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THE GALANIN TYPE 2 RECEPTOR: MOLECULAR BIOLOGICAL, HISTOCHEMICAL AND ELECTROPHYSIOLOGICAL STUDIES

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To my family

ABSTRACT

In the present thesis, the aim was to probe the distribution and functions of galanin type 2 receptor (GalR2) in the rodent brain. In order to overcome the lack of a reliable antibody to localize GalR2 by immunohistochemistry, we generated a construct which expresses GalR2 tagged with enhanced GFP (EGFP). We first studied the trafficking and function of this fusion protein in cell lines, including PC12 cells.. The results showed that GalR2-EGFP is predominantly localized on the plasma membrane with some intracellular fluorescent structures (vesicles), mainly in the perinuclear region. After activation by galanin, the GalR2-EGFP is able to induce a concentration-dependent increase in intracellular Ca^{2+} level, suggesting that the conjugate is functional. The results also indicate that GalR2 undergoes constitutive endocytosis and recycling, using the clathrin-dependent endocytic recycling pathway.

Then we examined the localization of the GalR2-EGFP in a transgenic mouse brain expressing the same GalR2-EGFP construct under the GalR2 promoter, using a GFP antibody. The immunostaining was mainly found in cell bodies and to a limited extent also in presumable nerve terminals. GalR2-EGFP positive processes and cell bodies were located in many brain regions, including the olfactory bulb, certain limbic cortical areas, the basal forebrain, amygdala, subregions of the hippocampal formation, thalamus, hypothalamus, periaqueductal grey, locus coeruleus, and some further areas in the midbrain and medulla oblongata. Using double-staining we analyzed some cell groups giving rise to major ascending systems, and could demonstrate presence of the GalR2-EGFP construct in noradrenergic/galaninergic locus coeruleus neurons. The GalR2-EGFP was also detected in calcium-binding protein- and GAD-positive cell bodies in the basal forebrain, but not in cholinergic neurons in this area, or in the 5-hydroxytryptamine neurons in the dorsal raphe nucleus. Our results support the concept that GalR2 primarily is a presynaptic autoreceptor in noradrenergic locus coeruleus neurons. Galanin is presumably involved in a wide range of brain functions, which are partly executed through GalR2.

Plasticity is an important characteristic of galanin signaling. The expression levels of galanin and its receptors are highly dynamic in various circumstances. In vitro electrophysiological study was done with hippocampal slices obtained from mice overexpressing galanin under the promoter for the platelet-derived growth factor-B (GalOE mice). Slices from young adult wild-type (WT) animals showed significant paired-pulse facilitation (PPF) of the 2nd excitatory postsynaptic field potential (fEPSP) evoked with paired-pulse stimuli, while PPF was less strongly expressed in slices from young adult GalOE mice, as well as aged WT mice, but were not observed at all in slices from aged GalOE animals. The results indicate that galanin is involved in hippocampal synaptic plasticity, in particular in age-related reduction of synaptic plasticity in the lateral perforant path input to the dentate gyrus.

Hypertrophic galanin-containing fibers engulf cholinergic basal forebrain neurons in Alzheimer's disease, a phenomenon that has been interpreted as a region-specific innate neuroprotection. We have developed an en masse cell isolation strategy based on the p75NTR to establish 90-95% homogenous cholinergic neuron cultures from dissociated embryonic basal forebrains. We exploited this in vitro system to dissect the cellular mechanisms of how galanin impacts cholinergic neurons. Cultured cholinergic neurons express GalR2 that is located in the soma and distributed to the axonal growth cone. Stimulation of cultured cholinergic neurons with galanin or GalR2 agonist ARM1896 induces ERK phosphorylation. Stimulation of GalR2 by galanin and its synthetic analogue resulted in increased neurite outgrowth. Furthermore, we demonstrate that GalR2 stimulation can protect cholinergic neurons against β-amyloid (Aβ) toxicity in vitro. Therefore, we speculate that the hypertrophy of galaninergic nerve endings in the basal forebrain of Alzheimer's patients may represent a signalling arrangement poised to activate specific GalR2-dependent intracellular signaling cascades to induce neuroprotection in situ as proposed previously.

In summary, the study with PC12 cell line elucidated the subcellular distribution and trafficking pattern of GalR2; this gave us some idea how the GalR2 receptor exerts its function at the cellular level. The immunohistochemical study with GalR2-EGFP transgenic animal provided the information about the distribution pattern of GalR2 in the brain, including its relation to some ascending systems. The study about the effect of galanin signaling in the aged mice and GalOE mice showed plasticity of the galanin signaling system and the effect of overexpressed galanin on neurotransmission at the presynaptic site. With the newly developed target-specific-isolated cholinergic basal forebrain neuron culture, we provide further evidence for a trophic effect of GalR2 activity and the plasticity of GalR2 related to Aβ toxicity. These studies may provide a basis for further discussion about the function of galanin signaling and potential therapeutic applications.

Keywords: Galanin, galanin receptor 2, PC12 cell, transgenic mouse, cholinergic basal forebrain neuron, target-specific-isolation, Alzheimer's disease

LIST OF PUBLICATIONS

This thesis is based on the following publications/manuscripts, which are referred to by their Roman numerals.

I. Xia S, Kjaer S, **Zheng K**, Hu PS, Bai L, Jia JY, Rigler R, Pramanik A, Xu T, Hökfelt T, Xu ZQ. (2004) Visualization of a functionally enhanced GFP-tagged galanin R2 receptor in PC12 cells: constitutive and ligand-induced internalization. Proc Natl Acad Sci U S A 101(42):15207-12.

II. **Zheng K**, Xia S, Stanić D, Dun X, Lu J, Gong XN, Xu ZQ, Hökfelt T. (2011) Galanin R2 receptor distribution in the brain of a GalR2-EGFP transgenic mouse (*Preliminary manuscript*).

III. **Zheng K**, Kuteeva E, Xia S, Bartfai T, Hökfelt T, Xu ZQ. (2005) Age-related impairments of synaptic plasticity in the lateral perforant path input to the dentate gyrus of galanin overexpressing mice. Neuropeptides 39(3):259-67.

IV. **Zheng K**, Mulder J, Keipema E, Berghuis P, Barde S, Dobszay MB, Luiten P G.M., Xu ZQ, Runesson J, Langel Ü, Lu B, Hökfelt T, Harkany T. (2011) Galanin receptor 2 induces trophic effects in cholinergic neurons isolated from the mouse basal forebrain (*Manuscript*).

CONTENTS

ABBREVIATIONS

5-HT	5-hydroxytryptamine, serotonin
7-TM	7-transmembrane
ABA	Allen Brain Atlas
ACh	acetylcholine
aCSF	artificial cerebrospinal fluid
AD	Alzheimer's disease
BSA	bovine serum albumin
cAMP	cyclic adenosine monophosphate
CBF	cholinergic basal forebrain
ChAT	choline acetyltransferase
CNS	central nervous system
DMEM	Dulbecco's modified eagle medium
DNA	deoxyribonucleic acid
DRN	dorsal raphe nucleus
EGFP	enhanced green fluorescent protein
FBS	fetal bovine serum
fEPSP	field excitatory postsynaptic potential
FITC	fluorescein isothiocyanate
FST	forced swim test
G418	geneticin antibiotic
GABA	γ-aminobutyric acid
GAD	glutamic acid decarboxylase
GALP	galanin-like peptide
GalR	galanin receptor
GFAP	glial fibrillary acidic protein
GI	gastrointestinal
GIRK	G-protein-regulated inwardly rectifying K^+ channel
GMAP	galanin message-associated peptide
GPCR	G-protein coupled receptor
HBSS	Hank's buffered salt solution
HiFo	hippocampal formation
i.c.v.	intracerebroventricular
LC	locus coeruleus
LDCV	large dense core vesicle
LPP	lateral perforant path
LRSC	lissamine rodamine
MAP2	microtubule-associated protein 2
MAPK	mitogen-activated protein kinase
mIPSC	miniature inhibitory postsynaptic current
mRNA	messenger ribonucleic acid
M β CD	methyl- β -cyclodextrin
NA	noradrenaline
NFT	neurofibrillary tangle
P75	low-affinity neurotrophin receptor
PBS	phosphate-buffered saline
PCR	polymerase chain reaction
PDGF-B	platelet-derived growth factor B
PFC	prefrontal cortex
PKC	protein kinase C
PLC	phospholipase C
PNA	peptide nucleic acid
PNS	peripheral nervous system
PPF	paired-pulse facilitation
PPS	perforant path stimulation
PTX	pertussis toxin
qPCR	quantitative PCR
REM	rapid eye movement
RT	room temperature
RT-PCR	reverse transcription PCR
SE	status epilepticus
SSRI	selective serotonin reuptake inhibitor
SV	synaptic vesicle
TH	tyrosine hydroxylase
TIP39	tuberoinfundibular peptide of 39 residues
TNB	Tris-sodium blocking buffer
TNT	Tris-sodium washing buffer
TSA	tyramide signal amplification
TST	tail suspension test
TTX	tetrodotoxin
VAChT	vesicular acetylcholine transporter
VGAT	vesicular GABA transporter
VGLUT	vesicular glutamate transporter
WT	wild type

1 INTRODUCTION

1.1 NEUROPEPTIDES

More than 100 small neuropeptides, in size ranging from 3 to >40 amino acid residues, have been identified over the last four decades (Burbach, 2010), and new members are still discovered. They not rarely have an amide group at the C-terminal (instead the usual carboxylic acid), which provides protection against enzymatic degradation and often is required for their physiological function. The 'first' neuropeptide, substance P, was discovered by von Euler and Gaddum in 1931 (von Euler and Gaddum, 1931), but the chemical structure was not described until 40 years later (Chang and Leeman, 1970). Even if neuropeptides can function as neurotransmitters, there are several characteristics that distinguish them from classic, small-molecule neurotransmitters (Hokfelt et al., 2003; Lundberg, 1996; Lundberg and Hokfelt, 1986; Merighi, 2002) (Figure 1). Classic, small-molecule neurotransmitters, like glutamate, γ-aminobutyric acid (GABA), monoamines and acetylcholine (ACh), are synthesised from a single amino acid-residue by enzymes mainly in the axonal terminal, then packed into small 'clear' synaptic vesicles (SV) (40-60 nm in diameter). Importantly, most SVs are generated by local recycling from the plasma membrane in the nerve terminals (Alberts et al., 2002). The release of the classic neurotransmitters from SVs can be triggered by low-frequency firing. Classic neurotransmitters activate various classes of receptors, both ionotropic receptors and G-protein coupled receptors (GPCRs), thus causing a fast and slow response, respectively. Terminating the activity of classic neurotransmitters mainly occurs by reuptake into the synaptic terminal via specific membrane transporters, alternatively by degradation by enzymes (Iversen, 2000).

