Design, Synthesis and Characterization of Galanin Receptor Selective Ligands

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Abstract
Galanin is a 29/30 amino acid long bioactive peptide discovered over 30 years ago when C-terminally amidated peptides were isolated from porcine intestines. The name galanin originates from a combination of the first and last amino acids - G from glycine and the rest from alanine. The first 15 amino acids are highly conserved throughout species, which indicates that the N-terminus is important for receptor recognition and binding. Galanin exerts its effects by binding to three different G-protein-coupled receptors, which all differ according to regional distribution, the affinity for shortened galanin fragments, as well as the intracellular G-protein signaling cascade used. When first discovered, galanin was found to cause muscle contraction as well as hyperglycemia. Over the years, galanin has been reported to be involved in a wide variety of biological functions, for example food intake and neurogenesis, and pathological functions, for example epilepsy and depression.

Determining the specific involvement of the three different galanin receptors in biological and pathological processes is limited by the small amount of galanin receptor selective-specific ligands available as research tools. Furthermore, the fast degradation of peptides limits the administration routes in animal studies.

This thesis aims at developing new galanin receptor-selective ligands to help delineate the involvement of the three different galanin receptors.

Paper 1 presents the shortest galanin fragment with a galanin receptor 2 specific binding preference where only a single amino acid substitution was made, Ala⁵Ser in galanin (2-11). In addition, G-protein coupled receptor signaling were evaluated through both a classical second messenger assay and a real-time label-free technique in cells overexpressing the receptor as well as low receptor expression.

Paper 2 demonstrates that the neuroprotective effects of galanin in a kainic acid-induced excitotoxic animal model were mediated through galanin receptor 1. Furthermore, a new robust protocol for evaluating G-protein signaling using a label-free real time impedance technique was presented and compared to two different classical second-messenger assays.

Paper 3 presents a series of systemically active galanin receptor 2 selective ligands subsequently evaluated in two different depression-like animal models.

Paper 4 investigates a mutated form of human galanin which was found in epilepsy patients and binding and signaling properties of the mutated associated ligand p(A39E) was examined.

In conclusion, this thesis presents the discovery of eight new galanin ligands, which can be used to evaluate the galaninergic system as well as to help investigate the possible use of peptides as pharmaceuticals in different diseases.

Keywords: galanin, GAL1R, GAL2R, depression, epilepsy.

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Department of Neurochemistry

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Kristin Webling
Cover picture: The chemical structure of human galanin (1-30) shaped as a heart. Drawn by hand by Kristin Webling

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Distributor: Department of Neurochemistry
To my surprise and my lovely family
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Publications

This thesis is based on the following publications

1) **Webling, K.**, Runesson, J., Lang, A., Saar, I., Kofler, B., Langel, Ü., 2016. Ala5-galanin(2-11) is a GAL2R specific galanin analogue. *Neuropeptides*, 60, 75-82.


* These authors contributed equally to the project
Additional publications

Publications not included in this thesis


- **Webling, K.E.,** Runesson, J., Bartfai, T., Langel, Ü., 2012. Galanin receptors and ligands. *Front Endocrinol (Lausanne).* 3:146


This PhD-thesis is an expansion of the work previously presented in my Licentiate thesis, ISBN: 978-91-7649-338-0, where Paper 2 and 3 were included.
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<tr>
<th>Abbreviation</th>
<th>Description</th>
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<tbody>
<tr>
<td>5-HT</td>
<td>Serotonin or 5-hydroxytryptamine</td>
</tr>
<tr>
<td>AC</td>
<td>Adenylyl cyclase</td>
</tr>
<tr>
<td>ACh</td>
<td>Acetylcholine</td>
</tr>
<tr>
<td>ARC</td>
<td>Hypothalamic arcuate nucleus</td>
</tr>
<tr>
<td>BBB</td>
<td>Blood-brain barrier</td>
</tr>
<tr>
<td>BSA</td>
<td>Bovine serum albumin</td>
</tr>
<tr>
<td>cAMP</td>
<td>Cyclic adenosine monophosphate</td>
</tr>
<tr>
<td>CHO</td>
<td>Chinese hamster ovary</td>
</tr>
<tr>
<td>CNS</td>
<td>Central nervous system</td>
</tr>
<tr>
<td>DIC</td>
<td>Diisopropylcarbodiimide</td>
</tr>
<tr>
<td>Dmt</td>
<td>2,6-dimethyl-L-tyrosine</td>
</tr>
<tr>
<td>DR</td>
<td>Dorsal raphe nucleus</td>
</tr>
<tr>
<td>DRG</td>
<td>Dorsal root ganglia</td>
</tr>
<tr>
<td>ECL</td>
<td>Extracellular loop</td>
</tr>
<tr>
<td>EDTA</td>
<td>Ethylenediaminetetraacetic acid</td>
</tr>
<tr>
<td>EEG</td>
<td>Electroencephalography</td>
</tr>
<tr>
<td>EM</td>
<td>Electron microscopy</td>
</tr>
<tr>
<td>ER</td>
<td>Endoplasmic reticulum</td>
</tr>
<tr>
<td>ERE</td>
<td>Estrogen responsive element</td>
</tr>
<tr>
<td>ESI-MS</td>
<td>Electrospray ionization mass spectrometry</td>
</tr>
<tr>
<td>FAM</td>
<td>5(6)-carboxyfluorescein</td>
</tr>
<tr>
<td>Fmoc</td>
<td>9-Fluorenylmethylxycarbonyl</td>
</tr>
<tr>
<td>GABA</td>
<td>Gamma-aminobutyric acid</td>
</tr>
<tr>
<td>GALP</td>
<td>Galanin-like peptide</td>
</tr>
<tr>
<td>GAL1R</td>
<td>Galanin receptor 1</td>
</tr>
<tr>
<td>GAL2R</td>
<td>Galanin receptor 2</td>
</tr>
<tr>
<td>GAL3R</td>
<td>Galanin receptor 3</td>
</tr>
<tr>
<td>GIRK</td>
<td>G protein-regulated inwardly rectifying K⁺</td>
</tr>
<tr>
<td>GPCR</td>
<td>G-protein coupled receptor</td>
</tr>
<tr>
<td>GTP</td>
<td>Guanosine triphosphate</td>
</tr>
<tr>
<td>HEK</td>
<td>Human embryonic kidney</td>
</tr>
<tr>
<td>HPLC</td>
<td>High-performance liquid chromatography</td>
</tr>
<tr>
<td>ICL</td>
<td>Intracellular loop</td>
</tr>
<tr>
<td>i.c.v.</td>
<td>Intracerebroventricularly</td>
</tr>
<tr>
<td>i.p.</td>
<td>Intraperitoneal</td>
</tr>
</tbody>
</table>
IP    Inositol phosphate
IP$_2$ Inositol 1,4-bisphosphate
IP$_3$ Inositol 1,4,5-trisphosphate
i.v. intravenous
KA Kainic acid
K_i Inhibitory dissociation constant
KO knockout
LC Locus coeruleus
LC/MS Liquid chromatography-mass spectrometry
LDCV Large dense core vesicles
LIF Leukemia inhibitory factor
MALDI Matrix-assisted laser desorption/ionization
MBHA p-Methylbenzhydrylamine
MRI Magnetic resonance imaging
mRNA Messenger ribonucleic acid
MS Mass spectrometry
NA Noradrenaline
NCI Normalized cell index
NGF Nerve growth factor
NMR Nuclear magnetic resonance
NPQ Neuropeptide Q or spexin
NPY Neuropeptide Y
OE Overexpression
PEI Polyethyleneimine
PKC Proteinkinase C
PLC Phospholipase C
PNA Peptide nucleic antisence oligonucleotide
PNS Peripheral nervous system
PTX Pertussis toxin
PTZ Pentylenetetrazol
RT-PCR Reverse transcript polymerase chain reaction
SE Status elepticus
SPPS Solid-phase peptide synthesis
SSSE Self-sustained status elepticus
SSV Small synaptic vesicle
SV2A Synaptic vesicle protein 2A
TFA Trifluoroacetic acid
TIS Triisopropylsilane
TLE Temporal lobe epilepsy
TOF Time of flight
VIP Vasoactive intestinal polypeptide
Wt Wildtype
Amino acids

Full name, three letter code, one letter code:

Alanine, Ala, A
Arginine, Arg, R
Asparagine, Asn, N
Aspartic acid, Asp, D
Asparagine, Asn, N
Glycine, Gly, G
Histidine, His, H
Isoleucine, Iso, I
Leucine, Leu, L
Lysine, Lys, K
Methionine, Met, M
Phenylalanine, Phe, F
Proline, Pro, P
Serine, Ser, S
Threonine, Thr, T
Tryptophan, Trp, W
Tyrosine, Tyr, Y
Valine, Val, V
1. Introduction

For all higher organisms composed of multiple cells, cellular communication is essential for survival. The specialized information signaling cells, the neurons, make up the nervous system and consist of more than a thousand different cell types. The nervous system is further divided into the central nervous system (CNS), namely brain and spinal cord, and the peripheral nervous system (PNS), consisting of cranial and spinal nerves.

Trying to understand the nervous system, down to molecular level, is one of the greatest hurdles in modern science and has attracted massive interest. This doctoral thesis focuses on one of those information carrying neurotransmitter molecules, namely the bioactive peptide galanin, in order to delineate its effects in biological and pathological functions.

1.1 Neurotransmission

The cellular communication is diverse and can result from either direct interaction or by the exchange of chemical messengers. Direct interaction is when a neuron transfers information electronically through gap junctions. These gap junctions connect the two neurons so that they behave as one single neuron through channel proteins that allows direct cell-to-cell transfer of ions, i.e. the electrical information that is an action potential. An example of this type of transmission is for example found in the heart.

Chemical messengers are used when neurons are separated by a synaptic cleft. The synaptic cleft creates a physical barrier for the electric signal and the electrical signal is therefore converted to a chemical signal using neurotransmitters. This neurotransmission is wider spread and can be found in both the peripheral- and the central nervous systems.

Focusing on the chemical messengers, they will only induce a response in cells that express specific recognition proteins in their cell surface, i.e. receptors.
1.2 Neurotransmitters

Neurotransmitters can be further divided into three categories depending on chemical characterization i.e. functional groups:

- Monoamines: Acetylcholine (ACh), adrenaline, dopamine, histidine, noradrenaline (NA) and serotonin (5-HT, after the older name 5-hydroxytryptamine).
- Amino acids: Glutamate, glycine and gamma-aminobutyric acid (GABA).
- Peptides: Enkephalins, galanin, neuropeptide Y (NPY), substance P, vasoactive intestinal polypeptide (VIP).

Monoamines and amino acids are also referred to as ‘classical neurotransmitters’. The classical neurotransmitters are stored in small synaptic vesicles (SSV), also denoted as small clear vesicles due to the transparent look in electron microscopy (EM) images and appear very closely associated with the active zone in the neuronal synapse. Upon stimulation, the vesicles are released into the synaptic cleft (Ludwig and Leng, 2006). The small volume in the synaptic cleft results in a high concentration of neurotransmitters, even when just a few vesicles are released. The classical neurotransmitters bind to ligand-gated ion channels, giving rise to very fast responses. Monoamines and amino acids are quickly degraded, recaptured by specific transporters situated on both neurons and glia cells and thereafter quickly recycled (Eiden et al., 2004; Y. Liu and Edwards, 1997). Classical neurotransmitters can also be synthesized in nerve endings and not only in the cell soma.

However, neuropeptides differ in several aspects from the classical neurotransmitters (summarized in Table 1). Neuropeptides are only synthesized in the soma and contained in large dense core vesicles (LDCV) which are transported to the synapse and stored further away from the synaptic cleft compared to the SSV (Mains et al., 1987). The nerve terminal generally requires higher frequency stimulation or burst firing in order to release the LDCV and the receptors are almost exclusively G-protein coupled receptors (GPCRs) (Adrian et al., 1983; Dutar et al., 1989; Lundberg, 1996; Lundberg and Hökfelt, 1983). Peptide binding to GPCRs results in a slower, but long-lasting response compared to SSV. There are no reuptake mechanisms for neuropeptides and they are degraded by peptidases (Roques et al., 1993). Thus, neuropeptides must be synthesized de novo after release, followed by transportation to nerve terminals or dendrites. The difference between monoamines and neuropeptides in the synapse is described in Figure 1 and summarized in Table 1.
Figure 1. A Schematic illustration of a synapse and chemical neurotransmission. Arrival of the action potential from the axon triggers the release of presynaptically stored monoamines into the synaptic cleft (left) and in case of stronger stimulation can neuropeptides be released (right). Released transmitters act on pre- and post-synaptically located receptors. (Modified from Hökfelt et al., 2003)
Table 1. Summary of the differences between classical neurotransmitters and neuropeptides (Modified from Webling 2016).

<table>
<thead>
<tr>
<th></th>
<th>Classical neurotransmitters i.e. monoamines and amino acids</th>
<th>Neuropeptides</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Synthesis</strong></td>
<td>Both in the soma and at the nerve terminal</td>
<td>Soma</td>
</tr>
<tr>
<td><strong>Storage</strong></td>
<td>SSV (40-60 nm in diameter) in close proximity to the active zone and Ca(^{2+}) channels in the nerve terminal as high, local Ca(^{2+}) levels are needed for release</td>
<td>LDCV (90-250 nm in diameter) stored further away from the active zone in the nerve terminal and lower Ca(^{2+}) levels are needed for release.</td>
</tr>
<tr>
<td><strong>Release</strong></td>
<td>Directed specific release into the synapse cleft</td>
<td>Requires high frequency firing or burst firing. Can be released from dendrites and soma as well as the nerve terminal.</td>
</tr>
<tr>
<td><strong>Degradation and removal</strong></td>
<td>Enzymatic inactivation in the synaptic cleft, reuptake by specific transporters on neighboring neurons and also glia cells</td>
<td>Mostly by peptidases outside the synaptic cleft but also diffusion</td>
</tr>
<tr>
<td><strong>Reuptake</strong></td>
<td>Several reuptake mechanisms</td>
<td>No reuptake mechanism</td>
</tr>
<tr>
<td><strong>Receptors</strong></td>
<td>Ligand gated ion channels</td>
<td>GPCRs</td>
</tr>
<tr>
<td><strong>Signal duration</strong></td>
<td>Very short and rapid signal duration</td>
<td>A slower, long-lasting signal duration</td>
</tr>
</tbody>
</table>

1.3 **G-protein coupled receptors, GPCRs**

G-protein coupled receptors are the largest family of membrane protein receptors, including around 800 different receptors, all composed of a seven transmembrane spanning protein with three extracellular loops, ECL, and three intracellular loops, ICL (See Figure 2). The fact that the receptors have a seven membrane spanning protein also gave rise to an alternative naming, seven transmembrane receptors or 7TM receptors. Neuropeptides almost
exclusively bind to GPCR, even tough peptide-gated ion channels have been reported (Gründler and Assmann 2015).

