, 57.1 ml glacial acetic acid, 100 ml of 0.5 M EDTA, pH 8.0). The DNA fragment of interest was excised,

weighed, and placed in a microcentrifuge tube. The gel was dissolved in SALT solution by incubation at 55°C for 5-10 minutes. The melted solution was incubated with 5 μl of the PCR-Pure BIND at room temperature for 5 minutes and the contents were occasionally mixed to allow the DNA to bind to the BIND. The BIND/DNA complex was collected by centrifugation at 10,000 × g for 5 seconds and the supernatant was removed. After rinsing twice with WASH buffer, the DNA was eluted by resuspending the pellet in water and incubating for 5 minutes. The BIND was removed by centrifugation at 10,000 × g for 1 minute. The DNA concentration was determined spectrophotometrically.

3.2 *In situ* electroelution of DNA from agarose gel

The DNA samples were separated by electrophoresis until the fragment of interest was well separated from the others. A scalpel blade was used to excise a slice of gel immediately adjacent to the band of interest. The gel was then placed back into the electrophoresis tank and the buffer was removed until the level was just below the surface of the gel. A fresh buffer was placed in the slot adjacent to the DNA band. The current was applied to the gel until the fragment had migrated into the slot. The buffer was then collected into a new tube, precipitated with ethanol/NaAc, and rinsed with 70% ethanol. The DNA fragment of interest was then dissolved in TE buffer.

4. Subcloning of DNA Fragments

4.1 Mapping of lambda phage inserts

The A_{22} phage DNA was digested with various restriction endonucleases and *Sal* I for 3-4 hours. The digested products were resolved on 0.7-1.0% (w/v) agarose gels and transferred to nitrocellulose as described in the section 7. The Southern blots were hybridized with radiolabelled either the Gal 1 (sense) oligonucleotide primer or a rat galanin cDNA probe and then exposed to X-ray film. Once the location of the 5'-flanking region of the rat galanin gene was determined, suitable inserts for subcloning were identified based upon their size and the comparability of the digested ends with enzyme site in the multiple cloning region of the vectors used for subcloning.

4.2 Subcloning of the A_{22} lambda phage inserts

Based on the results of Southern blotting, a *Hind* III DNA fragment in the A_{22} phage DNA containing coding and 5'-flanking region of the rat galanin gene was identified. The DNA was resolved on an agarose gel, and the 8 kb-*Hind* III fragment was isolated as described. After purification, the 8 kb-*Hind* III fragment was ligated into the pGEM-7Z vector, which had been digested with *Hind* III and dephosphorylated with 5 U calf intestinal alkaline phosphatase (CIP) (Pharmacia Biotech Inc.) prior to ligation. The ligation was done overnight at 16°C with 1-2 U of T_4 DNA ligase.

4.3 Mapping and construction of luciferase reporter plasmids

A series of deletions in the 5'-flanking region of the rat galanin gene, ending in the first exon (Figure 3), were prepared by digestion of the 8 kb-*Hind* III pGEM-7Z clone with suitable combinations of restriction endonucleases. The DNA fragments were inserted into

the multiple cloning region of pXP_2, a vector containing a promoterless luciferase reporter gene (Nordeen 1988).

The plasmid pGlu-657/152 was constructed by digesting the purified 8 kb-*Hind* III fragment with *Kpn* I and subcloned the *Kpn* I/*Kpn* I fragment into the *Kpn* I site of pXP_2. To create the plasmid pGlu-486/152, the plasmid pGEM-657, which was constructed by ligation of the insert of the pGlu-657/152 with the pGEM-7Z vector, was digested by *Kpn* I and *Pvu* II, the ends were filled-in with the Klenow fragment of DNA polymerase I, and subcloned into the *Sma* I site of pXP_2. To prepare the pGlu-208/152 and the pGlu-126/152 constructs, the pGEM-657 was cut with *Kpn* I/*Rsa* I and *Kpn* I/*Ban* II, respectively, following the procedure described above for the pGlu-486/152.

Since convenient restriction endonuclease enzyme sites for subcloning the pGlu-4500/152 were not present in the multiple cloning region of pXP_2, the 6 kb *Hind* III/*Sac* I fragment containing sequences from approximately -4500 to +1600 was subcloned into the *Hind* III/*Sac* I site of the pXP_2 vector to generate the pGlu-4500/1600. The pGlu-4500/1600 was digested with *Kpn* I and *Sac* I to remove the *Kpn* I-*Sac* I fragment and the remaining *Hind* III-*Kpn* I of the pGlu-4500/1600 fragment was subcloned into the site of *Kpn* I/*Sac* I site of the pGEM-7Z. The pXP_2-pGEM plasmid was digested with *Xho* I to remove the pGEM and religated to generate the pXP_2-*Hind* III/*Kpn* I plasmid containing the sequences from -4500 to -657. The pXP_2-*Hind* III/*Kpn* I was cut with *Kpn* I and ligated to a *Kpn* I fragment (-657 to +152) prepared from the pGlu-657 in the sense orientation, generating the

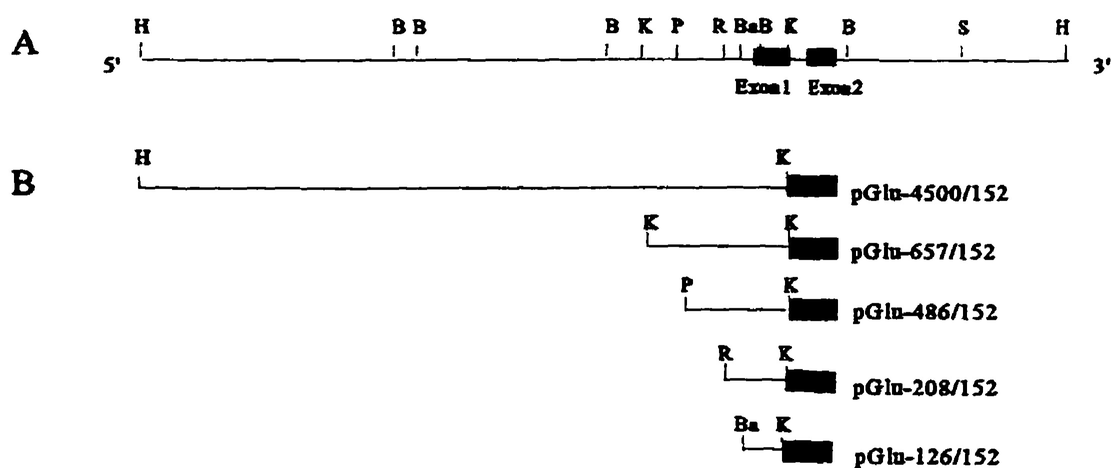


Figure 3. Deletion constructs and their locations in the rat galanin gene.
(A) Partial restriction enzyme map of the 8 kb-*Hind* III fragment of the rat galanin gene. (B) Luciferase fusion constructs of the 5'-flanking region of the rat galanin gene used for transfection experiments. The different sizes of the 5'-flanking region and the first exon of the rat galanin gene were fused into the pXP_2 expression vector. Enzyme abbreviations: B, *Bam*HI; Ba, *Ban* II; H, *Hind* III; K, *Kpn* I; P, *Pvu* II; R, *Rsa* I; S, *Sac* I.

pGlu-4500/152. All of the constructs were sequenced to assure the correct ligation products were obtained.

5. Transformation of Competent *E. coli* Cells

5.1 Preparation of TG1λ competent cells

LB medium, 5 ml, was inoculated with a single colony of TG1λ and grown overnight at 37°C with shaking. One ml of the overnight culture was used to inoculate 200 ml of LB medium and grown at 37°C for 2-4 hours with shaking to an OD_{600} of 0.4-0.5. Cell pellets were collected by centrifugation at 6,000 × g for 10 minutes at 4°C, and resuspended in 100 ml of ice-cold, sterile 50 mM $CaCl_2$ and left on ice for 15 minutes. The cells were centrifuged again at 6,000 × g for 10 minutes at 4°C and the $CaCl_2$ solution was removed. The cells were resuspended in 20 ml of ice-cold, sterile 50 mM $CaCl_2$. After aliquoted, the competent cells were stored at -80°C.

5.2 Transformation of TG1λ cells

Five μl of a ligation reaction, mixed with five μl of sterile water, were added to 100 μl of competent cells (previously thawed on ice) and incubated on ice for 45 minutes. The cells were heat shocked by placing them at 42°C for 90 seconds, and placed back on ice immediately. One hundred μl of LB medium was added and incubated for 30 minutes at 37°C. 10-100 μl of the transformation reaction was plated on selective media (LB/Amp) and incubated overnight at 37°C.

6. DNA Labelling

6.1 Random priming

Galanin cDNA fragments (700 bp) were labelled with ^{32}P-α-dATP by random priming. Two μg of random primers (hexadeoxyribonucleotides) and 25-250 ng of template were mixed in 14 μl dH_2O or TE buffer, denatured by boiling for 3 minutes, and cooled on ice for 5 minutes. After cooling, 2.5 μl of 10× Klenow buffer [0.5 M Tris-HCl, pH 7.5, 0.1 M $MgCl_2$, 10 mM dithiothreitol (DTT), 0.5 mg/ml BSA] and 2.5 μl of a dGTP, dTTP, and dCTP mixture (0.5 mM each) were added, followed by the addition of 1 μl of Klenow, and 5 μl of ^{32}P-α-dATP. This mixture was incubated at room temperature for 2 to 4 hours to allow synthesis. After incubation, 1 μl of 0.5 M EDTA (pH 8.0) was added to the mixture to stop the reaction. The mixture was then passed through a column of Sephadex G-50 (Pharmacia Biotech). Labelled probe was eluted from the column with 400 μl TE buffer. The total amount of radioactivity incorporated into the probe was determined by counting 2 μl of the probe in a liquid scintillation counter (Beckman, Irvine CA).

6.2 End labelling

6.2.1 Phosphate labelling with T_4 polynucleotide kinase

A reaction containing 5 pmol of oligonucleotide, 1×T_4 kinase buffer (250 mM imidazole-HCl, pH 6.4, 60 mM $MgCl_2$, 5 mM 2-mercaptoethanol, 350 μM ADP), 50 μCi (3000 Ci/mmol) γ-^{32}P-ATP, and 5 U T_4 polynucleotide kinase in a 25 μl volume was incubated at 37°C for 30 minutes. After incubation, the reaction was inactivated at 65°C for 10 minutes, following by ethanol/NaOAc precipitation at -20°C for 1 hour. The pellet was dried and

resuspended in TE buffer.

6.2.2 End-labelling with reverse transcriptase

A 50 μl reaction containing 50-100 ng of DNA fragment, 100 U moloney murine leukemia virus reverse transcriptase (MMLV, GIBCO-BRL), 1×RT buffer (0.5 M Tris-HCl, pH 8.3, 0.75 M KCl, 0.03 M $MgCl_2$), 20 mM DTT, 40 μM each of dGTP, dTTP and dCTP, and 60 μCi of α-^{32}P-dATP was incubated for 1 hour at 37°C. After fill-in incubation, 5 μl of 6 μg/μl yeast RNA were added to the reaction, following by ethanol precipitation. The pellet was dissolved in 20 μl TE buffer.

7. Southern Blot

7.1 Southern transfer of DNA

Southern transfer was carried out as follows: DNA mixed with DNA loading buffer (6 × stock: 0.25% bromophenol blue, 0.25% xylene cyanol, and 30% glycerol) was loaded onto a 0.8-2% agarose gel and separated by electrophoresis in 1×TBE for 1-16 hours. Molecular weight markers were run on the same gel to allow bands detected by hybridization to be sized. Gels were soaked in 0.5 N NaOH/1.5 M NaCl for 30 minutes at room temperature with gentle agitation, rinsed briefly in distilled H_2O, and then neutralized in 1 M Tris (pH 7.5)/1.5 M NaCl for 30 minutes at room temperature. Gels were transferred to nitrocellulose by capillary blotting using 20×SSC overnight. Blotting was carried out by placing the gel on a 3mm filter paper wick with the ends of the paper submerged in the SSC buffer. A

membrane, which was boiled in dH_2O for more than 5 minutes, was then placed on top of the gel and two pieces of filter paper were placed on top of the membrane. A stack of paper towels was then placed on the top of the filter paper and a weight was placed on the top of the paper towels to ensure that the SSC was drawn through the gel. After transfer, the membrane was air dried for 30 minutes, then baked at 80°C for 1 to 2 hours under vacuum.

7.2 Southern hybridization

For a probe > 100 bp:

Blots were prehybridized for 1 to 18 hours at 65°C in hybridization solution containing:

6 ×	SSC
0.5%	SDS
12.5 ×	Denhardt's solution (1 × solution contains 20 mg each of Ficoll, polyvinyl pyrrolidine, and bovine serum albumin in 100 ml water)
100 μg/ml	salmon sperm DNA (boiled 10 minutes before addition)

For hybridizations, 10^6 counts per minute (cpm)/ml of ^{32}P-labelled probe was boiled for 5 minutes, chilled on ice for 5 minutes, and then added to the hybridization solution. After incubation overnight at 65°C, the membranes were washed in 1× SSC containing 0.1% SDS at 65° C for 30 minutes. Washing was continued in 0.1 × SSC/0.1% SDS at 65°C for an additional 30 minutes to 1 hour if the blots were very radioactive. The blots were then placed in plastic wrap and exposed to x-ray film at -80°C.