Neuropeptides, in contrast, are synthesised in the cell body as larger precursor polypeptides (prepro-peptide), matured in the Golgi apparatus, packed into the large dense core vesicles (LDCVs, 90-250 nm in diameter), transported to the terminals by fast axonal transport and cut by specific 'cleavage' enzymes to produce the bioactive peptide(s) (Gainer et al., 1985). LDCVs are almost always found at some distance from the ready-to-release SV pool, that is outside of the synapse region. Therefore the release of neuropeptide packed in LDCVs is extrasynaptic and in general needs to be triggered by high frequency firing and subsequent increased cytoplasmic [Ca^{2+}] concentrations. The cognate receptors for peptides are almost always GPCRs leading to activation of various intracellular signaling cascades. The main effects of peptides include modulation of membrane excitability and regulation of gene expression, receptor dynamics and neurotransmitter secretion. The actions of neuropeptides are terminated by selective proteolytic enzymes, peptidases, and by diffusion (Iversen, 2000). These features allow neuropeptides to act both on close targets as well as to reach receptors far from the release site, often referred to as volume transmission (Fuxe and Agnati, 1991).

Neuropeptides are synthesized and released from neurons both in the central (CNS) and peripheral nervous (PNS) system; and mostly coexist with classic neurotransmitters (Hokfelt et al., 1980). Binding affinities of neuropeptides are commonly in the nanomolar range, which is a thousand–fold, or even higher, than the classical neurotransmitters, indicating high selectivity of neuropeptide ligand and receptor binding. This may possibly provide more precise pharmacological targeting with

analogues less prone to side-effects. These features and the abundance of neuropeptides and neuropeptide receptors have been thought to provide opportunities for development of new drugs and of novel treatment strategies for neuronal diseases (Hokfelt et al., 2003). A wide range of physiological processes and possibly associated pathologies have been associated with neuropeptides, for example: nociception (Xu et al., 2000), appetite (Kuhar and Dall Vechia, 1999; Leibowitz, 1995), mood regulation (Ogren et al., 2006; Refojo and Holsboer, 2009), alcohol dependence and drug addiction (Heilig and Thorsell, 2002; Picciotto, 2008; Van Ree et al., 2000), sleep and arousal (Kilduff and Peyron, 2000; Willie et al., 2001; Xu et al., 2004), learning and memory (Crawley, 2008; Ogren et al., 2010). In fact, much evidence indicates that neuropeptides are of particular importance, when the nervous system is challenged, e.g. by stress, traumatic events, injury, and drug abuse (Hokfelt et al., 2003).

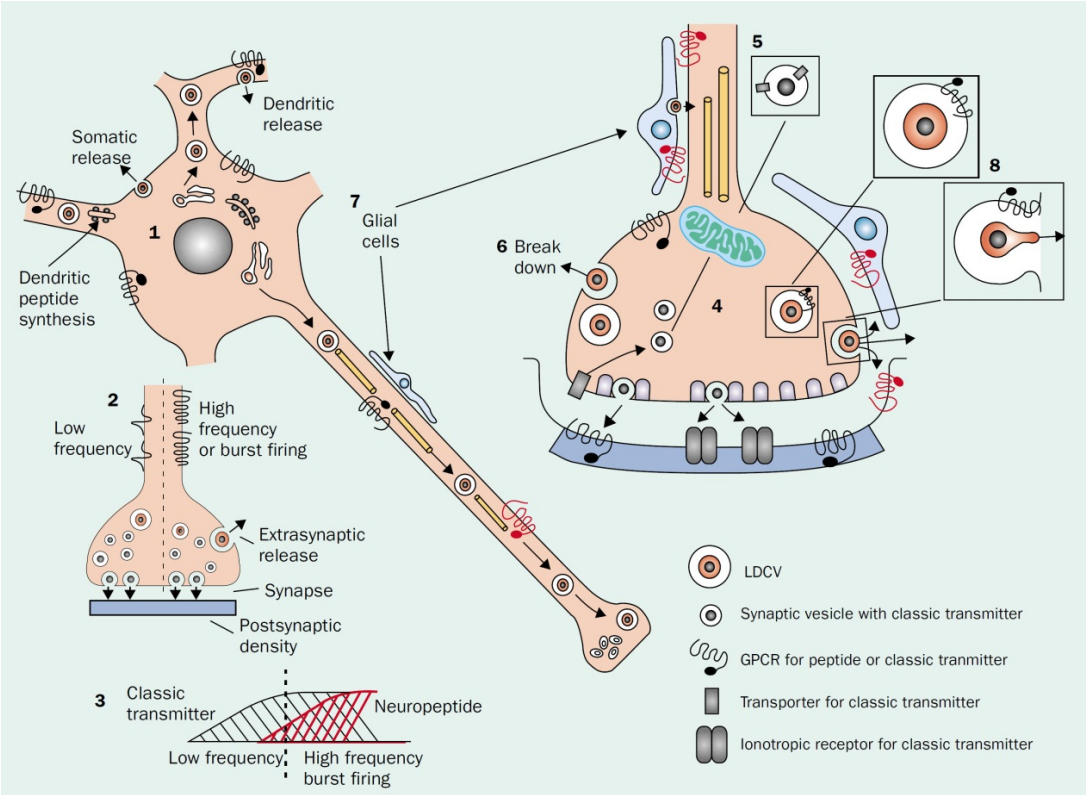

Figure 1. Neuropeptides are produced ribosomally in the cell body and dendrites, packaged in LDCVs and transported into axons and dendrites (1). LDCVs can also contain a classic transmitter (1, 2, 4). LDCVs contain processing enzymes (convertases) that release the bioactive peptide from the precursor. The peptides can be released both from nerve endings and dendrites/cell soma (1, 2, 4). The peptide receptors belong to the 7-TM GPCR family and are present on cell soma, dendrites, axons, and nerve endings (1, 4). Peptides are preferentially released extrasynaptically under burst or high frequency firing. Classic transmitters, stored in clear synaptic vesicles, are mostly released into the synaptic cleft (2–4), acting on ionotropic receptors and GPCRs in the synaptic cleft. Peptide receptors are mostly found outside the synapse (4). Classic transmitters can be produced in all parts of the neurons because the synthesising enzymes are present throughout the neuron (4). Classic transmitters, contrasting peptides, have reuptake mechanisms (transporter molecules) at both the cell and the vesicle membrane (5), leading to termination of the action as well as allowing recycling (4). Peptides are broken down by extracellular peptidases (6), and replacement has to occur via axonal transport. Also glial cells can produce peptides and their receptors (7). Receptors are mostly processed via the constitutive pathway, transported in small vesicles, and inserted into the cell membrane, but there is evidence that delta opioid receptors are localised in the membrane of LDCVs (8). From Hökfelt et al. (2003), with permission.

1.2 THE GALANIN FAMILY

Galanin was first isolated from extracts of porcine intestine by Tatemoto, Mutt and collaborators in 1983 at Karolinska Institutet (Tatemoto et al., 1983). Galanin has been evolutionally conserved as a 29 amino acid (aa)-residue peptide with an amidated C-terminal. An exception is human galanin consisting of 30 aa residues and lacking the C-terminal amidation (Evans and Shine, 1991). The N-terminal 1–15 amino acids of galanin are identical in all species (Evans and Shine, 1991). Galanin is proteolytically processed from a 123-(porcine, human) or 124-(murine) amino acid precursor pro-peptide along with a 59 or 60 amino acid peptide known as galanin message-associated peptide (GMAP) (Evans and Shine, 1991; Kaplan et al., 1988; Lang et al., 2007; Rokaeus and Brownstein, 1986; Vrontakis et al., 1987). The peptide precursor of galanin, preprogalanin, is encoded by a single-copy gene organized into six small exons spanning about 6 kb of genomic DNA, depending on the species (Lang et al., 2007) (Figure 2).