GPCRs are further divided into five classes, namely Rhodopsin-like (Class A), Secretin (Class B), Glutamate (Class C), Adhesion and Frizzled/Smoothened. GPCRs transduce signals over the cell membrane by a wide selection of ligands, from small ions, proteins, hormones and even light. The GPCR Class A is the largest class, and the galanin receptors are members of this class.

![Diagram of a 7 transmembrane spanning receptor](image)

**Figure 2. A general representation of a 7 transmembrane spanning receptor i.e. GPCR or 7TM receptors.** The green cylinders represent the α-helices that normally form in the hydrophobic environment of the cell membrane, which is a lipid bilayer. Extracellular loops are denoted ECL and intracellular loops are denoted ICL. (Modified from Runesson 2012).

The name “G-protein coupled receptors” is based on their ability to attract and activate guanosine triphosphate (GTP)-binding proteins, so called G-proteins, upon ligand binding. The identification and characterization of G-protein coupled receptors by crystallization, mainly by Robert J. Lefkowitz and Brian K. Kobilka respectively, led to them jointly being awarded the Nobel Prize in chemistry in 2012.

There are several common motifs in the class A GPCRs, i.e. common topology and saltbridges, that can be used to hypothesize ligand-receptor interaction based on crystal structures of class A GPCRs co-crystallized with both agonists and antagonists. However, a major finding was the crystallized state of an activated β-2-adrenergic receptor that showed minor conformational changes between active and inactive receptor in the extracellular spanning regions (Rasmussen et al., 2011), previously had also a high sequence similarity between different GPCRs in the intracellular regions been reported (Mirzadegan et al., 2003).
1.4 Neuropeptides

Neuropeptides are by definition small peptides, consisting of approximately 2-100 amino acid residues, abundant in neuronal tissues modulating neurotransmission. Neuropeptides bind specifically to their receptors within low nanomolar, nM, range. The specificity makes low doses sufficient for a prolonged response, which reduces possible side effects - an attractive attribute for possible pharmaceuticals. The ability of neuropeptides to modulate neurotransmission when the nervous system is stressed, challenged or dysfunctional further extends their possible use as pharmaceuticals. In contrast to classical neurotransmitters, which affect ligand gated ion channels, neuropeptides bind to G-protein coupled receptors (GPCRs). Modulation of GPCRs has been reported for 50-80% of the pharmaceuticals on the market today (Hill, 2006). The drawback of using peptides as pharmaceuticals is mainly the in vivo instability together with an extremely limited blood-brain barrier (BBB) penetration.

Neuropeptides are rapidly degraded by peptidases and the therapeutic pathway to evaluate their biological effects in animal models has so far mainly been via intracerebroventricular (i.c.v) administration. Since galanin also has been found in non-neuronal tissues e.g. in keratinocytes, eccrine sweat glands and macrophages (Bauer et al., 2010; Kofler et al., 2004), the question has been raised to define galanin as a bioactive peptide to describe its wider effects.

1.5 The galanin peptide family

Several bioactive peptides have been reported to be a part of the galanin peptide family, since different alternative splicing results of the preprogalanin sequence can be seen. The human genome exhibits a single copy of the galanin encoding gene localized on chromosome 11 at position q13.3-q13.5 (Evans et al., 1993). The galanin gene consists of six exons that generates a 123 (bovine, human and porcine) (Evans and Shine, 1991; Rökaeus and Brownstein, 1986; Rökaeus and Carlquist, 1988), or 124 (mouse and rat) (Kaplan et al., 1988; Kofler et al., 1996; Lundkvist et al., 1995; Vrontakis et al., 1987) amino acid precursor protein, namely preprogalanin. The N-termini of preprogalanin have a hydrophobic signaling sequence, the galanin sequence and further a 59/60 amino acid long flanking peptide at the C-termini named Galanin-message associated peptide or GMAP (See section 1.5.2). Flanking both galanin and GMAP are pairs of basic amino acids, lysine, Lys, and arginine, Arg, which are essential for generating the peptides after enzymatic processing (Kaplan et al., 1988).
A few years later, galanin-like peptide, GALP (see section 1.5.3) was reported which exhibited a binding capacity for galanin receptors and a splice variant named alarin (see section 1.5.4). Recently, an additional short peptide shown to bind to galanin receptors, namely spexin or neuropeptide Q (short NPQ) (See section 1.5.5). Three receptors have so far been identified to bind and interact with the peptides in the galanin family, galanin receptor 1, 2, and 3, GAL1R, GAL2R and GAL3R, named in order of their isolation and further characterization (see sections 1.6.1-1.6.3).

### 1.5.1 Galanin

Galanin was first discovered in 1983 by Professor Viktor Mutt and co-workers while isolating C-terminally amidated peptides from porcine intestines (Tatemoto et al., 1983). The 29 amino acid long peptide was named galanin, a name derived from the first glycine in the N-terminal and the last alanine in the C-terminal. C-terminally amidated peptides have a common characteristic of exerting biological effects and galanin was no exception. Galanin was shown to decrease blood-glucose levels in dogs as well as inducing contraction in smooth muscle preparations (Tatemoto et al., 1983).

Over the following years, galanin was discovered in a wide spectrum of species. A high sequence identity of the N-terminus was found throughout a wide range of species (Kakuyama et al., 1997), where the first 14 amino acids were conserved with the only two exceptions being galanin from two species of fish; tuna fish and cichlid (Habu et al., 1994; Hu et al., 2016) (see Table 2). Furthermore, human galanin and macaque galanin differed from other species by consisting of 30 amino acids and that the C-terminal was not amidated (Cunningham et al., 2002; Schmidt et al., 1991). The latest identified galanin sequence was found in African cichlid and revealed the longest galanin peptide found so far, a 32 amino acid long peptide with a C-terminal amidation (Hu et al., 2016).

The N-terminal of galanin has proven to be important for receptor binding and biological activity, whereas the C-terminal has been hypothesized to stabilize the peptide to prevent proteolytic degradation (Bedecs et al., 1995). Indeed, as reported in an *in silico* study by Kothandan et al., (2013) using both full length galanin as well as the two small molecule GAL3R antagonists, SNAP 37889 and SNAP 398299, the C-terminal was essential for additional ligand-receptor interactions (Kothandan et al., 2013).

Galanin expression was initially examined in rat, mouse and human brains and the bioactive peptide was found to be widely expressed throughout the CNS, PNS and endocrine system. Furthermore, galanin is expressed in keratinocytes, eccrine sweat glands, macrophages and surrounding blood vessels (Kofler et al., 2004; Bauer et al., 2010).
Galanin is co-expressed with several monoamines and amino acids, namely acetylcholine, dopamine, GABA, glutamate, serotonin and noradrenalin (Melander et al., 1986; Hökfelt et al., 1987; Xu et al., 1998; Liu et al., 2003). Galanin expression is induced by estrogens and three copies of estrogen responsive elements, ERE, were identified in the human galanin promoter region (Kaplan et al., 1988; Kofler et al., 1995; Vrontakis et al., 1987; 1989). Galanin upregulation was also reported in the development of sensory- and motorsystems (Gabriel et al., 1989; Xu et al., 1996) as well as after nerve injuries (Hökfelt et al., 1987) and in Alzheimer’s disease patients (Chan-Palay 1988a;b). Also leukemia inhibitory factor, LIF, have shown an upregulatory effect on galanin (Corness et al., 1996; Sun and Zigmond 1996). On the contrary, galanin depletion have been observed after epileptic seizures (Mazarati et al., 1998) and galanin is downregulated by nerve growth factor, NGF (Verge et al., 1995).

Galanin is also co-expressed with other bioactive peptides, for example neuropeptide Y, NPY, substance P, vasopressin, and encephalin (Rökaeus and Carlquist 1988; Merchenthaler et al., 1990; Zhang et al., 1993 a; b; 1995)

In most genetic studies, where galanin has been knocked out or down regulated, no reported birth defects or behavioral abnormalities of the animals were described. However, Schmidhuber and colleagues reported developmental deficits in Galanin-KO mice, which made it hard for them to continue with their study on microvasculature and inflammatory responses in murine skin (Schmidhuber et al., 2008a; 2008b).
Table 2. The amino acid sequences of galanin from various species. The N-terminal is highlighted in bold and highly conserved throughout species. (Modified from Webling 2016)

<table>
<thead>
<tr>
<th>Species</th>
<th>Sequence</th>
</tr>
</thead>
<tbody>
<tr>
<td>Porcinea</td>
<td>GWTLNSAGYLLGPHAIDSHRSFHDKYGLA-amide</td>
</tr>
<tr>
<td>Bovineb</td>
<td>GWTLNSAGYLLGPHALDNHRSQFDKHGLA-amide</td>
</tr>
<tr>
<td>Ratc</td>
<td>GWTLNSAGYLLGPHAINHRSFSDKHGLT-amide</td>
</tr>
<tr>
<td>Chickend</td>
<td>GWTLNSAGYLLGPAVDNHRSPFDKHGF-amide</td>
</tr>
<tr>
<td>Humane</td>
<td>GWTLNSAGYLLGPHAVGHRSFSDKNGLTS</td>
</tr>
<tr>
<td>Sheeff</td>
<td>GWTLNSAGYLLGPHAIDNHRSFHDKHGLA-amide</td>
</tr>
<tr>
<td>Alligatorg</td>
<td>GWTLNSAGYLLGPHAIDNHRSFNEKHDGIA-amide</td>
</tr>
<tr>
<td>Bowlfinb</td>
<td>GWTLNSAGYLLGPHAVDNHRSLNDKHGLA-amide</td>
</tr>
<tr>
<td>Dogi</td>
<td>GWTLNSAGYLLGPHAIDNHRSFHEKPG-amide</td>
</tr>
<tr>
<td>Troutf</td>
<td>GWTLNSAGYLLGPHAIDGHRTLSDKHGLA-amide</td>
</tr>
<tr>
<td>Tunak</td>
<td>GWTLNSAGYLLGPHAIDNHRSFNDKHGLA-amide</td>
</tr>
<tr>
<td>Mousec</td>
<td>GWTLNSAGYLLGPHAIDNHRSFSDKHGLT-amide</td>
</tr>
<tr>
<td>Frogd</td>
<td>GWTLNSAGYLLGPHAIDNHRSFNDKHGLA-amide</td>
</tr>
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<td>Quaill</td>
<td>GWTLNSAGYLLGPHAIDNHRSFNDKHGLT-amide</td>
</tr>
<tr>
<td>Turtleo</td>
<td>GWTLNSAGYLLGPHAIDNHRSFNDKHGLA-amide</td>
</tr>
<tr>
<td>Macaquep</td>
<td>GWTLNSAGYLLGPHAIDNHRSFNDKHGLA-amide</td>
</tr>
<tr>
<td>Cichlidq</td>
<td>GWTMNSAGYLLGPHGLDHGLHTRGLDKPGLA-amide</td>
</tr>
</tbody>
</table>

a (Tatemoto et al., 1983), b (Rökaeus and Carlquist, 1988), c (Kaplan et al., 1988), d (Norberg et al., 1991), e (Schmidt et al., 1991), f (Sillard et al., 1991), g (Wang and Conlon, 1994), h (Y. Wang and Conlon, 1994), i (Boyle et al., 1994), j (Anglade et al., 1994), k (Habu et al., 1994), l (Lundkvist et al., 1995), m (Langel and Bartfai, 1998), n (Tsutsui et al., 1998), o (Y. Q. Wang et al., 1999), p (Cunningham et al., 2002), q (Hu et al., 2016).

1.5.2 Galanin message associated peptide, GMAP

There are a very limited number of studies regarding the localization and function of GMAP. Using immunohistochemistry GMAP was shown to be localized mostly in parallel with galanin but heterogenous expression was also reported (Hökfelt et al., 1992). Regarding the bioactive effect of GMAP has it been reported to affect spinal nociception in rat (Andell-Jonsson et al., 1997; Hao et al., 1999; X. J. Xu et al., 1995a; 1995b). Later, reports accumulated of an antimicrobial activity of GMAP on several strains of Candida (Holub et al., 2011; Rauch et al., 2007). But the receptors involved in GMAPs molecular mechanisms of action are however still not fully characterized but no binding to the three galanin receptors could be seen.
1.5.3 Galanin-like peptide, GALP

Galanin-like peptide, GALP, was originally isolated from porcine hypothalamus and shown to induce GTP-binding in a GAL2R-transfected cell line (Ohtaki et al., 1999). Interestingly is the amino acid sequence of GALP-(9-21) is identical to the highly conserved N-terminal amino acids of galanin i.e. galanin (1-13). GALP was initially presented as a GAL2R preferential ligands (20 times) (Ohtaki et al., 1999) but was later presented as a GAL2R preferential ligand (3 times) (Lang et al., 2005). However, several studies have indicated that GALP might bind to other receptors than galanin receptors (Man and Lawrence, 2008a). Krasnow and colleagues (2004) presented that injections of GALP had the same effect on food intake and body weight in both GAL1R-KO, GAL2R-KO and wild type, wt, littermates (Krasnow et al., 2004). To further strengthen that other receptor/receptors are involved, as well as elucidate the effect of GAL2R, they reported that the GALP fragment (1-21) were unable to give the effect of full length GALP (Krasnow et al., 2004).

The CNS distribution of GALP was reportedly very restricted - compared to galanin – with expression in the hypothalamic arcuate nucleus (ARC), the median eminence and infundibular stalk, and the posterior pituitary for rat, mice and macaque (Cunningham et al., 2002; Fujiwara et al., 2002; Juréus et al., 2000; Kerr et al., 2000; Larm and Gundlach, 2000; Takatsu et al., 2001) as well as GALP-immunoreactive, IR, fibers projecting to several forebrain regions (Takatsu et al., 2001). The bioactive effects of GALP have been reported to stimulate male sex behavior when administered i.c.v. in rats independently of testosterone milieu (Fraley et al., 2004; Stoyanovitch et al., 2005) with the opposite effect reported in mice (Kauffman et al., 2005). Central administration of GALP increased interleukin-1α and -1β, which mediates an anorectic and febrile action (Man and Lawrence, 2008b). The amino acid sequence of GALP was used to generate a GAL2R-preferentially binding ligand, also showed that the N-terminus can be chemically modified without losing affinity to the galanin receptor 2 (Runesson et al., 2009).
1.5.4 Alarin

The 25 amino acid long splice variant of GALP presented by Santic et al., (2006) was named alarin in a similar fashion as Professor Mutt had previously done for all their bioactive peptides, namely from the first and last amino acid, N-terminal alanine and C-terminal serine (Santic et al., 2006). Alarin expression have been shown to be much broader found as compared to GALP, and found in the accessory olfactory bulb, hypothalamus and locus coeruleus, LC (Eberhard et al., 2012; Van Der Kolk et al., 2010). Alarin has no detectable affinity to neither of the three galanin receptors (Boughton et al., 2010) and lacks homology to galanin. The bioactive effects of alarin was first described to involve vasoconstriction and anti-edema activity (Santic et al., 2007) and no effect on neither body temperature or male sexual behavior in contradiction to GALP (Fraley et al., 2012; Van Der Kolk et al., 2010). Thus, i.c.v. injection of alarin was reported to increase food intake in a dose-dependent manner (Van Der Kolk et al., 2010; Boughton et al., 2010).

