Galanin receptor ligands

Johan Runesson

Licentiate thesis

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Close beneath our window sill the Earth is humming,
Like an eager Christmas child the day is coming,
Listen to the morning song it's singing.
..
Like an antique ballroom fan your eyelids flutter,
Sunlight streams across your eyes through open shutter.
Now I think you're ready for the journey.

Tom Paxton, from *I give you the morning*

List of publications

This thesis is based on the following publications, referred to in the text as paper I and paper II.

I Runesson, J., Saar, I., Lundström, L., Järv, J., Langel, Ü. (2009). A novel GalR2-specific peptide agonist. Neuropeptides, 43, 187-192.

II Sollenberg, U.E., Runesson, J., Sillard, R., Langel, Ü. (2009). Evaluation of chimeric peptides M617 and M871 binding to human galanin receptor type 3. Submitted

Abstract

In the nervous system galanin primarily displays a modulatory role. The galaninergic system consists of a number of bioactive peptides with a highly plastic expression pattern and three different receptors. The lack of receptor subtype selective ligands and antibodies have severely hampered the characterization of this system. Therefore, most of the knowledge has been drawn from experiments with transgenic animals, which has given some major conclusions, despite the compensatory effects seen in several animal studies. Therefore, the production of subtype selective ligands is of great importance to delineate the galanin system and slowly experimental data from receptor subtype selective ligand trials is emerging.

This thesis aims at studying galanin receptor-ligand interactions and to increase and improve the utilized tools in the galanin research field, especially the development of novel galanin receptor subtype selective ligands.

Paper I demonstrates the potential to N-terminally extend galanin analogues and the successful development of a galanin receptor 2 (GalR2) selective ligand. In addition, a cell line stably expressing galanin receptor 3 (GalR3) was developed, to improve and simplify future evaluations of subtype selective galanin ligands.

Paper II measures the affinities of M617 and M871 to GalR3 and demonstrates that M871 preferentially binds GalR2. Furthermore, the relatively high affinity of M617 was evaluated by assessing the contribution in receptor interaction of individual amino acid residues in the C-terminal part of the M617.

In conclusion, this thesis has provided a novel design strategy for galanin receptor ligands and increased the understanding of ligand interactions with the GalR3. Furthermore, M1145 has together with new analogues proven to be highly GalR2 specific, holding promises to future delineation of the galaninergic system as a therapeutic target.

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Abbreviations

5-HT	5-hydroxytryptamine, serotonin
AC	adenylate cyclase
AD	Alzheimer's disease
BSA	bovine serum albumin
cAMP	cyclic adenosine monophosphate
CCK	cholecystokinin
cDNA	complementary DNA
CHO	chinese hamster ovary
CMV	cytomegalovirus
CNS	central nervous system
CREB	cAMP regulatory element binding protein
DNA	deozyribonucleic acid
DRG	dorsal root ganglion
DRN	dorsal raphe nucleus
EAATs	excitatory amino acid transporters
FGF-1	fibroblast growth factor-1
Fmoc	9-fluorenylmethoxycarbonyl
FRT	flippase recognition target
GABA	gamma-aminobutyric acid
Gal	galanin
GALP	galanin-like peptide
GalR	galanin receptor
G-protein	GTP protein
GC	gas chromatography
GIRK	G protein-coupled inwardly-rectifying potassium channel
GMAP	galanin message associated peptide
GPCR	G-protein coupled receptor
GTP	guanosine triphosphate
HEK	human embryonic kidney
HPLC	high performance liquid chromatography
i.c.v.	intracerebroventricularly
IP	inositol phosphate
IP_2	inositol 1,4 bisphosphate
IP_3	inositol 1,4,5 trisphosphate
i.t.	intrathecally
K_i	inhibitory dissociation constant

KO	knock out
LC	locus coeruleus
LDCV	large dense core vesicles
MALDI	matrix-assisted laser desorption/ionization
MAPK	mitogen associated protein kinase
MBHA	*p*-methylbenzylhydrylamine
MS	mass spectrometry
NA	noradrenalin
NO	nitric oxide
NPY	neuropeptide Y
OE	over expression
PDK-1	phosphoinosotide-dependent protein-kinase I
PEI	polyethyleneimine
PIP_2	phosphatidylinositol 4,5-bisphosphate
PKC	protein kinase C
PLC	phospholipase C
PNS	peripheral nervous system
SCV	small clear vesicles
SPA	scintillation proximity assay
SPPS	solid phase peptide synthesis
$TetO_2$	*tet* operator 2
TFA	trifluoroacetic acid
TIS	triisopropylsilane
TOF	time of flight
VIP	vasoactive intestinal polypeptide

1. Introduction

The brain serves as the seat of human consciousness: it stores our memories and enables us to feel emotions. In short, the brain shapes our thoughts, hopes, dreams and imaginations. Ultimately, it dictates the behaviour which allows us to survive and makes us who we are. It underlies not only the personality of individuals, but also our laws, traditions, and social interactions. It is the foundation on which our civilization is built. Trying to understand the human nervous system represents one of the great frontiers in modern science.

The nervous system can be divided into the central nervous system (CNS) and the peripheral nervous system (PNS) and contains more than a thousand different cell types. To understand the diversified cellular communication taking place between all these cell types, is it pivotal to study the transmitter molecules responsible for this signal transduction.

This thesis focuses on one of these transmitter molecules, the neuropeptide galanin. For more than 25 years, the galanin system has been studied and much has been learned about its biochemical and physiological actions, and its roles in normal and pathological functions. The galanin research field has been enlarged with the addition of other related bioactive peptides and at least three receptor subtypes have been isolated and studied, and some selective pharmacological tools are emerging.

1.1 Neurotransmission

Communication between cells in the nervous system is essential for life. The word synapse was introduced in the late 19th century as a denotation of the specialized sites of neuronal interconnections. There are two main types of synapses, chemical and electrical, but mixed synapses have also been described. It is today established that neurons also use non-synaptic signalling to communicate, such as exocytosis at non-synaptic sites and transmembrane diffusion, for example the gaseous messenger nitric oxide (NO). Also emerging is the credit given to the importance of glial cells regarding cell communication in the nervous system. Nevertheless, the bulk of signal propagations in the nervous system is between neurons and is a result of

electrical impulses within the nerve cell generated by ions that flow through ion channels. Arrival of the electrical impulses to the presynaptic nerve terminal causes opening of calcium channels and hence, influx of Ca^{2+} ions, triggering neurotransmitter release from synaptic vesicles via exocytosis.

1.1.1 Neurotransmitters

Neurotransmitters are the information carrying molecules responsible for transferring neuronal signals from one cell to another. The major neurotransmitters can be divided into three categories 1) amines: acetylcholine, dopamine, adrenalin, noradrenalin (NA), serotonin (5-HT) and histamine 2) amino acids: glutamate, gamma-aminobutyric acid (GABA) and glycine or 3) peptides: cholecystokinin (CCK), dynorphin, enkephalins, neuropeptide Y (NPY), somatostatin, substance P, vasoactive intestinal polypeptide (VIP) and galanin. Group one and two are often referred to as the “classic neurotransmitters”.

Classic neurotransmitters are packed in small clear vesicles (SCVs, 40-60 nm in diameter) and undergo fast phasic release at the active zone of the nerve terminal. In contrast, neuropeptides are condensed in large dense core vesicles (LDCVs, 90-250 in diameter) and stored away from the active zone. LDCVs can be released in the soma as well as in the nerve terminal away from the active zone. Generally, LDCVs require higher frequency stimulation to undergo exocytosis than SCVs.

The majority of neuropeptides receptors are G-protein coupled receptors (GPCRs), giving a slow onset but a longer lasting signal transmission than the classical neurotransmitters, which often signals through ligand gated ion channels, mediating short and rapid responses.

Today, coexistence of multiple messenger molecules within a neuron is seen as a general feature of the nerve cells rather than an exception. And the neuropeptides do in most cases coexist with classical neurotransmitters, although coexistence between neuropeptides has also been reported.

1.2 Neuropeptides

Neuropeptides are small protein molecules composed of 3-100 amino acid residues. They are expressed in neuronal tissue, modulating neurotransmission, per definition. The first reported neuropeptide, substance P, was published as early as 1931 by von Euler and Gaddum (V Euler and Gaddum, 1931), and was recognized as a neurotransmitter in 1953 by Lembeck (Lembeck, 1953). However, the exact structure of substance P did remain unknown for almost 40 years after the first isolation (Chang and Leeman, 1970). The discovery of novel neuropeptides and orphan receptors are ongo-

ing, and so far more than 50 different neuropeptides have been identified and have been associated with more than 100 different GPCRs (Salio *et al.*, 2006).

Modulation of different neuropeptidergic systems are attractive targets for drug development based on their ability to modulate neurotransmission, particularly when the nervous system is stressed, challenged or dysfunctional.

1.3 Galanin

Galanin was discovered by Professor Viktor Mutt and colleagues in 1983 (Tatemoto *et al.*, 1983) at the Karolinska Institute in Stockholm by using a chemical method for identification of amidated peptides followed by a random search for peptides with this characteristic in porcine intestine. Using this approach, they found a 29 amino acid long peptide and named it galanin, after its N-terminal **g**lycine and its C-terminal **alanin**e. Galanin is a C-terminally amidated peptide in all species where it is identified so far, except in humans where galanin is 30 amino acids long and display a free acid at its C-terminal end. The N-terminal end of galanin is crucial for its biological activity and the first 15 amino acids are conserved in all species except in one, cf. Table 1 (Kakuyama *et al.*, 1997). Interesting, the C-terminal region (residue 17-29) varies and lacks receptor affinity, which also is true for N-terminal fragments shorter than galanin(1-11) (Land *et al.*, 1991b).

Galanin is proteolytically processed from a precursor pro-peptide called preprogalanin organized into 6 exons, giving rise to a signal peptide, galanin itself and to the galanin message-associated peptide (GMAP). The galanin-peptide family is now known to consist of galanin, GMAP, galanin-like peptide (GALP) and alarin (Table 1).

Table 1. Sequences of the galanin peptide family in various species.

Peptide	Sequence
Human galanin	GWTLNSAGYLLGPHAVGNHRSFSDKNGLTS
Rat galanin	GWTLNSAGYLLGPHAIDNHRSFSDKHGLT amide
Mouse galanin	GWTLNSAGYLLGPHAIDNHRSFSDKHGLT amide
Porcine galanin	GWTLNSAGYLLGPHAIDSHRSFHDKYGLA amide
Bovine galanin	GWTLNSAGYLLGPHALDNHRSFQDKHGLA amide
Tuna galanin	GWTLNAAGYLLGPHGIDGHRTLGDKPGLA amide
Human GMAP	ELRPEDDMKPGSFDRSIPENNIMRTIIEFLSFLHLKEAGAIDR- LLDLPAAASSEDIERS
Human GALP	APAHRGRGGWTLNSAGYLLGPVLHLPQMGDQDGKRE- TALEILDLWKAIDGLPYAHPPQPS
Porcine GALP	APVHRGRGGWTLNSAGYLLGPVLHPPSRAEGGGKGKTAL- GILDWKAIDGLPYPQSQLAS
Human alarin	APAHRSSTFPKWVTKTERGRQPLRS
Mouse alarin	APAHRSSPFPPRPTRAGRETQLLRS

1.3.1 Molecular mechanisms of action

All three galanin receptor subtypes are members of the G-protein coupled receptor (GPCR) superfamily, but the subtypes have substantial differences in their functional coupling and subsequent signalling activities (see Fig 1), which contributes to the diversity of possible physiological effects of galanin.