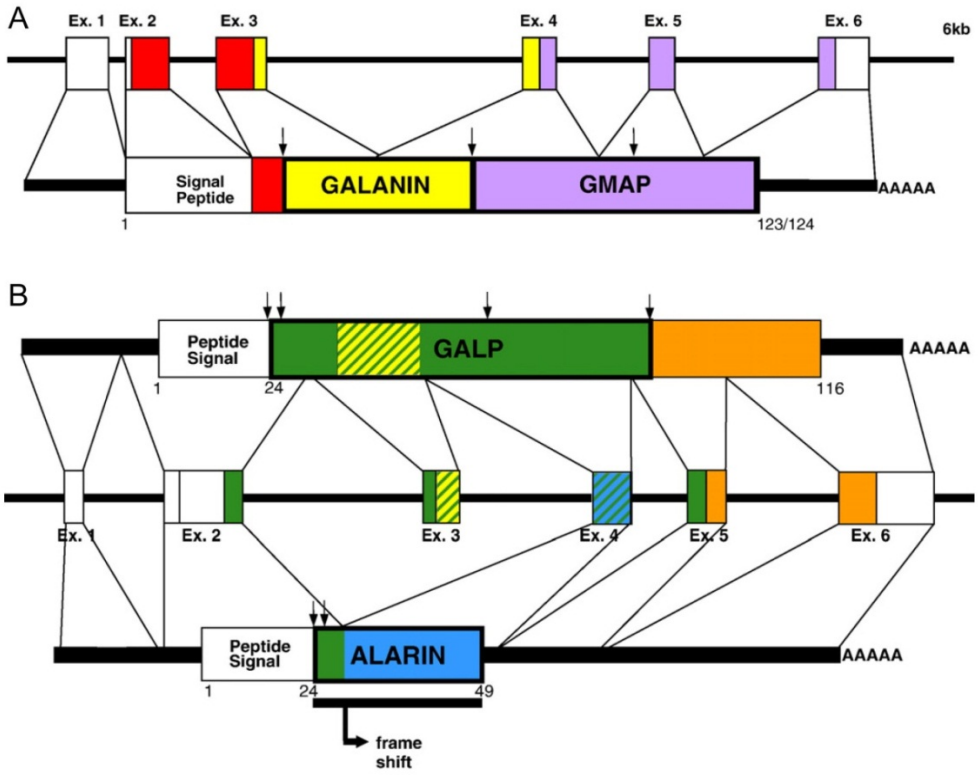

Figure 2. A. Organization of the preprogalanin gene. The first exon encodes only the 5′ -untranslated region of preprogalanin mRNA. Exon 2 starts with the translation initiation codon of the signal peptide and terminates before the proteolytic site preceding the mature galanin peptide. The first 13 amino acids of galanin are encoded by exon 3; the remaining 16 amino acids and most of GMAP by exons 4 and 5. The remaining portion of GMAP and the polyadenylation site are located in exon 6. Arrows indicate cleavage sites of endopeptidases. The first exon is noncoding. B. PreproGALP is encoded by exons 2–6 and the segment with galanin homology [(GALP (9–21)] is contained in exon 3. The mature peptide GALP (1–60) is encoded by exons 2–5. Post-transcriptional splicing leads to exclusion of exon 3 resulting in a frame shift and a novel precursor protein. This protein harbors the signal sequence of preproGALP and the first 5 amino acids of thematureGALP peptide followed by another 20 amino acids and proteolytic cleavage leads to alarin (1–25). Arrows indicate potential cleavage sites of endopeptidases. From Lang et al. (2007), with permission.

It was long thought that galanin is a unique peptide without any further family member(s), except then GMAP. However, in addition to the above mentioned GMAP, Ohtaki et al. (1999) discovered a 60 amino acid peptide termed galanin-like peptide (GALP). It was first found as an endogenous ligand for galanin receptors expressed in the porcine hypothalamus and gastrointestinal (GI) tract (Ohtaki et al., 1999). Since then, GALP mRNA expression has been identified in several other species, such as rat, mouse, macaque and human (Cunningham et al., 2002; Jureus et al., 2001; Lawrence and Fraley, 2010; Ohtaki et al., 1999). The GALP gene comprises six exons with a structural organization similar to that of galanin (Cunningham et al., 2002; Ohtaki et al., 1999). GALP (1–60) is cleaved from the precursor peptide preproGALP consisting of 115–120 amino acids, depending on the species, and GALP residues 9–21 are homologous to residues 1–13 of galanin (Figure 2). The newest member of this family - alarin - was discovered fairly recently, and is a splice variant of the GALP mRNA (Santic et al., 2006; Santic et al., 2007).

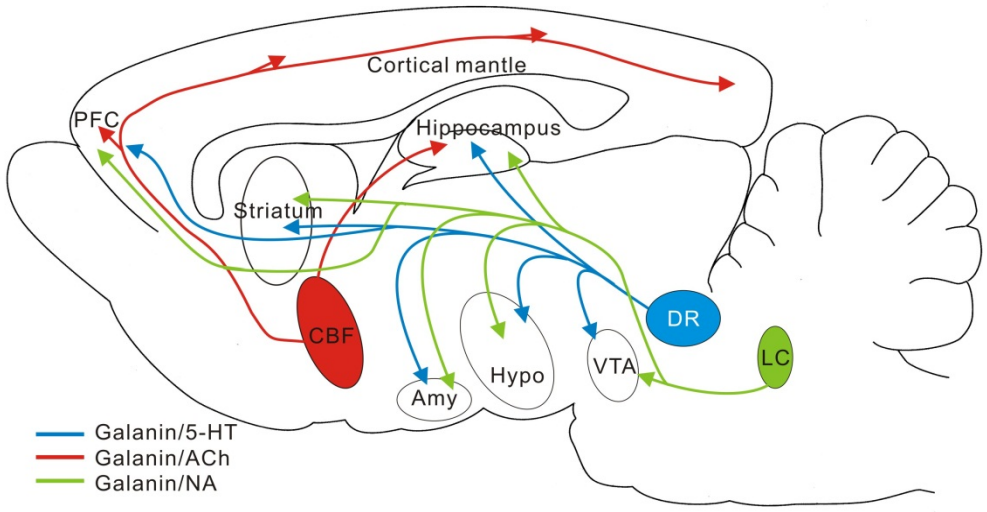

Figure 3. Galanin in some main ascending systems. PFC, prefrontal cortex; CBF, cholinergic basal forebrain; Amy, amygdala; Hypo, hypothalamus; VTA, ventral tegmental area; DR, dorsal raphe; LC coeruleus. Modified from Kuteeva (2007) with permission.

1.3 DISTRIBUTION OF GALANIN

Galanin like-immunoreactivity (LI) and galanin transcript are widely distributed throughout the CNS and PNS of different species, including rat (Jacobowitz et al., 2004; Melander et al., 1986a; Rokaeus et al., 1984; Ryan and Gundlach, 1996; Skofitsch and Jacobowitz, 1985; Skofitsch and Jacobowitz, 1986) , mouse (Cheung et al., 2001; Kuteeva et al., 2004; Lein et al., 2007; Perez et al., 2001), non-human primates (Beal et al., 1988; Kordower et al., 1992) and human (Benzing et al., 1993; Gentleman et al., 1989; Kordower et al., 1992; Kordower and Mufson, 1990). Of particular interest, in the CNS of mammals galanin is expressed in several subcortical ascending systems and coexists with various classic neurotransmitters and other neuropeptides. More precisely, in the rat galanin is expressed in 80% noradrenergic neurons in the locus coeruleus (LC) (Holets et al., 1988; Melander et al., 1986b; Xu et al., 1998b), in 60% serotonergic neurons in the dorsal (DRN) and median raphe nuclei (Larm et al., 2003; Melander et

al., 1986b; Xu et al., 1998b), in cholinergic neurons in bed nucleus of the stria terminalis, in the basal forebrain (Cheung et al., 2001; Jacobowitz et al., 2004; Melander et al., 1985a; Ryan and Gundlach, 1996; Senut et al., 1989). Galanin also coexists with GABA, substance P and enkephalin in the tuberomammilary nucleus (Kohler et al., 1986; Melander et al., 1986b), with dopamine (DA), growth hormone-releasing hormone and neurotensin in the hypothalamic arcuate nucleus (Meister et al., 1990) and with vasopressin in the bed nucleus of stria terminalis (Miller et al., 1993). Developmentally, in the embryonic, neonatal and even adult nervous system, galanin gene transcription can be found especially in the proliferative zones (Shen et al., 2003; Xia et al., 2005). Under certain circumstances galanin can also be found in glial cells (Shen et al., 2003; Ubink et al., 2003; Xu et al., 1992). Finally, there are also distinct species differences in galanin expression, for example there is much higher galanin expression in the basal forebrain and the HiFo in some primates as compared to rodents (Kordower et al., 1992; Melander et al., 1986a; Melander and Staines, 1986; Perez et al., 2001).

Galanin is present in several afferent systems to the hippocampal formation (HiFo) (Melander et al., 1985b). Especially the noradrenergic cell bodies in the LC (A6 group according to Dahlström and Fuxe, 1964) (Dahlstrom and Fuxe, 1964) have a robust galanin expression both in rat and mouse (Cheung et al., 2001; Holets et al., 1988; Jacobowitz DM, 1991; Kuteeva et al., 2004; Melander et al., 1986a; Perez et al., 2001; Rokaeus et al., 1984; Skofitsch and Jacobowitz, 1985; Skofitsch and Jacobowitz, 1986; Xu et al., 1998b); also subhuman primate (Kordower et al., 1992) and human (Chan-Palay, 1990; Chan-Palay and Asan, 1989) NA neurons in the LC synthesize this peptide.

The rat 5-hydroxytryptamine (5-HT) neurons in the DRN contain galanin (Araneda et al., 1999; Cheung et al., 2001; Cortes et al., 1990b; Jacobowitz DM, 1991; Larm et al., 2003; Melander et al., 1986b; Perez et al., 2001; Priestley et al., 1993; Xu and Hokfelt, 1997; Xu et al., 1998b). but cannot be detected in mouse DRN 5-HT neurons (Larm et al., 2003). Galanin can under certain circumstances be detected in rat cholinergic forebrain neurons (Melander et al., 1985b; Miller et al., 1999; Senut et al., 1989). In the owl monkey (Melander and Staines, 1986) and baboons (Beal et al., 1988), but not in apes or humans, galanin has been demonstrated in cholinergic forebrain neurons (Kordower and Mufson, 1990; Walker et al., 1989; Walker et al., 1991). Finally, histamine neurons in the tuberomamillary nucleus (Kohler et al., 1986; Melander et al., 1986a) can express galanin, Thus, although galanin systems often are conserved, interesting species differences exist. Taken together galanin can coexist with several important classic neurotransmitters, including ACh, NA, 5-HT (Figure 3) and histamine in ascending systems innervating among others the HiFo.