For oligonucleotide probes (< 100 bp):

Blots were prehybridized for at least 4-6 hours at a restrictive temperature in oligonucleotide hybridization solution containing:

6 ×	SSPE (3 M NaCl, 0.2 M NaH_2PO_4, 20 mM EDTA)
5 ×	Denhardt's solution
0.25%	SDS
100 μg/ml	salmon sperm DNA (boiled 10 minutes before addition)

The labelled oligonucleotide probes were boiled for 3 minutes, chilled on ice for 5 minutes, then added to the oligonucleotide hybridization solution to give a final of 2-5 × 10^6 cpm/ml. The hybridization was carried out at the specific hybridization temperature overnight.

The hybridization temperature (T) was determined by:

$$T\ (^{\circ}C) = 4(G+C) + 2(T+A) - 10$$

After hybridization, the blots were washed in 2× SSC/0.1% SDS at the same temperature and then 30 minutes in 1× SSC/0.1% SDS at the same temperature, if necessary. The membranes were exposed to x-ray film, placed between intensifying screens, at -80°C for 2-3 days.

8. Screening Genomic DNA Library by PCR

A Fisher rat liver genomic DNA library in the lambda DASH® vector was constructed by Stratagene (La Jolla, CA). Rat genomic DNA was partially digested with *Sau3A* I restriction endonuclease and 9-22 kb fragments were cloned into the *Bam*HI site of the lambda DASH® vector. The library was titered and amplified as described below, following the protocol of Israel, 1993 (Figure 4).

8.1 Titering the frequency of the original library for the rat galanin gene

The appropriate number of phage particles per tube used in the primary screen was determined by titering the experimental frequency of the rat galanin gene in the original library using PCR. The lowest number of phage that yielded a correct and detectable PCR product was defined the minimum experiment frequency of the gene in the library and was used to determined the number of initial phage to be screened. For the rat galanin gene in this labrary, we found a minimum of 10^6 plaque forming units (pfu) was required to yield a PCR product that was detectable by Southern hybridization to a rat galanin specific oligonucleotide Gal 3. Since the average size of the genomic insert in this library is 15.5 kb, PCR amplification of 10^6 phage represents 1.6×10^{10} bp of genomic DNA. The human genome size is about 3.26×10^9 bp. Therefore, the hypothetical frequency of the rat galanin gene is about 1/210,000.

8.2 Amplification of phage

A single colony of the host bacterial strain (XL1-Blue MRA-P2) was inoculated in a 50 ml

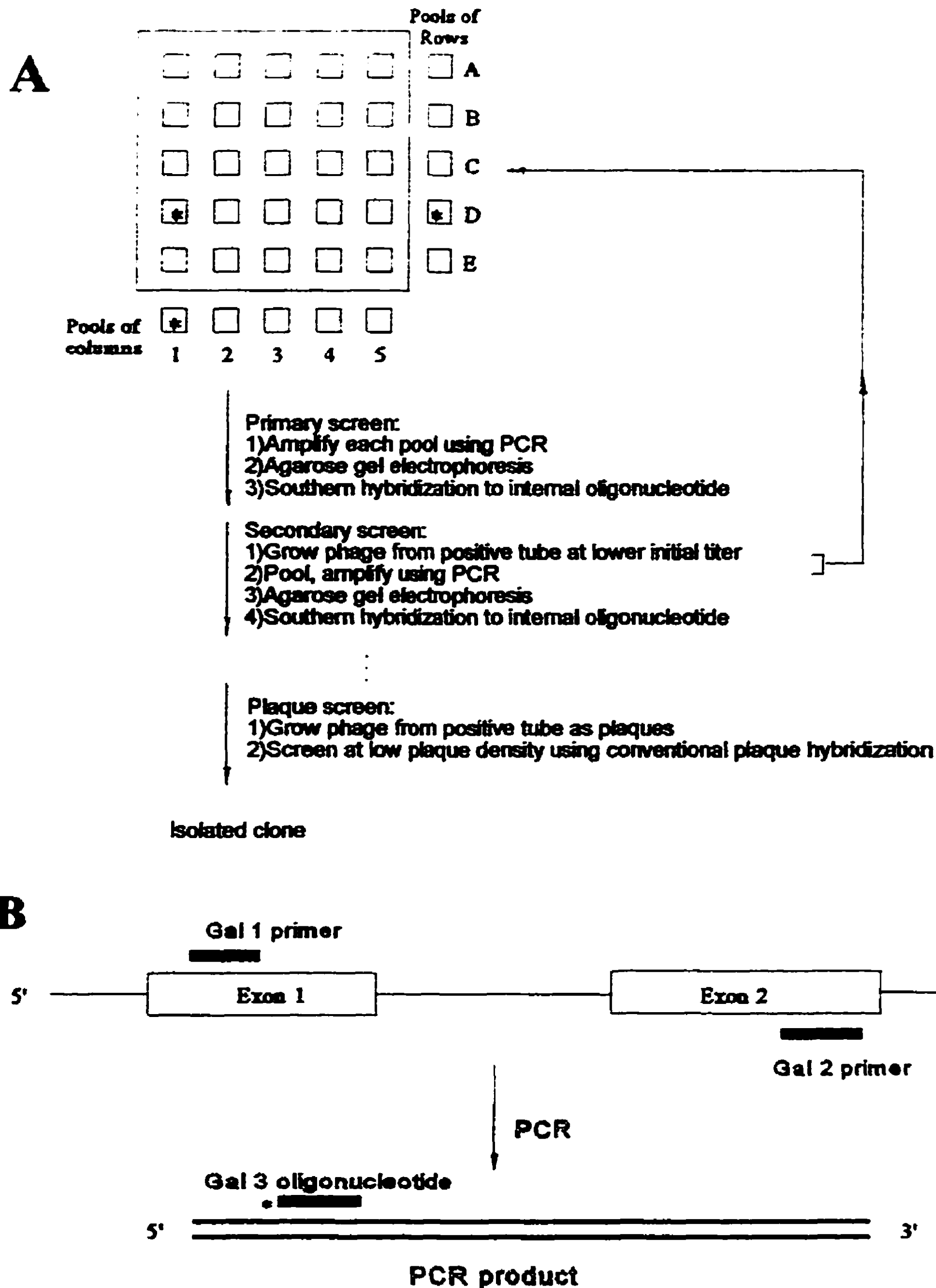


Figure 4. Schematic diagram of the PCR screening procedure and diagrammatic representation of the PCR product detectable by hybridization. Panel A: The genomic phage library is amplified and analyzed by PCR as described in the text. At each stage of screening, the number of initial phage particles per tube is decreased. After the library was highly enriched using the PCR screening protocol, the conventional plaque hybridization screening was used for a 150 mm plate. Panel B: The locations of the PCR primers and the hybridization oligonucleotide between exon 1 and exon 2 of the rat galanin gene are shown. The correct PCR product of 383 bp hybridizes to the Gal 3 hybridization oligonucleotide probe.

of L broth supplemented with 0.2% maltose and 10 mM $MgSO_4$. The culture was grown at 37°C with shaking (200 rpm) overnight. The cells were pelleted at 2,000 rpm for 10 minutes and then resuspended with sterile 10 mM $MgSO_4$ to OD_{600} =0.5 .

The rat genomic DNA library, containing about 10^6 pfu phage, was diluted in 300 μl of SM (50 mM Tris-HCl, pH 7.5; 100 mM NaCl; 8 mM $MgSO_4$; 0.01% gelatin) and then was used to infect 300 μl of the XL1-Blue MRA-P2 *E. coli*, which were resuspended in 10 mM $MgSO_4$, for 20 minutes at room temperature. The bacteria/phage mixture was diluted with 6 ml L broth/10 mM $MgSO_4$, put in 8 × 8 tube matrix (64 tubes, 100 μl/tube) and amplified at 37°C for 4-6 hours while shaking at 225 rpm.

The phage titer was typically 1-2 × 10^9/ml after amplification. For secondary and tertiary amplification, phage from a single positive tube were titered, and used to infect XL1-Blue MRA-P2 *E. coli* at approximately 4.4 × 10^3 initial pfu/100 μl (secondary screen, the results shown in Figure 5) or 180 initial pfu/100 μl (third screen) and amplified as described above.

8.3 Pooling of phage

Twenty five μl amplified phage from each of the tubes across a column or each of the tubes down a row were pooled together and diluted 1:1 with ddH_2O. The phage pooling mixture from tubes was to be used as templates for PCR analysis as described below.

The pooled phage, and amplified phage were kept at 4°C. At all steps, cross-contamination

of the samples was carefully avoided.

8.4 Design of primers and PCR reactions

Oligonucleotide primers for PCR were designed from exon 1 and 2 based on the rat galanin cDNA sequence using the OLIGO Primer Analysis version 5.0 software. The Gal 1, sense primer, (5'-GCCATGCAGTGAGCGACCCT-3') and the Gal 2, antisense primer, (5'-GCATCCCGAGCCCCAGAGTG-3') were located at nucleotides 20-39 and 184-203 of the rat galanin cDNA sequence, respectively (Vrontakis *et al.* 1987). The Gal 3 internal oligonucleotide, (5'-CCTGGACGGAGACACTTGGACCTGC-3'), corresponding to nucleotide 63-87 of the rat galanin cDNA sequence, which did not overlap either the sense or antisense primer, was used as a hybridization probe for the PCR product analysis.

Each PCR reaction was performed in a 25 μl volume and contained 20 pmol of the Gal 1 and Gal 2 primers, 0.25 μl Expand High Fidelity PCR System polymerase (Boehringer Mannheim), 0.2 mM each of dATP, dCTP, dTTP and dGTP, 1.5 mM $MgCl_2$, 1× PCR buffer (Boehringer Mannheim), and 0.5 μl template. For each round of screening, a negative control (without template) and a positive control (either 10 ng total rat genomic DNA or a phage population from a positive tube) were set up at the same time as the test samples. After disrupting the phage at 70°C for 5 minutes, and cooling on ice for 5 minutes, PCR was performed in a thermal MiniCycler (MJ Research) for 35 cycles. After an initial denaturation at 94°C for 1.5 minutes, each cycle consisted of 30 second at 94°C, 30 second at 57°C, and 1.5 minutes at 68°C. After the 35 cycles, samples were held at 15°C prior to electrophoretic

analysis.

8.5 Analysis of PCR products

PCR reaction products were separated by electrophoresis in a 1.2% agarose gel containing ethidium bromide and visualized with ultraviolet light. The DNA was transferred to a nitrocellulose membrane and hybridized with the Gal 3 internal oligonucleotide, end-labelled with γ-^{32}P-ATP, following the protocol described in Southern Blot. After washing at 50°C in 1 × SSC/0.1% SDS, the membrane was exposed to x-ray film.

8.6 Plaque screening

After three rounds of screening by PCR, the plaque screen was performed using a procedure modified from the instructions provided by Stratagene. Bacteriophage from a positive tube (300-500 pfu) were infected into XL1-Blue MRA-P2 *E. coli*, mixed with melted agarose, and plated on a 150 mm NZY (for per litre: 5 g NaCl, 2 g $MgSO_4 \cdot 7H_2O$, 5 g yeast extract, 10 g NZ Amine, 15 g agar, adjusted to pH 7.5) agar plate. The plated phage were incubated overnight and then chilled at 4°C for 2 hours. A 150 mm nitrocellulose membrane was laid onto the plaques formed in the agarose top layer. The disk was labelled with orientation markers, transferred for 2 minutes, and gently removed. The membrane was denatured by submersion in 0.5 M NaOH/1.5 M NaCl solution for 2 minutes, then neutralized in a pool of 0.5 M Tris-HCl (pH 8.0)/1.5 M NaCl for 2 minutes, and finally rinsed in 0.2 M Tris-HCl (pH 7.5)/2× SSC for 30 seconds. The membrane was then baked for 2 hours at 80°C under vacuum. The filter was hybridized (as described earlier) at 65°C overnight with a rat galanin

cDNA probe and positive clones were identified after autoradiograph.

The positive clones were picked and put in 1 ml SM buffer containing 20 μl of chloroform. This phage/SM mixture was titered and replated on 90 mm plates at a lower dilution (100 pfu/plate) and hybridized again, allowing a well isolated clone to be readily identified.

9. DNA Sequencing and Sequence Structure Analysis

The sequencing of plasmid DNA was carried out following the instructions of the dsDNA Cycle Sequencing System kit (GIBCO BRL) with slight modifications. Oligonucleotide primers were synthesized according to either vector-specific sequences (T7 and SP6) or specific sequences in exon 1 and exon 2 of the rat galanin gene (Gal 1 and Gal 2 primers as described in section 8.4).