1.5.5 Spexin or Neuropeptide Q, NPQ

Spexin is a 14 amino acid long peptide that was found to be able to bind GAL3R and GAL2R, with a preference for GAL3R (Kim et al., 2014). However, the GAL3R preference could not be replicated by the same group, rather they showed a weak GAL2R selectivity (Reyes-Alcaraz et al., 2016). Just like galanin is spexin C-terminally amidated in most examined species, thus having human and macaque as exceptions. Spexin and galanin share several identical amino acids, i.e. Trp^{2}, Thr^{4}, Tyr^{9}, Leu^{10} and Gly^{12} (Mirabeau et al., 2007; Schmidt et al., 1991; Cunningham et al., 2002) (See Table 5). Spexin is expressed in CNS, kidneys, heart, pancreas, liver, epithelial cells, skin, muscular tissue and to a smaller extent placenta (Mirabeau et al., 2007; Sonmez et al., 2009; Rucinski et al., 2010; Walewski et al., 2014; Gu et al., 2015). Interestingly, spexin have been described as mainly cytoplasmic for both rat and human (Porzionato et al., 2010). Spexin, just as galanin, has been shown to induce muscle contraction (Mirabeau et al., 2007). Furthermore, spexin has been shown to modulate cardiovascular functions, nociception and appetite (Rucinski et al., 2010; Toll et al., 2012; Wong et al., 2013; Walewski et al., 2014; Gu et al., 2015).
1.6 Molecular mechanisms of action

The three galanin receptors are members of the large superfamily of G-protein coupled receptors (GPCRs). However, each receptor differs in regional expression, affinity for different shortened galanin fragments (S. Wang et al., 1997b) and subsequent signal transduction pathways (See Figure 3). These differences between the galanin receptors indicate different possible physiological effects and subsequently plausible pharmacological relevance targeting the different galanin receptors.

When a galanin fragment binds to a galanin receptor, it could exert the same response as the parental peptide galanin and the fragment is called a full agonist. In case that only part of the parental peptide response is seen for the fragment, regardless if the fragment concentrations are increased, the fragment is denoted as a partial agonist. If no response could be detected even when the fragment is shown to bind to the receptor, the fragment is denoted as an antagonist. For some receptors with a basal level of activation, the fragment addition could result in a decrease of activation and subsequently be called inverse agonists.

The G-proteins are heterotrimeric and consists of $\mathrm{G}_\alpha,\beta\gamma$ upon activation will the heteromers disassociate into a $\mathrm{G}_\alpha$ and a $\beta\gamma$-subunits. $\mathrm{G}_\alpha$ is further subdivided into several subunits $\mathrm{G}_{\alpha_i},\mathrm{G}_{\alpha_o},\mathrm{G}_{\alpha_s},\mathrm{G}_{\alpha_q},\mathrm{G}_{\alpha_{11}},\mathrm{G}_{\alpha_{12}}$. Depending on which of these $\mathrm{G}_\alpha$ subunits that is present will the intracellular signaling cascade differ.

$\mathrm{G}_{\alpha_{i/o}}$ and $\mathrm{G}_{\alpha_s}$ modulate adenylate cyclase (AC) activity and subsequently increases cAMP levels. $\mathrm{G}_{\alpha_{q/11}}$ activates phospholipase C (PLC) which catalyzes the hydrolysis of phosphatidylinositol 4,5-biphosphate (PIP$_2$) to yield two intracellular second messengers, 1,2-diacylglycerol (DAG) and inositol 1,4,5-triphosphate (IP$_3$). IP$_3$ interacts with receptors on intracellular calcium stores on the ER which results in a cytosolic release of calcium while DAG can activate PKC. The $\mathrm{G}_{\alpha_{12/13}}$ activates the c-Jun N-terminal kinase (JNK), the $\mathrm{Na}^+$/H$^+$ exchanger and phospholipase D. The role of the $\mathrm{G}_{\beta\gamma}$ subunit is less studied, but it has been shown that the $\beta\gamma$-subunit was able to activate PLC and the G-protein coupled inwardly rectifying potassium channel, GIRK. The $\mathrm{G}_{\beta\gamma}$ subunit has also been reported to interact with arrestins that in turn have over 337 intracellular targets (Xiao et al., 2007).
Figure 3. Intracellular signaling pathways activated by the three galanin receptors (GAL1R-GAL3R). (Modified from Lang et al., 2015; 2007; Mons et al., 1998; Pan et al., 2014; Webling 2016; Wittau et al., 2000).

Abbreviations used: AC, adenylate cyclase; 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 1,4,5-triphosphate; MAPK, mitogen associated protein kinase; MEK, mitogen induced extracellular kinase; PDK-1, phosphoinosotide-dependent protein-kinase I; PIP2, phosphatidylinositol biphosphate; PIP3, phosphatidylinositol triphosphate; PI3K, phosphatidylinositol 3-kinase; PKB, protein kinase B; PLC, phospholipase C; RhoA, Ras homolog gene family member A.

One activated GPCR may in turn activate multiple G-proteins and each G-protein may activate a wide array of effector proteins, resulting in a considerable amplification and diversity of the signal. The heterotrimeric G-protein activation is further regulated by so called regulators of G-protein signaling (RGSs), G-protein receptor kinases (GRKs) and GPCR interacting proteins (GIPs).

Desensitization of GPCRs is a rather common phenomenon, that occurs through receptor phosphorylation by GRKs, enabling the recruitment of β-arrestins to the receptor, thereby blocking the interaction between the GPCR and G-proteins. β-arrestin itself can activate a wide array of different intracellular targets but also directs the GPCR to clathrin-coated pits, leading to receptor internalization and a second wave of signaling (Ferrie et al., 2013; Xiao et al., 2007). GPCR phosphorylation may also be mediated by second
messenger-dependent PKA or PKC (Luttrell, 2005). However, internalization of GPCRs have been considered a way to downregulate the receptors and end the signal. However, a third signaling wave have been shown to be generated from the internalized GPCRs in endosomes (Irannejad et al., 2013; Irannejan and von Zastrow 2014; Tsvetanova and von Zastrow 2014; Vilardaga et al., 2014; Tsvetanova et al., 2015; Gidon et al., 2016; Thomsen et al., 2016).

1.6.1 Galanin receptor 1, GAL1R

Galanin receptor 1 (GAL1R) was first cloned from human Bowes melanoma cells (Habert-Ortoli et al., 1994) and was followed by the rat GAL1R from Rin14B cells and cDNA from rat brain (Burgevin et al., 1995; Parker et al., 1995). Two years later, the mouse GAL1R was presented (Jacoby et al., 1997; S. Wang et al., 1997b). The species homology is high and the human GAL1R sequence compared to rat GAL1R was shown to be 93 % identical. GAL1R mRNA was first identified by northern blot in fetal brain and small intestinal tissues (Habert-Ortoli et al., 1994) and was later found in the gastrointestinal tract by using reverse transcript polymerase chain reaction (RT-PCR) (Lorimer and Benya, 1996). However, a later publication found GAL1R to be exclusively expressed in the CNS and PNS (Waters and Krause, 2000), where the highest levels were detected in the hippocampus, hypothalamus, amygdala, thalamus, cortex, brain stem (medulla oblongata, locus coeruleus), spinal cord, and dorsal root ganglia, DRG, (Gustafson et al., 1996). However, even wider central and peripheral tissue distributions of GAL1R have been reported (Sullivan et al., 1997).

Activation of GAL1R results in the pertussis toxin sensitive Gi/o-pathway activation where the enzyme adenylyl cyclase, AC, is inhibited, resulting in decreased levels of cAMP (Habert-Ortoli et al., 1994; Parker et al., 1995) (Fitzgerald et al., 1998; S. Wang et al., 1997b). Stimulation of the mitogen-activated protein kinase (MAPK) activity has also been presented with subsequent activation of G-protein-regulated inwardly rectifying K⁺ (GIRK) channels (Smith et al., 1998). The stimulation of the MAPK activity follows through a protein kinase C (PKC)-independent mechanism in concordance with the βγ-subunit being the mediator (S. Wang et al., 1998). GAL1R-KO mice were reported to exhibit spontaneous seizures (Fetissov et al., 2003; Jacoby et al., 2002; McColl et al., 2006) but other studies were not able to confirm these findings (Mazarati et al., 2004b).
1.6.2 Galanin receptor 2, GAL2R

Galanin receptor 2 (GAL2R) was first cloned from rat hypothalamus cells (Howard et al., 1997; Smith et al., 1997) followed by spleen cells in mice (Pang et al., 1998) and was cloned from a variety of human tissues (Bloomquist, 1998; Borowsky et al., 1998a). Comparing human GAL2R to the rat GAL2R reveals an 87% identity but with the difference that the human GAL2R has a 15 amino acid elongation at the C-terminus (Kolakowski et al., 1998; Waters and Krause, 2000).

Activation of GAL2R has been ascribed the ability to signal through several of the G-proteins (Wittau et al., 2000). The main signaling cascade is through Gq11 but signaling was also reported through Gi/o and G12/13. Thus, the Gs-pathway is not ruled out, indeed Gs proteins were even reportedly visible using photo affinity labeling on the examined H69 cells expressing GAL2R receptors only (Wittau et al., 2000).

Activating the PTX-insensitive Gq11 triggers the activity of phospholipase C (PLC) and intracellular phosphoinositol (IP) turnover, resulting in the release of Ca2+ into the cytoplasm from intracellular stores and subsequently opening of Ca2+-dependent channels (Kolakowski et al., 1998; Smith et al., 1997; S. Wang et al., 1998). GAL2R has also been shown to activate MAPK through a PKC dependent as well as through a Go class of G-protein dependent mechanism (S. Wang et al., 1998), resulting in a downstream phosphatidylinositol 3-kinase (PI3K)-dependent phosphorylation of protein kinase B (PKB). This in turn produces a suppression of caspase-3 and caspase-9 activity (Ding et al., 2006; Elliott-Hunt et al., 2007).

Activation of GAL2R was recently found to stimulate large conductance Ca2+-dependent K+ (BK) channels through the IP3 pathway (Pan et al., 2014). The activation of Gi/o class of G-protein pathways would inhibit forskolin stimulated cAMP production (Fathi et al., 1997; S. Wang et al., 1997a). Interestingly, a few studies have reported contradictory results of an increase in cAMP levels when cells were stimulated by full-length porcine galanin (Gu et al., 1994a; 1994b). Generally, an increase in cAMP levels is associated with activation of the Gs signaling pathway, leading to activation of AC followed by increased cAMP levels. Since the Gs-signaling route is presented as a very minor part of the signaling response, it has hardly been recognized in the literature. Instead, as peptide ligands displayed an increase in cAMP-levels, the result has been explained by the ligands acting as antagonists (Florén et al., 2000; Heuillet et al., 1994; Valkna et al., 1995), where galanin, used as a reference, showed a decrease in cAMP levels in the same studies. Instead, the high concentrations of Ca2+ intracellularly, resulting from the activation of the Gq11 signaling pathway, could stimulate AC leading to increased cAMP levels (Mons et al., 1998). Additionally, was the βγ
G-protein subunit and PKC was presented as a possible candidate to be able to stimulate AC and cause an increase in cAMP levels (Mons et al., 1998).

GAL\textsubscript{2}R has a wider expression pattern compared to GAL\textsubscript{1}R, due to the expression in multiple peripheral tissues including pituitary gland, gastrointestinal tract, skeletal muscle, heart, kidneys, uterus, ovary and testis as well as a wide spread in the CNS (Bloomquist, 1998; Smith et al., 1997; Waters and Krause, 2000). In the CNS, the highest concentrations of GAL\textsubscript{2}Rs are detected in hypothalamus, dentate gyrus, amygdala, piriform cortex, and mammillary nuclei (Mitchell et al., 1999; O'Donnell et al., 1999; Waters and Krause, 2000).

In contrast to the other galanin receptors, the expression levels of GAL\textsubscript{2}R have been found to vary during the development of the rat brain with a broad distribution that peaks at postnatal day 7, especially for the cortex and thalamus, and with significantly reduced levels after postnatal day 14 (Burazin et al., 2000). Lundström et al., (2005) performed an \textsubscript{L}-Ala-scan on the short galanin (2-11) subsequently highlighting Trp\textsuperscript{2}, Asn\textsuperscript{5}, Gly\textsuperscript{8} and Tyr\textsuperscript{9} as important amino acids for GAL\textsubscript{2}R interaction (Lundström et al., 2005a).

1.6.3 Galanin receptor 3, GAL\textsubscript{3}R

Galanin receptor 3, GAL\textsubscript{3}R, was first isolated from cDNA libraries of the rat hypothalamus (S. Wang et al., 1997a) followed by cDNA libraries from human cDNA (Kolakowski et al., 1998; Smith et al., 1998). Comparison of human GAL\textsubscript{3}R to rat GAL\textsubscript{3}R revealed a sequence identity of 90\% (Kolakowski et al., 1998). Despite that several cell lines are available with transfected GAL\textsubscript{3}R mRNA (Lang et al., 2005; Lu et al., 2005c) binding and signaling studies were not able to show receptor expression. In 2010, Runesson and co-workers presented a functional GAL\textsubscript{3}R tetracycline inducible cell line (Runesson et al., 2009) with affinity in concordance with literature (Branchek et al., 2000; Lu et al., 2005d). The GAL\textsubscript{3}R has been shown to involve several endoplasmic reticulum (ER) retention motifs (Robinson et al., 2013), which regulate intracellular trafficking of multiple GPCRs. The ER retention motifs would keep the receptor intracellularly in close association to the ER, and not in the cell membrane, which could explain the difficulty to achieve receptor binding results and subsequent signaling in the attempts to generate cell lines stably expressing GAL\textsubscript{3}R (Robinson et al., 2013).

Due to the limited cell models available to study this receptor, the signaling cascade of GAL\textsubscript{3}R is still rather ill-defined. Using oocytes or melanophores from the sub-saharan aquatic clawed frog (\textit{xenopus}), resulted in activation through \textsubscript{G}_{i/o} type of G-proteins inhibiting the AC and additionally opening of GIRK-channels (Kolakowski et al., 1998; Smith et al., 1998).
The distribution pattern of GAL3R was first thought to be very restricted compared to the other galanin receptors. Transcript levels of GAL3R were found at highest concentration in hypothalamus (Mennicken et al., 2002; Smith et al., 1998; S. Wang et al., 1997a) although other studies report a wider distribution through both central and peripheral tissues (Kolakowski et al., 1998; Waters and Krause, 2000).