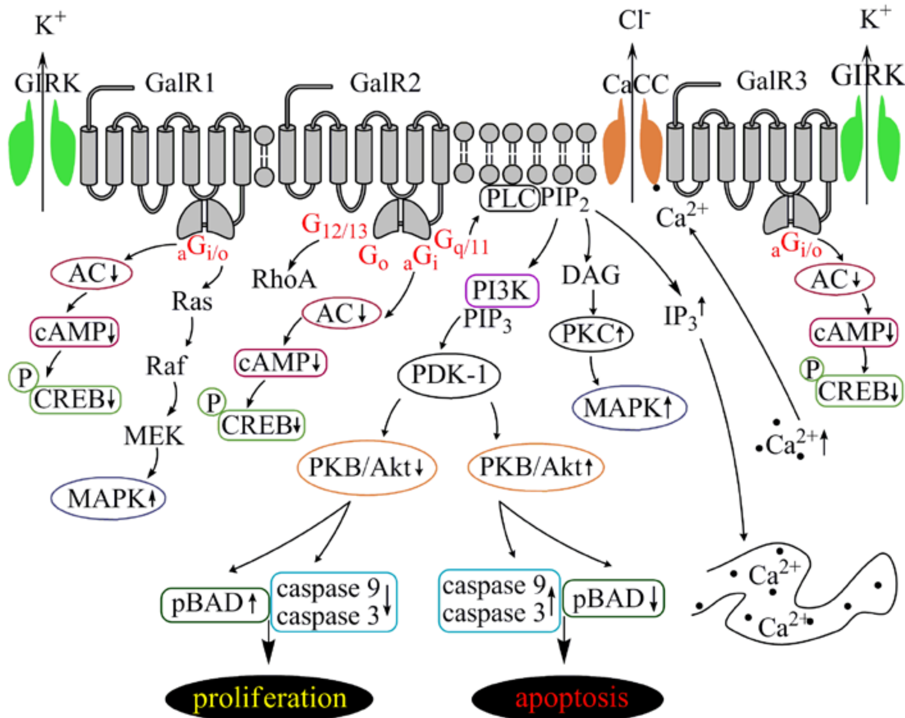

Figure 1. Intracellular signalling pathways activated by the galanin receptors (GalR1-3). AC, adenylate cyclase; (p)BAD, (phosphorylated) BCl-2 associated death promoter; CaCC, Ca^{2+} dependent chloride channel; cAMP, 3',5'-cyclic adenosine monophosphate; (p)CREB, (phosphorylated) cAMP response element binding protein; DAG, diacylglycerol; GIRK, G protein-coupled inwardly-rectifying potassium channel; IP_3, inositol triphosphate; MAPK, mitogen associated protein kinase; MEK, mitogeninduced extracellular kinase; PDK-1, phosphoinosotide-dependent protein-kinase I; PIP2, phosphatidylinositol biphosphate; PIP_3, phosphatidylinositol triphosphate; PI3K, phosphatidylinositol 3-kinase; PKB, protein kinase B; PLC, phospholipase C; RhoA, Ras homolog gene family, member A.

1.3.1.1 Galanin receptor type 1

Galanin receptor type 1 (GalR1) has been cloned from human Bowes melanoma cells (Habert-Ortoli *et al.*, 1994), Rin14B cells (Parker *et al.*, 1995),

rat brain (Burgevin *et al.*, 1995) and mouse (Jacoby *et al.*, 1997; Wang *et al.*, 1997a). The homology between species is high, as 92 % of the residues in rat GalR1 are identical to those of human GalR1. Activation of GalR1 results in pertussis toxin-sensitive inhibition of adenylate cyclase (AC) through interaction with $G\alpha_i/\alpha_o$ G-proteins (Habert-Ortoli *et al.*, 1994; Wang *et al.*, 1997a) and mitogen associated protein kinase (MAPK) activity (Wang *et al.*, 1998) (Fig 1).

The GalR1 expression is regulated by cyclic adenosine monophosphate (cAMP) through the transcription factor CREB (Zachariou *et al.*, 2001; Hawes *et al.*, 2005). GalR1 mRNA was initially reported to be found in fetal brain, small intestinal tissue and in the gastro-intestinal tract (Habert-Ortoli *et al.*, 1994; Lorimer and Benya, 1996). However, a later study identified the GalR1 expression to be exclusively in the central and peripheral nervous system (Waters and Krause, 2000), where it was detected in hypothalamus, amygdala, ventral hippocampus, thalamus, brainstem (medulla oblongata, locus coeruleus and lateral parabrachial nucleus) and dorsal horn of the spinal cord (Gustafson *et al.*, 1996), although broader central and peripheral tissue distribution has also been reported (Sullivan *et al.*, 1997). GalR1 expression does not fluctuate during the development of the brain (Branchek *et al.*, 2000; Burazin *et al.*, 2000).

1.3.1.2 Galanin receptor type 2

Galanin receptor type 2 (GalR2) was cloned from rat hypothalamus (Howard *et al.*, 1997a; Smith *et al.*, 1997; Ahmad *et al.*, 1998), mouse spleen (Pang *et al.*, 1998) and from various human tissues (Borowsky *et al.*, 1998; Bloomquist *et al.*, 1998). The hGalR2 protein has a high sequence identity (87 %) and similarity (92 %) to rGalR2, although there is one notable difference, which is the 15 amino acid extension of the C-terminal end in human GalR2 (Kolakowski *et al.*, 1998).

GalR2 has been ascribed the capacity to stimulate multiple intracellular pathways through multiple classes of G-proteins. GalR2 is able to activate the stimulatory pathway of $G\alpha_{q/11}$ G-proteins. This triggers phospholipase C (PLC) activity and intracellular phosphatidylinositol turnover, increasing intracellular Ca^{2+} and opening Ca^{2+}-dependent channels. GalR2 activates MAPK through a protein kinase C (PKC) dependent mechanism (Wang *et al.*, 1998). Furthermore, GalR2 affect phosphoinosotide-dependent protein-kinase I (PDK-1) activity and is only weakly coupled to AC inhibition (Smith *et al.*, 1997; Kolakowski *et al.*, 1998; Ding *et al.*, 2006) (Fig 1).

GalR2 mRNA is more widely distributed than GalR1. It is found in several peripheral tissues, including the pituitary gland, gastrointestinal tract, skeletal muscle, heart, kidney, uterus, ovary and testis, as well as in regions in the nervous system such as dentate gyrus, hypothalamus, and in the olfactory and cortical regions, cerebellum, and in the brainstem and spinal cord

(Ahmad *et al.*, 1998; Smith *et al.*, 1997; Bloomquist *et al.*, 1998; Waters and Krause, 2000; Xia *et al.*, 2005).

Unlike GalR1, GalR2 expression levels vary during the development of the rat brain with a broader distribution and a peak in expression before postnatal day 7, particularly in cortex and thalamus, and much reduced levels after postnatal day 14 (Burazin *et al.*, 2000).

1.3.1.3 Galanin receptor type 3

Galanin receptor type 3 (GalR3) was first isolated from rat hypothalamic cDNA libraries (Wang *et al.*, 1997b) and later from human cDNA (Kolakowski *et al.*, 1998; Smith *et al.*, 1998). The sequence of hGalR3 shares 36% amino acids identity with hGalR1 and 58% with hGalR2 and approximately 90% with rGalR3 (Kolakowski *et al.*, 1998). GalR3 actions are also mediated by $G\alpha_i/G\alpha_o$ G-proteins strongly coupled to the inhibition of AC (Kolakowski *et al.*, 1998) which results in an opening of the G protein-coupled inwardly-rectifying potassium channel (GIRK) (Smith *et al.*, 1998) (Fig 1).

There is some general acceptance of a weak and narrow GalR3 expression pattern relative to GalR1 and GalR2 transcript levels that is most prominent in the hypothalamus (Wang *et al.*, 1997b; Smith *et al.*, 1998; Mennicken *et al.*, 2002), although, some studies report a wider distribution of GalR3 throughout the central and peripheral tissues (Kolakowski *et al.*, 1998; Waters and Krause, 2000).

1.3.2 Galanin receptor ligands

Endogenous galanin has high affinity for all three galanin receptors, GalR1, GalR2 and GalR3 (Wang *et al.*, 1997b) (Table 2). The N-terminal region (residue 1-15) is conserved in all species studied except the tuna fish (Kakuyama *et al.*, 1997). The galanin fragment galanin(1-16) illustrates the importance of the N-terminal for receptor binding, since despite lacking half the complete sequence, it retains high affinity binding when compared to the striking reduction in receptor affinity observed for galanin(3-29) (Table 2). Several N-terminal truncated galanin fragments have shown to have a preference for GalR2 (Liu *et al.*, 2001; Wang *et al.*, 1997b) (Table 2), in concurrence with the fact that Gly^1 is of great importance for ligand binding to GalR1.

GALP was first isolated and cloned from porcine hypothalamus (Ohtaki *et al.*, 1999). GALP is a 60 amino acid, non-amidated peptide and is processed from a 115-120 amino acid precursor, depending on the species (cf. Table 1 and (Cunningham, 2004)). The amino acid sequence of GALP-(9-21) is completely identical to that of galanin(1-13). GALP has the lowest

affinity towards GalR1, although, it binds to all three galanin subtypes (Lang *et al.*, 2005; Ohtaki *et al.*, 1999) (Table 2).

The newest member of the galanin-peptide family, alarin, is a 25 amino acids long peptide originating as a splice variant of the GALP mRNA (Santic *et al.*, 2006). The alarin peptide, has been isolated from murine brain, thymus, skin (Santic *et al.*, 2007) and human neuroblastic tumors (Santic *et al.*, 2006) and has no detectable affinity towards the galanin receptor subtypes.

Table 2. Affinities of galanin, GALP and galanin fragments for the three galanin receptor subtypes, determined as K_i.

Ligand	K_i (nM)			Reference
	GalR1	GalR2	GalR3	
Rat galanin(1-29)	1.0	1.5	1.5	(Wang *et al.*, 1997b)
	0.3 (h)	1.6 (h)	12 (h)	(Borowsky *et al.*, 1998)
Human galanin(1-30)	0.4 (h)	2.3 (h)	69 (h)	(Borowsky *et al.*, 1998)
Porcine galanin(1-29)	0.23 (h)	0.95 (h)	9.8 (h)	(Borowsky *et al.*, 1998)
Galanin(1-16)	4.8	5.7	50	(Wang *et al.*, 1997b)
Galanin(2-11)	>5000 (h)	88	271	(Lu *et al.*, 2005a)
	879 [a] (h)	1.8 [a]	-	(Liu *et al.*, 2001)
Galanin(2-29)	85	1.9	12	(Wang *et al.*, 1997b)
Galanin(3-29)	>1000	>1000	>1000	(Wang *et al.*, 1997b)
Porcine GALP	4.3	0.24	-	(Ohtaki *et al.*, 1999)
Human GALP	77 [a] (h)	28 [a] (h)	10 [a] (h)	(Lang *et al.*, 2005)

Displacement is performed on the rat galanin receptor unless indicated otherwise. (h) human; [a] presented as IC_{50} values; - not determined.