9.1 End labelling of primer

One pmol of primer was mixed with 2 pmol of [γ-^{32}P]ATP and 1 U of T_4 polynucleotide kinase in 1 × kinase buffer (60 mM Tris-HCl, pH 7.8, 10 mM $MgCl_2$, 0.2 M KCl) in a total volume of 5 μl. The mixture was incubated at 37°C for 30 minutes and then at 55°C for 5 minutes to terminate the reaction.

9.2 Sequencing reactions

The DNA template (15 to 50 fmol) was mixed with 5 μl of end-labelled primer, 4.5 μl of 10

× *Taq* sequencing buffer [300 mM Tris-HCl, pH 9.0, 50 mM $MgCl_2$, 300 mM KCl, 0.5% (w/v) W-1], and 1.25 U of *Taq* DNA polymerase in a total volume of 36 μl. Eight μl of the template/primer cocktail mixture was mixed with 2 μl of each of the four dNTP/ddNTPs mixture (dATP/ddATP, dCTP/ddCTP, dGTP/ddGTP, and dTTP/ddTTP). The reactions were placed in a thermal cycler. The temperature cycling program was as follows: 95°C for 2 minutes, then 30 cycles of denaturation at 94°C for 30 seconds, annealing at 42°C or 59°C (42°C for T7 or SP6 primer and 59°C for Gal 1 or 2 primer) for 30 seconds, and extension at 72°C for 1 minute. The reactions were stopped by the addition of 5 μl of stop solution [95% (v/v) formamide, 10 mM EDTA (pH 8.0), 0.1% (w/v) bromophenol blue, 0.1% (w/v) xylene cyanol]. Each of the four reactions was denatured for 5 minutes at 90°C immediately before resolution on a denaturing acrylamide gel.

9.3 Acrylamide gel electrophoresis

The reactions were resolved on a 6% polyacrylamide gel containing 7 M urea and were run in 1 × Winter's TBE buffer (133 mM Tris, 45 mM boric acid, 3 mM EDTA) for 2 to 6 hours at 1500 V and 50 mA. Before samples were loaded, the gel was pre-run for 15-60 minutes until the temperature of the gel was above 45°C. The gel was lifted from the plate by absorption to a sheet of filter paper and vacuum dried at 80°C for 45 minutes. The dried gel was exposed to x-ray film overnight at -80°C.

The sequences were analyzed for regulatory element binding site sequences by using TFSEARCH computer software on the Internet.

10. Primer Extension Reaction

10.1 Labelling of primers

Twenty pmol of Radio-labelling Gal 5 primer (5'-AGGGTCGCTCACTGCATGGC-3'), antisense oligonucleotide and complementary to the regions +67 to +48 relative to the transcription start site of the rat galanin gene, was completed as described for end-labelled reactions, with the following changes: the final kinase reaction was resuspended in a 20 μl volume, and after incubation for 30 minutes at 37°C, the T_4 kinase was inactivated at 70°C for 10 minutes.

10.2 Primer extension

Total RNA from rat pituitaries was isolated by the guanidinium thiocyanate method (Chirgwin *et al.* 1979) and was hybridized with the end-labelled Gal 5 reverse oligonucleotide primer. Hybridization was carried out in 0.4 M NaCl, 10 mM Pipes (piperazine-N, N'-bis [2-ethanesulfonic acid]) pH 6.4 for 2 minutes at 85 °C and then 5 hours at 63°C. For the extension reaction, 0.5 mM dNTP and 1 RT × buffer (Promega), 20 U RNase Inhibitor (Promega), 1.5 µg BSA and 400 U of MMLV reverse transcriptase (Promega) were added and the incubation was at 42°C for 1 hr. After extension, phenol/chloroform extraction, and ethanol precipitation the DNA/RNA hybrids were heated for 4 minutes at 95°C and loaded on a 6% polyacrylamide urea gel. The size of the primer extension products was determined by comparison to a sequencing ladder using the same primer as that for primer extension but with the plasmid containing the 5'-flanking region of the gene as template.

11. Cell Culture

A human neuroblastoma cell line (SK-N-MC) was a generous gift from Dr. A. Nath (Department of Medical Microbiology, University of Manitoba), the Hela cell line was a gift from Dr. M. L. Duckworth (Department of Physiology, University of Manitoba), rat pheochromocytoma (PC_{12}) and rat pituitary GH_3 cell lines were purchased from the American Type Culture Collection (ATCC, Rockville Pyke, Maryland).

SK-N-MC cells were cultured in Dulbecco's modification of Eagle's medium (DMEM) with 2 mM L-glutamine, supplemented with 10% fetal calf serum (FCS), 1×antibiotic-antimycotic (GIBCO-BRL). Hela cells were cultured in DMEM medium plus 8% FCS and 1 × antibiotic-antimycotic (GIBCO-BRL). PC_{12} cells were cultured in DMEM medium and supplemented with 7% FCS, 7% horse serum and 1 × antibiotic-antimycotic (GIBCO-BRL). GH_3 cells were cultured in DMEM medium with 10% FCS and Bonus (200 mM L-glutamine and 1×10^4 units penicillin G, 1×10^5 μG/ml streptomycin sulphate in 0.85% saline). All of the cells were incubated at 37°C in a humidified atmosphere of 95% air and 5% CO_2.

17β-estradiol (E_2), 12-O-tetradecanoylphorbol-13-acetate (TPA) were purchased from Sigma Chemical Co. The stock solution of 10 mM estrogen (E_2) was prepared in ethanol and protected from light. The long-term stock solution of TPA was prepared at 10 mM in dimethyl sulfoxide (DMSO) and stored in the dark at -80°C. Before use, 0.1 mM TPA was prepared in ethanol from the 10 mM TPA stock. Nerve growth factor (NGF) was obtained from GIBCO-BRL (Grand Island, N.Y.).

The cells were plated at an initial density of 1-2 × 10^5/60 mm dish in medium. After transfection, fresh medium was added and all were treated with different drugs (either 100 nM TPA, 10 μM E_2, 50 ng/ml NGF supplemented with 0.1% BSA, or vehicle) for 12-36 hours.

12. Transfection

One or two days prior to transfection, SK-N-MC, PC_{12}, GH_3, and Hela cells were plated at a density of about 2 × 10^5 cells/60 mm dish. Two to four hours before transfection, the culture of the cells was replaced with 2 ml/dish of fresh medium. Transient transfections of the cells with plasmid DNA were performed using a modified version of the calcium phosphate DNA precipitation method (Cattini *et al.*, 1986). Briefly, 0.25 ml of 2 × calcium phosphate chloride (250 mM $CaCl_2$) - DNA solution, containing 6 μg of rat galanin/luciferase construct and 1 μg of β-galactosidase expression vector (pCMV) per 60 mm dish was bubbled with air into 0.25 ml of 2 × HEBS (280 mM NaCl, 50 mM HEPES, 1.5 mM Na_2HPO_4, pH 7.1) at room temperature. This mixture was left at room temperature for 30 minutes to allow the DNA to form calcium/DNA precipitates. The precipitation of $CaPO_4$/DNA was added drop by drop to the dish of cells (150 μl/dish) and then incubated for seven hours at 37°C. The medium was removed and rinsed twice with calcium and magnesium-free (CMF) Phosphate Buffered Saline (PBS) after transfection. Fresh medium containing either a different drug (TPA, NGF, and E_2) or vehicle was then added to each transfected plate and kept at 37°C for 24-36 hours.

13. Luciferase and β-Galactosidase Assays

13.1 Luciferase assays

Twenty-four to thirty-six hours after transfection, individual plates of cell cultures were lifted with either 1 mM EDTA/PBS or 1 mM EDTA/PBS/0.05% trypsin (for SK-N-MC cells only), rinsed twice with ice-cold CMF-PBS, and subjected to centrifugation at 1000 rpm for 10 minutes at 4°C. Cell pellets were lysed with 30-100 μl of 1 × lysis solution (2 mM DTT, 2 mM 1,2-diaminocyclohexane-N,N,N',N'-tetraacetic acid, 10% glycerol, 1% Triton X-100, 25 mM Tris-phosphate, pH 7.8) at room temperature for 15 minutes. Centrifugation was carried out at 3,500 × g for 15 minutes at 4°C and the soluble protein of the cell extract was collected and stored at -80°C.

Extracts containing equal amounts of protein were assayed by measuring the light emitted when cell extract, containing the luciferase enzyme, was mixed with the luciferase assay substrate (Promega). Briefly 10 μl of cell extract was mixed with 50 μl of luciferase assay substrate at room temperature. The reaction was placed in a scintillation counter and the light produced by the enzyme was measured for a period of 10 seconds to 1 minute.

13.2 β-galactosidase assays

The transfection experiments were corrected for transfection efficiency based on β-galactosidase activities which were expressed by the cotransfected β-galactosidase expression vector (pCMV). The levels of β-galactosidase were determined by measuring the light emitted from a mixture of Galacto-Light™ accelerator and Galacto™

chemiluminescent substrate which can be hydrolysed by β-galactosidase. Briefly, 2-5 μl of cell extract was incubated with 200 μl of the Galacto™ chemiluminescent substrate at room temperature for 15-60 minutes and then mixed with 300 μl of the Galacto-Light™ accelerator in a scintillation counter. The light was measured for a period of 1 minute.

13.3 Transfection efficiency and modification of luciferase activity

As indicated above, the cell extracts were collected by lysis of the transfected cells as described in 13.1. For each transfection the highest β-galactosidase activity was given a value of 100%. The β-galactosidase activities in the other samples were expressed as a percentage of the highest value.

The luciferase activities of the samples were corrected for transfection efficiency by dividing by the percentage of β-galactosidase activity. The final calculation was to determine the average luciferase activity of duplicates of the same transfection and the standard error of the mean.

14. Preparation of Nuclear Extract

Nuclear extracts of cells were prepared from cells of Hela, SK-N-MC, and SK-N-MC treated with 100 nM TPA for 36 hours following published protocols (Dignam *et al.* 1983). Briefly, cells were harvested and rinsed with PBS three times. The cells were resuspended and swelled with buffer A [10 mM HEPES, pH 7.9, 1.5 mM MgCl, 0.5 mM DTT and 1 mM

phenylmethylsulphonyl fluoride (PMSF)]. The cells were then broken with a Dounce homogenizer and centrifuged at 3,000 × g for 10 minutes at 4°C. After removing the supernatant, the nuclei were recovered by centrifugation again at 25,000 × g for 20 minutes at 4°C. The nuclei were broken with a Dounce homogenizer in buffer C (20 mM HEPES, pH 7.9, 25% glycerol, 0.42 M NaCl, 1.5 mM MgCl, 0.2 mM EDTA, 1.0 mM PMSF, 0.5 mM DTT) and the nuclear membranes were removed by centrifugation at 25,000 × g for 30 minutes at 4°C. The nuclear extracts were dialysed in buffer D (20 mM Hepes, pH 7.9 at 4°C, 20% glycerol, 0.1 M KCl, 0.2 mM EDTA, 0.5 mM DTT and 1 mM PMSF) and centrifuged at 25,000 × g at 4°C to remove all insoluble debris. The nuclear extracts were aliquoted and stored at -80°C.

15. Gel Mobility Shift Assay

Nuclear extracts from cells were used to specifically retard [^{32}P]-labelled DNA fragments which contain binding sites for proteins in the extract. The binding reactions contained 1 μg of polydeoxyinosinic-deoxycytidylic acid (poly [dI-dC]), 6 μg of nuclear extract, 10 mM Hepes, pH 7.9, 10% (v/v) glycerol, 0.1 M KCl, 0.2 mM EDTA, 0.5 mM DTT. Reactions were incubated for 15 minutes at room temperature with or without competitor DNA, the incubations were continued for additional 20 minutes after 5' end [^{32}P]-labelled DNA fragments (10^4 cpm) were added. Competition assay was carried out using 10-, 40-fold molar excess of unlabelled probe. Samples were separated by electrophoresis on 6% nondenaturing polyacrylamide gels at 4°C in 0.5 × TBE buffer (45 mM Tris, 45 mM boric acid, 2 mM

EDTA, pH 8.0). After electrophoresis, the gel was exposed to X-ray film.