An *in silico* study by Runesson et al., (2010) reported that docking of the short galanin fragment galanin (2-6) to human GAL3R identified Trp2, Thr3, Ser6 as important amino acids for ligand binding (Runesson et al., 2010). In 2013, Kothandan and colleagues performed an in silico study of full length human galanin binding to human GAL3R and through their results could highlight Gly1, Trp5, Asn5, Gly8, and Tyr9 to be of importance for receptor binding (Kothandan et al., 2013). Additionally, the C-terminal has been hypothesized as important for binding to GAL3R and to stabilize the peptide from proteolytic degradation, and Kothandan et al., (2013) were first to show additional agonistic interactions of Arg20, Lys25, Leu28 and Thr29 in the C-terminal of galanin with the GAL3R (Kothandan et al., 2013).

1.7 Galanin receptor ligands

Endogenous galanin binds with similar high affinity i.e. low nanomolar, nM, range to all three receptors (S. Wang et al., 1997a). The first 15 amino acids in the N-terminal part of galanin are highly conserved among species and are of essence for receptor interaction and biological activity (See Table 2). Exemplifying the importance of the N-terminal portion of galanin for receptor binding is galanin (1-16), which retains its high receptor affinity despite lacking almost half of the galanin sequence (See Table 3). An important advance in the field was the discovery of the galanin fragment, galanin (2-11), as a non-GAL1R ligand (H. X. Liu et al., 2001; Lu et al., 2005b). This finding that the receptors could be distinguished by the fact that GAL1R does not tolerate N-terminal deletions of the first glycine in comparison to the other two receptors has successfully been applied in the design of several peptides, including the GAL1R selective antagonist M871 (Sollenberg et al., 2006; 2010) and the GAL2R specific agonist M1145 (Runesson et al., 2009). Two GAL1R preferential ligands have also been developed, the M617 and the Gal-B2, with a modest 25-fold and a 15-fold selectivity for GAL1R compared to GAL3R (Bulaj et al., 2008; Lundström et al., 2005b). The GAL3R interaction for M617 has also been characterized, showing a 200-fold difference when compared with the preferential binding to GAL3R (Sollenberg et al., 2010).

Even though the N-terminal is important for receptor binding, some N-terminal alterations have been shown to retain high receptor binding affinity,
e. g. addition of amino acids (Runesson et al., 2009) and N-terminal methylation (Bulaj et al., 2008). Through 1-Ala-scans, where each amino acid in the sequence has been replaced by alanine, one at a time, and all the new ligands were tested for binding, several important amino acids for receptor binding have been revealed. When tested to hippocampal cell preparation, containing a mixture of all three galanin receptors, the following amino acids were highlighted as important for receptor binding

Table 3. Important amino acids in galanin receptors for ligand binding determined by either receptor mutation studies or in silico studies.

<table>
<thead>
<tr>
<th>Galanin receptor</th>
<th>Residue</th>
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<tbody>
<tr>
<td>GAL1R</td>
<td>Phe115&lt;sup&gt;a,b&lt;/sup&gt;, Phe186&lt;sup&gt;b&lt;/sup&gt;, His264&lt;sup&gt;a&lt;/sup&gt;, His267&lt;sup&gt;a&lt;/sup&gt;, Glu271&lt;sup&gt;a,b&lt;/sup&gt;, Phe282&lt;sup&gt;a&lt;/sup&gt;</td>
</tr>
<tr>
<td>GAL2R</td>
<td>His252&lt;sup&gt;c&lt;/sup&gt;, His253&lt;sup&gt;c&lt;/sup&gt;, Ile256&lt;sup&gt;c&lt;/sup&gt;, Phe264&lt;sup&gt;c&lt;/sup&gt;, Tyr271&lt;sup&gt;c&lt;/sup&gt;</td>
</tr>
<tr>
<td>GAL3R</td>
<td>Gln79&lt;sup&gt;d,e&lt;/sup&gt;, Ile82&lt;sup&gt;d,e&lt;/sup&gt;, Leu85&lt;sup&gt;d&lt;/sup&gt;, Asp86&lt;sup&gt;e&lt;/sup&gt;, Trp88&lt;sup&gt;e&lt;/sup&gt;, Phe90&lt;sup&gt;d&lt;/sup&gt;, Ala92&lt;sup&gt;d&lt;/sup&gt;, His99&lt;sup&gt;e&lt;/sup&gt;, Ile102&lt;sup&gt;e&lt;/sup&gt;, Tyr103&lt;sup&gt;d,e&lt;/sup&gt;, Tyr161&lt;sup&gt;d&lt;/sup&gt;, Tyr166&lt;sup&gt;d&lt;/sup&gt;, Glu170&lt;sup&gt;d&lt;/sup&gt;, Pro174&lt;sup&gt;e&lt;/sup&gt;, Ala175&lt;sup&gt;e&lt;/sup&gt;, Asp185&lt;sup&gt;e&lt;/sup&gt;, His251&lt;sup&gt;d&lt;/sup&gt;, Phe263&lt;sup&gt;d&lt;/sup&gt;, Tyr270&lt;sup&gt;d&lt;/sup&gt;, Arg273&lt;sup&gt;d,e&lt;/sup&gt;, His277&lt;sup&gt;d,e&lt;/sup&gt;, Tyr281&lt;sup&gt;e&lt;/sup&gt;</td>
</tr>
</tbody>
</table>

<sup>a</sup> (Berthold et al., 1997); <sup>b</sup> (Church et al., 2002); <sup>c</sup> (Lundström et al., 2005a); <sup>d</sup> (Runesson et al., 2010); <sup>e</sup> (Kothandan et al., 2013); <sup>f</sup> (Parthiban and Shanmughavel, 2007)
Table 4. Summarizing the affinity, Ki-values, for some commonly used galanin ligands of the three galanin receptors. (Modified from Webling et al., 2012)

<table>
<thead>
<tr>
<th>Ligand</th>
<th>GAL₁R</th>
<th>GAL₂R</th>
<th>GAL₃R</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>Rat galanin (1-29)</td>
<td>1.0 (r)</td>
<td>1.5 (r)</td>
<td>1.5 (r)</td>
<td>(S. Wang et al., 1997b)</td>
</tr>
<tr>
<td></td>
<td>0.3 (h)</td>
<td>1.6 (h)</td>
<td>12 (h)</td>
<td>(Borowsky et al., 1998a)</td>
</tr>
<tr>
<td></td>
<td>0.9 (h)</td>
<td>1.2 (h)</td>
<td>7.4 (h)</td>
<td>(Lu et al., 2005c)</td>
</tr>
<tr>
<td>Human galanin (1-30)</td>
<td>0.4 (h)</td>
<td>2.3 (h)</td>
<td>69 (h)</td>
<td>(Borowsky et al., 1998a)</td>
</tr>
<tr>
<td>Porcine galanin (1-29)</td>
<td>0.23 (h)</td>
<td>0.95 (h)</td>
<td>9.8 (h)</td>
<td>(Borowsky et al., 1998a)</td>
</tr>
<tr>
<td>Rat galanin (1-29)</td>
<td>1.5 (r)</td>
<td>1.5 (r)</td>
<td>1.5 (r)</td>
<td>(S. Wang et al., 1997b)</td>
</tr>
<tr>
<td></td>
<td>0.3 (h)</td>
<td>1.6 (h)</td>
<td>12 (h)</td>
<td>(Borowsky et al., 1998a)</td>
</tr>
<tr>
<td></td>
<td>0.9 (h)</td>
<td>1.2 (h)</td>
<td>7.4 (h)</td>
<td>(Lu et al., 2005c)</td>
</tr>
<tr>
<td>Human galanin (1-30)</td>
<td>0.4 (h)</td>
<td>2.3 (h)</td>
<td>69 (h)</td>
<td>(Borowsky et al., 1998a)</td>
</tr>
<tr>
<td>Porcine galanin (1-29)</td>
<td>0.23 (h)</td>
<td>0.95 (h)</td>
<td>9.8 (h)</td>
<td>(Borowsky et al., 1998a)</td>
</tr>
<tr>
<td>Galanin (1-16)</td>
<td>4.8</td>
<td>5.7</td>
<td>50</td>
<td>(S. Wang et al., 1997b)</td>
</tr>
<tr>
<td>Galanin (2-29)</td>
<td>85</td>
<td>1.9</td>
<td>12</td>
<td>(S. Wang et al., 1997b)</td>
</tr>
<tr>
<td>[w²]-galanin (1-29)</td>
<td>407</td>
<td>28</td>
<td>&gt; 1000</td>
<td>(Smith et al., 1998)</td>
</tr>
<tr>
<td>Galanin (3-29)</td>
<td>&gt; 1000</td>
<td>&gt; 1000</td>
<td>&gt; 1000</td>
<td>(S. Wang et al., 1997b)</td>
</tr>
<tr>
<td>Human GALP</td>
<td>77²</td>
<td>28²</td>
<td>10⁰</td>
<td>(Lang et al., 2005)</td>
</tr>
<tr>
<td>M15</td>
<td>0.65</td>
<td>1.0</td>
<td>1.0</td>
<td>(Smith et al., 1998)</td>
</tr>
<tr>
<td>M35</td>
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<td>2.0 (h)</td>
<td>nt</td>
<td>(Borowsky et al., 1998a)</td>
</tr>
<tr>
<td>M40</td>
<td>2.4 (h)</td>
<td>4.1 (h)</td>
<td>nt</td>
<td>(Borowsky et al., 1998a)</td>
</tr>
<tr>
<td>Galanin (2-11)</td>
<td>879⁴</td>
<td>1.8⁴</td>
<td>nt</td>
<td>(H. X. Liu et al., 2001)</td>
</tr>
<tr>
<td></td>
<td>&gt; 5000</td>
<td>88</td>
<td>271</td>
<td>(Lu et al., 2005b)</td>
</tr>
<tr>
<td>M1145</td>
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<td>6.55</td>
<td>497</td>
<td>(Runesson et al., 2009)</td>
</tr>
<tr>
<td>M1153</td>
<td>1890</td>
<td>4.98</td>
<td>230</td>
<td>(I. Saar et al., 2011a)</td>
</tr>
<tr>
<td>M1160</td>
<td>15 500</td>
<td>33.3</td>
<td>&gt; 10 000</td>
<td>(I. Saar et al., 2013)</td>
</tr>
<tr>
<td>M617</td>
<td>0.23 (h)</td>
<td>5.7 (h)</td>
<td>nt</td>
<td>(Lundström et al., 2005b)</td>
</tr>
<tr>
<td></td>
<td>nt</td>
<td>nt</td>
<td>49 (h)</td>
<td></td>
</tr>
<tr>
<td>M871</td>
<td>420 (h)</td>
<td>13 (h)</td>
<td>nt</td>
<td>(Sollenberg et al., 2006)</td>
</tr>
<tr>
<td></td>
<td>nt</td>
<td>nt</td>
<td>&gt; 10 000(h)</td>
<td>(Sollenberg et al., 2010)</td>
</tr>
<tr>
<td>Gal-B2</td>
<td>3.5 (h)</td>
<td>51.5 (h)</td>
<td>nt</td>
<td>(Bulaj et al., 2008)</td>
</tr>
<tr>
<td>[N-Me, des-Sar]Gal-B2</td>
<td>364 (h)</td>
<td>20 (h)</td>
<td>nt</td>
<td>(Robertson et al., 2010a)</td>
</tr>
<tr>
<td>NAX 409-9</td>
<td>242</td>
<td>60.4</td>
<td>nt</td>
<td>(Metcalf et al., 2015; L. Zhang et al., 2013)</td>
</tr>
<tr>
<td>NAX 810-2</td>
<td>494</td>
<td>31.7</td>
<td>nt</td>
<td>(Bialer et al., 2013; 2015; Metcalf et al., 2017)</td>
</tr>
</tbody>
</table>

w = D-tryptophan; h = human; r = rat; a = reported as IC₅₀-values; nt = not tested
1.8 Non-peptide galanin ligands

Galnon was identified after screening a combinatorial peptidomimetic library. It acts as an agonist in functional studies both in vitro and in vivo (Bartfai et al., 2004; K. Saar et al., 2002). It has been evaluated in models for anxiety and depression (Rajarao et al., 2007), feeding (Abramov et al., 2004) and pain (W.-P. Wu et al., 2003).

Galmic is a non-peptide agonist with micromolar, uM, affinity for GAL1R and a very low – if non- affinity for GAL2R (Bartfai et al., 2004), which under conditions of intrahippocampal administration was 6-fold more potent than galnon in inhibiting self-sustaining status epilepticus, in 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 which results in unwanted physiological effects (Florén et al., 2005; Lu et al., 2005d).

The metabolite Sch 202596, originated from an Aspergillus sp. culture found in an abandoned uranium mine in Tuolumene County California, was found to have a modest affinity to GAL1R in vitro (Chu et al., 1997). Based on these findings, were several 1,4-dithiins and dithiipine-1,1,4,4-tetroxides with binding affinity to GAL1R identified (M. K. Scott et al., 2000). The compound 2,3-dihydro-2-(4-methylphenyl)-1,4-dithiepine-1,1,4,4-tetroxide was reported to be an antagonist with high affinity for GAL1R with an IC50 of 190 nM for GAL1R and above the highest tested concentration (30 μM) for GAL2R. 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 (Kozoriz, 2006; Mahoney et al., 2003).

A series of 3-imonio-2-indolones were identified as specific GAL3R antagonists, with Kᵢ values for GAL3R as low as 17 nM and above tested 10 μM for the other receptors studied (Konkel et al., 2006a). One of these was referred as SNAP37889 in (Swanson et al., 2005a). One drawback of the above mentioned indolones is the low aqueous solubility (less than 1 μg/ml) which resulted in the identification of a compound with an increased water solubility and selectivity, 1,3-dihydro-1-[3-(2-pyrrolidinylethoxy)phenyl]-3-[[3-(trifluoromethyl)phenyl]imino]-2H-indol-2-one, referred as SNAP398299 (Konkel et al., 2006b; Swanson et al., 2005a). Both of these SNAP compounds have been used in several studies to delineate the effect of GAL3R.

A series of 2,4,6-triaminopyrimidines were present as both GAL1R and GAL2R selective compounds with Kᵢ-values starting from 330 nM (Sagi et al., 2011). Studies from the same institute led to characterization of the first
identified allosteric modulator, named CYM2503, for the galanin receptor family, i.e. GAL2R (Lu et al., 2010). CYM2503 failed to displace galanin in binding studies and showed no detectable signaling by itself, but potentiated the effect of galanin when administered simultaneously and is presented as a GAL2R positive allosteric modulator (Lu et al., 2010). Among these non-peptide galanin ligands have the SNAP compounds, SNAP 37889 and SNAP 398299, been the most widely used in research. Thus, Koller et al., (2016) reported that SNAP 37889 was cytotoxic in several cell lines regardless of the galanin receptor expressed which subsequently limits the usefulness of these compounds (Koller et al., 2016).