1.3.2.1 Chimeric peptides

Several chimeric ligands have been synthesized, conjugating galanin(1-13) to other bioactive molecules (Table 3), M15 (also called galantide) (Bartfai *et al.*, 1991), M32 (Wiesenfeld-Hallin *et al.*, 1992b), M35 (Ögren *et al.*, 1992; Wiesenfeld-Hallin *et al.*, 1992b; Kask *et al.*, 1995), C7 (Langel *et al.*, 1992) and M40 (Langel *et al.*, 1992; Bartfai *et al.*, 1993). Although, they all maintain antagonistic properties *in vivo* at doses between 0.1 to 10 nmol when delivered intracerebroventricularly (i.c.v.) or intrathecally (i.t.) (Parker *et al.*, 1995; Lu *et al.*, 2005b), they all have a partial agonistic nature *in vivo* at doses higher than 10 nmol when delivered i.c.v. or i.t. (Kask *et al.*, 1995; Lu *et al.*, 2005b). Several other peptides have been synthesized and compared with the above mentioned ligands by various groups (Yanaihara *et al.*, 1993; Kakuyama *et al.*, 1997; Pooga *et al.*, 1998).

The first introduced chimeric peptide which acts as an antagonist of the galanin receptor family was M15 (Bartfai *et al.*, 1991). Here, the galanin(1-13) fragment, was coupled to a C-terminal fragment in substance P (residue

5-11), reported to have agonistic effect on the substance P receptor. M15 showed an about 10-fold higher affinity than the endogenous galanin to unspecified subtypes of the galanin receptor family in membrane preparations of rat tissues. Later, M35 was synthesized (Ögren *et al.*, 1992) with an improved *in vivo* stability (Wiesenfeld-Hallin *et al.*, 1992b). M15, M32, M35 and M40 have similar affinity as galanin and have been valuable tools in galanin research, but are limited by their relative non-specificity towards the different galanin receptors (Ögren *et al.*, 1992) and by their weak interactions with other receptors than the galanin receptors (Wiesenfeld-Hallin *et al.*, 1992a).

M617 (Table 5) resembles the M35 peptide, with the substitution of proline at position 14 to a glutamine, which results in a 25-fold selectivity for GalR1 over GalR2 *in vitro* (Table 3). This was later confirmed in several distinct *in vivo* models (Lundström *et al.*, 2005a; Blackshear *et al.*, 2007). M617 has been shown to produce anti-nociceptive effects (Jimenez-Andrade *et al.*, 2006) and to delay the development of seizure in an animal model (Mazarati *et al.*, 2006). The M871 peptide (Sollenberg et al. 2006) is N-terminally truncated and has two additional amino acid residues compared to the M40 peptide (Table 5). M871 function as a partial agonist, selective for GalR2 (Table 3), which was later confirmed *in vivo* (Jimenez-Andrade *et al.*, 2006; Alier *et al.*, 2007; Kuteeva *et al.*, 2008). The importance of the development of M617 and M871 and other subtype selective agonists and antagonists can almost not be overestimated and is the key to a successful delineation of galaninergic system and to identify its potential as a therapeutic target. Recently, several galanin analogues, all modified by introducing several cationic amino acid residues and a palmitoyl moiety was shown to exhibit improved bioavailability after systemic administration (Bulaj *et al.*, 2008; White *et al.*, 2009). One of these, the NAX5055, was shown to have anticonvulsant effect in several tested animal models and further characterization will probably identify other potential application of NAX5055 and other systemically active galanin analogues (White *et al.*, 2009).

Table 3. Affinities of commonly utilized peptidergic galanin receptor ligands at the three galanin receptor subtypes, determined as K_i.

Ligand	K_i (nM)			Reference
	GalR1	GalR2	GalR3	
M15	0.65	1.0	1.0	(Smith *et al.*, 1998)
M35	4.8	8.2	4.7	(Lu *et al.*, 2005b)
M40	1.8	5.1	63	(Lu *et al.*, 2005b)
M617	0.23 (h)	5.7 (h)	-	(Lundström *et al.*, 2005a)
M871	420 (h)	13 (h)	-	(Sollenberg *et al.*, 2006)
NAX 5055	3.5 (h)	51.5 (h)	-	(Bulaj *et al.*, 2008)

Displacement is performed on the rat galanin receptor unless indicated otherwise. (h) human; - not determined.

1.3.2.2 Non-peptide ligands

Galnon was identified after screening a combinatorial peptidomimetic library. It acts as an agonist in functional studies both *in vitro* and *in vivo* (Table 4) (Saar *et al.*, 2002; Bartfai *et al.*, 2004). It has been evaluated in models of anxiety and depression (Rajarao *et al.*, 2007), feeding (Abramov *et al.*, 2004) and pain (Wu *et al.*, 2003). Galmic is a non-peptide agonist with higher affinity for GalR1 compared to GalR2 (Table 4), which under conditions of intrahippocampal administration was 6-fold more potent than galnon in inhibiting self-sustaining status epilepticus, an *in vivo* model for epilepsy (Bartfai *et al.*, 2004; Ceide *et al.*, 2004). Nevertheless, both galnon and galmic potentials are limited by the fact that they have multiple sites of interactions, i.e. D2 dopamine receptors, grehlin and melanocortin receptors, which produce unwanted physiological effects (Florén *et al.*, 2005; Lu *et al.*, 2005b).

The metabolite Sch 202596, originated from an *Aspergillus* sp. culture found in an abandoned uranium mine in Tuolemene County California, was found to have a modest affinity to GalR1 *in vitro* (Table 4) (Chu *et al.*, 1997). Sch 202596 was characterized as a molecule with a spirocoumaranone skeleton and has only partly been synthesized so far (Katoh *et al.*, 2002). Several 1,4-dithiins and dithiipine-1,1,4,4-tetroxides with binding affinity to GalR1 were identified at the R. W. Johnson Pharmaceutical Institute (Scott *et al.*, 2000). The compound 2,3-dihydro-2-(4-methylphenyl)-1,4-dithiepine-1,1,4,4-tetroxide was shown to be a submicromolar antagonist. It has an IC_{50} of 190 nM for GalR1 (Table 4) and above the highest tested concentration (30 μM) for GalR2, however, its reactive nature and its low solubility makes it unattractive from a therapeutic point of view. Nevertheless, it has been used and evaluated in several studies (Mahoney *et al.*, 2003; Kozoriz *et al.*, 2006).

A series of 3-imonio-2-indolones was identified as specific GalR3 antagonists, with K_i values for GalR3 as low as 17 nM and above tested 10 μM for the other receptors tested (Konkel *et al.*, 2006a). One of these was referred as SNAP 37889 in Swanson et al. (2005). One drawback of the above mention indolones is theirs low aqueous solubility (less than 1 μg/ml) which motivated further studies, leading to the identification of a compound with an increased water solubility and selectivity (Table 4), 1,3-dihydro-1-[3-(2-pyrrolidinylethoxy)phenyl]-3-[[3-(trifluoromethyl)phenyl]imino]-2H-indol-2-one (Swanson *et al.*, 2005; Konkel *et al.*, 2006b). Another of the synthesized indolones was evaluated *in vivo* by Barr and colleagues (2006), which together with the other articles and several patent applications (Konkel *et al*, 2004) indicate that specific GalR3 ligands are in development.

Table 4. Affinities of commonly utilized non-peptid galanin receptor ligands at the three galanin receptor subtypes, determined as K_i.

Ligand	K_i (nM)			Reference
	GalR1	GalR2	GalR3	
Galnon	11700 (h)	34100 (h)	-	(Bartfai *et al.*, 2004)
Galmic	34200 (h)	>100000 (h)	-	(Bartfai *et al.*, 2004)
Sch 202596	1700 (h)	-	-	(Chu *et al.*, 1997)
Dithiepine-1,1,4,4-tetroxide	190[a] (h)	>30000[a] (h)	-	(Scott *et al.*, 2000)
SNAP 37889	>10000 (h)	>10000 (h)	17.4	(Swanson *et al.*, 2005)
SNAP 398299	>1000 (h)	>1000 (h)	5.3	(Swanson *et al.*, 2005)
GalR3ant	>10000 (h)	>10000 (h)	15	(Barr *et al.*, 2006)

Displacement is performed on the rat galanin receptor unless indicated otherwise. (h) human; [a] presented as IC_{50} values; - not determined.

1.3.3 Clinical applications

The galanin system has been ascribed a number of diverse functions. This requires the galanin system to have a highly plastic expression pattern, which has been portrayed numerous times in the literature. Galanin was early shown to be induced by estrogens (Kaplan *et al.*, 1988; Howard *et al.*, 1997b), and its expression has thereafter been shown also to be upregulated by the leukemia inhibitory factor (LIF) (Corness *et al.*, 1996) and downregulated by the nerve growth factor (NGF) (Verge *et al.*, 1995). Furthermore, an extensive upregulation is seen during development of sensory and motor systems (Gabriel *et al.*, 1989; Xu *et al.*, 1996).

The first quarter of a century of galanin research did not produce any galaninergic therapeutic beyond the animal model stage. Even though, several areas of research have given promising results; by which some, will shortly be outlined in the sections below.

1.3.3.1 Mood disorders

Several studies have documented that galanin affects noradrenalin (NA) and serotonin (5-HT) release in locus coeruleus (LC) and dorsal raphe nucleus (DRN), respectively (Razani *et al.*, 2000; Kehr *et al.*, 2002), two neurotransmitter systems known to be important in mood disorders. Furthermore, galanin downregulates the $5\text{-}HT_{1a}$ receptor (Kuteeva *et al.*, 2008).

Administration of galanin i.c.v. reduces the efficacy of antidepressant therapy (Yoshitake *et al.*, 2003). Together with data from a wide variety of studies in transgenic animals, this has boosted the interest for the galanin systems involvement in mood disorders lately. By utilizing one of several GalR3 antagonists taken from a patent application (Konkel *et* al., 2004), a number of papers have highlighted GalR3 as a promising target for antidepressive treatment (Swanson *et al.*, 2005; Barr *et al.*, 2006). Furthermore,

antidepressant effects of galanin have been shown in a clinical study on patients diagnosed with depression (Murck *et al.*, 2004).

1.3.3.2 Epilepsy

Another active area of galanin research was early established when it was shown that galanin modulates hippocampal excitability by inhibiting glutamate release, the major neurotransmitter responsible for epileptic seizures. Galanin knockout mice have, as well as mice succumbed to pharmacological blockade of the galanin receptors, lower threshold to induced seizures. Furthermore, galanin overexpressing transgenic mice as well as mice transiently expressing galanin show an increased resistance to induced seizures (Mazarati *et al.*, 2000; Kokaia *et al.*, 2001).