RESULTS

1. Isolation and Cloning of the Rat Galanin Gene

A rat liver genomic DNA library was screened for the galanin gene using a PCR-based strategy as described in Materials and Methods. At each stage of screening (primary, secondary, or tertiary), the number of initial phage particles per tube was decreased (from 1.6×10^4 pfu/tube to 4.4×10^3 pfu/tube then to 180 pfu/tube). In the primary screen, 64 tubes, each containing 16,000 phage, were amplified and pooled in an 8×8 matrix. The pooled phage from columns and rows were screened using PCR. In the second screen, 36 tubes, containing 4,400 pfu/tube, were amplified in 6×6 matrix, following the same approach. Finally 25 tubes, containing 180 pfu/tube, were carried out in the tertiary screen. As shown in Figure 5, in the tertiary screen pools from rows a and b and from column E gave a 383 bp PCR product, which also was positive by Southern hybridization to the GAL internal oligonucleotide located between the Gal 1 (sense) and Gal 2 (antisense) primers (Figure 5A & B). Phage from the positive tubes were further grown as plaques and screened with the full length galanin cDNA using conventional plaque hybridization. Four positive clones were obtained by hybridization with the full length rat galanin cDNA probe. The positive clones were fully purified and isolated (one of them is shown in Figure 6). Restriction endonuclease digestion with *Sal* I and Southern analysis (data not shown) revealed that one clone (A_{22}) contains the largest insert (~25 kb) and therefore, this clone was selected for

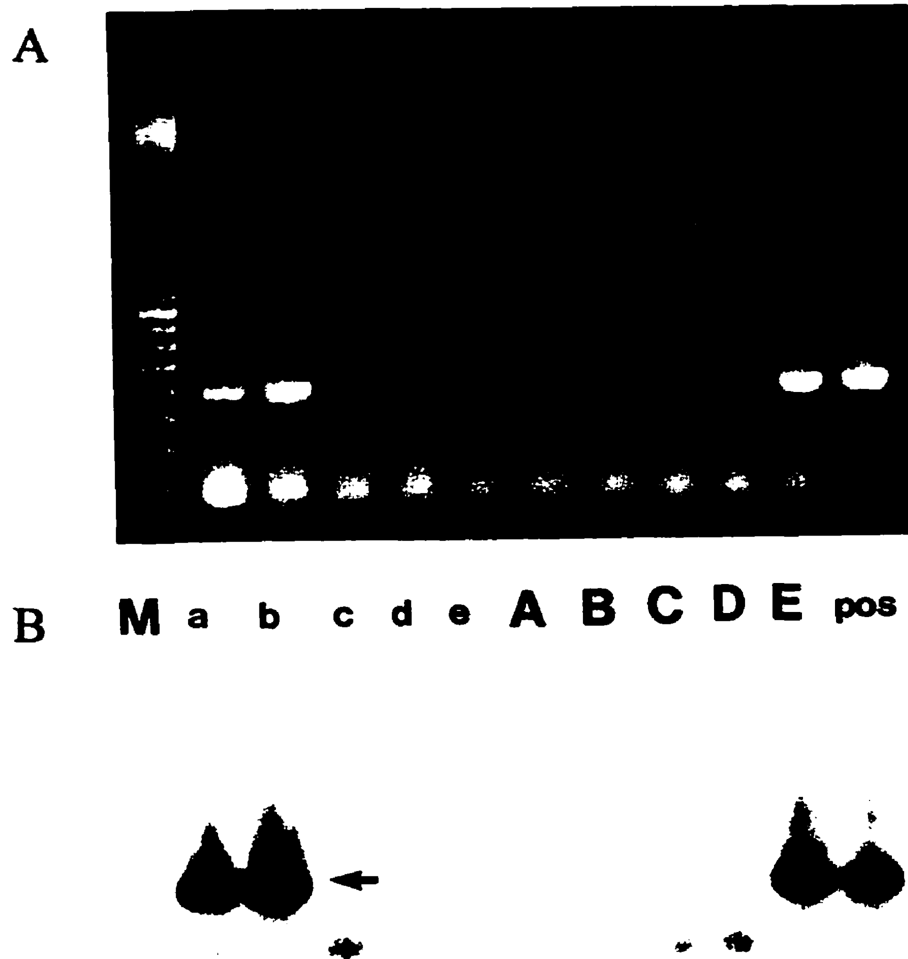
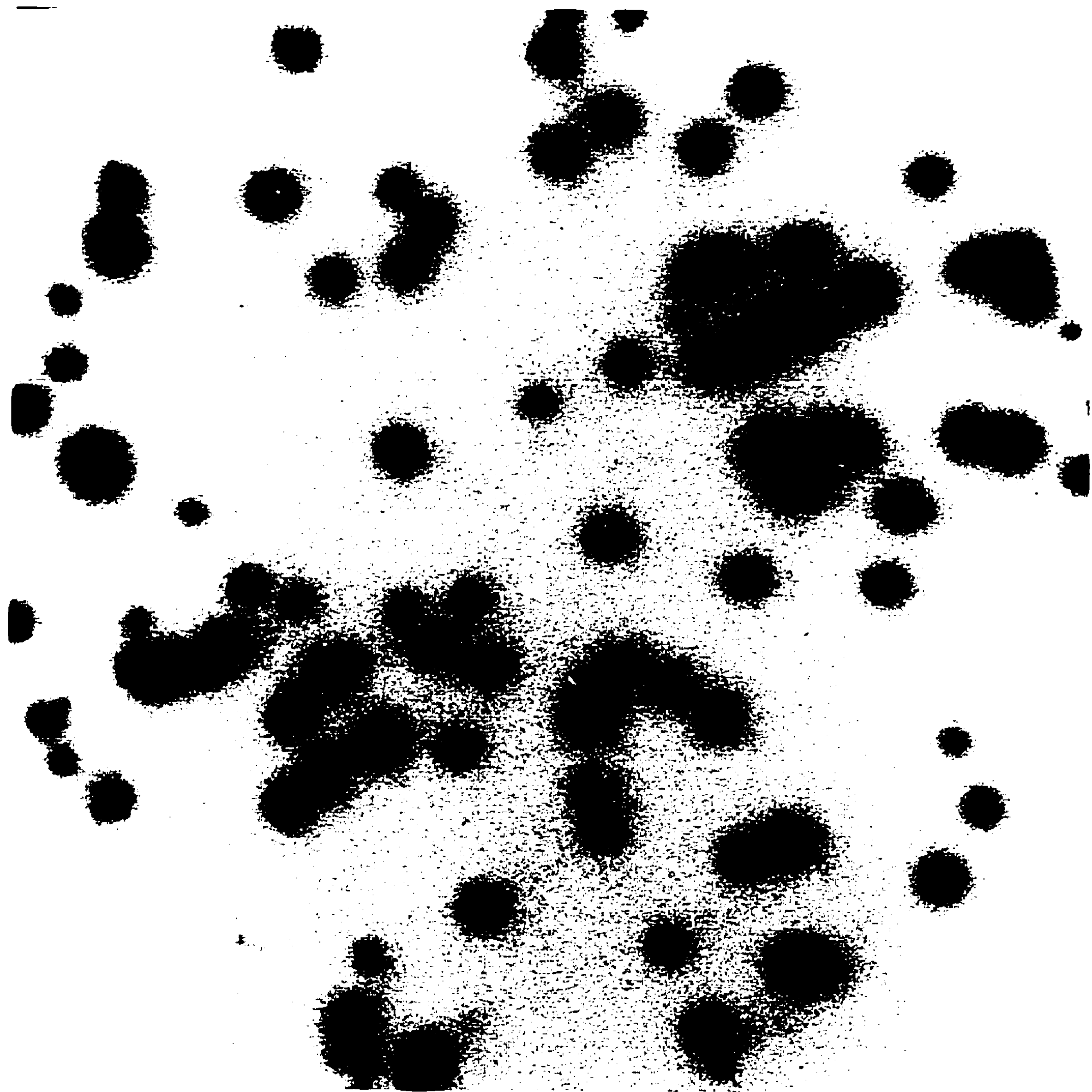


Figure 5. PCR screening of a rat genomic library for the galanin gene. 25 tubes were inoculated with 180 phage/well, amplified and pooled in an 5 × 5 matrix. Pools from columns and rows were screened using PCR and Southern hybridization. Panel A, ethidium bromide staining of PCR products. Panel B, hybridization with Gal 3 oligonucleotide shows a positive 383 bp PCR product. The templates for each reaction were: lane M, marker; lanes a-e, pools of rows; lanes A-E, pools of columns; lane pos, 20 ng rat genomic DNA (positive control). The arrows denote the 400 bp positive PCR products.

Figure 6. **Isolation of the rat genomic clone.** A rat liver genomic library was screened and positive plaques were identified by hybridization with rat galanin cDNA probe. The figure shows an autoradiogram of a fully purified plaque filter (clone A_{22}) hybridized with the rat galanin cDNA probe.

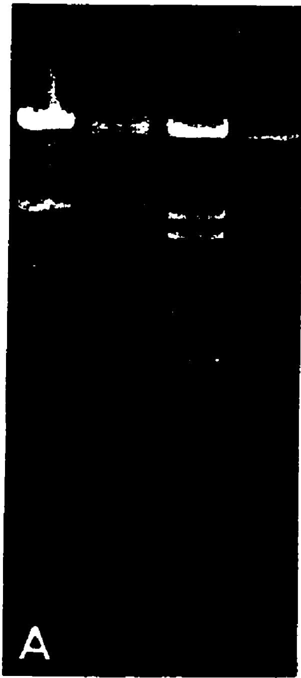


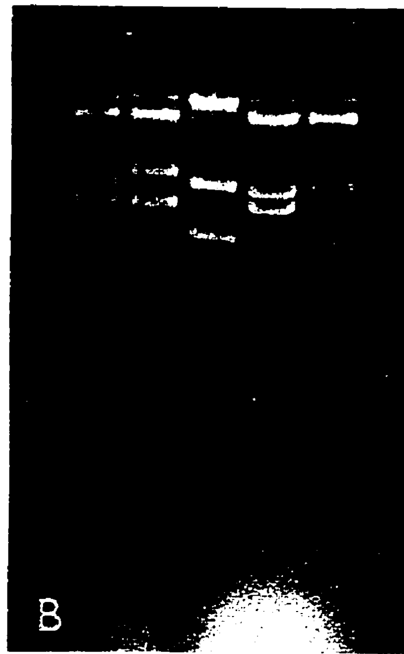
further analysis. Restriction enzyme mapping of the positive clone together with Southern analysis using the full length rat galanin cDNA as a probe revealed two *Hind* III fragments (8 kb and 3 kb) contained rat galanin cDNA sequence. Hybridization with the Gal 3 oligonucleotide primer, that is located in the first exon, indicated that the 8 kb fragment contains more 5' end sequences (Figure 7). The 8 kb-*Hind* III fragment was subcloned into the plasmid pGEM-7Z and characterized by restriction enzyme analysis as it is illustrated in Figure 8. Two *Bam*HI fragments (Figure 8) containing 1 kb of the 5'-flanking region and exon 1 and 2 were fully sequenced, following the strategy illustrated in Figure 8. Further restriction analysis of the 8 kb *Hind* III fragment and partial sequence of its fragments showed that it contains a 4.5 kb 5'-flanking region of the rat galanin gene as well as intronic sequences including intron 4. The second 3 kb *Hind* III fragment contains the two other exons (5 and 6) and introns (Data not shown).

The sequence extending 1005 bp upstream of the transcription start site is shown in Figure 9. Analysis of the sequence revealed the presence of a TATA box-like element (TATAAATA) at position of -32 to -26. Exon 1 consists entirely of non-coding sequences (+1 to +152) which is separated from the exon 2 by a 200 bp intron. Exon 2 (+352 to +432) begins with the site of translation initiation and codes for the first 27 amino acids including the signal peptide of the galanin precursor. Exon 1 and 2 sequences are in agreement with the rat cDNA sequence (Vrontakis *et al.* 1987).

The DNA sequence, which has been submitted to the Gene Bank with the accession number

Figure 7. Restriction mapping of the genomic clone A_{22}. Phage DNA from the A_{22} clone was digested with *Hind* III and *Sal* I. The digested DNA was separated by electrophoresis in a 0.6% agarose gel (panel A and B) and transferred to nitrocellulose. The Gal 1 primer (20 bp) (as a probe in panel C) or rat galanin cDNA (~700 bp) (as a probe in panel D) were used for hybridization. Lane m, marker; lane 1, 2, and 3, clone A_{22} digested with *Hind* III *and Sal* I; lane a and c, clone A_{22} digested with *Hind* III and *Sal* I; lane b, clone A_{22} digested with *Hind* III; lane d, clone A_{22} digested with *Sal* I. Panel A and B, gels stained by ethidium bromide. Panel C, the gel of panel A was hybridized with Gal 1 primer. Panel D, the gel from panel B was hybridized with rat galanin cDNA probe.