1.4 GALANIN RECEPTORS

Up till now, three cognate receptor subtypes have been identified, galanin receptor 1 (GalR1), galanin receptor 2 (GalR2), and galanin receptor 3 (GalR3). They belong, as virtually all other neuropeptidergic receptors, to the 7-transmembrane (7-TM), GPCR superfamily. Stimulation of these receptor subtypes will result in activation of different intracellular signaling cascades (Table 2; Figure 4), mediating the diversity of different physiological effects of galanin. The galanin receptors have a wide-spread distribution, first shown with ligand binding autoradiography (Melander et al., 1988; Skofitsch et al., 1986). Subsequently in situ hybridisation studies allowed visualization of the discrete localization of the three receptor subtypes in rat (Burazin et al., 2000a; O'Donnell et al.,

1999; O'Donnell et al., 2003) and mouse (Lein et al., 2007) brain. Because of lack of reliable antibodies for either of these receptor subtypes (Lu and Bartfai, 2009b), definitive information about the subcellular location of the receptor protein is still sparse.

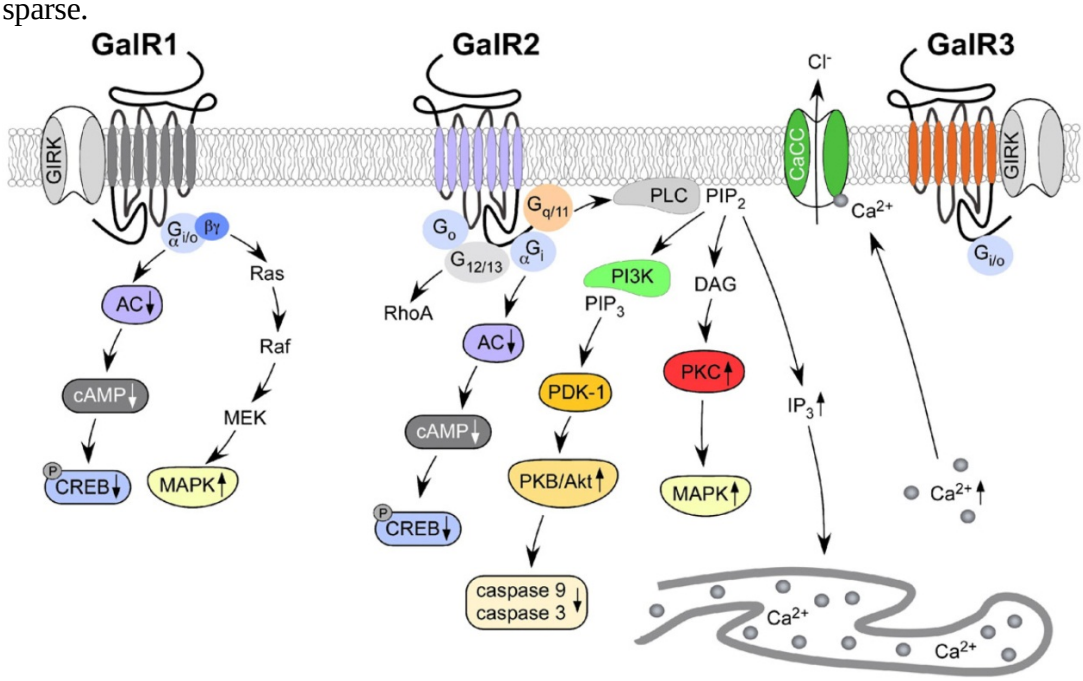

Figure 4. Signaling pathways of galanin receptor subtypes. Abbreviations: AC, adenylate cyclase; CaCC, Ca2+-dependent chloride channel; cAMP, 3′,5′-cyclic adenosine monophosphate; (p)CREB, (phosphorylated) cAMP response element binding protein; 3′,5′-cAMP response element-binding protein; DAG, diacylglycerol; IP3, inositol triphosphate; MEK, mitogen-induced extracellular kinase; PDK-1, phosphoinosotide-dependent protein-kinase 1; PIP2, phosphatidylinositol bisphosphate; PIP3, phosphatidylinositol trisphosphate; PI3K, phosphatidylinositol 3-kinase; PKB, protein kinase B; PLC, phospholipase C. From Lang et al. (2007), with permission.

1.4.1 GALR1

GalR1 was isolated from the human Bowes melanoma cell line (Burgevin et al., 1995; Habert-Ortoli et al., 1994; Parker et al., 1995). The distribution of GalR1 was first revealed by northern blot analysis and reverse transcription polymerase chain reaction (RT-PCR) and appeared relatively restricted (Habert-Ortoli et al., 1994; Lorimer and Benya, 1996). In situ hybridization studies on rat brain have revealed a wide distribution with GalR1 transcript detected in the hypothalamus, amygdala, ventral hippocampus, thalamus, medulla oblongata and spinal cord (Burazin et al., 2000b; Landry et al., 1998; O'Donnell et al., 1999; O'Donnell et al., 2003). Several transduction mechanisms have been reported. Thus, GalR1 activation can via coupling to G_i/G_o G-proteins, (i) inhibit forskolin-stimulated cAMP production in a pertussis toxin (PTX)-sensitive manner (Fitzgerald et al., 1998; Habert-Ortoli et al., 1994; Parker et al., 1995; Smith et al., 1997; Wang et al., 1998b); (ii) open G-protein-regulated inwardly rectifying K^+ (GIRK) channels (Smith et al., 1998); and (iii) activate mitogen-activated protein kinase (MAPK) signaling pathway in

Brain region	*GalR1*	*GalR2*	*GalR3*
Cerebral cortex			
Neocortex	-	(+)	-
Insular cortex	++	-	-
Piriform cortex	++	+	-
Entorhinal cortex	++	-	-
Limbic and basal forebrain			
Lateral septum	++	-	-
Medial septum	+	-	-
Bed nucleus of stria terminalis	++	+	+
Nucleus accumbens (shell)	+	-	-
Amygdaloid nuclei	++	+	(+)
Hippocampus			
Dorsal CA field	-	-	-
Dorsal dentate gyrus	-	+++	-
Ventral CA1	+++	+	-
Ventral CA2, CA3	+	+	-
Ventral dentate gyrus	-	++	-
Hypothalamus			
Paraventricular nucleus	+++	++	(+)
Midbrain			
Ventral tegmental area	-	++	-
Dorsal raphe	+	+	-
Pons			
Locus coeruleus	++	+	(+)

Table 1. Relative levels of galanin receptor subtype mRNAs in several brain areas related to regulation of mood and mood disorders. '+' indicates relative level of expression (both the number of cells and the intensity of signal by cell), '(+)' indicates a very low labelling or very few cells, '-' indicates absence of signal. Modified from O'Donnell et al. (2003) with permission.

a protein kinase C (PKC)-independent fashion (Wang et al., 1998b). The binding of galanin induces internalization of GalR1 (Wang et al., 1998b; Xia et al., 2008), which may be a mechanism for regulating the 'sensitivity' of this receptor. Physiologically, GalR1 receptor is believed to mediate several inhibitory actions of galanin in brain and the GI system (Branchek et al., 2000; Iismaa and Shine, 1999; Wang et al., 2000). The distribution of GalR1 mRNA in the mouse brain is reported in the Allen Brain Atlas (ABA) (Lein et al., 2007). Intrerestingly this receptor was not detected in the LC.

1.4.2 GALR2

The second identified galanin receptor, GalR2, was isolated from the rat hypothalamus (Howard et al., 1997; Smith et al., 1997; Wang et al., 1997a). By coupling with different classes of G-proteins, GalR2 can activate several intracellular pathways. Much focus has been on activation of phospholipase C (PLC) increasing IP3 production and intracellular release of Ca^{2+} from intracellular endoplasmatic reticulum stores. The elevated intracellular [Ca^{2+}] then induces multiple downstream events, such as

induction of a Ca^{2+}-dependent chloride current (Borowsky et al., 1998; Fathi et al., 1997; Pang et al., 1998; Smith et al., 1997; Wang et al., 1998a). These GalR2-mediated effects are PTX-insensitive and mediated by $G_{q/11}$ G-protein. GalR2 can also couple to G_o G-protein, inducing MAPK phosphorylation in a PTX-sensitive, PKC-dependent fashion (Elliott-Hunt et al., 2007; Hawes et al., 2006; Hobson et al., 2006; Wang et al., 1997a). This can subsequently activate the Akt signaling pathway and suppress caspase-3 and caspase-9 activity (Ding et al., 2006; Elliott-Hunt et al., 2007; Hobson et al., 2006). Consequently galanin can mediate neuronal survival by GalR2. The trafficking manner of GalR2 was studied in PC12 cells, showing constitutive internalization, providing evidence for a highly dynamic scenario (Xia et al., 2004).

The GalR2 receptor transcription can be detected widely throughout CNS and peripheral tissues like heart, stomach, intestine, uterus, ovary, prostate and others (Howard et al., 1997; O'Donnell et al., 1999; Smith et al., 1997; Waters and Krause, 2000). In CNS, using RT-PCR and in situ hybridization, the highest levels of GalR2 mRNA expression are detected in the hypothalamus, dentate gyrus, amygdala, piriform cortex and mammillary nuclei (Burazin et al., 2000a; O'Donnell et al., 1999; O'Donnell et al., 2003; Waters and Krause, 2000) (Table 1).