It seems as if both GalR1 and GalR2 are involved in the anticonvulsant effect of galanin, where GalR1 affects the initiation phase while GalR2 affects the maintenance phase of status epilepticus (Mazarati *et al.*, 2004; Mazarati *et al.*, 2006), implicating a putative role for galanin receptor agonists as antiepileptic therapeutics (see (Lerner *et al.*, 2008)).

1.3.3.3 Neurogenesis

Increased galanin concentration has shown to promote neuroprotection (Mahoney *et al.*, 2003) and to play a trophic role in neurons (Hobson *et al.*, 2008). GalR2-KO mice have a reduced number of neurons in the dorsal root ganglion (DRG) and have an impaired regeneration after nerve crush injury, and cultured DRG mutant neurites is both less in numbers and in length (Hobson *et al.*, 2006). Addition of exogenous galanin or galanin(2-11) can protect against glutamate, staurosporine and kainite induced hippocampal cell death (Elliott-Hunt *et al.*, 2004). Galanin and galanin(2-11) also attenuates β-amyloid induced cell death (Ding *et al.*, 2006), implicating a potential for a GalR2 agonist to reduce neuronal damage in Alzheimer's disease (AD).

1.3.3.4 Alzheimer's disease

Galanin markers have been shown to be increased in basal forebrain structures of persons who are succumbed to AD, initially interpreted as an indication of that galanin potentiated the cognitive and behavioural impairments in AD (Chan-Palay, 1988). However, the growing evidence of the neurotrophic properties (see section Neurogenesis) of the galanin family suggests that the hyperinnervation may be an attempt to re-establish some equilibrium as the pathology progresses (Counts *et al.*, 2006).

1.3.3.5 Cancer

Expression of galanin has been detected in a variety of tumors, e.g., small cell lung cancer, prostate carcinomas, breast cancer and oral squamous cells (see (Berger *et al.*, 2003; Berger *et al.*, 2005)). Galanin has been shown to stimulate proliferation in lung cancer cells (Seufferlein and Rozengurt, 1996), whereas it acts as an antiproliferative molecule in colon cancer (El-Salhy and Sitohy, 2002).

2. Aims of the study

This thesis is primarily focusing on designing and evaluating galanin receptor ligands as a means of expanding the number of tools available for studies regarding the galanin system.

Paper I: This paper presents a novel galanin receptor ligand, selective for GalR2. Furthermore, a stable GalR3 cell line was developed to facilitate the screening of subtype selective galanin ligands.

Paper II: Here we evaluate the binding of existing galanin receptor ligands towards galanin receptor 3. Furthermore, this study was aiming at characterizing the net influence of individual amino acid residues located in the C-terminal part of the galanin receptor ligand M617 on receptor-ligand interaction.

3. Methodological considerations

The methods used in this thesis are described in each contributed paper; consequently, merely theoretical and methological aspects will be discussed here. The selections below are valid for both papers when nothing else is stated.

3.1 Solid phase peptide synthesis

All peptides used in this thesis were synthesized by solid phase peptide synthesis (SPPS), a strategy introduced in 1963 by Bruce Merrifield (Merrifield, 1963). SPPS strategy is based on anchoring the peptide to an insoluble support follow by coupling and deprotection of protected amino acids in repeated cycles. This enables the step wise peptide synthesis procedure to occur without purification and characterization steps of intermediates needed in solution based peptide synthesis.

All peptides utilized in this thesis were synthesized anchored to a *p*-methylbenzylhydrylamine (MBHA) resin to produce C-terminal amidated peptides, and all reactions were synthesized with fmoc protection chemistry. Peptides were thereafter cleaved from the resin using 95 % trifluoroacetic acid (TFA), 2.5 % triisopropylsilane (TIS) and 2.5 % H_2O. Following cleavage and extraction, peptides were subsequently freeze-dried. Crude peptide products were purified using semi-preparative reversed-phase high performance liquid chromatography (HPLC) and analyzed using a matrix-assisted laser desorption/ionization-time of flight (MALDI-TOF) mass spectrometer (MS).

3.2 Design of galanin receptor ligands

The M1145 peptide is a chimeric ligand constructed from fragments of GALP and M40 (Table 5). The GALP(9-21) is identical to galanin (1-13), wherefore the M1145 peptide could also be seen as a chimeric construct of the N-terminal fragment (RGRGN), galanin(2-13), fragment (VL) and the C-terminal part from M40. The C-terminal part of M40 utilized in M1145 has also been utilized in the earlier published peptide, M871 (Table 5).

The Gly[1] residue in galanin has been shown to be of great importance for binding to GalR1, since N-terminal truncated forms of galanin fragments and chimeric peptides has reduced GalR1 affinity (Lu *et al.*, 2005b; Sollenberg *et al.*, 2006). Therefore, the glycine residue at the putative position 5 in M1145 (corresponding to residue 1 in galanin) was replaced with an aspargine residue to potentially disturb the binding to GalR1 (Table 5).

Table 5. List of the discussed and synthesized peptides in Paper I.

Peptide	Sequence
Rat galanin	GWTLNSAGYLLGPHAIDNHRSFSDKHGLT amide
Porcine GALP	APVHRGRGGWTLNSAGYLLGPVLHPPSRAEGGGKGK-TALGILDWKAIDGLPYPQSQLAS
Galanin(2-11)	GWTLNSAGYLL amide
M35	Galanin(1-13)-bradykinin(2-9)-amide GWTLNSAGYLLGPPPGFSPFR amide
M40	Galanin(2-13)-PPALALA-amide GWTLNSAGYLLPPPALALA amide
M617	Galanin(1-13)-Gln^{14}-bradykinin(3-9)-amide GWTLNSAGYLLGPQPGFSPFR amide
M871	Galanin(2-13)-EHPPPALALA-amide WTLNSAGYLLGPEHPPPALALA amide
M1145	GALP(5-23)-PPPALALA-amide RGRGNWTLNSAGYLLGPVLPPPALALA amide

In Paper II, amino acid residues in the C-terminal part of the M617 sequence was replaced by L-alanine to determine the importance of the chemical and physical properties of these particular amino acid residue side-chains in the interaction with the human GalR3. The side chain of L-alanine carries neither charge, hydrogen bonding ability, aromatic properties nor other reactive groups. Although, the relative small size of the alanine side chain is it large enough to at least partially avoid the introduction of flexibility which would occur by the alternative introduction of glycine. Therefore, L-alanine substitution strategy was utilized to delineate important pharmacophores in the M617 peptide.

3.3 Ligand binding to Galanin receptors

Measuring the rate and the extent of binding by the aid of a radiolabeled ligand can provide information about the number of binding sites, and their affinities and accessibility for various novel ligands. Radiolabeling of galanin has been performed through the iodination of tyrosine residues in the galanin peptide from different species. Human ^{125}I-galanin is available; how-

ever, it is rarely used since it is labelled on a residue which participates in receptor interactions (Tyr^9). Instead, the commonly used species is the porcine galanin (see Table 1), since the unique presence of an additional tyrosine residue (Tyr^{26}) makes it more favourable to use (Land *et al.*, 1991a).

Throughout the displacement studies performed in this thesis, silanized plastic has been used to minimize peptide adsorption to the plastic. Protease inhibitors have been added to reduce receptor and ligand degradation and addition of bovine serum albumin (BSA) to block unspecific binding. Finally, polyethyleneimine (PEI) was used to minimize peptide adsorption to the glass fiber filters (Land *et al.*, 1991a).

3.3.1 Competition binding assay

Displacement assay with a radioligand has been a commonly used option in the first screening step for pharmacological drugs for decades. For studies of ligand interaction to the galanin receptors, competition displacement of ^{125}I-labeled galanin has been used as the primary screening tool and was introduced only a few years after the first galanin publication (Fisone *et al.*, 1989).

During preparation of this thesis, two variants of the protocol have been utilized, although with very minor differences. Cells have been detached by scraping in PBS, lysed on ice in EDTA containing buffers and collected through centrifugation, 8500 x g (Paper I) or 45000 x g (Paper II). The displacement reaction was performed on 30 µg (Paper I) or 35 µg (Paper II) with 0.1-0.15 nM radiolabeled galanin. The binding reaction was incubated for 30 min at 37°C while shaking and terminated by rapid filtration over glass fiber filter plates.

3.3.2 Saturation binding assay (Paper I)

In order to compare receptor density between the utilized cell lines, B_{max} for the three cell lines were determined. The most accurate way to determine this is to keep the ligand concentration constant while the (hot) radioligand varies. This is commonly called hot saturation binding. An alternative is to keep the radioligand concentration constant while the (cold) ligand varies. This is commonly referred to as homologous competitive binding or cold saturation binding. The large amount of radioligand required for hot saturation binding experiments makes it expensive and used to a less extent. However, cold saturation binding is only applicable if the labelled and unlabelled ligand has the same affinity to the receptor. The five residues difference, in the C-terminal part of the rat and porcine galanin peptides (see Table 1) is insignificant for receptor interactions (Borowsky *et al.*, 1998), wherefore this

first assumption is valid. In addition, a second requirement is also essential; the concentration of the radioligand must be less than half the IC_{50} of the cold ligand. All data analysis for displacement studies was performed with Prism 5.0 (GraphPad Software Inc., San Diego, CA, USA).

3.4 Construction of an inducible GalR3 cell line

The Flip-In TREx-293 cell line is a commercial available expression system for recombinant proteins, and here we developed a stable Flip-In TREx cell line with an inducible expression of hGalR3. Among others, this expression system has been used to stably express excitatory amino acid transporters (EAATs) (Melzer *et al.*, 2005) and fibroblast growth factor-1 (FGF-1) (Widberg *et al.*, 2009). The integrated gene of interest is controlled by the strong cytomegalovirus (CMV) immediate early enhancer/promoter in combination with two inserted *tet* operator 2 ($TetO_2$) sequence, wherefore the presence of the Tet repressor inhibits the expression of the gene of interest. Consequently, the addition of Tetracycline, results in an inhibition of this expression repression since it binds to the Tet repressor, resulting in a complex that is unable to bind the tet operator (Yao *et al.*, 1998).

3.4.1 Cloning of hGalR3 (Paper I)

The human GalR3 sequence (GB Acc No: AY587582) was ligated into pcDNA5/FRT/TO plasmid cleaved by *Bam*HI and *Xho*I. Thereafter, full length sequencing was performed, to demonstrate the presence of the correct sequence of the inserted human GalR3 gene.

3.4.2 Transfection and selection (Paper I)

The hGalR3 containing plasmid was transfected into Flip-In TREx-293 cells utilizing a transfection agent (Fu-GENE). Thereafter, the hGalR3 integrates into the genome using the Flp recombinase which inserts the hGalR3 gene into the flippase recognition target (FRT) site present in these cells. Following transfection, positive clones were selected with Blasticidin and Hygromycin B. Although not performed, additional selection is possible by utilizing the zeocin sensitivity or the lack of β-galactosidase activity in the positive clones.