1.9 Clinical applications

The different distribution patterns of the three galanin receptors in the CNS and in some peripheral tissues as well as the different binding affinities for shortened galanin fragments indicate specific involvement in different biological and pathological functions (Borowsky et al., 1998; Lu et al., 2005d; Smith et al., 1998; S. Wang et al., 1997b). Several studies using knockout (KO)-animals along with overexpression (OE)-animals and the use of specific and selective galanin receptor ligands have started to delineate the galaninergic system and highlighted a possible use of galanin receptor agonists and antagonists in several biological as well as pathological functions (Wibling et al., 2012). For example, agonists for GAL1R and GAL2R (Bulaj et al., 2008; Mazarati et al., 2004a; Mazarati, 2004; Robertson et al., 2010b) have been reported as possible antidepressants and anxiolytics. Additionally, GAL2R agonist (Kuteeva et al., 2008) or antagonists for either GAL1R or GAL2R (Ash et al., 2010; Kuteeva et al., 2008; Swanson et al., 2005a) are reported as possible antidepressants. The acute feeding behavior was shown to be increased when administrating a GAL1R agonist (Anderson et al., 2013; I. Saar et al., 2011b) and a GAL1R antagonist was reported to reduce alcohol consumption (Ash et al., 2013).

1.9.1 Depression and mood disorders

The findings that galanin is co-localized with and affects noradrenaline (NA) release in the locus coeruleus (LC) and in addition also affects serotonin (5-HT) release in the dorsal raphe nucleus (DR) in rat raised the question that galanin could be involved in mood disorders like depression (Hedlund et al., 1991; Kehr et al., 2002; Melander et al., 1986; Razani et al., 2000; Skofitsch and Jacobowitz, 1985; Z. Xu et al., 1998a; 1998b). Furthermore, galanin was also reported to downregulate the 5-HT1A receptor (Kuteeva et al., 2008) and
that increased levels of NA and 5-HT through the use of imipramine and citalopram, two commonly used antidepressants, were significantly hindered by intracerebroventricularly (i.c.v.) administration of galanin (Yoshitake, 2003). Both GAL1R antagonists (Kuteeva et al., 2008), GAL2R agonists (Kuteeva et al., 2008) and GAL3R antagonists (Ash et al., 2010; Barr et al., 2006; Kuteeva et al., 2008; Swanson et al., 2005a) have been reported as potential antidepressants and antianxiety agents. Furthermore, galanin has been shown to exert antidepressant properties when administered intravenously, i.v, in a clinical study on diagnosed depressed patients (Murck et al., 2004).

### 1.9.2 Neuroprotection

Several studies have reported up-regulation of both galanin and mRNA levels of galanin in neurons exposed to different sorts of lesions studied in both the CNS and the PNS. The first report of an increase of galanin mRNA after peripheral axotomy in DRG neurons was published already in 1987 (Hökfelt et al., 1987). Subsequently, have several studies confirmed these results and several groups have raised the hypothesis that galanin exerts neuroprotective effects. Using transgenic galanin-KO mice, exposure to kainic acid (KA) intraperitoneally (i.p.) resulted in a more prominent cell death and in concordance with this, galanin-OE mice showed a decrease in cell death for i.p. administration of KA (Elliott-Hunt et al., 2004). Hippocampal cultures from the galanin OE mice have shown that endogenous galanin decreased excitotoxicity and apoptosis (Elliott-Hunt et al., 2004). Furthermore, a non-selective galanin receptor antagonist administered into hippocampal areas after systemic administration of KA significantly increased cell death (Schauwecker, 2010).

### 1.9.3 Epilepsy

Among the earliest reports of biological effects of galanin were the decreased excitability of myenteric neurons (Tamura et al., 1988) and cardiac ganglia (Konopka et al., 1989). Additionally, the hippocampus, which is an important brain structure involved in the initiation and maintenance of seizures, have a large galaninergic innervation (Lu et al., 2005d; Webling et al., 2012). Epileptic seizures were also shown to deplete galanin (Mazarati et al., 1998). Galanin immunoreactivity in hippocampal areas were significantly depleted for up to a week after performing performant path model to obtain self-sustaining status epilepticus, SSSE, in rats (Mazarati et al., 1998).
Using a picrotoxin-kinedeled seizure model, it was shown that galanin had an anticonvulsant effect (Mazarati et al., 1992). Furthermore, administration of galanin i.c.v. were reported to be an anticonvulsant in several animal studies either using pentylenetetrazol, PTZ, kainic acid, KA, or Li-Pilocarpine induced seizures (Chepurnov et al., 1998; Kong et al., 2008; Mazarati et al., 1998; 2000; Schauwecker, 2010; Wilson et al., 2005), or performant path stimulation (Mazarati et al., 2004b; 1998). The galanin receptors expressed in the hippocampus is both GAL1R and GAL2R at relatively high levels while the expression of GAL3R is still not well characterized (Burazin et al., 2000; O'Donnell et al., 1999; Hohmann et al., 2003; Lu et al., 2005d; Welling et al., 2012). Galanin-KO mice reportedly had a lower threshold for developing seizures in both performant path stimulation and KA induced seizure model, with neuronal damage in hippocampal regions (Mazarati et al., 2000). Galanin-OE mice had a higher threshold for seizures induced by performant path stimulation, KA- or PTZ- induced seizures (Lin et al., 2003; Mazarati et al., 2000) as well as being less affected in hippocampal kindling, a human complex partial epilepsy model (Kanter-Schlifke et al., 2007; Kokaia et al., 2001).

Several techniques have been used to delineate the role of the different galanin receptors in their role in seizures. GAL1R-KO mice showed an increased severity of seizures when seizures were induced by either Li-pilocarpine or performant path stimulation, but this could not be seen when seizures were induced by kainic acid (Mazarati et al., 2004b). Inbred mice with a lower expression of GAL1R compared to wildtype, wt, showed a greater cell death in several hippocampal regions when exposed to KA (Kong et al., 2008; Schauwecker, 2010). Studies have also shown that GAL1R-KO mice display spontaneous seizures (Fetissov et al., 2003; Jacoby et al., 2002; McColl et al., 2006) even though other research groups could not replicate these findings (Mazarati et al., 2004b). GAL2R-KO mice showed no difference in seizure susceptibility (Gottsch et al., 2005), but when peptide nucleic antisense, PNA, nucleotides were used to downregulate the receptor was an increased severity of seizures seen as well as cellular damage in hippocampal regions i.e. GABAergic hilar interneurons (Mazarati et al., 2004b). Further, Lu and co-workers (2010) reported that CYM2503, an allosteric positive modulator of GAL2R, when injected i.p. to rats and mice decreased the seizure time and prolonged the latency time before seizures were initiated in two different seizure models, namely Li-pilocarpine induced seizures and electroshock-induced seizures (Lu et al., 2010). The use of neuropeptides as possible pharmaceuticals indicates several ad-
vantages since they are highly potent as concentrations in low nanomolar range and prolonged action periods is needed. One limiting aspect of neuropeptides as possible pharmaceuticals is their fast degradation which subsequently limits the administration routes. However, systemically active galanin analogues with effects seen when administered i.v. have been presented with preference for either GAL1R (Bulaj et al., 2008) or GAL2R (Metcalf et al., 2017; Robertson et al., 2010a). Both galanin receptor 1 and 2 analogues were shown to prevent seizures in mice using a 6 Hertz, Hz, seizure model of pharmaco-resistant epilepsy (Bulaj et al., 2008; Robertson et al., 2010b) and other seizure models (Metcalf et al., 2017; White et al., 2009).
2. Aim

The general aim was to design and generate new galanin ligands that selectively/specifically binds to one or two of the three galanin receptors by chemically modify the galanin sequence in order to provide tools for delineating the galaninergic system.

♥ Paper 1: The aim of this paper was to investigate the binding profile of a short galanin fragment, galanin 2-11, where the Ala5Ser amino acid substitution originally found in tuna fish was included. Additionally, two classical signaling assays used to measure GPCR signaling were compared with two real-time label-free techniques on both galanin receptor overexpressing cells and cell lines with a low galanin receptor expression.

♥ Paper 2: The aim of this study was to design a new galanin ligand selective for galanin receptor 1 and 2 and to compare two classical second messenger assays, with a real-time label-free impedance-based technique. Furthermore, investigate whether the neuroprotective effect after KA-induced cell death in the hippocampus was mediated through GAL1R or GAL2R in an animal model.

♥ Paper 3: The aim of this study was to chemically stabilize peptide ligands by using addition of cationic charges, addition of a fatty acid and by introducing non-natural amino acids and/or non-proteinogenic amino acids. To evaluate the pharmacological properties of the stabilized peptide ligands including toxicity, biodistribution and chronic administration. Furthermore, compare the stabilized peptide ligands alongside the commonly used antidepressant imipramine in two screening animal models for possible antidepressants.
Paper 4: The aim of this study was to examine the role of galanin in epilepsy. A genetic mutation found in the preprogalanin gene found in temporal lobe epilepsy patients which resulted in an amino acid substitution in the N-termini of galanin, namely p. (A39E). The aim was to evaluate the binding and signaling properties of the mutation variant to clarify the role of galanin in temporal lobe epilepsy, TLE.
3. Methodological considerations

The detailed description of all methods used in this thesis can be found in each included paper and the theoretical background of the methods will be discussed herein.

3.1 Solid phase peptide synthesis

All peptides used were synthesized using solid phase peptide synthesis (SPPS) (See Table 5 for sequences). This is the most common method for producing peptides, both for research and therapeutic purposes. The SPPS method was invented by Merrifield (Merrifield, 1963), who was awarded the Nobel Prize in chemistry in 1984 for his findings. SPPS is an iterative process of coupling, washing, deprotection and subsequent washing (See Figure 4). The peptide is attached to an insoluble resin at its C-terminus and the peptide grows in a stepwise manner towards the N-terminus. All byproducts that result from the coupling and deprotection reactions are eliminated through filtration during the washing steps. Amino acids used in SPPS contain protecting groups at both their α-amino group and at their possible reactive side chains. In each step, the carboxylic group of the new amino acid is activated, in all papers is this activation obtained by transforming the amino acids to active esters which results in a better leaving group compared to carboxylic acids, this allows a nucleophilic attack by the amino group of the previously coupled amino acid. In order to make the reaction specific the α-amino group is protected by a 9-fluorenylmethoxycarbonyl (Fmoc) group that easily can be removed by the addition of a weak base, most commonly used is piperidine.

To obtain the C-terminally branched peptides with stearic acid, a special protection group was used on the side chain of lysine: Fmoc-Lys (Mtt)-OH. The 4-methyltrityl, (Mtt), protection group was removed manually with dichloromethane (DCM) containing 1% trifluoroacetic acid (TFA) and 2% triisopropylsilane (TIS). After the assembly of the desired peptide and possible modifications was complete, the peptide was cleaved from the resin and
all side chain-protecting groups were removed. In Fmoc chemistry the cleavage reaction uses a mixture composed of concentrated TFA and low amounts of scavengers, such as water and TIS that react with the released side-chain protecting groups. The peptide was then precipitated using ice-cold diethyl ether. For peptides in paper 1, 2 and 3, were p-methylbenzylhidrylamine (MBHA) used, generating C-terminally amidated peptides after final cleavage whereas in paper 4 was a HMPB-ChemMatrix resin used to obtain a free acid at the C-terminus. The peptides were synthesized on a Syro multiple peptide synthesizer (MultiSynTech GmbH).

All peptides were purified through reverse-phase high performance liquid chromatography (RP-HPLC) where the solvent, in this case acetonitrile and water containing 0.1% TFA, was used to elute the peptides at high pressure through a silica column, which could be a BioBasic C8 column (octylsilane) or a C4 column (Sigma-Aldrich, Sweden).

Finally, the identities of the peptides were confirmed by either Electrospray ionization mass spectrometry ESI-MS or Matrix-assisted laser desorption/ionization time-of-flight mass spectrometry (MALDI-TOF) (Voyager-DE STR, Applied Biosystems).

Electrospray ionization is a gentle method to generate charged molecules of non-volatile samples. In electrospray ionization mass spectrometry, small charged droplets of the sample are generated by spraying the sample at atmospheric pressure through a capillary in a strong electrical field. By evaporation of the solvent, the size of the droplets decrease, and the droplets are led through a region of low pressure towards and ion source. At this stage, the droplets are small enough for the electrostatic repulsion between ions at the surface of the droplets to exceed the liquid surface tension and thereby generating free ions. These ions are separated according to their mass-to-charge, m/z, ratio, before reaching the detector.

In matrix-assisted laser desorption/ionization time-of-flight mass spectrometry, a small sample of the purified peptide is mixed and co-crystallized with a large quantity of matrix. The matrix is excited by pulsed laser UV light and a small part of the matrix quickly heats and desorbs from the surface, together with the sample. Charge is transferred from the ionized matrix to the analyte, which generates free analyte ions. These ions travel due to a potential difference V₀, which is constant with respect to all ions. The law of conservation of energy can be used to determine the velocity of the attracted ions. Ions with smaller mass-to-charge ratio (m/z), thus monocharged ions of lower mass and more highly charged ions, move faster. Consequently, the
time of ion flight differs according to the m/z value of the ion.

Figure 4. A schematic representation of solid phase peptide synthesis, where the amino acids are coupled in a stepwise manner from the C-terminal towards the N-terminal with repeated cycles of washing and deprotection. Depending on the resin used, the C-terminal can have a free carboxyl acid or be C-terminally amidated. Abbreviations used: AA, amino acid; P, protection group; R, Fmoc-protection group. (Modified from Palomo J. M. 2014).
Table 5. Amino acid sequence of the ligands used or discussed in the four papers.