A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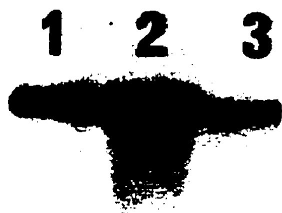
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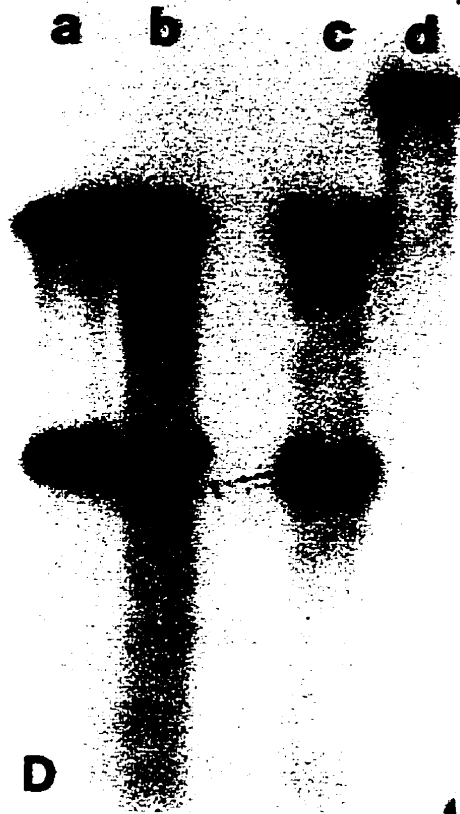
C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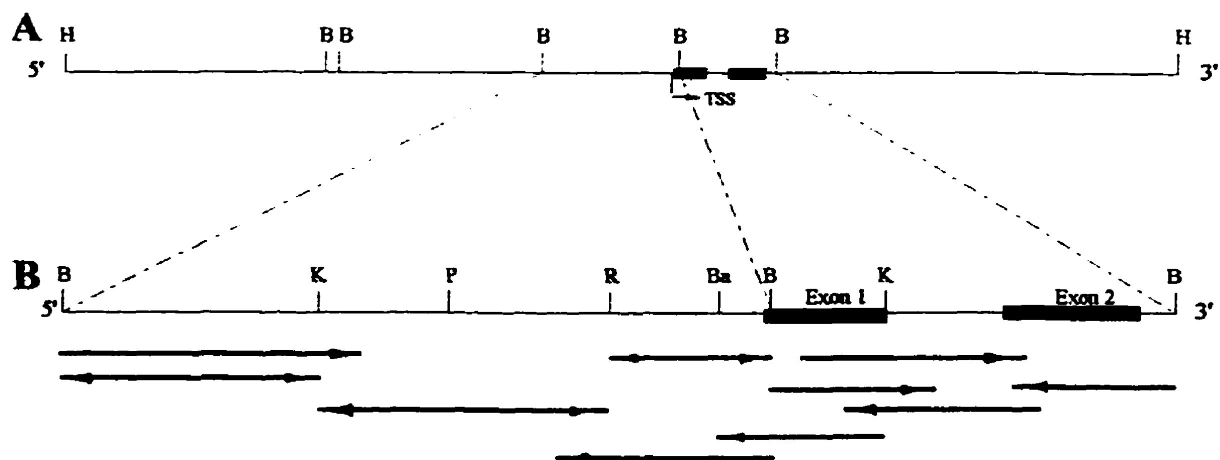

Figure 8. Restriction map of the 5'-flanking region of the rat galanin gene and sequence strategy of the 8 kb-*Hind* III fragment. Panel A, restriction enzyme mapping of the 8 kb-*Hind* III fragment of the rat galanin gene clone with *Hind* III and *Bam* HI. TSS, transcription start site. Panel B, restriction enzyme analysis of portions of the 8 kb-*Hind* III fragment and the sequence strategy after subcloning into the pGEM-7Z. The extent and direction of the sequencing is indicated with arrows under the restriction map. Enzyme abbreviations: B, *Bam* HI; Ba, *Ban* II; H, *Hind* III; K, *Kpn* I; P, *Pvu* II; R, *Rsa* I.

of Babklt118411 AF006690, of the rat galanin gene clone was searched using the TFSEARCH version 1.3 database for possible consensus DNA binding sites of transcription factors. The results of the analysis are shown in Figure 9. The following elements were found in the proximal promoter region: a TATA box-like element and some putative *cis*-elements including a CREB, three half-palindrome EREs, several AP-1, AP-4, SP1, GATA, Nkx-2, cEts. (Figure 9).

2. Determination of the Transcription Start Site

Primer extension analysis was employed to localize a transcription start site for the rat galanin gene (Figure 10) using total RNA isolated from rat pituitary and uterus. A 20-mer oligonucleotide Gal 5 (5'-AGGGTCGCTCACTGCATGGC-3') based on the sequence in exon 1 region of the rat galanin cDNA, and complementary to the sequence from +48 to +67 of the genomic clone, yielded an extension product of 67 bp in length (Figure 10). The transcription start site is located at a G nucleotide, 26 bp downstream of the TATA-like box.