3.5 Characterization of the human GalR3 cell line

A series of initial experiments was performed to clarify the hGalR3 expression and the functional integrity of the transcript. Membrane preparation for receptor binding and GTPγS function were done with a series of centrifugations and the use of EDTA containing buffers which aimed at collecting receptor containing membranes, although several parameters differs from the parameters used in the rest of the thesis (see section 3.3.1 and section 3.5.3).

3.5.1 Western blot (Paper I)

Western blot was used to detect protein levels of hGalR3 after tetracycline induction and also the absence of protein before induction. The Western blot assay assesses the protein level of a specific protein in a given sample. The protein of interest is detected by the use of specific primary antibodies. Thereafter, a secondary antibody is used for visualizing were the primary antibody interacts with the protein of interest. Size of the protein is given by the aid of a standard with known molecular weights. The antibody used in this thesis was not galanin receptor subtype selective, as shown in the paper. This is a well know problem which also is discussed in the paper.

3.5.2 Functional assay, GTPγS (Paper I)

The functionality of the expressed protein was determined with assessing the GTP levels after stimulation by galanin and galanin in combination with the GalR3 antagonist, SNAP37889. Experiments with the selective GalR3 ligand were performed to demonstrate the cloned galanin receptor subtype identity.

The [^{35}S]-GTPγS-binding assay is based on the fact that this non-hydrolysable analogue of GTP traps the Gα subunit in an activated state. Therefore, without receptor stimulation, the Gα subunit with a GDP molecule bound has none or only low interaction with the receptor and no exchange of GDP to GTP occurs. As the receptor is activated, GDP dissociates from the Gα subunit and [^{35}S]-GTPγS will be able to bind at a higher level.

Several functional assays based on radiolabeled GTP analogues are available, and in the assay used in this thesis, the use of scintillation proximity assay (SPA) beads makes it easy to separate the receptor-G-protein-[^{35}S]-GTPγS complex from free [^{35}S]-GTPγS.

3.5.3 Galanin receptor displacement (Paper I)

Cells were homogenized using a polytron at 10000 rpm for 10 sec in cold buffer containing 10 mM EDTA, and collected in cold buffer containing 0.1 mM EDTA through centrifugation twice at 48000 x g. The displacement reaction was performed on 20 µg with 0.25 nM radiolabeled galanin. The binding reaction was incubated for 120 min at RT and terminated by rapid filtration over glass fiber filter plates.

3.6 Cell cultures

Three characterized cell lines have been used as model system when studying galanin receptor binding and signalling in this thesis; human bowes melanoma cells (Bowes), epithelial chinese hamster ovary (CHO) cells and human embryonic kidney (HEK) cells (Flp-In TREx-293).

Bowes cells endogenously express galanin receptors and has been shown to exclusively express GalR1 (Heuillet *et al.*, 1994), with a large number of high-affinity binding sites, 540 fmol/mg protein (Paper I). The CHO cell line is considered a good expression system for recombinant proteins, and the human GalR2 stable transfection cell line used in this study, is widely used for functional studies.

Cells with a stable expression of the GalR3 have not been available and this has halted the ability to test galaninergic ligands. Therefore it was an important step when we succeeded in producing a stable human GalR3 cell line.

3.7 Intracellular signalling by galanin receptors

After confirmation of a novel receptor-ligand interaction, intracellular signalling assays are an indispensable step in the work flow. This is to determine the ability of the ligand to activate intracellular signalling cascades (agonist) or the ability to inhibit the induction from a known agonist (antagonist).

One point should be made regarding the usage of cell lines with expressed GPCRs at abnormal high level in the cell. Likely, the normal ratio between GPCR and G-proteins are disturbed, the high level of GPCR could lead to a deficit in G-proteins available for receptor interactions. Consequently, this could result in limited G-protein coupling or even interactions with other G-protein subunits.

3.7.1 Inositol phosphate accumulation (Paper I)

To measure the activation of G_q-proteins, we exploited the production of tritium labelled inositol phosphate (IP) in the cells after stimulation. This strategy does not require any advanced equipments, therefore, is it naturally appealing in its simplicity. The method has been useful in pharmacological screening (Borowsky *et al.*, 1998) although the lower sensitivity compared to analytical methods like gas chromatography/mass spectrometry (GC/MS).

The cells were prelabeled with ^{3}H-myoinositol, which is incorporated into phosphatidylinositol 4,5-bisphosphate (PIP_2) and subsequently into its hydrolyzed product IP_3 and downstream metabolites, all which thus are ^{3}H-labelled. Downstream events of PIP_2 occur at a high rate; consequently, this makes the detection of IP_3-molecules unfavourable. The last step, where IP is recycled to myo-inositol can fortunately be blocked by addition of LiCl, wherefore accumulation can occur. The three forms of IPs: IP_3, inositol 4,5-bisphosphate (IP_2) and IP were extracted in the presence of perchloric acid and separated on an anion exchange column. Finally, radioactivity is measured in a β-counter and normalized to the total count in each sample.

4. Results and discussion

The two papers in this thesis cover different parts of the development and characterization of novel galanin receptor analogues. Paper I, involved the development of a new stable recombinant cell system and the development of a new subtype specific galanin receptor ligand. Paper II, explores the opportunities that arises from the new cell system to characterize important pharmacophores in binding to GalR3.

4.1 Development of a GalR2 selective agonist (Paper I)

In Paper I we aimed to design and characterize a galanin analogue with receptor subtype selectivity for GalR2 over GalR1 and GalR3. We aimed at exploring the possibility to N-terminally extend the galanin analogue, a strategy which in the past have had very low success rate, given the nonappearance of end products in the literature. Although, the presence of the endogenously GALP reveal the plausibility of this design strategy.

After confirming the preferential binding towards GalR2 (see Table 6), M1145 evoked effects on intracellular signalling was elucidated in signalling through the preferential G-protein for the GalR2, the G_q. M1145 was found to stimulate IP formation via GalR2, in concurrence with galanin. In addition, stimulation of the receptor by M1145 in the presence of galanin, resulted in an additive effect.

In conclusion, we have characterized a new galanin analogue with preferential binding for only one galanin receptor subtype. In addition, the agonistic effect of M1145 *in vitro* was conclusively shown, both in absence and presence of galanin. However, contradictory results regarding receptor stimulus have earlier been reported for galanin ligands, when tested *in vitro* and *in vivo* (Florén *et al.*, 2000; Lundström *et al.*, 2005b). Consequently, delineating the evoked intracellular response by M1145 *in vivo* is of great importance.

Preliminary results from experiments performed after publication of Paper I reveals that the new design strategy employed in Paper I can further be optimized to achieve galanin analogues with significantly higher receptor subtype selectivity.

Table 6. List of K_i values for galanin, galanin(2-11) and the developed subtype selective ligand on the galanin receptors.

Peptide	K_i (nM)			K_i (GalR1) /	K_i (GalR3) /
	GalR1	GalR2	GalR3	K_i (GalR2)	K_i (GalR2)
Rat galanin	1.75	2.98	4.49	0.6	1.5
Galanin(2-11)	n.t.	16.6	199	n.t.	12
M1145	587	6.55	497	90	76

n.t., not determined.

4.2 Development of a stable GalR3 cell line (Paper I)

In paper I, we also aimed at improving the possibilities for receptor subtype screening of novel galanin analogues. The presentation of a cell system expressing GalR3 would be a well appreciated step forward in the galanin research field.

We utilized a commercially available expression system into which we cloned the human GalR3 sequence. The accuracy of the sequence of the plasmid was confirmed by full length sequencing. Thereafter, transfection of the correct plasmid was made and positive clones were selected in the presence of Hygromycin B according to the manufacturer's instructions.

Western blot analysis showed both a clear induction of the GalR3 protein after 24 and 48h of tetracycline treatment and showed no expression of the GalR3 protein in non-induced cells. In addition, the ability of galanin and the selective GalR3 antagonist SNAP37889 to inhibit ^{125}I-galanin binding in receptor displacement studies, indispensably confirms the presence of a functional galanin receptor, and furthermore confirms the subtype identity.

In order to further elucidate the functionality of the GalR3 transcript, receptor signalling was assessed by the GTPγS assay. Galanin was able to stimulate the GTPγS activity 75% over basal activity. Furthermore, SNAP37889 was able to dose-dependently inhibit activation induced by galanin, again, indicating that the transcribed GalR3 protein had the characteristic that earlier has been ascribed the GalR3.

Finally, the level of protein expression was assessed in the new cell system. When comparing the levels with the other cell systems used in this thesis, no conclusive difference could be seen, indicating that these cell systems are highly compatible with each other in screening for novel galanin analogues. However, one should keep in mind that, using the method hot saturation binding instead of the used homologues competitive binding, may have given more accurate results regarding receptor expression.

In summary, we could present a stable hGalR3 cell line which exhibits affinities (see also table 6) and properties in line with the literature (Branchek *et al.*, 2000; Lu *et al.*, 2005a).

4.3 Characterization of GalR3-binding (Paper II)

This study contains a GalR3 binding assessment of earlier published M617 and M871 peptides. The M617 peptide has been used as a GalR1 preferential agonist in several papers, despite the absence of GalR3 binding experiments. M617 shows affinity towards the GalR3 and has a K_i around 50 nM (see Table 7). Furthermore, when comparing the GalR3 result with earlier published affinities towards GalR1 and GalR2, M617 could still be seen as a GalR1 preferential agonist. However, since the affinity difference is quite small between the receptor subtypes, the design of the experimental setup for experiments with M617 is of great importance. Furthermore, when interpretations of the seen M617 effects are made, consideration should be made regarding the possibility of diminutive effects mediated through the other receptor subtypes.

The affinity of the M871 peptide towards GalR3 was not analyzed in the original article, mainly due to the absence of a cell system expressing GalR3. Although, it was at that time thought that N-terminal truncated galanin analogues retained a high affinity towards GalR3, mainly due to the fact that galanin(2-11) bind to GalR2 and GalR3 with similar affinity. Therefore, interpretation of results from studies performed with M871 has been done with the notion that the GalR3 evoked effects could not be excluded. Interestingly, M871 appears to have no distinct GalR3 affinity (see Table 7).

Table 7. Sequence and receptor affinities for peptides tested in Paper II.

Peptide	Sequence	K_i (nM)	K_i (M617 analogue) / K_i (M617)
M871	WTLNSAGYLLGPEHPPPALALA amide	>10000	-
M617	GWTLNSAGYLLGPQPGFSPFR amide	49.2	1.00
Ala^{16} M617	GWTLNSAGYLLGPQP**A**FSPFR amide	45.0	0.91
Ala^{17} M617	GWTLNSAGYLLGPQPG**A**SPFR amide	71.3	1.45
Ala^{18} M617	GWTLNSAGYLLGPQPGF**A**PFR amide	56.5	1.15
Ala^{19} M617	GWTLNSAGYLLGPQPGFS**A**FR amide	85.7	1.74
Ala^{20} M617	GWTLNSAGYLLGPQPGFSP**A**R amide	224	4.55
Ala^{21} M617	GWTLNSAGYLLGPQPGFSPF**A** amide	296	6.01

In order to elucidate the mechanism behind the relative high affinity towards GalR3 for M617 compared to M871, a number of alanine substitutions were exploited. Substitution of the $phenylalanine^{20}$ and $arginine^{21}$ for alanine lowers the GalR3 affinity markedly. Consequently, the identity and order of amino acid residues in C-terminal part of galanin analogues, at least for the M617 peptide, are important for receptor binding. This may be seen as an expected result, although, interactions of galanin and galanin analogues

with galanin receptors are often simplified to be the result of interaction only made by the N-terminal part of galanin, i.e. galanin(1-16).