<table>
<thead>
<tr>
<th>Name</th>
<th>Sequence</th>
</tr>
</thead>
<tbody>
<tr>
<td>M35</td>
<td>GWTLNSAGYLLGPFGSPFR-amide</td>
</tr>
<tr>
<td>rat Galanin (1-16)</td>
<td>GWTLNSAGYLLGPHAI-amide</td>
</tr>
<tr>
<td>Galanin (2-11)</td>
<td>WTLNSAGYLL-amide</td>
</tr>
<tr>
<td>Human GALP</td>
<td>APAHRGRGGGWTLNSAGYLLGPVLHPQMGDQDKRETAEILDWLWKAIDQLPYSHPQPS</td>
</tr>
<tr>
<td>M617</td>
<td>GWTLNSAGYLLGPQGFSPFR-amide</td>
</tr>
<tr>
<td>M871</td>
<td>WTLNSAGYLLGPEHPMPALALA-amide</td>
</tr>
<tr>
<td>M1145</td>
<td>RGRGNWTLNSAGYLLGPVLPMPALALA-amide</td>
</tr>
<tr>
<td>M1153</td>
<td>RGRGNWTLNSAGYLLGPK(εNH.C(O)Glu)-amide</td>
</tr>
<tr>
<td>M1154</td>
<td>GWTLNSAGYLLPQGFSPFA-amide</td>
</tr>
<tr>
<td>J17</td>
<td>RGRGNWTLNSAGYLLGPKKK(εNH.C(O)stearic acid)-amide</td>
</tr>
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<td>J18</td>
<td>RGRGNWTLNSAGYLLGPKKK(εNH.C(O)stearic acid)k-amide</td>
</tr>
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<td>J19</td>
<td>RGRGNWTLNSAGYLLGPOOK(εNH.C(O)stearic acid)O-amide</td>
</tr>
<tr>
<td>J20</td>
<td>RGRGNWTLNSAGYLLGPXXK(εNH.C(O)stearic acid)K-amide</td>
</tr>
<tr>
<td>J21</td>
<td>OGOGNWTLNSAGYLLGPOOK(εNH.C(O)stearic acid)O-amide</td>
</tr>
<tr>
<td>Ala5-galanin (2-11)</td>
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<td>Phe9-galanin (1-15)</td>
<td>GWTLNSAGFLLGPHA-amide</td>
</tr>
<tr>
<td>Pro11-galanin (1-15)</td>
<td>GWTLNSAGYLPGPHA-amide</td>
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<td>Phe9-galanin (2-15)</td>
<td>WTLNSAGFLLGPHA-amide</td>
</tr>
<tr>
<td>Phe9, Pro10-galanin (2-15)</td>
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</tr>
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<td>Human spexin</td>
<td>NWTPQAMLYLKGAQ-amide</td>
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<td>GWTLNSAGYLLGPHAVGNHRSFDKNGLTS</td>
</tr>
<tr>
<td>Glu1-galanin 1-30</td>
<td>GWTLNSEGYLLGPHAVGNHRSFDKNGLTS</td>
</tr>
</tbody>
</table>

Non-proteinogenic amino acids used: O = ornithine; X = 6-aminohexanoic acid; ε-amino acids are denoted with lower-case letters, here ε-lysine is k.
3.2 Cell cultures

Several different cell lines have been characterized as good expression systems for the study of galanin receptors. Bowes melanoma cells are epithelial cells endogenously expressing only GAL1R with a high concentration of binding sites (540 fmol/mg protein) (Heuillet et al., 1994). However, a later publication highlighted an additional low expression of GAL2R in Bowes melanoma cells (Lang et al., 2001). Two stable over-expression cell lines were used when characterizing the GAL2R – Chinese hamster ovary (CHO)-K1 cells for human GAL2R and human embryonic kidney (HEK) cells for rat GAL2R. Studies have shown that the high sequence similarity between the rat and human galanin receptors also leads to similar binding affinities for both rat and human galanin (Borowsky et al., 1998a; S. Wang et al., 1997a).

In 2009, Runesson and colleagues presented a tetracycline inducible human GAL3R cell line in HEK 293 cells (Flp-In T-REx 293 cells) (Runesson et al., 2009). These cell lines have been extensively used in our lab throughout the years and have shown to express all three galanin receptors with similar concentrations. Additionally, SH-SY5Y cells, a human neuroblastoma cell line, was used which had a stable expression of either rat GAL1R or rat GAL2R with a tetracycline inducible overexpression.

The use of tetracycline for an induction of expression is based on an expression system where the gene of interest is controlled by the cytomegalovirus, CMV, promoter in combination with two inserted tet operator sequences (TetO2), that inhibits the expression of the gene of interest. Upon addition of tetracycline, the Tet repressor is no longer able to bind the tet operator and the gene of interest is expressed (Yao et al., 1998).

3.3 Galanin receptor binding studies

Membrane preparations using the cell lines presented above were used. Cells were cultured in petri dishes and detached by scraping in phosphate buffered saline (PBS) lysed on ice in ethylenediaminetetraacetic acid (EDTA) supplemented buffer and collected after centrifugation at 8500 x g. The displacement reaction was performed using 30 μg protein with 0.1 nM galanin labeled with the radioisotope, ¹²⁵I. The binding reaction was allowed to incubate for 30 minutes at 37 °C while gently shaking before being terminated by rapid filtration over glass fiber filters.

The ¹²⁵I-labeled galanin was from porcine origin even though human ¹²⁵I-galanin is available. The radiolabeling is substituted onto the side chain of the amino acid tyrosine. In human galanin, the tyrosine is placed at position 9, whereas in porcine galanin it is placed at position 26. Since the N-terminus has been presented as strongly related to the receptor binding por-
cine galanin is most commonly used to avoid possible interference from the labeling (Land et al., 1991).

### 3.4 cAMP measurements

All three galanin receptors activate the $G_{i/o}$ subunit of G-proteins, which leads to an inhibition of adenyl cyclase, AC, and a subsequent decrease of cAMP levels (Branchek et al., 2000). Forskolin was added as an AC stimulator to facilitate the examination of a ligand induced decrease of cAMP. Degradation of cAMP was further inhibited by the addition of phosphodiesterase inhibitor 3-isobutyl-1-methylxanthine (IBMX).

### 3.5 Inositol phosphate measurements

To examine the activation of $G_q$ proteins, the intracellular production of tritium labeled inositol phosphate (IP) after stimulation was measured. This strategy is a commonly used method to measure second messengers and does not require advanced equipment (Borowsky et al., 1998b; Mei et al., 1989). Lithium chloride (LiCl) is used to block the subsequent recycling of inositol phosphate (IP) to myo-inositol, which leads to accumulation of IP. The three forms of IPs, namely $IP_3$, $IP_2$ and IP, were extracted using perchloric acid and separated on an anion exchange column. The IPs were eluted and the radioactivity was measured in a $\beta$-counter and normalized to the total count of each sample.

### 3.6 Galanin receptor signaling studies using real-time label free assays (Paper 1 and 2)

Recent findings that several GPCRs naturally exist in heteromers (Birdsall, 2010; Fuxe et al., 2012; 2014; Goupil et al., 2013) and using several different signaling cascades (Lang et al., 2015; 2007; Wittau et al., 2000) have pointed out the classical second messenger assays as inadequate to measure GPCR signaling. Galanin has been ascribed the ability of being a biased ligand i.e. prefers one signaling cascade over several others. However, this property will make it difficult to distinguish the contribution of each single pathway using specific inhibitors. Furthermore, the signaling cascades have also proven far more complex, where activated $\beta$-arrestins trigger a second wave of cell signaling (Ferrie et al., 2013) alongside a third wave of GPCR signaling from internalized receptors in endosomes (Irannejad et al., 2015; Irannejad and Zastrow, 2014; Irannejad et al., 2013; Tsvetanova et al., 2015). New real-time label-free techniques have been introduced by a number of
companies for studying cell adhesion, toxicity, cell migration and receptor-mediated signaling, in particular GPCR signaling. When labeling of neither receptor nor ligand is required, the assay design is facilitated and possible artifacts from the labeling process can be avoided.

Used herein are two real-time label-free techniques: an impedance based real-time label-free technique developed by ACEA Bioscience Inc. (San Diego, CA, USA) and an optical biosensor developed by Perkin Elmer, (Austria). Briefly, for the impedance based technique the 96-well plates with evenly distributed gold electrodes throughout the bottom were used to culture cells onto. A weak current was added and the local ionic environment at the electrode surface was detected. Small changes in the physical contact between cell and electrode, such as cell number, adhesion, and morphology changes were detected.

For the optical based technique, cells were grown in specific Enspire LFC-348 well plates with a patented optical biosensor integrated in each well. The cells were exposed to light and the reflected wavelengths were detected. Changes in light refraction results from changes in dynamic mass redistribution, DMR, upon receptor activation regardless of G-protein signaling pathway. One difference between these techniques is that the experiments performed with the impedance based technique, xCELLigence, are performed in cell culture conditions i.e. inside a cell culture incubator, whereas the optical based technique, EnSpire, experiments are not. Both techniques have been ascribed the ability to detect small cellular changes that would not be detected in normal assay conditions which may not provide adequate signal-to-background readings.

### 3.7 Animal studies

In Paper 2, adult male Sprague-Dawley rats were used, which were allowed to adapt to the animal facility for 7 days prior to experiments. In Paper 3, Balb/c (Harlan) mice of both sexes were used with ages ranging between 8-10 weeks by the start of the experiment. The GAL2R-KO animals were generated on a 129/Sv genetic background (Shi et al., 2006) and backcrossed into C57BL/6 for eight generations and both males and females were used in the experiment.

Both rats and mice were kept in a temperature controlled environment with a 12-hour light/dark cycle and ad libitum access to food and water. Animal studies were performed in accordance to the NIH Guide for Care and Use of Laboratory Animals approved by the University of Georgia Animal Care and Use Committee (Paper 2) and Estonian laboratory animal ethics committee (Paper 3).
3.8.1 Cell counts in the hippocampus (Paper 2)

Rats were sacrificed and brains collected. The brains were fixed in a 4% formalin solution and stored at -20 °C. Sections of 20 μm from both ventral and dorsal hippocampus were thaw mounted onto slides and Nissl-stained to visualize the cell nucleus. Nissl staining is a method to visualize the nucleus by adding basic dyes such as anilin, cresyl violet, methylene blue, thionine or toluidine blue that binds to negatively charged DNA or RNA (Kádár et al., 2009). Lastly, the cell numbers in the CA3 region were counted under microscope.

3.8.2 Behavioral characterization

Administration of kainic acid was used to induce seizures in mice in paper 2 and the severity of the seizures were graded according to the Racine’s scale (Racine, 1972a; b), which is one of the most commonly used. However, a small modification was made because the injection method itself occasionally resulted in behavior that might be confused with the seizure activity (Reiss et al., 2009). For statistical analysis, the average seizure score was used.

3.8.3 Cannulation

Both rats (Paper 2) and mice (Paper 3) were cannulated for the i.c.v administration of peptide ligands. The mice were anesthetized and cannulated by stainless steel hypodermic tubing using the co-ordinates from Bregma. The cannula was kept in place by stainless screws and dental cement and the mice were allowed to recover from the surgery for a minimum of 7-10 days before used in experiments. After the experiments, animals were decapitated under deep anesthetic and the correct placing of the cannula was verified by visual inspection.

The rats were anesthetized and mounted in a stereotactic frame and cannulated according to the coordinates from Bregma. The cannula was held in place by stainless steel screws and epoxy. The rats were allowed to recover for one week before used in experiments and were subsequently euthanized and the correct placement of the cannula was confirmed by injection of fast green dye upon visual inspection. Naïve rats used as controls did not undergo cannulation surgery or administration of any pharmaceuticals.
3.8.4 Induced cell death by kainic acid administration (Paper 2)

Previous studies have shown that galanin could reduce cell death. However, studies about the galanin receptors responsible for the neuroprotective effects have resulted in rather contradictory data. Both GAL1R and GAL2R have been ascribed neuroprotective effects (Elliott-Hunt et al., 2007; Mazarati and Lu, 2005; Schauwecker, 2010; Webling et al., 2012) To delineate the neuroprotective effects of galanin, a previously characterized model in Professor P. V. Holmes laboratory was used (Reiss et al., 2009). Intracerebroventricular administration of KA induced neuronal death since KA is an undegradable analogue of the excitatory amino acid glutamate. A constant excitatory stimulation leads to cell death, so called excitotoxicity. The administration of the excitotoxic KA results in prominent neuronal cell death, primarily in the CA3 area of the hippocampus and depending on dose it can also cause epileptic seizures. Herein, a previously optimized dose of KA was administered i.c.v to ensure neuronal cell death by direct excitotoxic mechanisms (Reiss et al., 2009), subsequently allowing the neuroprotective effects of the peptide ligands to be evaluated.

3.8.5 Mouse model for screening for novel antidepressants (Paper 3)

Two commonly used models to screen for new antidepressant substances were used in this study, namely the tail suspension test and the forced swimming test. For both these models the immobility time was measured. The immobility time was defined as no moving other than respiration when mice were either swimming or hanging upside down. A general characteristic of approved well-known antidepressants is a decrease in immobility time. The simplicity of these two depression models has made them commonly used for screening and evaluating leads for new antidepressants. In the tail suspension test, mice were suspended by the tail to a wooden rod using adhesive tape and studied for 6 minutes. For statistical evaluation, only the last 4 minutes were used.

The forced swimming test was performed by placing one mouse at a time in a water-filled glass cylinder and studying their movements during 6 minutes. Similarly to the tail suspension test, only the last 4 minutes were used for statistical evaluation. The used peptides, and the commonly used imipramine used as a positive control, were all dissolved in saline and administered intravenously, i.v., intraperitoneal, i.p., or intracerebroventricularly, i.c.v.

Administration of solutions through i.v. and i.p. injection was performed 15 minutes prior to experiments whereas the i.c.v. injection was administrated immediately prior to experiments. For experiments regarding chronic administration, stearylated peptides were administrated i.p. on a daily basis for a total period of 17 days.
3.8.6 **Biodistribution (Paper 3)**

The stearylated J18 and non-stearylated GAL2R specific ligand M1145 were both labeled with $^{125}$I according to the Iodo-Gen method and later mixed with non-labeled peptide in a 1:10 ratio and diluted in saline. The tissues examined for radioactivity were blood, brain, duodenum, heart, kidneys, liver, lungs, muscles, spleen, testis and thyroid. The result was presented as percentage of injected dose per weight of the tissue sample (% ID/g), thus considering background radiation and radioisotope decay.

3.8.7 **Toxicological analysis (Paper 3)**

The animals were monitored throughout the 17 days of chronic i.p. administration of J18 with focus on weight and general look/behavior. After the experiment all rats were put under deep anesthetic and blood was collected through hearth punctation and subsequently analyzed for indications of inflammation by measuring the levels of interleukin-6 and C-reactive protein. The analyses were performed in the United Laboratories at Tartu University Hospital (Tartu, Estonia).

3.8.8 **Galanin mutation analysis (Paper 4)**

Whole exome sequencing was performed using DNA extracted from blood samples of a six membered family including twins affected by temporal lobe epilepsy. All patients involved in the study were examined by EEG and MRI to rule out developmental malformations of the brain as a cause for epilepsy. The family members were all examined by the same neurologist and clinical data was collected. Both affected twins started to experience seizures at the age of 13 and are now seizure-free due to medications. Neither of the affected twins had any signs of hippocampal sclerosis or other abnormalities when examined by MRI.

Exome capture was carried out on genomic DNA (2µg) using a SureSelect Human ALL Exon v5 kit. The sequences were aligned to the human reference genome NCBI build (GRCh37/hg19) and duplicated reads were removed using SAMtools. Potential pathogenic variants were identified using VariantMaster (Santoni et al., 2014) which estimated the probability of the remaining variants to be present in parents and siblings.