3. Reporter gene constructs.

To determine the role of transcriptional elements in the functional activity of the galanin promoter and to locate potential tissue-specific positive and negative control elements, a series of deletions of the promoter region were constructed. Fragments containing various lengths of the 5'-flanking sequence of the rat galanin gene including the entire first exon

```
-1005                                          ggatc ccagatgggt cttaggagaa gagggggaca  acttggctca
                                                  GATA
-960   gggtcatgca gtgttcatca ggacattggt tagcccgagt gactgtggtc tcttgtgatc cgctgctcct  tgctccagca
       ERE1/2
-880   acggagtcat gttaagccgt gatcagaacc ctcttgggtg aacaaggctc ctcaggacct gggtcaccat  gctagtgggg
         AP-1                                                              AP-1, ERE ½
-800   ttcacttctg ggccaggtga cagagtgctc ctgatgagcc ccaaaggcta acacgttcat cagagctgcg ccctggttaa
                        AP-1          GATA
-720   atatctcacg ctttgggagt ccggagttgc tagctctgtg tctgtcctga ggtgagattt cagggtacct gtatgctgat
       GATA                                                                          GATA
-640   gtgctgctcc tgcacaggtt ggctaaactc cctgggtgca gacagctctg caccttggca agaatctggc ctggggctct
                                                                        GATA
-560   gcaggactca accacagtca cgacagagac cactccagaa acggggctct aagggaaaat ggatggttgg ggcacagctg

-480   ccagccaccc tctacccagc cctcagccct gaatggctgc acccctcccc ctttttcccc agcaaaagag gaatggagga
                    SP1
-400   ccctggacca gggtagggaa gctgcagtaa catggtgcaa agcagtcctg ggaatttggt ttctcaggag gtgtccgtga

-320   ctggccttgc ttgggctttg gggtggtcca ttccagcccc agccctggga aggagagcaa gacctcctcg ccagcctcag

-240   gatggggtg tcggggactc attcttgtgt gagtacgggg cagaacagtg ggaagtgact ctgtgatgca gggttggccg
       GATA                                                              AP-1
-160   ggagatagtc tgggactgtg ggtggtcctc tcctgagccc caggagcggg agcgggttcc ggtcacagcg gcccttggga
         GATA                                                            cEts  ERE ½
-80    ctcgcaggag gcggcgctga gcgggtgacg cggcagctcc caccgggtat aaatagcggc agcagcgcgg ctcctgcggc
              SP1              CREB1/2                  TATA box
+1     GGACACGTCG AGGGATCCTC GTGCGCTTCC CTACGCCGCT GATCTGCGCC

+51    ATGCAGTGAG CGACCCTCGC GCCCGCCACT CTACGCCACG CCTGGACGGA

+101   GACACTTGGA CCTGCACTAA CCAGCTACGC CCGGTTCCCA CCACTGCTCA
            Nkx-2
+151   AGgtacccgc gtcccaccga ggcttgcctg gccctagtcc tcctgcggtt tgtagcccca tcccctgcccc tgcaccccctc
                                                                 GATA   SP1
+231   acagctctgt tcccatcacc cagccactcc catgccaatg ccttcgcagt ccaagtgccc cagacatgtg cgtgtgcagg
                   GATA   SP1                          NKx-2
+311   actgctcagg tgcgtccact catccacttc tttccttcca gATGGCCAGG GGCAGCGTTA TCCTGCTAGC
                                                     GATA                GATA
+381   CTGGCTCCTG TTGGTTGCAA CCCTGTCAGC CACTCTGGGG CTCGGGATGC
                                                        GATA
+431   CAgtaagtac tggggacagc tgacttgtaa agagggctaa gggtgtcaga tctgaagatc agcctggaag aaggatggtt
                   AP-4
+511   cattgtcccc atagcaggaa tagggtgggg ggacatgtcc ctgaagctgc tggagggtgg ggaggatcc
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Figure 9. Nucleotide sequence of the proximal promoter region of the rat galanin gene and exon 1, exon 2, and the first intron of the gene. A TATA-like box and consensus sequences for known protein binding factors are indicated in bold or underlined. ERE, estrogen receptor complex binding site; CREB, site for the binding protein mediating the response to cAMP and/or calcium; GATA, zinc finger trans-regulator binding site; AP-4 and cEts, transcription factor binding sites; AP-1, TPA response element; SP1 and Nkx-2, transcriptional regulator response elements. The upper-case letters represent exon 1 and exon 2 of the gene. The sequence has been submitted to the Gene Bank with the accession number of BankIt118411 AF006690.

Figure 10. Determination of the transcription start site of the rat galanin gene by primer extension analysis. About 10 μl of total RNA were hybridized to the ^{32}P-labelled oligonucleotide Gal 5 (+48 to +69) and then extended by MMLV reverse trancriptase. Lane 1, total RNA isolated from rat pituitary; lane 2, total RNA isolated from estrogen treated rat pituitary; lane t, c, g, a, sequence using the same ^{32}P-labelled oligonucleotide as primer for sequencing; lane 3, total RNA isolated from rat uterus. The main extended products are indicated by arrows.

were introduced upstream of the translation start site of a luciferase reporter sequence in the promoter less luciferase plasmid pXP_2 (Nordeen, 1988) to generate hybrid rat galanin/luciferase pGlu-4500/152, pGlu-657/152, pGlu-486/152, pGlu-208/152, and pGlu-126/152 constructs as indicated in Figure 3.

4. Basal Rat Galanin Promoter Activity in Neuronal and Endocrine Cell Lines

Each construct was transfected into neuronal, endocrine and non-neuronal cell lines (SK-N-MC, PC_{12}, GH_3, and Hela). The luciferase activities of different cell lines were measured. After correcting their transfection efficiency using the co-expressed β-galactosidase activity, the promoter activities of the different constructs in the above mentioned cell lines were calculated as fold-increase above the pXP_2 basic activity. Every measurement was in duplicate or triplicate and repeated in at least three independent experiments. The results of the luciferase assay showed that the galanin promoter is actively transcribed in neuronal and endocrine cell lines but not in Hela cells (Figure 11). The highest transcriptional activity was detected in the neuroblastoma cell line SK-N-MC. Data shown in Figure 11 indicate that 0.2 kb of the 5'-flanking rat galanin sequence are enough to obtain the basal transcriptional activity in neuronal and endocrine cell lines. Significantly lower activity was obtained when 4.5 kb of up stream sequence was included suggesting the presence of strong suppressor elements in this region. In contrast, no differences in basal promoter activity among the different constructs was noticed in Hela cells.

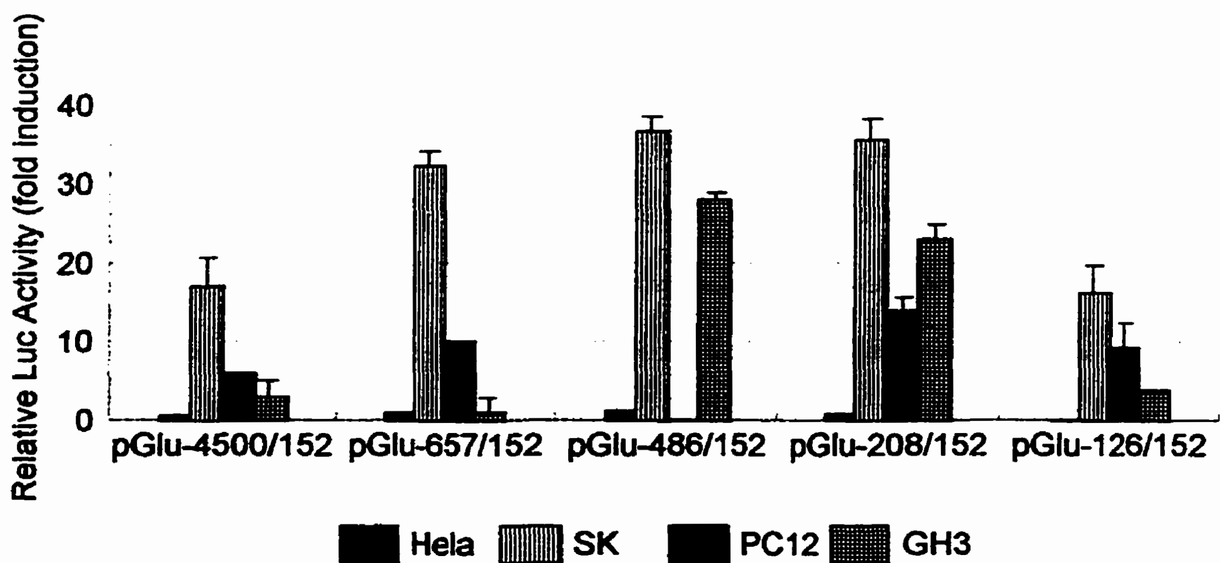

Figure 11. Rat galanin gene promoter activity in the Hela, SK-N-MC, PC_{12}, and GH_3 cell lines. Galanin luciferase reporter gene constructs were transiently transfected into the different cell lines. Variations in transfection efficiencies were corrected by co-transfection of the pCMV reporter plasmid. Thirty six hours after transfection, cell extracts were assayed for luciferase (Luc) and β-galactosidase activities. Results shown are representative experiments of triplicate transfection. Values are expressed as fold increase over the basic activity (Luc activity of promoterless reporter construct pXP_2) The promoter activity of pGlu-486/+152 in PC_{12} is unavailable.

As shown in Figure 11 in both endocrine and neuronal cell lines, positive regulatory elements are located in less than 200 bp of the proximal promoter region while repressor(negative) regulatory elements are located 657 bp in the case of neuronal cells and 486 bp in the case of endocrine cells, upstream from the transcriptional start site. The repressor elements are much stronger in the endocrine cells (7 fold decrease) compared to neuronal cells (2 fold decrease). In neuronal cells luciferase activity was still in high level in the 126 bp of the galanin promoter construct but not in the endocrine cell line.

5. Induction of Galanin Promoter Activity by TPA

The 5'-flanking rat galanin gene constructs were transfected into the GH_3, PC_{12}, SK-N-MC, and Hela cell lines. After 36 hours treatment with 100 nM TPA, the cells were harvested and their activities were measured.

Comparing with the basal activity of the untreated cells, TPA increased the activity of the rat galanin promoter in the SK-N-MC cells. Treatment of the transfected cells with 100 nM TPA induced a 2-4 fold stimulation of luciferase activity over the untreated controls.
High activity was sustained from the pGlu-4500/152 construct through the pGlu-126/152 construct suggesting that a strong TPA responsive element is located within the last 126 bp 5'-flanking region with the highest response within 200 bp of the 5'-flanking of the rat galanin gene. Similarly in the GH_3 cell line, a strong TPA responsive element was located within 200 bp of the proximal promoter region (Figure 13) with a 2 fold increase of

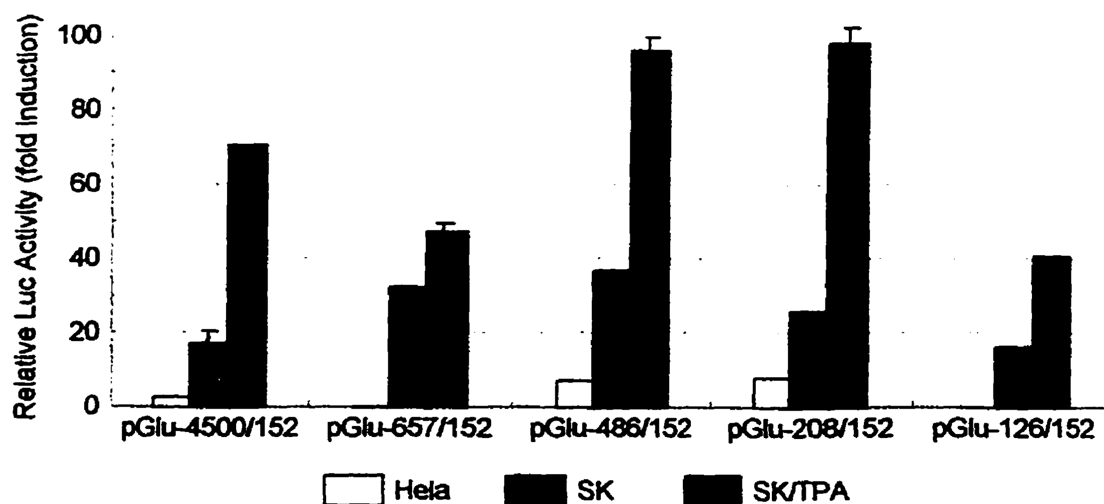

Figure 12. Rat galanin gene promoter activity in the Hela and SK-N-MC cells. The cells were transfected with the galanin constructs containing luciferase reporter gene (pXP_2). Variations in transfection efficiencies were corrected by co-transfection of the pCMV reporter plasmid. After transfection and treatment with TPA or vehicle for 36 hours, cell extracts were assayed for luciferase (Luc) and β-galactosidase activities. Results shown are representative of duplicate transfection experiment. Values are expressed as fold increase over the basic pXP_2 activity. No data is available in Hela for pGlu-657/152 and pGlu-126/152.

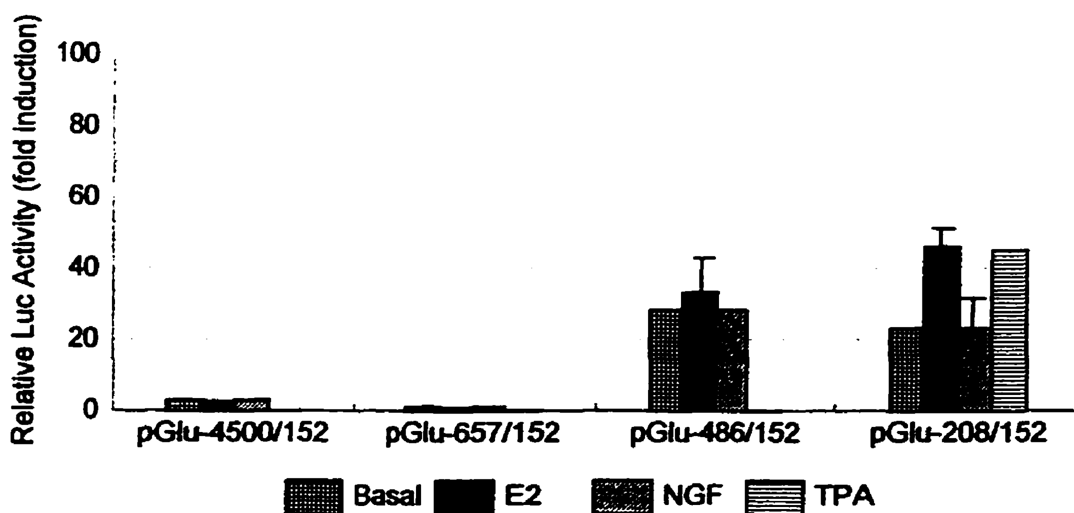

Figure 13 . Rat galanin gene promoter activity in the GH_3 cell line. Cells were transfected with the plasmid containing luciferase reporter gene (pXP_2). Variations of transfection efficiencies were corrected by co-transfection of the pCMV reporter plasmid. After transfection and treatment with estrogen (100 μM), NGF (50 ng/ml), TPA (100 nM), or vehicle for 36 hours, cell extracts were assayed for luciferase (Luc) and β-galactosidase activities. Results shown are representative of duplicate transfection experiments. Values are expressed as fold increase over the basic pXP_2 activity. No data is available for pGlu-657/152 treatment with estrogen and for pGlu-486/152 treatment with TPA.

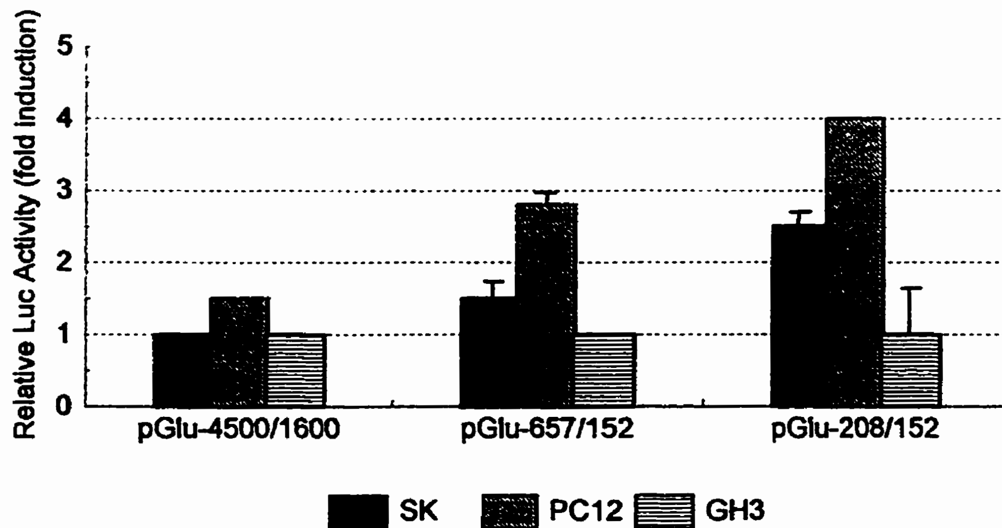

Figure 14. Stimulation of the rat galanin gene promoter activity by NGF in the SK-N-MC, PC_{12}, and GH_3 cells. Cells were transfected with the plasmid containing the luciferase reporter gene (pXP_2). Variations in transfection efficiencies were corrected by co-transfection of the pCMV reporter plasmid. After transfection and treatment with 50 ng/ml NGF and 0.1% BSA for 36 hours, cell extracts were assayed for luciferase (Luc) and β-galactosidase activities. Results shown are representative of duplicate transfection experiments. Values are expressed as fold increase over the basal activity of untreated controls.

luciferase activity over and above the untreated controls, while no significant response was noted in the PC_{12} cells (data not shown).

6. Induction of Galanin Promoter Activity by NGF

The constructs of the 5'-flanking rat galanin gene were transiently transfected into the GH_3, PC_{12}, and SK-N-MC cell lines. Thirty six hours after treatment with 50 ng/ml NGF, the cells were harvested and their luciferase activities were determined.

Results from these experiments showed that NGF stimulated galanin promoter activity in both neuronal cell lines (SK-N-MC and PC_{12}) with the highest stimulation in the PC_{12} cell line. The strongest response was located within the 200 bp of the proximal promoter region (Figure 14) of the galanin gene (4 fold and 2.5 fold increase of luciferase activity over and above the untreated control in SK-N-MC and PC_{12} cells respectively).

7. Induction of Galanin Promoter Activity by Estrogen