5. Conclusions

This thesis has provided new data regarding interactions between galanin analogues and the galanin receptor subtypes. We clearly demonstrate that N-terminal extension analogues of galanin are doable and may be even preferential when designing galanin receptor subtype specific ligands. Hopefully, this knowledge will allow us to take further steps towards unlocking the potential that resides in the galanin.

Paper I: Introduces a new subtype selective ligand that is a promising tool to be used in future studies delineating the interplay between galanin and its receptors. Furthermore, a new cell system expressing GalR3 was introduced and characterized, simplifying future galanin receptor ligand screening studies.

Paper II: Determines the GalR3 interaction for earlier published peptide ligands M617 and M871. It furthermore, characterizes the relative importance of the C-terminal amino acid residues in M617 in binding to GalR3.

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7. References

Abramov, U., Florén, A., Echevarria, D.J., Brewer, A., Manuzon, H., Robinson, J.K., Bartfai, T., Vasar, E. & Langel, Ü. (2004) Regulation of feeding by galnon. Neuropeptides, **38**, 55-61.

Ahmad, S., O'Donnell, D., Payza, K., Ducharme, J., Menard, D., Brown, W., Schmidt, R., Wahlestedt, C., Shen, S.H. & Walker, P. (1998) Cloning and evaluation of the role of rat GALR-2, a novel subtype of galanin receptor, in the control of pain perception. Ann. N. Y. Acad. Sci., **863**, 108-119.

Alier, K. A., Chen, Y., Sollenberg, U.E., Langel, Ü. & Smith, P.A. (2007) Selective stimulation of GalR1 and GalR2 in rat substantia gelatinosa reveals a cellular basis for the anti- and pro-nociceptive actions of galanin. Pain, .

Barr, A. M., Kinney, J.W., Hill, M.N., Lu, X., Biros, S., Rebek, J.,Jr & Bartfai, T. (2006) A novel, systemically active, selective galanin receptor type-3 ligand exhibits antidepressant-like activity in preclinical tests. Neurosci. Lett., **405**, 111-115.

Bartfai, T., Lu, X., Badie-Mahdavi, H., Barr, A.M., Mazarati, A., Hua, X.Y., Yaksh, T., Haberhauer, G., Ceide, S.C., Trembleau, L., Somogyi, L., Krock, L. & Rebek, J.,Jr (2004) Galmic, a nonpeptide galanin receptor agonist, affects behaviors in seizure, pain, and forced-swim tests. Proc. Natl. Acad. Sci. U. S. A., **101**, 10470-10475.

Bartfai, T., Langel, Ü., Bedecs, K., Andell, S., Land, T., Gregersen, S., Ahren, B., Girotti, P., Consolo, S. & Corwin, R. (1993) Galanin-receptor ligand M40 peptide distinguishes between putative galanin-receptor subtypes. Proc. Natl. Acad. Sci. U. S. A., **90**, 11287-11291.

Bartfai, T., Bedecs, K., Land, T., Langel, Ü., Bertorelli, R., Girotti, P., Consolo, S., Xu, X.J., Wiesenfeld-Hallin, Z. & Nilsson, S. (1991) M-15: High-affinity chimeric peptide that blocks the neuronal actions of galanin in the hippocampus, locus coeruleus, and spinal cord. Proc. Natl. Acad. Sci. U. S. A., **88**, 10961-10965.

Berger, A., Santic, R., Hauser-Kronberger, C., Schilling, F.H., Kogner, P., Ratschek, M., Gamper, A., Jones, N., Sperl, W. & Kofler, B. (2005) Galanin and galanin receptors in human cancers. Neuropeptides, **39**, 353-359.

Berger, A., Santic, R., Almer, D., Hauser-Kronberger, C., Huemer, M., Humpel, C., Stockhammer, G., Sperl, W. & Kofler, B. (2003) Galanin and galanin receptors in human gliomas. Acta Neuropathol., **105**, 555-560.

Berglund, M. M., Saar, K., Lundell, I., Langel, Ü. & Larhammar, D. (2001) Binding of chimeric NPY/galanin peptides M32 and M242 to cloned neuropeptide Y receptor subtypes Y1, Y2, Y4, and Y5. Neuropeptides, **35**, 148-153.

Blackshear, A., Yamamoto, M., Anderson, B.J., Holmes, P.V., Lundström, L., Langel, Ü. & Robinson, J.K. (2007) Intracerebroventricular administration of galanin or galanin receptor subtype 1 agonist M617 induces c-fos activation in central amygdala and dorsomedial hypothalamus. Peptides, **28**, 1120-1124.

Bloomquist, B. T., Beauchamp, M.R., Zhelnin, L., Brown, S.E., Gore-Willse, A.R., Gregor, P. & Cornfield, L.J. (1998) Cloning and expression of the human galanin receptor GalR2. Biochem. Biophys. Res. Commun., **243**, 474-479.

Borowsky, B., Walker, M.W., Huang, L.Y., Jones, K.A., Smith, K.E., Bard, J., Branchek, T.A. & Gerald, C. (1998) Cloning and characterization of the human galanin GALR2 receptor. Peptides, **19**, 1771-1781.

Branchek, T. A., Smith, K.E., Gerald, C. & Walker, M.W. (2000) Galanin receptor subtypes. Trends Pharmacol. Sci., **21**, 109-117.

Bulaj, G., Green, B.R., Lee, H.K., Robertson, C.R., White, K., Zhang, L., Sochanska, M., Flynn, S.P., Scholl, E.A., Pruess, T.H., Smith, M.D. & White, H.S. (2008) Design, synthesis, and characterization of high-affinity, systemically-active galanin analogues with potent anticonvulsant activities. J. Med. Chem., **51**, 8038-8047.

Burazin, T. C., Larm, J.A., Ryan, M.C. & Gundlach, A.L. (2000) Galanin-R1 and -R2 receptor mRNA expression during the development of rat brain suggests differential subtype involvement in synaptic transmission and plasticity. Eur. J. Neurosci., **12**, 2901-2917.

Burgevin, M. C., Loquet, I., Quarteronet, D. & Habert-Ortoli, E. (1995) Cloning, pharmacological characterization, and anatomical distribution of a rat cDNA encoding for a galanin receptor. J. Mol. Neurosci., **6**, 33-41.

Ceide, S. C., Trembleau, L., Haberhauer, G., Somogyi, L., Lu, X., Bartfai, T. & Rebek, J.,Jr (2004) Synthesis of galmic: A nonpeptide galanin receptor agonist. Proc. Natl. Acad. Sci. U. S. A., **101**, 16727-16732.

Chang, M. M. & Leeman, S.E. (1970) Isolation of a sialogogic peptide from bovine hypothalamic tissue and its characterization as substance P. J. Biol. Chem., **245**, 4784-4790.

Chan-Palay, V. (1988) Galanin hyperinnervates surviving neurons of the human basal nucleus of meynert in dementias of alzheimer's and parkinson's disease: A hypothesis for the role of galanin in accentuating cholinergic dysfunction in dementia. J. Comp. Neurol., **273**, 543-557.

Chu, M., Mierzwa, R., Truumees, I., King, A., Sapidou, E., Barrabee, E., Terracciano, J., Patel, M.G., Gullo, V.P., Burrier, R., Das, P.R., Mittelman, S. & Puar, M.S. (1997) A new fungal metabolite, sch 202596, with inhibitory activity in the galanin receptor GALR1 assay. Tetrahedron Lett., **38**, 6111-6114.

Corness, J., Shi, T.J., Xu, Z.Q., Brulet, P. & Hökfelt, T. (1996) Influence of leukemia inhibitory factor on galanin/GMAP and neuropeptide Y expression in mouse primary sensory neurons after axotomy. Exp. Brain Res., **112**, 79-88.

Counts, S. E., Chen, E.Y., Che, S., Ikonomovic, M.D., Wuu, J., Ginsberg, S.D., Dekosky, S.T. & Mufson, E.J. (2006) Galanin fiber hypertrophy within the cholinergic nucleus basalis during the progression of alzheimer's disease. Dement. Geriatr. Cogn. Disord., **21**, 205-214.

Cunningham, M. J. (2004) Galanin-like peptide as a link between metabolism and reproduction. J. Neuroendocrinol., **16**, 717-723.

Ding, X., MacTavish, D., Kar, S. & Jhamandas, J.H. (2006) Galanin attenuates beta-amyloid (abeta) toxicity in rat cholinergic basal forebrain neurons. Neurobiol. Dis., **21**, 413-420.

Elliott-Hunt, C. R., Marsh, B., Bacon, A., Pope, R., Vanderplank, P. & Wynick, D. (2004) Galanin acts as a neuroprotective factor to the hippocampus. Proc. Natl. Acad. Sci. U. S. A., **101**, 5105-5110.

El-Salhy, M. & Sitohy, B. (2002) Triple therapy with octreotide, galanin and serotonin induces necrosis and increases apoptosis of a rat colon carcinoma. Regul. Pept., **108**, 55-62.

Fisone, G., Langel, Ü., Carlquist, M., Bergman, T., Consolo, S., Hökfelt, T., Unden, A., Andell, S. & Bartfai, T. (1989) Galanin receptor and its ligands in the rat hippocampus. Eur. J. Biochem., **181**, 269-276.

Florén, A., Sollenberg, U., Lundström, L., Zorko, M., Stojan, J., Budihna, M., Wheatley, M., Martin, N.P., Kilk, K., Mazarati, A., Bartfai, T., Lindgren, M. & Langel, Ü. (2005) Multiple interaction sites of galnon trigger its biological effects. Neuropeptides, **39**, 547-558.

Florén, A., Land, T. & Langel, Ü. (2000) Galanin receptor subtypes and ligand binding. Neuropeptides, **34**, 331-337.

Gabriel, S. M., Kaplan, L.M., Martin, J.B. & Koenig, J.I. (1989) Tissue-specific sex differences in galanin-like immunoreactivity and galanin mRNA during development in the rat. Peptides, **10**, 369-374.

Gustafson, E. L., Smith, K.E., Durkin, M.M., Gerald, C. & Branchek, T.A. (1996) Distribution of a rat galanin receptor mRNA in rat brain. Neuroreport, **7**, 953-957.

Habert-Ortoli, E., Amiranoff, B., Loquet, I., Laburthe, M. & Mayaux, J.F. (1994) Molecular cloning of a functional human galanin receptor. Proc. Natl. Acad. Sci. U. S. A., **91**, 9780-9783.

Hawes, J. J., Brunzell, D.H., Wynick, D., Zachariou, V. & Picciotto, M.R. (2005) GalR1, but not GalR2 or GalR3, levels are regulated by galanin signaling in the locus coeruleus through a cyclic AMP-dependent mechanism. J. Neurochem., **93**, 1168-1176.