The distribution of GalR2 mRNA in the mouse brain is reported in the Allen Brain Atlas (ABA) (Lein et al., 2007) but appears limited when comparing with the GalR2 transcript in the rat brain (O'Donnell et al., 1999), possibly reflecting low abundancy expression (cf. **Paper II**).

A role for GalR2 promoting survival and protecting against neuronal injury is supported by GalR2 upregulation following nerve injury or inflammation (Burazin and Gundlach, 1998; Sten Shi et al., 1997) and in the HiFo after seizure (Elliott-Hunt et al., 2004; Elliott-Hunt et al., 2007; Mazarati et al., 2004a). GalR2 also enhances neurite outgrowth (Mahoney et al., 2003) suggesting neurotrophic effects.

1.4.3 GALR3

The last identified galanin receptor, GalR3, was cloned from rat and human tissue (Smith et al., 1998; Wang et al., 1997b). The information about signaling properties of GalR3 is sparse, one option being coupling to $G_{i/o}$ G-protein inducing an outward K+ current in a PTX-sensitive manner (Smith et al., 1998), similar to GalR1, leading to hyperpolarization, reduced firing and presumably inhibition of neurotransmitter release (Branchek et al., 2000; Lapsys et al., 1999; Wang and Gustafson, 1998). GalR3 transcription is widely detectable in peripheral tissues such as heart, spleen, testes, liver, kidney and stomach (Smith et al., 1998; Wang et al., 1997b; Waters and Krause, 2000), versus a limited distribution in CNS, including hypothalamus, pituitary, olfactory bulb, cerebral cortex, caudate putamen, cerebellum, medulla and spinal cord, as shown both with RT-PCR and in situ hybridization (Mennicken et al., 2002; O'Donnell et al., 2003; Smith et al., 1998; Waters and Krause, 2000). No results on the distribution of GalR3 mRNA is reported in the ABA (Lein et al., 2007), so to our knowledge no information is available on this receptor in the mouse brain.

Species	Test system	G-proto	Effector	Response	Refs
Rat	HEK293	$G_{q/11}$	PLC	IPs ↑	[1]
Rat	Xenopus oocyte	$G_{q/11}$	PLC	$[Ca^{2+}]i$ ↑ ; activates CaCCs	[2]
	CHO			$[Ca^{2+}]i$ ↑ ; IPs ↑	
	HEK293				
Human	Xenopus oocyte	$G_{q/11}$	PLC	$[Ca^{2+}]i$ ↑ ; activates CaCCs	[3]
	CHO			IPs ↑ ; $[Ca^{2+}]i$ ↑	
Mouse	COS-7	$G_{q/11}$	PLC	IPs ↑	[4]
Rat	COS-7	$G_{o/i}$	Adenylate cyclase	cAMP ↓	[5]
Human	HEK293	$G_{q/11}$	PLC	IPs ↑ ; $[Ca^{2+}]i$ ↑	[6]
		$G_{o/i}$	Adenylate cyclase	cAMP ↓	
Mouse	Hippocampal slice	$G_{o/i}$	Adenylate cyclase	cAMP ↓ ; pCREB ↓	[7]
Rat	CHO	G_o	PKC	pMAPK ↑	[8-11]
		G_i	Adenylate cyclase	cAMP ↓	[8]
	CHO; COS-7	G_q	PLC	IPs ↑	
Human	SCLC (H69,H510)	$G_{q/11}$	PLC β	IPs ↑ ; $[Ca^{2+}]i$ ↑ ; pMAPK ↑	[12]
		G_i	?	?	
		G_{12}	?	Rho ↑	
Rat	Basal forebrain neuron culture	$G_{q/11}$	PKC	Akt ↑	[13]
Mouse	DRG neuron culture	$G_{q/11}$	PKC	pAkt ↑	[10]
Mouse	Hippocampal slice culture	$G_{q/11}$	pAkt ↑	pAkt ↑	[11]

Table 2. Summary of studies investigating the GalR2 intracellular signaling pathways, the coupling G-protein subtype and effector and activity consequence in different biological models.

1. Fathi, Z., et al., Brain Res Mol Brain Res, 1997. 51(1-2): 49-59. **2.** Smith, K.E., et al.,J Biol Chem, 1997. 272(39): 24612-6. **3.** Borowsky, B., et al., 1998. 19(10): 1771-81. **4.** Pang, L., et al., J Neurochem, 1998. 71(6): 2252-9. **5.** Wang, S., et al., J Biol Chem, 1997. 272(51): 31949-52. **6.** Fathi, Z. et al., Brain Res Mol Brain Res, 1998. 58(1-2): 156-69. **7.** Badie-Mahdavi, H., et al., Neuroscience, 2005. 133(2): 591-604. **8.** Wang, S., et al., Biochemistry, 1998. 37(19): 6711-7. **9.** Hawes, J.J.et al., Eur J Neurosci, 2006. 23(11): 2937-46. **10.** Hobson, S.A., et al., J Neurochem, 2006. 99(3): 1000-10. **11.** Elliott-Hunt, C.R., et al., J Neurochem, 2007. 100(3): 780-9.**12.** Wittau, N., et al., Oncogene, 2000. 19(37): 4199-209. **13.** Ding, X., et al., Neurobiol Dis, 2006. 21(2): 413-20.

2 AIMS OF THE THESIS

The overall aim of the thesis is to obtain a better understanding of the localization and function of the GalR2 receptor.

Specific aims

- To study the trafficking of the GalR2 receptor in a PC12 cell line, using a GalR2-EGFP construct. To observe the possible effects of the C-terminal EGFP tag on the functionality of GalR2 receptor (**Paper I**).

- To generate and select (based on Paper I) a transgenic mouse line expressing the novel GalR2-EGFP construct under the GalR2 promoter; with this GalR2-EGFP transgenic mouse line, to explore the cellular localization pattern of GalR2 by immunostaining with GFP antibody (**Paper II**).

- To investigate the effect of galanin on glutamatergic synaptic transmission by pair pulse facilitation (PPF) in the lateral perforant pathway (LPP) of the hippocampal dentate gyrus region (**Paper III**).

- To develop a pure cholinergic basal forebrain neuron primary culture using target-specific isolation and together with p75 primary antibody and a magnet bead secondary antibody. To study with this cholinergic primary culture, the possible plasticity of galanin and galanin receptors in response to beta-amyloid toxiticity. (**Paper IV**).

3 MATERIAL AND METHODS

3.1 ANIMALS

Both adult mice and rats were used in the experiments. GalR2-EGFP mice were generated in our laboratory as described below. At the time of experiments the mice were between 3-18 months old. Female Sprague-Dawley timed pregnant rats (E16-17) were obtained from Harlan (Netherlands) or Charles River (USA). Transgenic and WT mice were housed in groups of two to six in standard plastic cages (Macrolon® Type A3). Rats were housed in groups of four in standard plastic cages (Macrolon® Type A4) until surgery. All animals were kept in colony under standardized conditions (12 h light/dark cycle, lights on at 7 a.m.; temperature of 22±0.5°C and 40-50% relative humidity). Food and water were provided ad libitum up to the time of the experiment. Animals were allowed to habituate to the animal facility for at least five days before starting the experiments. Animal housing and experimental procedures followed the provisions and general recommendations of the Swedish animal protection legislation. All experimental procedures in this thesis were approved by the local Animal Ethics committee (Stockholm norra djurförsöksetiska nämnd) and in special cares by other authorities (see below).

3.2 GENERATION OF A GALR2-EGFP MOUSE

The GalR2 with its promoter was amplified by PCR from C57BL/6N mouse genomic DNA (chr 11:116,138,89 to 11619938). The PCR product was ligated and fused with EGFP in pEGFP-1(Clontech, Mountain View, CA). The construct was injected into pronuclei from fertilized mouse oocytes (Hogan et al., 1986). After the transgenic mice had been back-crossed to C57BL/6N strain for seven generations, the copy number inserted into the genomic DNA was revealed by qPCR analysis. The result showed that one extra copy of GalR2-EGFP had been integrated into the genome (Figure 5A, B).

3.3 GENOMIC DNA PREPARATION AND GENOTYPING

Biopsies from mouse-tail were used for genomic DNA extraction, and DNA was amplified using PCR with specific primers for GALR2-EGFP: forward 5´ GTCAACCCCATCGTTTATGCTC TGGTCTCC-3´, and reverse 5´-TGGGTGCTCAGGTAGTGGTTGTCGG-3´ and Taq DNA polymerase (2.5 units; Sigma, St. Louis, MO). The thermo cycle was at 94℃ for 20 s, at 62℃ for 30s, and at 72℃ for 1 min for 30 cycles and, finally, at 72℃ for 10 min. The PCR product showed an 880-bp EGFP-GALR2 band (Figure 5C).

3.4 COLCHICINE INJECTION

Some mice (n=6) were injected intracerebroventricularly (i.c.v.) with colchicine under Hypnorm/Midazolam anesthesia (contains Midazolam 12.5 mg/kg, fentanyl citrate 0.78 mg/kg and fluanisone 25 mg/kg i.p.). Colchicine (Sigma) was dissolved in 0.9% NaCl to a final concentration of 30 ug in 5 ul and slowly infused i.c.v. into the right ventricle using a Hamilton syringe with 26-gauge needle attached. Injection coordinates were AP -0.2 mm from bregma, L -0.9 mm from midline, and V -2.0 mm deep from the surface of the brain, according to the Atlas of Paxinos and Franklin (Paxinos and Franklin, 2007). The syringe was left in the brain for three min after injection. Animals received

repeated intraperitoneal (i.p.) saline/5% glucose injections. Twenty-four hours after injection, animals were perfused and processed for immunohistochemistry.