The constructs of the 5'-flanking rat galanin gene were transiently transfected into the GH_3, PC_{12}, and SK-N-MC cell lines. Thirty six hours after treatment with estrogen (E_2), the cells were harvested and luciferase activity was determined. In the SK-N-MC and PC_{12} cell lines, estrogen had a very weak effect on the promoter activity of the rat galanin gene which was not significant. In GH_3 cell line the promoter activity was significantly increased after

estrogen treatment, in the pGlu-208/152 and the pGlu-126/152 constructs (almost 2 fold over the basal activity of the untreated controls) although not always consistent (Figure 13).

8. Identification of Transcription Factor-binding Sites in the 5'-flanking Region

Since the high activity of the galanin promoter (both basal and drug induced) was demonstrated in the first 208 bp of the 5'-flanking region, we examined the ability of this fragment to bind putative transcription factors in nuclear extracts. Gel mobility shift assay was carried out and the 208 bp fragment of the rat galanin gene 5'-flanking region was labelled with ^{32}P to examine binding activities of this region to proteins presented in nuclear extracts prepared from SK-N-MC (with or without TPA treatment) and Hela cells as well. As shown in Figure 15, when the fragment from -208 to +13 was tested, a prominent retarded band was observed from both SK-N-MC and Hela cell extracts. The retarded band was much stronger in Hela cell extract than that in SK-N-MC cell extract. Competition experiments, in which a 10-fold and 40-fold molar excess of the same unlabelled fragment was used, showed a proportional decreased in the intensity of this band indicating the specificity of binding. In order to further identify transcription factor-binding site in the 5'-flanking region of the rat galanin gene, we generated two other fragments in the region, the -208 to -126 and the -126 to +13 fragments in the 5'-flanking of the rat galanin gene. Gel shift assay with the -208 to -126 fragment gave a strong retarded band with the Hela extracts with less prominent band with the SK-N-MC extract while treatment of SK-N-MC cells with TPA abolished this band. Gel shift assay with the -126 to +13 fragment generated as

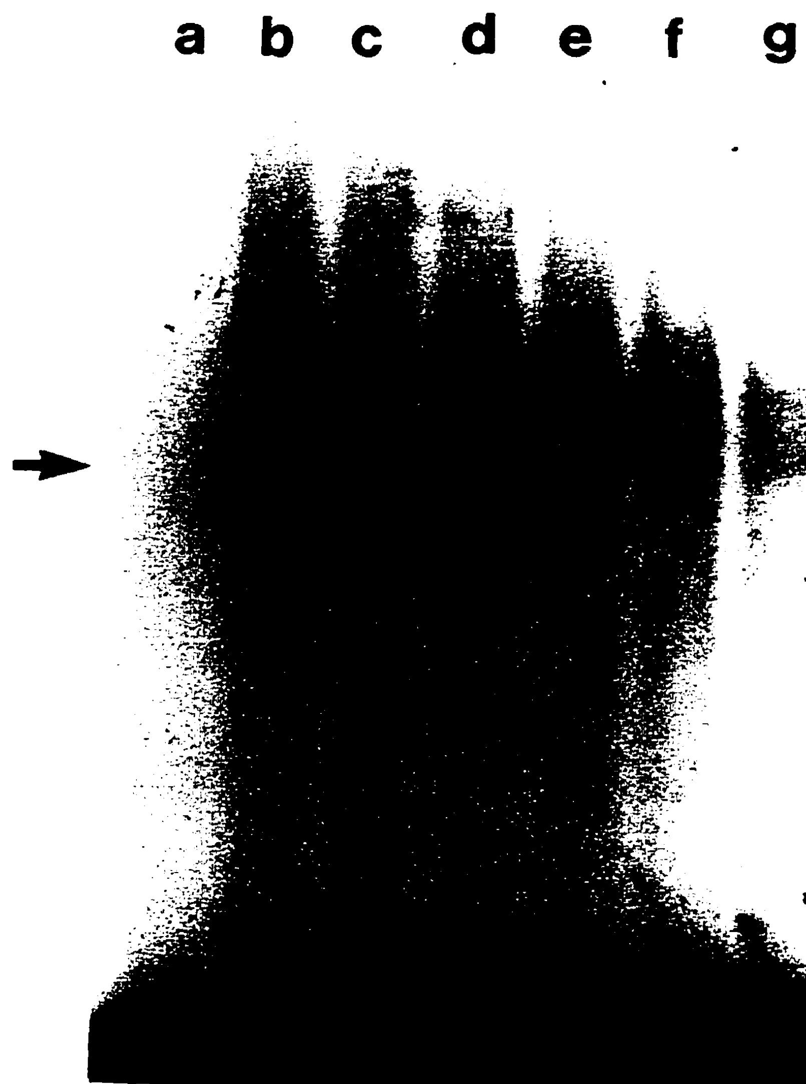

Figure 15. Gel mobility shift and competition assay of the fragment -208 to +13 5'-flanking region of the rat galanin gene. The positions of the protein-DNA complexes are denoted by arrows. Specific competition with increasing amounts (as fold molar excess) of the unlabelled homologous oligonucleotide (lane d to g) is shown. Lane a, the labelled probe in the absence of the protein; lane b, the labelled probe in the presence of the Hela nuclear extract; lane c, the labelled probe in the presence of the SK-N-MC cell nuclear extract; lane d, the labelled probe with 10-fold molar excess of the unlabelled homologous oligonucleotide in the presence of the Hela nuclear extract; lane e, the labelled probe with 10-fold molar excess of the unlabelled homologous oligonucleotide in the presence of the SK-N-MC nuclear extract; lane f, the labelled probe with 40-fold molar excess of the unlabelled homologous oligonucleotide in the presence of the Hela nuclear extract; lane g, the labelled probe with 40-fold molar excess of the unlabelled homologous oligonucleotide in the presence of the SK-N-MC nuclear extract.

well a retarded band that was unique in the SK-N-MC extract and that was increased after TPA treatment (Figure 16, lower arrow in lane 4).

Figure 16 Gel mobility shift assay of the fragments -208 to -126 (lane 5-8) and -126 to +13 (lane 1-4) 5'-flanking regions of the rat galanin gene. Gel shift assays were performed with cell nuclear extracts of either Hela or SK-N-MC or SK-N-MC treated with 100 nM of TPA. The positions of the protein-DNA complexes are denoted by arrows. Lane 1, the labelled probe of -126/+13 in the absence of proteins; lane 2, the labelled probe of -126/+13 in the presence of nuclear extract of Hela; lane 3, the labelled probe of -126/+13 in the presence of nuclear extract of SK-N-MC; lane 4, the labelled probe of -126/+13 in the presence of nuclear extract of SK-N-MC/TPA; lane 5, the labelled probe of -208/-126 in the absence of proteins; lane 6, the labelled probe of -208/-126 in the presence of nuclear extract from Hela; lane 7, the labelled probe of -208/-126 in the presence of nuclear extract of SK-N-MC; lane 8, the labelled probe of -126/+13 in the presence of nuclear extract of SK-N-MC/TPA.

DISCUSSION

1. Cloning and Characterization of the 5'-Flanking Region of the Rat Galanin Gene

The focus of this investigation was to examine the transcriptional activation of the rat galanin gene and to determine the location and characteristics of functionally active regulatory elements within the 5'-flanking region of the rat galanin gene.

Galanin is a highly conserved neuropeptide, expressed in both neuronal and endocrine tissues and is up-regulated in a tissue specific manner. In order to identify potential galanin transcriptional regulatory elements we have isolated a genomic clone (25 kb) containing the whole rat preprogalanin gene including at least 4.5 kb of 5'-flanking region.

Sequence analysis of the rat galanin gene (including the sequence from exon 3 to the 3' end of the rat galanin gene which is Dr. Vrontakis' unpublished data) revealed several interesting features. The rat galanin gene extends for approximately 5 kb (the human galanin gene spans 6.5 kb) from the start of exon 1 to the site of poly (A) tail and contains six exons and five introns (the same as human). The first exon of the human, rat, and bovine galanin genes encodes the 5' untranslated region. The preprogalanin peptide starts with the first codon of the translation initiation methionine in exon 2 and to the middle of the exon 6 (Figure 17). The observiation that the coding region of a gene starts from exon 2 is also common to

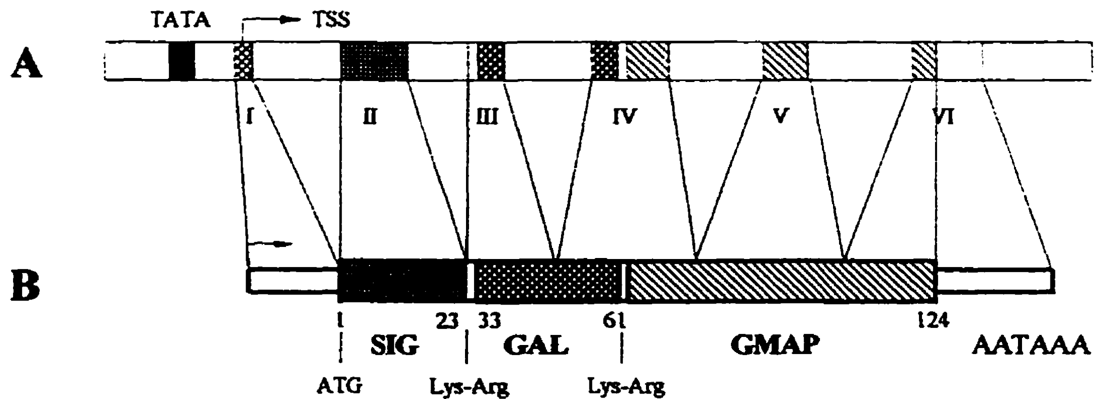

Figure 17 Organization of the rat preprogalanin gene. A: Schematic representation of the rat preprogalanin gene B: The position of the six exons with respect to the pituitary preprogalanin cDNA are shown. The numbers indicate the amino acid position in the preprogalanin. Abbreviations are as follows: ATG, the translation initiation site; AATAAA, the poly (A); TATA, TATA box; TSS, transcription start site; SIG, signal peptide; GAL, galanin; GAMP, galanin messenger-associated peptide (Vrontakis *et al.* 1987 and Vrontakis' unpublished observation).

several other neuroendocrine peptides, including neuropeptide Y (NPY), peptide-YY (PYY), and pancreatic polypeptide (Leiter *et al.* 1985, Minth *et al.* 1986 and Krasinski *et al.* 1991).

The sequence of the 5'-flanking region of the rat galanin gene revealed a single atypical TATA-box (ATAAATA, -33 to -26) (Figure 9). The modified TATA-box in the rat galanin gene is homologous to that in the bovine galanin and mouse galanin gene (Rokaeus *et al.* 1994, Kofler *et al.* 1996). The rat galanin promoter region contains some consensus sequences for known transcription factors. Upstream of the TATA box, there is a conserved half-element (TGACG) for the protein CREB, which typically mediates gene expression by binding to the cyclic AMP response element (CRE) and is induced by protein kinase A (Ryseck and Bravo, 1991). The CREB motif also could serve as the binding site for different PMA-inducible members in the *Jun/Fos* protein families (Karin *et al.* 1992a and1992b, Anouar *et al.* 1994). In the rat galanin promoter region, there are alson several AP-1 binding sequences for the *Jun/Fos* protein families, which control gene expression by binding to the TPA response element (TRE) and activated by the protein kinase C pathway (Angel *et al.* 1991). Upstream from the TATA box and downstream from the exon 1 there are three GC-rich stretches, which are generally thought to have an important role in transcription initiation and for binding of the transacting factor SP1 (Gidoni *et al.* 1984). With regard to estrogen receptor binding sites, three perfect $ERE_{1/2}$ (GGTCA) were found upstream from the TATA box in the rat galanin gene. In the chicken ovalbumin gene promoter, this half-palindromic ERE is enough to mediate estrogen responsiveness if stereoaligned with

the TATA box along the DNA helix (Tora *et al.* 1988). Clusters of GATA-like motifs were found in the 5'-flanking and the first intron and exon 2 of the rat galanin gene. GATA *trans*-regulators are thought to regulate a large number of lineage-restricted target genes in vertebrates (Orkin *et al.* 1990, Bockamp *et al.* 1994). Upstream of the CREB binding site there is a c-Ets element for the Ets factors which is a transcriptional activator involved in controlling cellular growth and differentiation (Nuchprayoon *et al.* 1997) (Figure 9 and Table 3).

2. Identification of Transcriptional Activating Elements in the 5'-Flanking Region of the Rat Galanin Gene.

Our transfection data indicated that the rat galanin gene transcription is activated in both neuronal and endocrine cell lines, while no activity was detected in Hela cells. The highest basal activity was detected in SK-N-MC cells indicating that the basal promoter activity of the galanin gene is neuronal type specific (Figure 11). This could acount for the wide distribution of galanin in the central and peripheral nervous system.

2.1 Basal activity of the rat galanin promoter

Previous reports show that there are some cell-type-specific silencers between -5 kb to -131 bp of the human and bovine galanin gene (Kofler *et al.* 1995, Rokaeus *et al.* 1994, and Anouar *et al.* 1994). Here with my studies, I also found that there are some tissue specific

Table 3. Analysis of the 5'-flanking and the first two exons and introns of the rat galanin gene for potential binding sites of known transcriptional factors. Relative locations of the putative *cis*-elements in the sense (+) or antisense (-) strands are indicated with reference to the transcription initiation site (+1).

Cis-element	Consensus binding site	Sequence in the rat galanin gene	Strand	Location
AP-1	TGA$^{C}_{G}$T$^{C}_{A}$A	GGAGTCA TGGGTCA TGACAGA TGACTCT	+ + + +	-878/-872 -821/-815 -783/-777 -185/-179
AP-4	$^{T}_{C}$CAGCTG$^{T}_{C}$	ACAGCTGA	-	+446/+453
CREB	TGACG	TGACG	+	-55/-50
$ERE_{1/2}$	GGTCA	GGTCA GGTCA GGTCA	+ + +	-959/-955 -819/-815 -100/-96
Ets	$^{C}_{G}$$^{A}_{C}GGA^{A}_{T}G^{C}_{A}$	GGTTCCGG	-	-106/-99
GATA	WGATR	CCAGATGGG CCTGATGAG AATATCTCA GCTGATGTG AGAATCTGG CAGGATGGG GGAGATAGT CCCATCCCT CCCATCACC CCAGATGGC GTTATCCTG CGGGATGCC	+ + - + - + + - - + - +	-1000/-992 -771/-763 -721/-713 -656/-638 -580/-572 -243/-235 -160/-152 +207/+215 +242/+250 +348/+356 +367/+375 +423/+431
SP1	$^{C}_{A}$$^{T}_{C}CCG^{C}_{A}$$^{C}_{T}$$^{T}_{C}$$^{A}_{C}$	ACCCAGCCC CCCTGCCCC CCCAGCCAC	+ + +	-467/-459 +212/+220 +249/+257