To further examine the role of galanin mutations in temporal lobe epilepsy, blood samples were taken and the galanin gene was sequenced in additionally 530 individuals with temporal lobe epilepsy together with 52 patients with other forms of focal epilepsy. All patients involved in this study were examined by EEG and MRI. These studies were performed in collabo-
ration with research groups in Italy, Switzerland, Germany, France, USA, and Algeria.
4 Results and Discussion

4.1 Design and evaluation of the peptide ligands

The major aim of all four papers was to design receptor selective ligands.

In the first paper, Ala5-galanin (2-11) was tested for binding preference to all three receptors due to the finding that it was the only naturally occurring amino acid substitution found in the first 13 amino acids of galanin at the time (Habu et al., 1994). Interestingly, no binding for neither GAL1R nor GAL3R could be seen even at concentrations as high as 0.1 mM. The single amino acid substitution to alanine instead of serine at position 5 in galanin (2-11) is the shortest galanin receptor specific ligand known to date. Runesson et al., (2009) showed in an in silico study using galanin (2-6) that the sidechain of serine interacts with GAL3R through hydrogen bonds (Runesson et al., 2010). The importance of serine at position 5 for binding to GAL3R was in this study experimentally verified. Thus, affecting the affinity of the GAL2R 14-fold, this amino acid substitution was tolerated by GAL2R, which was not the case for neither GAL1R nor GAL3R.

The novel peptide M1154 (Paper 2) was designed to minimize affinity to the GAL3R as a mixed GAL1/2 ligand has been implicated as a putative therapeutic in several cases. For the design of M1154, particular interest was turned to the M617 peptide and its high affinity for GAL1R. Deletion of Gly12 in several galanin analogs severely affects the interaction with GAL3R relative to GAL1R and GAL2R, possibly due to the narrow binding pocket in GAL3R (Runesson et al., 2010), which does not tolerate the relative movement of the Pro13 induced kink in these galanin analogous. Furthermore, the Ala21Arg mutation from an L-Ala-scan of M617 revealed a 6-fold reduction in the GAL3R affinity (Sollenberg et al., 2010). The M1154 peptide combines these two modifications known to reduce GAL3R affinity relative to the other two galanin receptor subtypes, creating a GAL3R non-interacting galanin receptor ligand. The 125I-galanin competitive binding study showed that M1154 had a GAL1/2 selectivity with similar Ki values to GAL1R and GAL2R (see Table 6), whereas no binding could be detected to GAL3R even at concentrations of 10 μM. These results reveal a GAL1/2R selective ligand with a 1000-fold difference to GAL3R.

The focus on GAL2R selective agonists is due to the fact that GAL2R tolerates most modifications and retains reasonably high binding affinity compared to the other galanin receptors. GAL2R agonists have also been reported
to be neuroprotective in Alzheimer’s disease (Pirondi et al., 2010), have anxiolytic and antidepressant effects (Kuteeva et al., 2008) and acts as anti-convulsants by reducing the severity of seizures (Robertson et al., 2010c; Webling et al., 2012). The sequences of J17-21 (Paper 3) are all based on the previously published GAL₂R specific ligands M1145 (Runesson et al., 2009) and M1153 (I. Saar et al., 2011a). M1145 introduced an addition of four amino acids from the GAL₂R preferring galanin-like peptide (GALP) sequence to the N-terminal (Ohtaki et al., 1999). However, GALP was presented as GAL₂R preferential ligand by 3-fold compared to GAL₂R (Lang et al., 2005) and Boughton and co-workers have reported GALP to preferentially bind GAL₂R 10-fold in comparison to GAL₂R (Boughton et al., 2010).

To further minimize the binding to GAL₁R, the first glycine was replaced by asparagine. The first glycine was shown to be important for binding to GAL₁R (H. X. Liu et al., 2001), hypothetically by stabilizing an alpha-helix (Morris et al., 1995; Öhman et al., 1998; Wennerberg et al., 1990). M1153 is based on M1145, however, with the introduction of a branched C-terminal where a glutamic acid is coupled to the amino group on the side chain of lysine. Commonly used strategies to improve the stability to degradation and also to improve the CNS activity is to elongate the ligand with amino acids with positively charged side chains, so called cationization. Moreover, the stability can also be improved through the addition of a fatty acid, a process called lipidization, for example coupled to the side chain of lysine (Bulaj et al., 2008). Both of these stabilizing strategies were applied to a series of ligands, namely J17-21, in order to enhance the bioavailability in CNS for galanin ligands. To further stabilize the peptides from degradation, some non-proteinogenic amino acids were added, D-lysine as indicated by lowercase letters, ornithine which is an amino acid with similar chemical structure to lysine and also the use of 6-aminohexanoic acid, which could act as a spacer to create a longer distance between the cationic modified C-terminal and the receptor interacting N-terminal of the ligands. All the sterically modified ligands showed a modest GAL₂R preference (See Table 6). The best subtype selectivity for GAL₂R among the stearylated peptides J17-J21 was shown for J18 with a moderate six to seven-fold selectivity for GAL₂R compared to GAL₁R and GAL₃R respectively. The best binding affinity toward GAL₂R was demonstrated by J20 with a $K_i$ of 4.9 nM, similar to that of full-length rat galanin, which has a $K_i$ of 2.98 nM. Worth mentioning is that a somewhat decreased receptor binding affinity or a lower receptor selectivity of the peptides do not indicate much about their in vivo usefulness since increased stability might compensate for lower affinity.

After confirming the preferential binding to GAL₁R and GAL₂R for M1154, the GPCR signaling properties were investigated. For the modest GAL₂R preferential binding J17-21 on the other hand, focus was to develop in vivo usable peptide-based ligands and to evaluate the clinical potential.
Table 6. The affinities, $K_i$-values in nM, for all three galanin receptors in a competitive $^{125}$I-galanin binding study for the designed ligands tested + SEM in the four discussed papers along with previously published ligands used as controls. (Modified from Webling 2016).

<table>
<thead>
<tr>
<th>Ligand Type</th>
<th>GAL$_1$R</th>
<th>GAL$_2$R</th>
<th>GAL$_3$R</th>
</tr>
</thead>
<tbody>
<tr>
<td>Rat galanin 1-29</td>
<td>1.75 ± 7.2</td>
<td>2.98 ± 1.4</td>
<td>4.49 ± 0.8</td>
</tr>
<tr>
<td>Galanin 1-16</td>
<td>0.78 ± 0.26</td>
<td>2.44 ± 0.57</td>
<td>8.98 ± 3.8</td>
</tr>
<tr>
<td>M1154</td>
<td>11.7 ± 7.2</td>
<td>14.4 ± 4.1</td>
<td>&gt; 10,000</td>
</tr>
<tr>
<td>J17</td>
<td>156 ± 102</td>
<td>29 ± 18</td>
<td>125 ± 94</td>
</tr>
<tr>
<td>J18</td>
<td>138 ± 46</td>
<td>20 ± 8.0</td>
<td>112 ± 14</td>
</tr>
<tr>
<td>J19</td>
<td>231 ± 120</td>
<td>83 ± 23</td>
<td>114 ± 70</td>
</tr>
<tr>
<td>J20</td>
<td>25 ± 5.5</td>
<td>4.9 ± 1.6</td>
<td>13 ± 7.0</td>
</tr>
<tr>
<td>J21</td>
<td>138 ± 60</td>
<td>41 ± 22</td>
<td>65 ± 8.6</td>
</tr>
<tr>
<td>Ala$^5$-galanin (2-11)</td>
<td>&gt;100,000</td>
<td>258 ± 68</td>
<td>&gt;100,000</td>
</tr>
<tr>
<td>Galanin (2-11)</td>
<td>&gt;100,000</td>
<td>14.6 ± 2.2</td>
<td>186 ± 74</td>
</tr>
<tr>
<td>Phe$^7$-galanin (1-15)</td>
<td>5.72 ± 1.36</td>
<td>21.23 ± 2.93</td>
<td>1.46 ± 0.11</td>
</tr>
<tr>
<td>Pro$^{11}$-galanin (1-15)</td>
<td>350.5 ± 90.5</td>
<td>580.9 ± 121.4</td>
<td>3.74 ± 0.6</td>
</tr>
<tr>
<td>Phe$^8$, Pro$^{10}$-galanin (2-15)</td>
<td>&gt;100,000</td>
<td>&gt;10,000</td>
<td>9.56 ± 2.16</td>
</tr>
<tr>
<td>human Glu$^7$-galanin 1-30</td>
<td>3.00 ± 0.84</td>
<td>24.9 ± 7.0</td>
<td>780 ± 337</td>
</tr>
<tr>
<td>human galanin 1-30</td>
<td>6.07 ± 2.13</td>
<td>1.78 ± 0.68</td>
<td>80.90 ± 27.0</td>
</tr>
</tbody>
</table>

In Paper 4, a mutated galanin ligand was synthesized, which was originally found in twins with temporal lobe epilepsy, where alanine in position 7, Ala$^7$, was substituted by glutamic acid, Glu, in the 30 amino acid long galanin sequence and the binding and signaling were compared to wt human galanin. This single amino acid substitution, Glu$^7$-galanin (1-30), had similar or slightly higher affinity to GAL$_1$R, whereas the affinities to both GAL$_2$R and GAL$_3$R were lower compared to full length human galanin. Interestingly, the mutated ligand was found to exert the opposite effect on GAL$_1$R compared to full length galanin when examined in the signaling assay.
4.2 Classical second messenger assays compared to a label-free real-time impedance technique (Paper 1 and 2)

To assess if galanin receptor stimulation could be detected by the impedance based technology, cells were exposed to the full length galanin or galanin (1-16) as controls. Substrate addition induced a fast concentration-dependent response in normalized cell index (NCI) with different concentration dependent profiles for each receptor subtype. Two different GAL2R cell lines were characterized, CHO cells expressing human GAL2R and HEK cells expressing rat GAL2R, displaying similar profiles for galanin, although the signal to noise ratio was significantly higher in the HEK cells and therefore continuously used. Because the impedance changes elicited by receptor activation is registered in real time, it is possible to generate concentration-activity curves in a variety of ways, dependent on the time point or period of time chosen and if peak values or area under curve, AUC, is used. The aim was to generate a robust protocol that could be used independent of the type of activated G-protein and eliminate the need for a clear peak maximum, since that is often not seen in the literature (Peters and C. W. Scott, 2009; Schroeder et al., 2010). Qualification of NCI signals for concentration effect curves and the subsequent calculation of EC50 were therefore performed by calculation of the AUC between 0 and 3600 s. The EC50-values from the classical signaling assays, cAMP measurements and IP turnover, were compared to the EC50-values obtained by the real-time label-free technique (summarized in Table 7). Generally, a slightly lower EC50-value was obtained with the xCELLigence system compared to classical second messenger assays used i. e. cAMP measurements and IP turnover.

Notably, M617 displays an EC50-value of 2840 ± 1090 nM for GAL3R which results in a signaling profile for the galanin receptor subtypes more in concordance with the binding profile. Here, the ability of M1145 to activate GAL1R and GAL3R was characterized for the first time. M1145 acts as an agonist in all receptor subtypes, although the potency varies significantly, with a more than 70 times difference in the calculated EC50-values. To address the efficacy of M1154, the maximal response induced by M1154 was compared to the maximal response (E\text{max} normalized to 100 percentage) induced by galanin. M1154 behaves as a full agonist, with a similar E\text{max} value as galanin.

In Paper 1, two different cell lines expressing GAL2R were used, CHO K1 cells with high GAL2R expression and SH-SY5Y cells with a 10-fold lower expression of GAL2R compared to the CHO K1 cell line. The SH-SY5Y cell line shows a tetracycline induced overexpression of the GAL2R, but for these experiments tetracycline was not added.

The sensitivity of the techniques was tested by not adding tetracycline and comparing the result on the same cell lines examined using classical assay conditions. Ala5-galanin (2-11) selectively activates GAL2R, which uses several G\alpha pathways for intracellular signaling. The major signaling path-
way is \( \text{Ga}_q \), which generates an increase in inositol phosphate, \( \text{IP}_3 \). This could be seen with both the classical assay, \( \text{IP}_3 \) turnover, as well as with the real-time label-free technique, xCELLigence, with similar results. \( \text{EC}_{50} \) values of Ala\(^5\)-galanin (2-11) were 1,010±180 nM using classical second messenger assays and 191±49 nM using xCELLigence. Full length rat galanin (1-29) had in the same study \( \text{EC}_{50} \) values in the low nanomolar range with 1.1±0.11 nM towards GAL\(_1\)R and 8.26±1.9 nM towards GAL\(_2\)R. For full length galanin on GAL\(_3\)R, the \( \text{EC}_{50} \) values were 412±38 nM, which was surprisingly high. However, using a GTP\(\gamma\)-assay on the same cell line by Runesson et al., (2009) showed \( \text{EC}_{50} \) values of 530 nM (Runesson et al., 2009).

### Table 7. Comparison of \( \text{EC}_{50} \) values obtained from classical signaling assays, namely cAMP measurements and inositol phosphate turnover, reported in bold and the \( \text{EC}_{50} \) values obtained from label-free impedance based xCELLigence reported in parenthesis + SEM. n.t. = not tested

<table>
<thead>
<tr>
<th>Ligand</th>
<th>GAL(_1)R</th>
<th>GAL(_2)R</th>
<th>GAL(_3)R</th>
</tr>
</thead>
<tbody>
<tr>
<td>Rat galanin</td>
<td>31.6 (1.1 + 0.11)</td>
<td>173 (8.26 + 1.9)</td>
<td>530 (412 + 38)</td>
</tr>
<tr>
<td>M1145</td>
<td>n.t (1260)</td>
<td>38 (16 + 4.7)</td>
<td>n.t (2670 + 502)</td>
</tr>
<tr>
<td>M617</td>
<td>104 (11.4 + 0.67)</td>
<td>304 (24.6 + 3.8)</td>
<td>121 (2840 + 1090)</td>
</tr>
<tr>
<td>M1154</td>
<td>159 (124 + 47)</td>
<td>1530 (26 + 1.1)</td>
<td>n.t (&gt;31 600)</td>
</tr>
<tr>
<td>Ala(^5)-galanin (2-11)</td>
<td>n.t non tested</td>
<td>1,010 (191 + 49)</td>
<td></td>
</tr>
</tbody>
</table>

### 4.3 GAL\(_{1/2}\)R selective ligand, M1154, in an KA-induced excitotoxicity animal model (paper 2)

Administration of kainic acid, KA, has been utilized as a model for both neurodegeneration and status epilepticus depending on dose (Ben-Ari and Cossart, 2000; Q. Wang et al., 2005). The CA3 pyramidal cells in the hippocampus have been reported as particularly vulnerable to KA (Ben-Ari and Cossart, 2000; Reiss et al., 2009). In this study, an i.c.v. administration of KA was used to ensure a robust excitotoxic effect within the CA3 region of the hippocampus.