3.5 IMMUNOHISTOCHEMISTRY

All animals were deeply anesthetized with sodium pentobarbital (60 mg/kg i.p.) and perfused through the heart via the ascending aorta with 20 ml Ca^{2+}-free Tyrode's buffer (37°C), followed by 20 ml of a picric acid-formalin fixative (4% paraformaldehyde and 0.2% picric acid diluted in 0.16 M phosphate buffer, pH 6.9) (Stefanini et al., 1967) and 50 ml of the same fixative at 4°C, the latter for approximately 5 min. The brains were dissected out and postfixed in the same fixative for 90 min at 4°C, then transferred into 10% sucrose solution containing 0.01% sodium azide (Sigma) and 0.02% Bacitracin (Sigma) at 4°C. After immersion in 10% sucrose solution for about 48 h, the brains were rapidly frozen by CO_2. Sections were cut in a cryostat (Microm, Heidelberg,

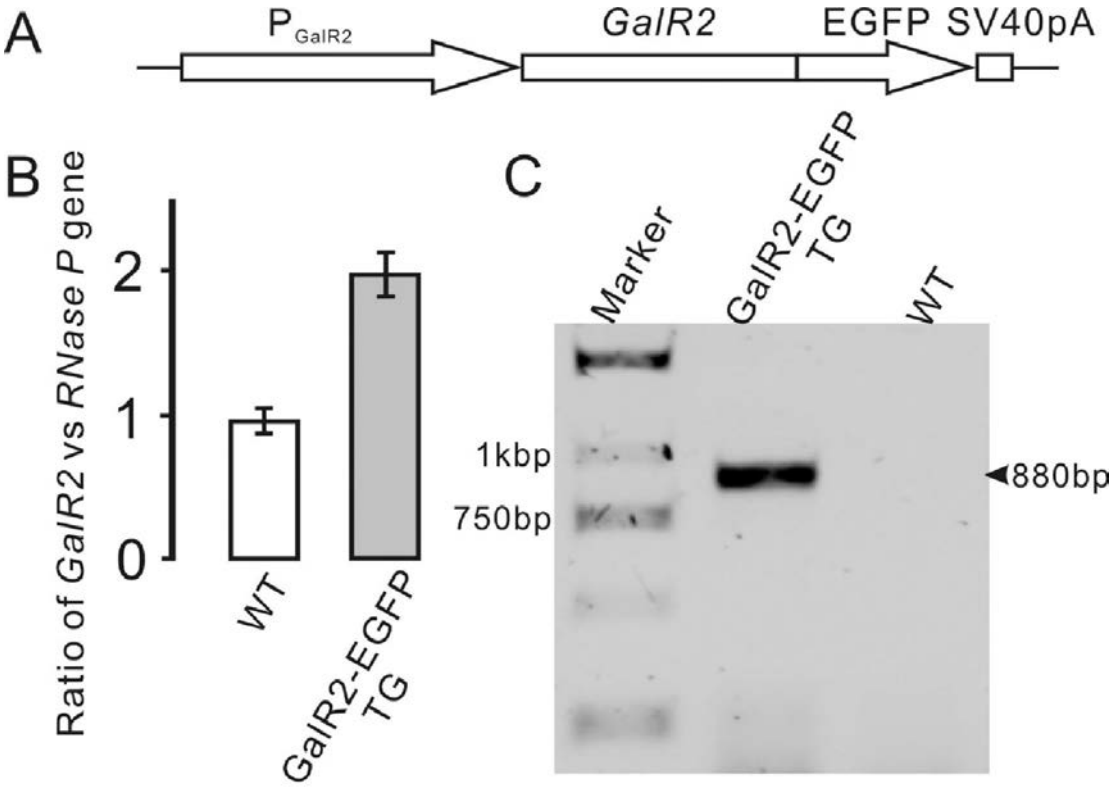

Figure 5. Generation, verification and genotyping of GalR2-EGFP transgenic mouse. Schematic drawing of the GalR2-EGFP construct consisting of the GalR2 promoter, and EGFP in the 3' end (A). CalR2 gene copy number was calculated by compare to the *RNase P* gene (1.97±0.16, B). The PCR product showed an 880-bp EGFP-GALR2 band in the transgenic animal (C).

Germany) at a thickness of 14 µM, and thaw-mounted on to aluminum gelatin-coated slides. Every tenth section was selected for immunohistochemistry, and an adjacent section was stained with 0.25% cresyl violet (Merck, Darmstadt, Germany) in distilled water.

3.5.1 SINGLE-STAINING TSA+

Sections were rinsed for 2-10 min in 0.01 M PBS and incubated in 0.03% H2O2 in PBS for 15 min. After further 2-10 min washes in PBS, sections were incubated for 24 h at 4°C with a rabbit antibody against GFP (1:2,000; Invitrogen, Carlsbad, CA) diluted in 0.01 M PBS containing 0.3% Triton X-100 and 0.5% bovine serum albumin (BSA). To visualize immunoreactivity, sections were processed with a commercial kit (TSA+; NEN Life Science Products, Boston, MA; PerkinElmer Inc., Waltham, MA). Briefly, sections were washed in TNT buffer (0.1 M Tris-HCl, pH 7.5; 0.15 M NaCl; 0.05% Tween-20; Sigma) for 10 min, incubated with TNB buffer (0.1 M Tris- HCl, pH 7.5; 0.15 M NaCl; 0.5% blocking reagent) for 30 min at room temperature (RT), and incubated with a swine anti-rabbit/horseradish peroxidase conjugate (Dako, Copenhagen, Denmark) diluted 1:200 in TNB buffer for 30 min at RT. Sections were then washed 4x in TNT buffer and incubated for 10 min at RT in a biotinyl tyramide-fluoroscein isothiocyanate (BT-FITC) conjugate (NEN EkinElmer) diluted 1:100 in amplification diluent. Sections were washed for 2-10 min in TNT and coverslipped with 2.5% 1,4-diazabicyclo[2.2.2]octane (DABCO; Sigma) in glycerol (Sigma).

3.5.2 DOUBLE IMMUNOHISTOCHEMICAL STAINING

Some sections stained with GFP antibody were processed for double-staining. Here the second primary antibody was diluted and processed as described in Table 3 in 0.01 M PBS containing 0.3% Triton X-100 and 0.5% BSA, that is using the 'routine' immunofluorescence method of Coons and collaborators (1958). After incubation overnight the sections were washed with 0.01 M PBS at RT for 30 min, incubated for 30 min at 37℃ with appropriate secondary antibodies labeled with lissamine rodamine (LRSC) (Jackson ImmunoResearch, West Grove, PA). Finally, the sections were washed in 0.01 M PBS at RT and coverslipped as above.

3.6 IMAGE PROCESSING

After processing, brain sections were examined with a Nikon Eclipse E600 fluorescence microscope (Nikon, Tokyo, Japan), equipped with a darkfield condenser and epifluorescence with appropriate filter combinations, and with objective lenses of X2, X4, X10, and X20. Photographs were taken with a Hamamatsu ORCA-ER C4742-80 digital camera attached to the Nikon Eclipse microscope, using Hamamatsu Photonics Wasabi 150 software.

For confocal analysis, we used a Bio-Rad Radiance Plus confocal scanning device installed on a Nikon Eclipse E600 fluorescence microscope equipped with X10 (N.A. 0.45), X20 (N.A. 0.75), and X60 (N.A. 1.40) oil objectives. The FITC labeling was excited at a 488-nm argon laser and its signal detected with the HQ 530/60 (Bio-Rad Hercules, CA) emission filter. Digital images from the microscopy were optimized for resolution, brightness, and contrast in Adobe Photoshop 7.0 (Adobe Systems Inc., San Jose, CA).

Specificity	*Origin*	*Staining method*	*Dilution ratio*
TH	Incstar Corp	Routine immuno	1:1,000
GAD67	Millipore (Chemicon)	TSA+	1:20,000
Parvalbumin	Swant	Routine immuno	1:200
Calretinin	Dr. Mathias Uhlen	Routine immuno	1:1,000
Calbindin	Swant	Normal immuno	1:200
5-HT	Dr. A.A.J. Verhofstad	Routine immuno	1:400
Galanin	Dr. A.A.J. Verhofstad	Routine immuno	1:1,000
ChAT	Millipore (Chemicon)	Routine immuno	1:200
GFP	Invitrogen	TSA+	1:2,000

Table 3. List of antibodies and staining method used in this study. ("Routine" means according to Coons (1958)).

3.7 PC12 CELLS CULTURE AND TRANSFECTION

The PC12 cells were obtained from the American Type Culture Collection (Manassas, VA) and cultured in DMEM (Invitrogen) supplemented with 10% horse serum (Invitrogen) and 5% FBS (Invitrogen) at 37°C in a 5% CO_2 incubator. Cells were transfected by using 1 μg of DNA with an Effectene Transfection Reagent kit (Qiagen, Hilden, Germany). For transient expression, cells were used for experiments 24–48 h after transfection. To generate stable transfectants, the transfected cells were selected in the presence of the antibiotic Geneticin (G418) (Invitrogen) at 800 μg/ml. Transfected PC12 cells were subcultured into eight-well chamber slides (Lab-Tek) (Nunc, Roskilde, Danmark) and starved for 4 h before experiments.