regulatory elements in rat galanin gene. As we have shown in section 4 of Results (Figure 11), the first 208 bp of the 5'-flanking rat galanin gene sequence is enough to obtain the full basal activity in both neuronal and endocrine cells, while the inclusion of 4.5 kb upstream 5'-flanking sequence exhibited a significant reduction of the basal activity suggesting the presence of a cell specific silencer (s).Thus both negative and positive regulatory elements exist in the rat galanin gene. These result are consistent with reports for the human (Kofler *et al.* 1995) and bovine galanin gene (Rokeus *et al.* 1994 and Anouar *et al.* 1994) which contain negative regulatory elements as well. Our experiments have also shown that in the rat galanin gene, the negative regulatory elements appeared to be tissue-specific since they are located differently in the SK-N-MC and GH_3 cells (activity at the pGlu-657/152 versus pGlu-486/152 in the two cell lines) with a stronger tissue specific silencer element in the GH_3 cells (7-fold decrease of activity between the pGlu-657/152 and pGlu-486/152). The minimum segment conferring neuronal activity in both the SK-N-MC and PC_{12} is the 126 bp of the galanin promoter. These differences in the location of the negative elements could account for the differential expression and regulation of the galanin gene in neuronal and endocrine tissues. Furthermore these negative regulatory elements could account for the transient expression of galanin during development. In both neuronal and endocrine tissues galanin is transiently increased during fetal and neonatal life (Xu *et al.* 1996 and Giorgi *et al.* 1995).

Thus these observations are important as they suggest that negative regulation is also utilized by cells to restrict expression of certain genes to subpopulation of cells. Identification of

similar inhibitory factors or negative regulatory elements which seem to repress gene expression has been described before for rat PRL and GH genes (Nachtigal *et al.* 1993 and Jackson *et al.* 1992) and also for the type II sodium channel gene (Kraner *et al.* 1992). It has been suggested (Renkawitz *et al.* 1990) that silencers (transcriptional repressors) may be of importance in: i) reducing the level of gene transcription in a tissue-specific way, ii) rapidly inactivating transcription after removal of a stimulus, or iii) downregulating the expression of a gene during development. With respect to this latter function, galanin expression is transiently increased in rabbit paraganglia just before birth and gradually disappears over 10 days (Wikstrom *et al.* 1993).

2.2 TPA

The protein kinase signal transduction pathways are known to regulate gene expression by the modulation of the activity of sequence-specific transcription factors (Karin *et al.* 1992a and 1992b). Protein kinase C (PKC) induces the transcriptional regulatory activity of AP-1 complexes, which correspond to *fos/jun* family of transcription factors and interact with a consensus TPA-responsive element (TGACTCA) (Angel *et al.* 1991) or closely related sequences in the promoter regions of a variety of genes, permitting *cis*-activation of gene transcription (Karin *et al.* 1992a and 1992b).

In order to identify potential transcription control elements within the rat galanin gene that may respond to TPA stimulation, deletion analysis of rat galanin promoter/reporter constructs in neuronal and endocrine tissues were undertaken. The results showed that the

rat galanin promoter responded very strongly to TPA in both the SK-N-MC and the GH_3 cells (Figure 12 and 13). Although the high level of promoter activity was obtained with all constructs, the highest activity and highest fold increase over and above the basal activity (4-fold in SK-N-MC cells and 2-fold in GH_3 cells) was achieved with the pGlu-208/152 construct. These results would suggest that the sequences on the first 208 bp of the rat galanin promoter or on the first exon are responsible for the TPA induction of galanin activity in those cells. As indicated in Figure 9, the consensus sequences for AP-1 and CREB binding are located in this region. Some transcription regulatory factors, which bind to these consensus elements, might be induced by TPA. In addition the removal of the AP-1 element (pGlu-208/152 to pGlu-126/152) diminished the TPA response by 2.5 fold, indicating that the cooperation between the both elements of AP-1 and CREB increases the TPA response. TPA response element has also been found in the promoter region of the bovine galanin gene (Anouar *et al.* 1994), suggesting that the response to TPA is conserved among species. In that study (Anouar *et al.* 1994), it was shown that the bovine galanin promoter region contains a GTRE (galanin TPA responsive element) motif (TGACG) in the proximal promoter, that has the consensus sequence for CREB (cAMP responsive element) binding site. The rat galanin gene sequence (Figure 9) also has the TGACG (CREB site) motif in the same region indicating that the GTRE element is identical in these two species. Mutation analysis of this motif will confirm the functional activity of this GTRE in the rat galanin promoter.

Gel mobility shift further revealed the possible presence of both silencer(s) and enhancer(s)

in the 5'-flanking region of the rat galanin gene. Since the pGlu-208/152 construct produced the highest activity in both basal SK-N-MC cells and SK-N-MC cells treated with TPA (Figure 12), this olignucleotide (oligo -208 to +13) was selected to be used with nuclear extracts prepared from the SK-N-MC and Hela cells in a gel mobility shift assay (Figure 15). A prominent retarded band was observed with both extracts. This binding activity was consistently increased in the Hela nuclear extracts compared to equivalent amount of the SK-N-MC nuclear extracts. In addition competition experiment with 10 to 40 fold molar excess of the unlabelled same fragment decreased the intensity of the shift band proportionally, indicating the specificity of the binding. Since galanin promoter is inactive in Hela cells and yet the intensity of the shift band is more prominent, it is possible that this binding protein is a repressor protein that negatively regulates galanin promoter activity. Furthermore the mobility shift assay using a -208 to -126 and -126 to +13 oligonucleotide (Figure 16) revealed that aside from the silencer element, another enhancer element (at the position -126 to +13) which binding activity increased in SK-N-MC cells compared to Hela cells. Furthermore the expression of this enhancer element was attenuated by TPA treatment, indicating the presence of a binding protein which may correspond to a positively regulated transcription factor. As shown in the Figure 9, there are cEts, ERE $_{1/2}$ and CREB putative cis-elements located between positions -126 and +13. It is known that cEts is a transcription factor which controls the expression of a number of genes involved in extracellular matrix remodelling and has been postulated to play a role in cell migration, proliferation, differentiation, and tumour invasion (Lewin, 1991). An AP-1 element has been located 70 bp upstream of the cEts element in the 5'-flanking region of rat galanin gene. According to

previous reports, cEts has been shown to synergise with AP-1 binding site (Wasylyk *et al.* 1990 and Noti *et al.* 1995). Based on the these studies, it is not unreasonable to propose that the positive regulatory element in the region position between -126 and +13 is related to the cEts and AP-1 elements.

2.3 NGF

NGF, the classical neurotrophic factor, confers differentiation and proliferation upon precursor cells. These cells have receptors for NGF and respond to NGF by extending neurite or increased proliferation (Janet *et al.* 1995 and Kaplan *et al.* 1991). NGF stimulated rat galanin promoter activity significantly in both neuronal cell lines (Figure 14). The highest response was observed with the pGlu-208/152 construct indicating again that strong negative NGF responsive elements are located upstream of the 200 bp 5'-flanking region of the rat galanin gene. Differential regulation of the negative and positive NGF responsive elements of the galanin promoter may account for the either positive or negative effect of NGF on galanin mRNA depending on the tissue and the environmental conditions. For instance, while NGF induces galanin gene expression in the rat basal forebrain (Planas *et al.* 1997), it has no effect on galanin expression in DRG (dorsal root ganglia) cell culture or in *vivo* after axotomy (Kerekes *et al.* 1997 and Ji *et al.* 1996).

2.4 Estrogen

The response of the galanin promoter constructs to estrogen is particularly relevant because this hormone dramatically up regulates galanin mRNA in the rat pituitary (Vrontakis *et al.*

1987, 1989a, 1989b and Kaplan *et al.* 1988b). Estrogen acts through two response elements: the short half-palindromic ERE (GGTCA) (Jost *et al.* 1990) and the full ERE (GGTCAGTAG) (Martinez *et al.* 1987). My results showed that no difference in transcriptional activity of the promoter constructs was seen in the neuronal cell lines, SK-N-MC and PC_{12} cells treated with estrogen, compared to untreated cells. In GH_3 cells there was a 2-fold increase over the basal level for the pGlu-208/152 constructs, but these results were not significant in a series of exprements (Figure 13).Studies have also showed no direct regulation of galanin by estrogen on cultured pituitary cells (Ottlecz *et al.* 1986, Meister *et al.* 1987, Cella *et al.* 1988 and Kofler *et al.* 1995). The GH_3 cells have been previously used to demonstrate estrogen response *in vitro* without co-transfection of the estrogen receptor (Nowakowski *et al.* 1994 and Day *et al.* 1990), indicating that these cells are normally responsive to estrogen. By the time this study had been done, Corness group also reported the rat galanin gene promoter activity did not respond to estrogen in Neuro 2A cells (Cornees *et al.* 1997). Therefore, the regulation of galanin gene expression by estrogen might be mediated via indirect pathway which involves cooperating with adjacent regulatory binding proteins. Gaub *et al.* (1990) reported that the sequence of TGGGTCA, containing the half-palindromic ERE, is responsible for induction by phorbol esters of the ovalbumin promoter and is a target for *c-fos* and *c-jun trans*-activation. The *c-fos*, *c-jun* and ER (estrogen receptor) coactivate the ovalbumin promoter. In the rat galanin gene promoter region, there is a $ERE_{1/2}$ (Figure 9) overlapping with an AP-1 element, which contains the sequence of TGGGTCA. It is possible that the ER may not bind directly to the half-palindromic ERE motif, but rather that its effect could be mediated by forming a complex,

containing both ERE and AP-1, with both *fos* and *jun* oncoproteins and transcription stimulation triggered by estrogen could be mediated by this complex. The pGlu-4500/152 (-4500 to +152) construct, which contains the ERE sequence (located at around -820 bp upstream of the promoter) may contain other negative elements which inhibit the stimulation of the ERE/AP-1 complex. That could explain the lack of estrogen induction of galanin promoter activity.

In summary, stimulation of the rat galanin gene transcription in neuro-endocrine cells appears to be mediated by both negative and positive regulatory elements. Identification of such elements confers that neuronal specific and TPA response elements have been located within the first 200 bp of the rat galanin promoter. Characterization of these binding proteins might provide means of identifying the exact involvement of galanin in some conditions such as nerve regeneration or Alzheimer's disease.

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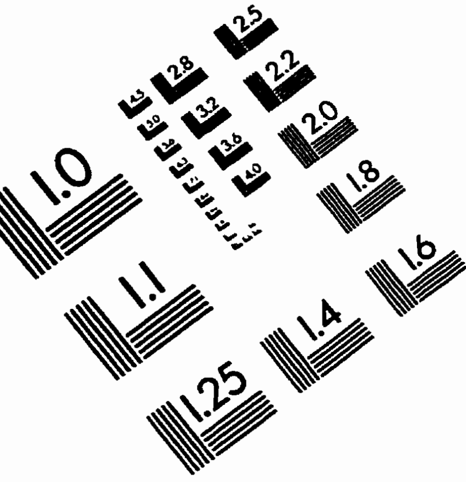
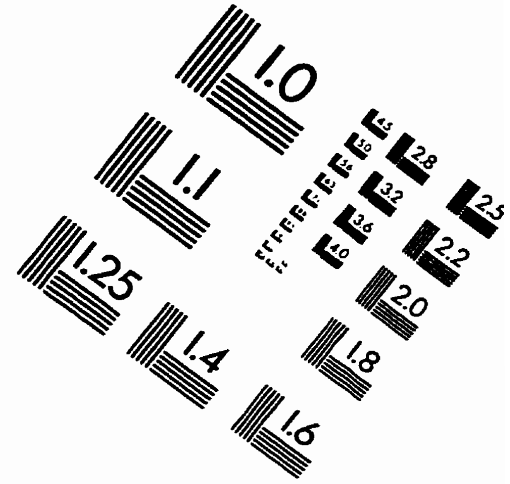
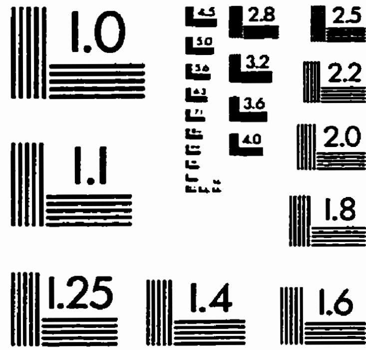
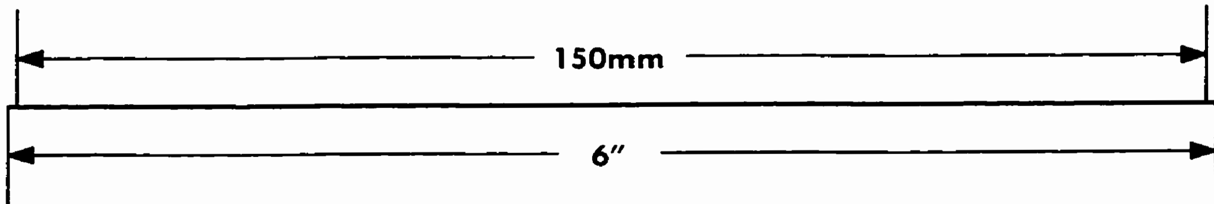
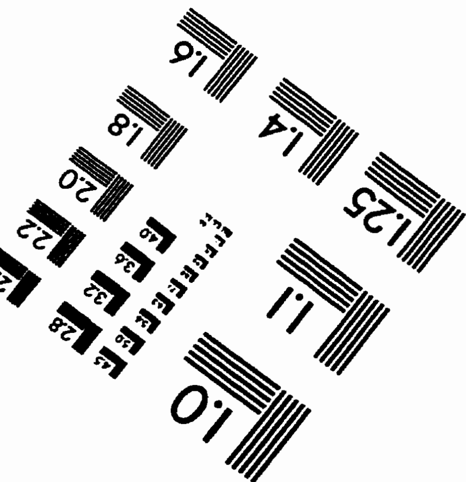
IMAGE EVALUATION TEST TARGET (QA-3)





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