Animals treated with the GAL\(_2\)R selective ligand M1145, compared to vehicle, showed similar amounts of cell death, whereas administration of...
either the GAL₁R selective ligand, M617, or the novel mixed GAL₁₂R selective ligand, M1154, resulted in a reduction of cell death. These results are in concordance with earlier studies where low GAL₁R expression in inbred mice resulted in increased cell death when exposed to KA (Kong et al., 2008; Schauwecker, 2010), which strengthens the hypothesis that the observed neuroprotective effect is mediated through GAL₁R.

### 4.4 Stearylated peptide ligands in two depression-like animal models (paper 3)

The series of stearylated ligands, J17-21, were all administrated intravenously and screened using the previously described forced swimming test (see section 3.8.5). All ligands showed a small decrease in immobility time and exerted antidepressant effects, in accordance to being preferential GAL₂R agonistic ligands. From these results, J18 was chosen to be further characterized due to the highest galanin receptor selectivity, high affinity and a significant decrease in the immobility time, as the effect was comparable to that of the commonly used antidepressant imipramine (15 mg/kg administered i.p.). However, a linear dose-response effect was not seen but rather a U-shaped curve where J18 at a dose of 0.5 mg/kg was most effective. Evaluating J18 in the tail suspension test showed a decrease in immobility time with a similar U-shaped dose-response curve. However, the effect was smaller compared to imipramine in this study. To evaluate whether the effect would decrease after chronic administration, J18 was administered i.p. for 17 days. A decreased effect may be adopted by receptor desensitization, but the tail suspension test, TST, showed retained effect.

To further test whether the antidepressant effects seen for J18 were indeed mediated through GAL₂R, the non-selective receptor antagonist M35 was administered i.c.v. together with J18 administered i.p. and no effect of J18 could be observed. Thus, M35 by itself did not influence the immobility time in accordance with Kuteeva and co-workers (Kuteeva et al., 2007). Furthermore, imipramine significantly reduced the immobility time in GAL₂R-KO mice, but no effects could be seen for J18 which further indicates that GAL₂R acts as a mediator of the antidepressant effect. These results are in concordance with the previous literature reporting GAL₂R agonists to decrease depression-like behavior, while agonists for either GAL₁R or GAL₃R would increase depression-like behavior (Barr et al., 2006; Kuteeva et al., 2008; Lu et al., 2005a; Swanson et al., 2005a). However, the modest selectivity for GAL₂R could explain the U-shaped dose-response curve by activating either GAL₁R or GAL₃R resulting in pro-depressive...
effects, possibly counteracting the antidepressant effects mediated through GAL2R (Kuteeva et al., 2008; Swanson et al., 2005a).

The effects observed at these low doses strengthens the hypothesis that peptides might be useful as pharmaceuticals due to high receptor specificity, potency and low toxicity, proposed to result in decreased risk of adverse side-effects.

The chemical stabilization of the peptides with fatty acids, non-proteinogenic amino acids and positive charges has been presented to dramatically increase serum stability of the peptide from minutes to several hours (Bulaj et al., 2008). Recently, a new galanin ligand was presented by Metcalf and coworkers, namely NAX 810-2, which included both lysine and the non-proteinogenic encoded amino acid, 4-amino-phenylalanine, as positively charged amino acids (Bialer et al., 2015; 2013; Metcalf et al., 2017). NAX 810-2 is based on the previously presented GAL1R preferential ligand NAX 5055 (Bulaj et al., 2009). However, the N-terminus was changed to a methylated tryptophan instead of the previously used sarcosine, i.e. N-methylglycine, in order to limit the binding to GAL1R. NAX 5055 had a GAL1R preference and showed promising anticonvulsant effects in several epilepsy rat models, but when administered systemically, it increased blood glucose levels, impaired glucose handling as well as reduced insulin response in an acute glucose challenge (Flynn and White, 2015). These results were verified when the GAL1R agonist, M617, was shown to cause increased insulin sensitivity in diabetic rats (Z. Zhang et al., 2016).

4.5 Pharmacological properties of stearylated peptide J18 (Paper 3)

The addition of a fatty acid, i.e. lipidization, together with several positively charged amino acids, i.e. cationization, to stabilize peptides was presented by Bulaj and co-workers for galanin ligands (Bialer et al., 2013; Bulaj et al., 2008), and the serum stability of their palmitoylated galanin ligands was presented in hours instead of minutes as for unmodified peptides. These modifications also generated ligands that could pass the BBB and exert their effect. The biodistribution of the stearylated J18 was examined in several tissues, using the unmodified GAL2R selective ligand M1145 as reference (Runesson et al., 2009).

Overall, the levels of J18 were significantly higher at 1 hour and 3 hours after administration, compared to the unmodified M1145, which was found to be eliminated after 3 hours. The level of J18 was eliminated first after 24 hours and it was thus found to have significantly higher stability to degrada-
tion compared to M1145. J18 levels in the brain were found to be significantly higher than for unmodified M1145, in concordance with the similar effect of J18 and i.c.v. administrated GAL2/3R agonist, galanin (2-11) (Kuteeva et al., 2008).

Antidepressant drug screening in animal models utilizes acute administration of drug leads. However, commonly used antidepressants modulate their effect by inhibiting reuptake of serotonin. These selective serotonin reuptake inhibitors (SSRI) need to be administered during 2-4 weeks for antidepressant effects. Here, the observed effect was fast and at notably low doses that indicates a low risk of possible side effects. To examine whether galanin ligands administered over a longer period would result in a decreased effect, due to possible feedback mechanisms or receptor desensitization, J18 was administered daily for 17 days. However, no decreased effect could be seen when evaluated in TST.

4.6 Mutations in the galanin gene and temporal lobe epilepsy, TLE (paper 4)

In both twins affected by temporal lobe epilepsy, a mutation could be found in the galanin gene in position 39 in preprogalanin. This mutation generated a glutamic acid instead of an alanine in position 7 in the 30 amino acid long human galanin sequence. When comparing the binding affinity of the mutated variant, p.(A39E), to human full length galanin, the affinity to GAL1R was similar, or slightly better for the mutated variant, whereas the mutated ligand had an almost 14-fold less affinity towards GAL2R and a 10-fold less affinity to GAL3R. Thus, the most striking difference was found in the signaling studies where the mutated ligand had the opposite effect as full length human galanin on human GAL1R expressing cells.

All family members were screened for the p.(A39E) mutation using sanger sequencing and it was confirmed that the wild type, wt, allele was present in both parents and unaffected siblings, whereas the mutated allele was present in both affected twins. Since none of the 530 other patients diagnosed with either temporal lobe epilepsy or other forms of epilepsy had any pathogenic mutation in the galanin gene, it is suggested to be a rare cause of TLE.

Both affected twins had seizures under control by either Carbamazepin (600 mg/day) or Levetiracetam (1g/day), respectively. Carbamazepine, 5H-Dibenz[b,f]azepine-5-carboxamide, has a structure similar to tricyclic antidepressants with a mechanism of action that is not completely understood. However, tricyclic antidepressants are proven to elevate the levels of serotonin, noradrenalin and in some instances also dopamine and acetylcholine. Galanin, among other neuropeptides, has been shown to modulate noradrenaline, serotonin and dopamine (Le Maître et al., 2013). Galanin has also been shown to increase the released levels of both dopamine (Melnikova et al., 2006) and noradrenaline (Robinson and Brewer,
A GAL2,R agonist, namely galanin (2-11), has also been reported to reduce glutamate toxicity in neural hippocampal cells (Pirondi et al., 2005). Levetiracetam, S-alpha-ethyl-2-oxo-1-pyrrolidin acetamide, also has a mechanism of action that is not completely understood, but it has been shown to partially reduce Ca\(^{2+}\) release from intracellular storages in the endoplasmic reticulum, ER, and to inhibit N-, P- and Q-type Ca\(^{2+}\) channels (Deshpande and Delorenzo, 2014). Levetiracetam binds to the synaptic vesicle protein 2A, SV2A, involved in vesicular fusion and subsequent exocytosis of neurotransmitters (Berkley A Lynch, 2004). SV2A modulates the release of neuropeptides from vesicles and SV2A-deficient mice have been shown to be unaffected by Levetiracetam (Kaminski et al., 2009). Levetiracetam is also reported to stimulate GABA and glycine mediated currents, the two major inhibitory neurotransmitters.

Interestingly, Metcalf et al., (2017) have in several animal epilepsy models shown that Levetiracetam has a similar preclinical seizure profile as a moderately GAL2,R preferring analogue named NAX 810-2. Levetiracetam showed protective effects in PTZ-kinned seizures model, and corneal kindling model, but no effect could be seen in acute maximal electroshock seizures (Klitzgaard et al., 1998; Löscher and Hönack, 1992; Metcalf et al., 2017). Moreover, both GAL1,R and GAL2,R have been highlighted as important and even GAL1,R/GAL2,R heteroreceptors have been reported for the dorsal hippocampus and dorsal raphe nucleus (Borroto-Escuela et al., 2014; Millón et al., 2016).

Even though the exact mechanism of action for either of the pharmaceuticals used by the TLE affected twins, is not completely understood, and the fact that several neuropeptides have modulating effects just like galanin, one can compare the effects of the pharmaceuticals with those of a galanin agonist and note similarities.

The finding that a mutation in the galanin peptide could result in temporal lobe epilepsy is the first pathogenic report on galanin in humans. However, it has recently been reported that a single nucleotide mutation in the promoter region of the galanin gene could be linked to an increased risk of multiple sclerosis in men, but this increased risk could not be seen for women (Lioudyno et al., 2016), and a single nucleotide mutation in the promoter region of galanin has also been positively correlated with an increased risk of major depressive disorder (Wang et al., 2013).
5 Conclusions

♥ Ala⁵-galanin (2-11) is the shortest GAL₂R specific ligand known to date.

♥ Ser⁶ in galanin was experimentally verified to be of importance for binding to GAL₃R.

♥ A protocol for evaluating the signaling properties using both real-time label free impedance based techniques, xCELLigence, and optical based techniques, EnSpire, for all three galanin receptor was presented.

♥ The new label-free real-time technique, namely xCELLigence, to examine G-protein signaling was shown to be sensitive enough for detecting signaling on cells with low receptor expression that classical second-messenger methods were not able to detect.

♥ A novel GAL₁₂R selective agonist was designed and evaluated. No detectable binding to GAL₃R, nor receptor activation, could be shown.

♥ GAL₁R agonists were shown to be of importance in the prevention of the KA-induced cell death in the rat hippocampus.

♥ A series of novel systemically active GAL₂R preferring ligands were designed and evaluated in animal depression models. Antidepressant effects were seen at notably low doses.

♥ Lipidization and cationization was shown to increase the peptide stability and also the ability to penetrate the blood-brain barrier.

♥ The first pathogenic mutation in the galanin gene, which resulted in a disturbed galanin receptor binding and signaling, was found in patients with temporal lobe epilepsy.
The amino acid substitution of Glu⁷-galanin 1-30 in human full length galanin found in patients with temporal lobe epilepsy resulted in a ligand with GAL₁R antagonistic effects, a 14-fold reduced agonistic affinity for GAL₂R, and an almost 10-fold reduced affinity for GAL₃R. Subsequently, signaling studies revealed antagonistic effects on human GAL₁R.

Mutations in the galanin gene can cause temporal lobe epilepsy, but is not one of the major causes.
6 Populärvetenskaplig sammanfattning på svenska

Galanin är ett kort protein, så kallad peptid, som först upptäcktes när Professor Viktor Mutt och hans kollegor utvann peptider ur stora mängder gristarmar som de kommit över från ett närliggande slakthus. En peptid består av ihopkopplade aminosyror, 2-50 aminosyror långa. Galanin är 29 aminosyror lång i de flesta arter, dock 30 aminosyror lång hos människa och fick sitt namn genom att ta den första bokstaven från dess första aminosyra, glycine, och resten från dess sista aminosyra, alanin. Galanin har visat sig vara involverad i en rad olika sjukdomar - såsom depression, epilepsi, cancer och Alzheimers sjukdom för att nämna några.

Effekten man kan se från galanin resulterar från att den binder in till galaninreceptorer och det finns tre olika sådana. Dessa tre galaninreceptorer skiljer sig från varandra genom vilka signaleringsvägar som aktiveras inuti cellen men även var i kroppen de finns. Detta talar starkt för att de olika galaninreceptorerarna har olika effekter. Galanin binder lika bra till alla tre receptorerna så det övergripande målet med detta projekt har varit att göra förändringar i galanin på ett sådant sätt att den bara kan binda till en eller två av receptorererna och att man då ska kunna använda dessa förändrade peptider i vidare studier för att undersöka effekten av var och en av de tre receptorerarna. I denna avhandling summeras arbetet att få fram galaninligander som designas för att binda till någon av de tre receptorerna för att undersöka dess roll i olika sjukdomar eller biologiska skeenden.

I den första artikeln presenteras den kortaste peptiden, bara tio aminosyror lång, som endast kan binda till galanin receptor 2. Här har bara en aminosyra ändrats vilket visar vikten av denna aminosyra för inbindning till GAL3R.

I artikel 2 efterfrågas en peptid som kan binda in till galanin receptor 1 och 2 då flera studier pekade ut dessa som skyddande vid långvarig nervstimulering - det som sker vid ett epileptiskt anfall. En ligand som endast kunde aktivera galanin receptor 1 och 2 togs fram och testades i en rättmodell tillsammans med tidigare framtagna ligander som bara binder galanin receptor 1, M617, samt en ligand som bara binder till galanin receptor 2, M871.

I artikel tre låg fokus på depression. Aktivering av galanin receptor 2 har visat sig ge antidepressiva effekter i flera studier. Till skillnad från flera van-

I artikel fyra hittades en mutation i galaninsekvensen hos två enäggstvillingar med epilepsi. När vi jämförde galanin och den muterade galaninliganden upptäckte vi att bindningen till de tre receptornas skiljde sig åt. Den muterade liganden band in sämre till galanin receptor 2 (14 gånger) och till galanin receptor 3 (10 gånger) men det mest väsentliga var att den muterade liganden hade motsatt effekt som galanin då vi studerade signaleringen från galanin receptor 1. Tvillingarna håller sin epilepsi i schack med mediciner som har samma effekt som en fungerande galaninreceptor 1 ligand skulle ge. Då över 500 patienter med epilepsi undersöktes för mutationer i galaninliganden kunde man inte hitta något avvikande, så en icke-fungerande galaninligand kan ge upphov till epilepsi men är inte en av de större orsakerna till sjukdomen.

Sammanfattningsvis presenteras åtta nya galaninligand som kan användas för att klargöra vad var och en av galaninreceptorernas roller i olika biologiska och patologiska skeenden och i modeller för exempelvis vissa sjukdomar.
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- John Bingham

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