3.8 IN VITRO ELECTROPHYSIOLOGY

Following decapitation mice brains were rapidly removed, and transverse slices (300 μM thick) were cut on a Vibratome in ice-cold artificial cerebrospinal fluid (aCSF) containing (mM): 124 NaCl, 2.5 KCl, 1.3 $MgSO_4$, 1.24 NaH_2PO_4, 2.4 $CaCl_2$, 25 $NaHCO_3$ and 10 glucose; bubbled with a mixture of 95% O_2 and 5% CO_2 for more than 15 min. After recovery in aCSF in RT for about 1 h, slices were transferred to a submerged chamber continuously perfused (at a speed of 2 ml/min) with oxygen-enriched aCSF at RT. Extracellular field potential recordings were made using glass microelectrodes (1–2 MΩ) filled with aCSF. DC-coupled signals were amplified using an EPC9-2 amplifier (HEKA, Lambrecht/Pfalz, Germany), and the resulting signal was then low-pass filtered at 5 kHz. For synaptic plasticity at lateral perforant path (LPP)-dentate gyrus cell synapses, bipolar stimulating electrodes were positioned in the outer third of the molecular layer, adjacent to the hippocampal fissure, and distal from the dentate granule cell layer. In every series of experiments, stimuli comprised square-wave pulses (100 μs; 3–10 V) delivered at a fixed intensity with a frequency of 0.1 Hz. Field excitatory postsynaptic potentials (fEPSPs) were recorded from the dentate gyrus. Paired-pulse facilitation (PPF) of fEPSPs at the LPP-dentate gyrus synapses was elicited in dentate gyrus after paired stimulation with two different interpulse intervals (25 or 50 ms). Baseline recordings were made for a minimum of 20 min to ensure stationarity of responses before experiments were initiated. All data were stored on a

personal computer for on-line and off-line analysis with Igor software (WaveMetrics, Lake Oswego, OR). Statistical analysis of differences between groups was performed by using Student's unpaired t-test. Differences were considered significant at $P < 0.05$. Data are presented as mean ± SEM.

3.9 TARGET-SPECIFIC ISOLATION OF CHOLINERGIC NEURONS

Female Sprague Dawley rats were deeply anesthesized with isoflurane (5% [v/v%]; in O_2/N_2O flow rate: one l/min) on day 17 of pregnancy (E17), and embryos were removed and kept in ice-cold Hank's buffered salt solution (HBSS) (Invitrogen) with added 100 U/ml penicillin/streptomycin (Invitrogen) and glucose (0.6% [v/v%]) (dissociation medium). Procedures relevant to animal care, treatment and killing methods were approved by relevant local ethical committees of the University of Groningen, Karolinska Institutet and National Institutes of Health. Whole brains were removed; the basal forebrain region was dissected out and collected in ice-cold dissociation medium. Tissue blocks were gently triturated (< 1 mm^3) and then enzymatically dissociated by trypsin (0.1%, dissolved in dissociation medium; Invitrogen) for 10 min at 37℃. The reaction was stopped by using Neurobasal medium supplemented with BSA (0.4%, Sigma) and DNAse (1000 U/ml; Promega, Fitchburg, WI) as replacement medium, and dissociated cells were filtered through 40 µM cell strainer (BD, Franklin Lakes, NJ) and collected by centrifugation (500 rpm, 10 min). Cell debris was removed from the resultant cell pellet by repeated re-suspension/centrifugation in Neurobasal medium with 5% BSA. A monoclonal antibody raised against the N-terminus of the rat p75NGFR (Clone: 192IgG, Millipore, Billerica, MA) was coupled to paramagnetic beads coated with goat anti-mouse IgG (Dynal Biotech/Invitrogen). Dissociated cells from the fetal basal forebrain were exposed to stable antibody constructs in suspension and under continuous agitation at RT for 90 min. Target cells were isolated using a magnetic particle concentrator (Dynal/Invitrogen). Antibody linkers were broken down by a brief trypsin treatment (0.1%, 5 min, 37°C), and released cells were plated in poly-D-lysine (15 µg/ml)-coated wells at a proper density. Isolated neurons were cultured in Neurobasal medium conditioned by forebrain astroglia for 24h (1:1) (Berghuis et al., 2004; Guerri et al., 1989) supplemented by B27 (2%; Invitrogen) and penicillin/streptomycin (100 U/ml). Half of the culture medium was replaced every other day with Neurobasal medium. Cultures were maintained in a humidified incubator (37°C) gassed with 95% air/5% CO_2.

4 RESULTS

4.1 TRAFFICKING AND FUNCTION OF C-TERMINAL EGFP-FUSED GALR2 (PAPER I)

Little was known about trafficking of galanin receptors some ten years ago, one important reason being the lack of reliable antibodies against these receptors. To circumvent this problem, we decided to take a molecular biological approach, and create a GalR2-EGFP construct for transfection into cell lines (**Paper I**) and for generating a transgenic mouse expressing this construct (**Paper II**).

4.1.1 SUBCELLULAR DISTRIBUTION, TRAFFICKING AND FUNCTION OF GALR2 EXPRESSED IN PC12 CELL

Using a stably expressed GalR2-EGFP fusion protein we were able to directly examine the subcellular localization of GalR2 and its intracellular trafficking at steady state and upon ligand stimulation. After expression of GalR2-EGFP cDNA in PC12 cells followed by fixation and imaging in the confocal microscope, a strong green fluorescent signal was observed. GalR2-EGFP predominantly appeared on the plasma membrane with some intracellular fluorescence localized mostly in presumable vesicles in the perinuclear region.

The GalR2-EGFP conjugate was found functional and capable of coupling to the phosphatidylinositol hydrolysis signaling pathway. PC12 cells stably expressing GalR2-EGFP were treated with galanin, and $[Ca^{2+}]_i$ was measured. Galanin application caused a concentration-dependent increase in $[Ca^{2+}]_i$ levels in GalR2-EGFP-transfected cells, but not in untransfected cells, with an EC50 at 25 nM. Application of AR-M1986, a selective GalR2 agonist, induced a similar increase of $[Ca^{2+}]_i$.

4.1.2 CONSTITUTIVE RECYCLING OF GALR2-EGFP

To define the primary source of intracellular GalR2-EGFP, the distribution of GalR2-EGFP was monitored after continuous treatment with cycloheximide, an inhibitor of protein synthesis. This treatment did not change the distribution of GalR2-EGFP (Figure 6B), Moreover, when GalR2-EGFP-transfected cells were incubated with Texas red-conjugated transferrin, a marker for early and recycling endosomes, a substantial proportion of intracellular GalR2-EGFP colocalized with transferrin (Figure 6B). To further examine a possible constitutive internalization, we blocked endocytosis with methyl- β -cyclodextrin (M β CD) (10 mM), which inhibits clathrin-mediated endocytosis but does not affect intracellular receptor recycling and steady-state distribution of clathrin. After incubation with M β CD, there appeared to be an increased amount of GALR2-EGFP on the surface and a concomitant decrease in intracellular GalR2-EGFP (Figure 6 C). After blocking endocytic recycling for 1 h with monensin (50 μM), a potent inhibitor of recycling, GalR2-EGFP accumulated in intracellular vesicles and decreased on the plasma membrane (Figure 7 D). The FDm/FDc ratio was decreased (Figure 6E). Application of cytochalasin D which inhibits recycling by blocking actin polymerization, also increased intracellular accumulation and decreased membrane expression of GalR2-EGFP (Figures 6 E and 7 G-I).

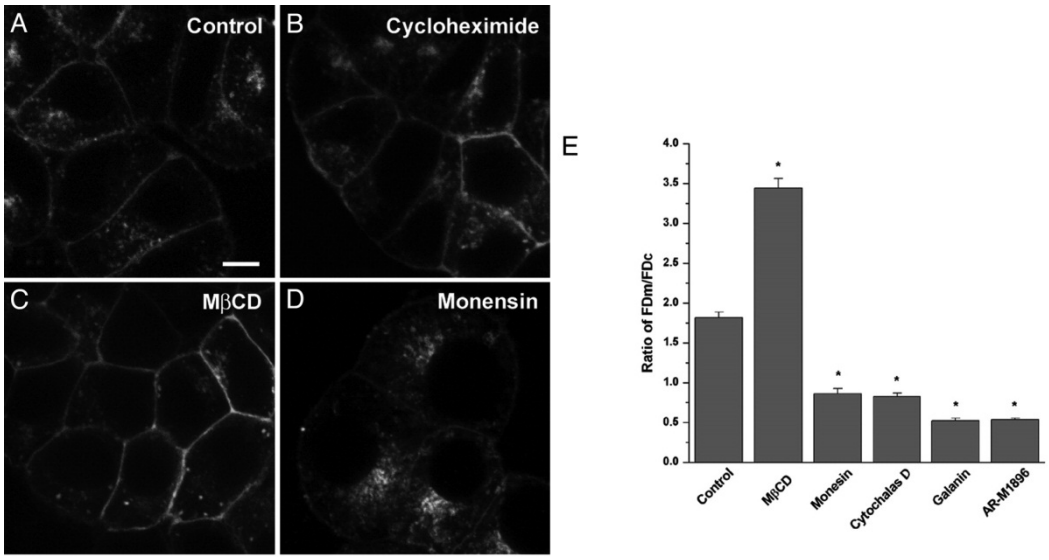

Figure 6. Effect of monensin, M β CD, cycloheximide, galanin, and AR-M1896 on the subcellular distribution of GALR2-EGFP in transfected PC12 cells. Cells transfected with GalR2-EGFP were incubated with cycloheximide (25 µM) for 4 h (B) or M β CD (10 mM) (C) or monensin (50 µM) for 1 h (D) at 37℃. (Scale bar: 8 µM, for A-D.) (E) Transfected cells were examined with confocal microscopy after treatment with M β CD (10 mM), monesin (50 µM), cytochalasin D (4 µg/ml), galanin (1 µM), or AR-M1896 (1µM). Confocal images were collected and quantified, and the corresponding FDm/FDc ratio was calculated in each case (n = 20). Results are expressed as mean ± SEM. Asterisks indicate significant difference from control ($P < 0.001$).