Heuillet, E., Bouaiche, Z., Menager, J., Dugay, P., Munoz, N., Dubois, H., Amiranoff, B., Crespo, A., Lavayre, J. & Blanchard, J.C. (1994) The human galanin receptor: Ligand-binding and functional characteristics in the bowes melanoma cell line. Eur. J. Pharmacol., **269**, 139-147.

Hobson, S. A., Bacon, A., Elliot-Hunt, C.R., Holmes, F.E., Kerr, N.C., Pope, R., Vanderplank, P. & Wynick, D. (2008) Galanin acts as a trophic factor to the central and peripheral nervous systems. Cell Mol. Life Sci., **65**, 1806-1812.

Hobson, S. A., Holmes, F.E., Kerr, N.C., Pope, R.J. & Wynick, D. (2006) Mice deficient for galanin receptor 2 have decreased neurite outgrowth from adult sensory neurons and impaired pain-like behaviour. J. Neurochem., **99**, 1000-1010.

Howard, A. D., Tan, C., Shiao, L.L., Palyha, O.C., McKee, K.K., Weinberg, D.H., Feighner, S.D., Cascieri, M.A., Smith, R.G., Van Der Ploeg, L.H. & Sullivan, K.A. (1997a) Molecular cloning and characterization of a new receptor for galanin. FEBS Lett., **405**, 285-290.

Howard, G., Peng, L. & Hyde, J.F. (1997b) An estrogen receptor binding site within the human galanin gene. Endocrinology, **138**, 4649-4656.

Jacoby, A. S., Webb, G.C., Liu, M.L., Kofler, B., Hort, Y.J., Fathi, Z., Bottema, C.D., Shine, J. & Iismaa, T.P. (1997) Structural organization of the mouse and human GALR1 galanin receptor genes (galnr and GALNR) and chromosomal localization of the mouse gene. Genomics, **45**, 496-508.

Jimenez-Andrade, J. M., Lundström, L., Sollenberg, U.E., Langel, Ü., Castaneda-Hernandez, G. & Carlton, S.M. (2006) Activation of peripheral galanin receptors: Differential effects on nociception. Pharmacol. Biochem. Behav., **85**, 273-280.

Kakuyama, H., Kuwahara, A., Mochizuki, T., Hoshino, M. & Yanaihara, N. (1997) Role of N-terminal active sites of galanin in neurally evoked circular muscle contractions in the guinea-pig ileum. Eur. J. Pharmacol., **329**, 85-91.

Kaplan, L. M., Gabriel, S.M., Koenig, J.I., Sunday, M.E., Spindel, E.R., Martin, J.B. & Chin, W.W. (1988) Galanin is an estrogen-inducible, secretory product of the rat anterior pituitary. Proc. Natl. Acad. Sci. U. S. A., **85**, 7408-7412.

Kask, K., Berthold, M., Bourne, J., Andell, S., Langel, Ü. & Bartfai, T. (1995) Binding and agonist/antagonist actions of M35, galanin(1-13)-bradykinin(2-9)amide chimeric peptide, in rin m 5F insulinoma cells. Regul. Pept., **59**, 341-348.

Katoh, T., Ohmori, O., Iwasaki, K. & Inoue, M. (2002) Synthetic studies on sch 202596, an antagonist of the galanin receptor GalR1: An efficient synthesis of (±)-geodin, the spirocoumaranone part of sch 202596. . Tetrahedron, **58**, 1289-1299.

Kehr, J., Yoshitake, T., Wang, F.H., Razani, H., Gimenez-Llort, L., Jansson, A., Yamaguchi, M. & Ögren, S.O. (2002) Galanin is a potent in vivo modulator of mesencephalic serotonergic neurotransmission. Neuropsychopharmacology, **27**, 341-356.

Kokaia, M., Holmberg, K., Nanobashvili, A., Xu, Z.Q., Kokaia, Z., Lendahl, U., Hilke, S., Theodorsson, E., Kahl, U., Bartfai, T., Lindvall, O. & Hökfelt, T. (2001) Suppressed kindling epileptogenesis in mice with ectopic overexpression of galanin. Proc. Natl. Acad. Sci. U. S. A., **98**, 14006-14011.

Kolakowski, L. F.,Jr, O'Neill, G.P., Howard, A.D., Broussard, S.R., Sullivan, K.A., Feighner, S.D., Sawzdargo, M., Nguyen, T., Kargman, S., Shiao, L.L., Hreniuk, D.L., Tan, C.P., Evans, J., Abramovitz, M., Chateauneuf, A., Coulombe, N., Ng, G., Johnson, M.P., Tharian, A., Khoshbouei, H., George, S.R., Smith, R.G. & O'Dowd, B.F. (1998) Molecular characterization and expression of cloned human galanin receptors GALR2 and GALR3. J. Neurochem., **71**, 2239-2251.

Konkel, M. J., J. Wetzel and I. J. Talisman (2004). "3-Imino-2-indolones for the treatment of depression and /or anxiety." United States patetn 514/323.

Konkel, M. J., Lagu, B., Boteju, L.W., Jimenez, H., Noble, S., Walker, M.W., Chandrasena, G., Blackburn, T.P., Nikam, S.S., Wright, J.L., Kornberg, B.E., Gregory, T., Pugsley, T.A., Akunne, H., Zoski, K. & Wise, L.D. (2006a) 3-arylimino-2-indolones are potent and selective galanin GAL3 receptor antagonists. J. Med. Chem., **49**, 3757-3758.

Konkel, M. J., Packiarajan, M., Chen, H., Topiwala, U.P., Jimenez, H., Talisman, I.J., Coate, H. & Walker, M.W. (2006b) Amino substituted analogs of 1-phenyl-3-phenylimino-2-indolones with potent galanin Gal3 receptor binding affinity and improved solubility. Bioorg. Med. Chem. Lett., **16**, 3950-3954.

Kozoriz, M. G., Kuzmiski, J.B., Hirasawa, M. & Pittman, Q.J. (2006) Galanin modulates neuronal and synaptic properties in the rat supraoptic nucleus in a use and state dependent manner. J. Neurophysiol., **96**, 154-164.

Kuteeva, E., Wardi, T., Lundström, L., Sollenberg, U., Langel, Ü., Hökfelt, T. & Ögren, S.O. (2008) Differential role of galanin receptors in the regulation of depression-like behavior and Monoamine/Stress-related genes at the cell body level. Neuropsychopharmacology, .

Land, T., Langel, Ü. & Bartfai, T. (1991a) Hypothalamic degradation of galanin(1-29) and galanin(1-16): Identification and characterization of the peptidolytic products. Brain Res., **558**, 245-250.

Land, T., Langel, Ü., Low, M., Berthold, M., Unden, A. & Bartfai, T. (1991b) Linear and cyclic N-terminal galanin fragments and analogs as ligands at the hypothalamic galanin receptor. Int. J. Pept. Protein Res., **38**, 267-272.

Lang, R., Berger, A., Santic, R., Geisberger, R., Hermann, A., Herzog, H. & Kofler, B. (2005) Pharmacological and functional characterization of galanin-like peptide fragments as potent galanin receptor agonists. Neuropeptides, **39**, 179-184.

Langel, Ü., Land, T. & Bartfai, T. (1992) Design of chimeric peptide ligands to galanin receptors and substance P receptors. Int. J. Pept. Protein Res., **39**, 516-522.

Lembeck, F. (1953) Zur frage der zentralen übertragung afferenter impulse. III. das vorkommen and die bedeutung der substanz P in den dorsalen. Naunyn Schmiedebergs Arch Exp Pathol Pharmakol, **219**, 197-213.

Lerner, J. T., Sankar, R. & Mazarati, A.M. (2008) Galanin and epilepsy. Cell Mol. Life Sci., **65**, 1864-1871.

Liu, H. X., Brumovsky, P., Schmidt, R., Brown, W., Payza, K., Hodzic, L., Pou, C., Godbout, C. & Hökfelt, T. (2001) Receptor subtype-specific pronociceptive and analgesic actions of galanin in the spinal cord: Selective actions via GalR1 and GalR2 receptors. Proc. Natl. Acad. Sci. U. S. A., **98**, 9960-9964.

Lorimer, D. D. & Benya, R.V. (1996) Cloning and quantification of galanin-1 receptor expression by mucosal cells lining the human gastrointestinal tract. Biochem. Biophys. Res. Commun., **222**, 379-385.

Lu, X., Lundström, L. & Bartfai, T. (2005a) Galanin (2-11) binds to GalR3 in transfected cell lines: Limitations for pharmacological definition of receptor subtypes. Neuropeptides, **39**, 165-167.

Lu, X., Lundström, L., Langel, Ü. & Bartfai, T. (2005b) Galanin receptor ligands. Neuropeptides, **39**, 143-146.

Lundström, L., Sollenberg, U., Brewer, A., Kouya, P.F., Zheng, K., Xu, X.J., Sheng, X., Robinson, J.K., Wiesenfeld-Hallin, Z., Xu, Z.Q., Hökfelt, T., Bartfai, T. & Langel, Ü. (2005a) A galanin receptor subtype 1 specific agonist. Int. J. Peptide Res. Ther., **11**, 17-27.

Lundström, L., Elmquist, A., Bartfai, T. & Langel, Ü. (2005b) Galanin and its receptors in neurological disorders. Neuromolecular Med., **7**, 157-180.

Mahoney, S. A., Hosking, R., Farrant, S., Holmes, F.E., Jacoby, A.S., Shine, J., Iismaa, T.P., Scott, M.K., Schmidt, R. & Wynick, D. (2003) The second galanin receptor GalR2 plays a key role in neurite outgrowth from adult sensory neurons. J. Neurosci., **23**, 416-421.

Mazarati, A., Lundström, L., Sollenberg, U., Shin, D., Langel, Ü. & Sankar, R. (2006) Regulation of kindling epileptogenesis by hippocampal galanin type 1 and type 2 receptors: The effects of subtype-selective agonists and the role of G-protein-mediated signaling. J. Pharmacol. Exp. Ther., **318**, 700-708.

Mazarati, A., Lu, X., Kilk, K., Langel, Ü., Wasterlain, C. & Bartfai, T. (2004) Galanin type 2 receptors regulate neuronal survival, susceptibility to seizures and seizure-induced neurogenesis in the dentate gyrus. Eur. J. Neurosci., **19**, 3235-3244.

Mazarati, A. M., Hohmann, J.G., Bacon, A., Liu, H., Sankar, R., Steiner, R.A., Wynick, D. & Wasterlain, C.G. (2000) Modulation of hippocampal excitability and seizures by galanin. J. Neurosci., **20**, 6276-6281.

Melzer, N., Torres-Salazar, D. & Fahlke, C. (2005) A dynamic switch between inhibitory and excitatory currents in a neuronal glutamate transporter. Proc. Natl. Acad. Sci. U. S. A., **102**, 19214-19218.

Mennicken, F., Hoffert, C., Pelletier, M., Ahmad, S. & O'Donnell, D. (2002) Restricted distribution of galanin receptor 3 (GalR3) mRNA in the adult rat central nervous system. J. Chem. Neuroanat., **24**, 257-268.

Merrifield, R. B. (1963) Solid phase peptide synthesis. I. the synthesis of a tetrapeptide. J. Am. Chem. Soc., **85**, 2149-2154.

Murck, H., Held, K., Ziegenbein, M., Kunzel, H., Holsboer, F. & Steiger, A. (2004) Intravenous administration of the neuropeptide galanin has fast antidepressant efficacy and affects the sleep EEG. Psychoneuroendocrinology, **29**, 1205-1211.

Ögren, S. O., Hökfelt, T., Kask, K., Langel, Ü. & Bartfai, T. (1992) Evidence for a role of the neuropeptide galanin in spatial learning. Neuroscience, **51**, 1-5.

Ohtaki, T., Kumano, S., Ishibashi, Y., Ogi, K., Matsui, H., Harada, M., Kitada, C., Kurokawa, T., Onda, H. & Fujino, M. (1999) Isolation and cDNA cloning of a novel galanin-like peptide (GALP) from porcine hypothalamus. J. Biol. Chem., **274**, 37041-37045.

Pang, L., Hashemi, T., Lee, H.J., Maguire, M., Graziano, M.P., Bayne, M., Hawes, B., Wong, G. & Wang, S. (1998) The mouse GalR2 galanin receptor: Genomic organization, cDNA cloning, and functional characterization. J. Neurochem., **71**, 2252-2259.

Parker, E. M., Izzarelli, D.G., Nowak, H.P., Mahle, C.D., Iben, L.G., Wang, J. & Goldstein, M.E. (1995) Cloning and characterization of the rat GALR1 galanin receptor from Rin14B insulinoma cells. Brain Res. Mol. Brain Res., **34**, 179-189.

Pooga, M., Jureus, A., Razaei, K., Hasanvan, H., Saar, K., Kask, K., Kjellen, P., Land, T., Halonen, J., Maeorg, U., Uri, A., Solyom, S., Bartfai, T. & Langel, Ü. (1998) Novel galanin receptor ligands. J. Pept. Res., **51**, 65-74.

Rajarao, S. J., Platt, B., Sukoff, S.J., Lin, Q., Bender, C.N., Nieuwenhuijsen, B.W., Ring, R.H., Schechter, L.E., Rosenzweig-Lipson, S. & Beyer, C.E. (2007) Anxiolytic-like activity of the non-selective galanin receptor agonist, galnon. Neuropeptides, **41**, 307-320.

Razani, H., Diaz-Cabiale, Z., Fuxe, K. & Ögren, S.O. (2000) Intraventricular galanin produces a time-dependent modulation of 5-HT1A receptors in the dorsal raphe of the rat. Neuroreport, **11**, 3943-3948.

Saar, K., Mazarati, A.M., Mahlapuu, R., Hallnemo, G., Soomets, U., Kilk, K., Hellberg, S., Pooga, M., Tolf, B.R., Shi, T.S., Hökfelt, T., Wasterlain, C., Bartfai, T. & Langel, Ü. (2002) Anticonvulsant activity of a nonpeptide galanin receptor agonist. Proc. Natl. Acad. Sci. U. S. A., **99**, 7136-7141.

Salio, C., Lossi, L., Ferrini, F. & Merighi, A. (2006) Neuropeptides as synaptic transmitters. Cell Tissue Res., **326**, 583-598.

Santic, R., Schmidhuber, S.M., Lang, R., Rauch, I., Voglas, E., Eberhard, N., Bauer, J.W., Brain, S.D. & Kofler, B. (2007) Alarin is a vasoactive peptide. Proc. Natl. Acad. Sci. U. S. A., **104**, 10217-10222.

Santic, R., Fenninger, K., Graf, K., Schneider, R., Hauser-Kronberger, C., Schilling, F.H., Kogner, P., Ratschek, M., Jones, N., Sperl, W. & Kofler, B. (2006) Gangliocytes in neuroblastic tumors express alarin, a novel peptide derived by differential splicing of the galanin-like peptide gene. J. Mol. Neurosci., **29**, 145-152.

Scott, M. K., Ross, T.M., Lee, D.H., Wang, H.Y., Shank, R.P., Wild, K.D., Davis, C.B., Crooke, J.J., Potocki, A.C. & Reitz, A.B. (2000) 2,3-dihydro-dithiin and -dithiepine-1,1,4,4-tetroxides: Small molecule non-peptide antagonists of the human galanin hGAL-1 receptor. Bioorg. Med. Chem., **8**, 1383-1391.

Seufferlein, T. & Rozengurt, E. (1996) Galanin, neurotensin, and phorbol esters rapidly stimulate activation of mitogen-activated protein kinase in small cell lung cancer cells. Cancer Res., **56**, 5758-5764.

Smith, K. E., Walker, M.W., Artymyshyn, R., Bard, J., Borowsky, B., Tamm, J.A., Yao, W.J., Vaysse, P.J., Branchek, T.A., Gerald, C. & Jones, K.A. (1998) Cloned human and rat galanin GALR3 receptors. pharmacology and activation of G-protein inwardly rectifying K+ channels. J. Biol. Chem., **273**, 23321-23326.

Smith, K. E., Forray, C., Walker, M.W., Jones, K.A., Tamm, J.A., Bard, J., Branchek, T.A., Linemeyer, D.L. & Gerald, C. (1997) Expression cloning of a rat hypothalamic galanin receptor coupled to phosphoinositide turnover. J. Biol. Chem., **272**, 24612-24616.

Sollenberg, U. E., Lundström, L., Bartfai, T. & Langel, Ü. (2006) M871 - A novel peptide antagonist selectively recognizingthe galanin receptor type 2. Int. J. Peptide Res. Ther., **12**, 115-119.

Sullivan, K. A., Shiao, L.L. & Cascieri, M.A. (1997) Pharmacological characterization and tissue distribution of the human and rat GALR1 receptors. Biochem. Biophys. Res. Commun., **233**, 823-828.

Swanson, C. J., Blackburn, T.P., Zhang, X., Zheng, K., Xu, Z.Q., Hökfelt, T., Wolinsky, T.D., Konkel, M.J., Chen, H., Zhong, H., Walker, M.W., Craig, D.A., Gerald, C.P. & Branchek, T.A. (2005) Anxiolytic- and antidepressant-like profiles of the galanin-3 receptor (Gal3) antagonists SNAP 37889 and SNAP 398299. Proc. Natl. Acad. Sci. U. S. A., **102**, 17489-17494.

Tatemoto, K., Rökaeus, A., Jornvall, H., McDonald, T.J. & Mutt, V. (1983) Galanin - a novel biologically active peptide from porcine intestine. FEBS Lett., **164**, 124-128.

V Euler, U. S. & Gaddum, J.H. (1931) An unidentified depressor substance in certain tissue extracts. J. Physiol., **72**, 74-87.

Verge, V. M., Richardson, P.M., Wiesenfeld-Hallin, Z. & Hökfelt, T. (1995) Differential influence of nerve growth factor on neuropeptide expression in vivo: A novel role in peptide suppression in adult sensory neurons. J. Neurosci., **15**, 2081-2096.

Wang, S., Hashemi, T., He, C., Strader, C. & Bayne, M. (1997a) Molecular cloning and pharmacological characterization of a new galanin receptor subtype. Mol. Pharmacol., **52**, 337-343.

Wang, S., He, C., Hashemi, T. & Bayne, M. (1997b) Cloning and expressional characterization of a novel galanin receptor. identification of different pharmacophores within galanin for the three galanin receptor subtypes. J. Biol. Chem., **272**, 31949-31952.

Wang, S., Hashemi, T., Fried, S., Clemmons, AL. & Hawes, BE. (1998) Differential G-protein-coupling profiles of the GalR1 and GalR2 galanin receptors. Biochemistry, **37**, 6711-6717.

Waters, S. M. & Krause, J.E. (2000) Distribution of galanin-1, -2 and -3 receptor messenger RNAs in central and peripheral rat tissues. Neuroscience, **95**, 265-271.

White, H. S., Scholl, E.A., Klein, B.D., Flynn, S.P., Pruess, T.H., Green, B.R., Zhang, L. & Bulaj, G. (2009) Developing novel antiepileptic drugs: Characterization of NAX 5055, a systemically-active galanin analog, in epilepsy models. Neurotherapeutics, **6**, 372-380.

Widberg, C. H., Newell, F.S., Bachmann, A.W., Ramnoruth, S.N., Spelta, M.C., Whitehead, J.P., Hutley, L.J. & Prins, J.B. (2009) Fibroblast growth factor receptor 1 is a key regulator of early adipogenic events in human preadipocytes. Am. J. Physiol. Endocrinol. Metab., **296**, E121-31.

Wiesenfeld-Hallin, Z., Bartfai, T. & Hökfelt, T. (1992a) Galanin in sensory neurons in the spinal cord. Front. Neuroendocrinol., **13**, 319-343.

Wiesenfeld-Hallin, Z., Xu, X.J., Langel, Ü., Bedecs, K., Hökfelt, T. & Bartfai, T. (1992b) Galanin-mediated control of pain: Enhanced role after nerve injury. Proc. Natl. Acad. Sci. U. S. A., **89**, 3334-3337.

Wu, W. P., Hao, J.X., Lundström, L., Wiesenfeld-Hallin, Z., Langel, Ü., Bartfai, T. & Xu, X.J. (2003) Systemic galnon, a low-molecular weight galanin receptor agonist, reduces heat hyperalgesia in rats with nerve injury. Eur. J. Pharmacol., **482**, 133-137.

Xia, C. Y., Qi, Y.T., Zhang, Y. & Yuan, C.G. (2005) The expression of galanin and galanin receptors in olfactory ensheathing cells and effects of galanin on cells proliferation. Shi Yan Sheng Wu Xue Bao, **38**, 98-104.

Xu, Z. Q., Shi, T.J. & Hökfelt, T. (1996) Expression of galanin and a galanin receptor in several sensory systems and bone anlage of rat embryos. Proc. Natl. Acad. Sci. U. S. A., **93**, 14901-14905.

Yanaihara, N., Mochizuki, T., Takatsuka, N., Iguchi, K., Sato, K., Kakuyama, H., Li, M. & Yanaihara, C. (1993) Galanin analogues: Agonist and antagonist. Regul. Pept., **46**, 93-101.

Yao, F., Svensjo, T., Winkler, T., Lu, M., Eriksson, C. & Eriksson, E. (1998) Tetracycline repressor, tetR, rather than the tetR-mammalian cell transcription factor fusion derivatives, regulates inducible gene expression in mammalian cells. Hum. Gene Ther., **9**, 1939-1950.

Yoshitake, T., Reenila, I., Ögren, S.O., Hökfelt, T. & Kehr, J. (2003) Galanin attenuates basal and antidepressant drug-induced increase of extracellular serotonin and noradrenaline levels in the rat hippocampus. Neurosci. Lett., **339**, 239-242.

Zachariou, V., Georgescu, D., Kansal, L., Merriam, P. & Picciotto, M.R. (2001) Galanin receptor 1 gene expression is regulated by cyclic AMP through a CREB-dependent mechanism. J. Neurochem., **76**, 191-200.