4.1.3 LIGAND-INDUCED INTERNALIZATION OF GALR2-EGFP

Incubation of GalR2-EGFP-transfected cells with galanin resulted in a dramatic redistribution of GalR2-EGFP fluorescence from the plasma membrane to an intracellular compartment, leading to a rapid and extensive decrease in surface GalR2-EGFP and with a multitude of punctate structures appearing in the cytoplasm in a dose-dependent manner. Internalization was detectable after 3-5 min, but appeared maximal at 10-15 min and was maintained for at least 30 min. After removal of galanin (washout), GalR2-EGFP was again seen on the plasma membrane, even in the presence of cycloheximide. The reinsertion of GalR2 after washout was blocked by cytochalasin D. Restimulation with galanin after washout induced reinternalization of GalR2-EGFP. Application of the GalR2 agonist AR-M1986 also caused internalization of GalR2-EGFP from the plasma membrane to the cytoplasm. Preincubation with the putative galanin antagonists M35 or M40 induced internalization of GalR2-EGFP by themselves and did not prevent either AR-M1986- or galanin-induced internalization of GalR2-EGFP. In fact, both M35 and M40 increased $[Ca^{2+}]_i$ levels in GALR2-EGFP-transfected cells.

4.1.4 TRAFFICKING OF GALR2-EGFP IN TRANSFECTED PC12 CELLS

When transfected cells were treated with Texas red-transferrin alone, GALR2-EGFP and Texas red-transferrin were separately compartmentalized at the membrane level, not in the perinuclear area. Thus, GALR2-EGFP was located on the plasma membrane, whereas transferrin was distributed in the cytoplasm (Figure 7 A–C). However, after coincubation with galanin and Texas red-transferrin for 30 min, the internalized GALR2-EGFP overlapped strongly with Texas red transferrin, the marker of the

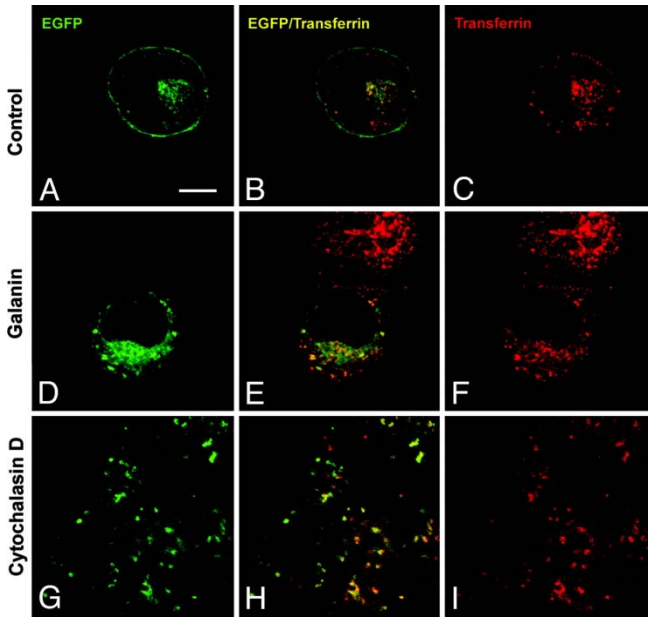

Figure 7. Colocalization of GalR2-EGFP with transferrin. Transfected cells were incubated with Texas red-transferrin alone for 30 min (A-C), plus galanin (D-F), or plus cytochalasin D (G-I). The distribution of GalR2-EGFP (green) and transferrin (red) was examined with a confocal microscope. Colocalization of GalR2-EGFP with transferrin (yellow) can be observed in the merged images. (Scale bar: 8 µM, for A-I.)

clathrin endocytic pathway (Figure 7 D–F). Moreover, after treatment with 0.4M sucrose, which is known to block internalization processes that use clathrin-coated pits and vesicles, for 5 min before coincubation, internalization of GALR2-EGFP did not occur. All those data indicate that the clathrin pathway is involved in GALR2 endocytosis.

Taken together, these results suggest that GalR2, stably fused with EGFP and expressed in PC12 cells, (i) is mainly localized at the cell membrane, (ii) is internalized by ligand using the clathrin-dependent, endocytic recycling pathway, (iii) is functional and (iv) undergoes ligand-independent, constitutive internalizatio.

4.2 GALANIN R2 RECEPTOR DISTRIBUTION IN THE BRAIN OF A GALR2-EGFP TRANSGENIC MOUSE (PAPER II).

In order to overcome the lack of a reliable antibody (Lu and Bartfai, 2009a) to localize GalR2 by immunohistochemistry, we examined the expression of a GalR2- EGFP) construct in the mouse brain using a GFP antibody.

The expression of EGFP fused on to the C-terminus of GalR2 controlled by the GalR2 promoter was not strong enough for direct visualization of the construct in the fluorescence microscope in the brain of the transgenic C57BL mouse. Therefore we used a GFP antibody to visualize the EGFP tag using the highly sensitive modification of the original Coons method, the TSA+ technique (Adams, 1992).

The results showed a wide distributtion of GalR2-EGFP in neuronal cell bodies but only to a limited extent in nerve terminals/nerve endings. Colchicine treatment markedly increased the number of immunoreactive cell bodies, and the results are therefore mainly based on colchicine-treated mice. The staining patterns were specific

in the sense that they could not be observed in sections from wild-type (WT) mice present on the same slides as those from the transgenic mice, sections which thus were processed under the same conditions. Exceptions were staining in the olfactory tubercle and fiber-like staining in substantia nigra, reticular part (SNR), where staining also appeared in the tissue from WT animal. The results in WT mice will therefore not be further mentioned. In the following we will only describe areas with strongly and moderately stained cell bodies, and areas containg fiber staining.

GalR2-EGFP positive cell bodies and processes were located in many brain regions, including the olfactory bulb, certain limbic cortical areas, the basal forebrain, amygdala, subregions of the hippocampal formation, thalamus, hypothalamus, periaqueductal grey, LC, and some further areas in the midbrain and medulla oblongata. Using double-staining we analyzed some cell groups giving rise to major ascending systems, and could demonstrate presence of the GalR2-EGFP construct in noradrenergic LC neurons most of which also express galanin. The GalR2-EGFP was also detected in calcium-binding protein- and GAD-positive cell bodies in the basal forebrain neurons, but not in cholinergic neurons in this area, nor in the 5-HT neurons in the DRN. Our results support the concept that GalR2 primarily is a presynaptic receptor, and an autoreceptor in noradrenergic LC neurons. Galanin is presumably involved in a wide range of brain functions, partly executed through GalR2.

4.2.1 TELENCEPHALON

In the olfactory system, GFP-positive (GFP^+) cell bodies and processes were found in the granular layer of the main and accessory olfactory bulb and in the anterior olfactory nucleus.

High numbers of GFP^+ cell bodies were observed in the entorhinal cortex with moderate numbers in the endopiriform claustrum, primary and secondary somatosensory cortex and piriform cortex.

GFP^+ cell bodies were found in several subregions of the basal forebrain: the medial septum nucleus and the vertical and horizontal limbs of the diagonal band nucleus. Lower numbers were found in the medial forebrain bundle area, medial ventral pallidum and the bed nucleus of the stria terminalis. Strong and patchy GFP^+ processes were found in the periventricular and medial part of caudate putamen and in accumbens nucleus, core. Moderate GFP^+ fiber staining was found in the medial part of accumbens nucleus and the septohippocampal nucleus.

In the amygdaloid complex, moderate numbers of GFP^+ cell bodies were found in the medial amygdaloid nucleus, amygdalo-hippocampal nucleus; and amygdalopiriform nucleus and transition area.

The GFP staining in HiFo was mainly seen in the ventral part. GFP^+ cell bodies were found in field CA3, dentate gyrus and polymorph layer of dentate gyrus. Dense staining of processes was found in the parasubiculum, and in the most ventral aspects of the dentate gyrus surrounding the strongly stained cell bodies. Also in the CA3 pyramidal cell layer a weak fiber staining was observed.

4.2.2 DIENCEPHALON

Strong GalR2-GFP^+ cell bodies staining were seen in moderate numbers in many hypothalamic nuclei: the lateral preoptic nucleus; the periventricular region; the anterior hypothalamic area; the lateral hypothalamic area/perifornical region; the dorsomedial nucleus; and the posterior hypothalamic area. Among the mammillary nuclei, strongly/moderately stained cell bodies were observed in the retro- and premammillary nuclei.

GalR2-EGFP$^+$ cell bodies were widely distributed in the thalamic nuclei. A particularly strong staining was found in the border area between the lateral and medial habenular nuclei, the former being weakly stained versus lack of staining in the latter. Dense cell body staining was found in the anterior dorsal nucleus. Strongly/moderately immunoreactive cell bodies were located in the zona incerta. Moderately stained cell bodies were seen in the paraventricular thalamic nucleus and in the medial aspects of the parafascicular nucleus.

4.2.3 MESENCEPHALON

Numerous strongly stained cell bodies were found in the periaqueductal gray, including the laterodorsal tegmental nucleus, extending laterally to the medial geniculate body and the DRN, caudal part; the interpeduncular nucleus, dorsomedial subnucleus; the ventral aspects of the mesencephalic reticular area, dorsal to the substantia nigra, pars compacta; and the pre-Edinger-Westphal nucleus. Moderate cell body staining was found the median raphe nucleus; the parabigeminal nucleus; the superficial gray layer of the superior colliculus extending into the inferior colliculus; and the rostral linear nucleus.

4.2.4 CEREBELLUM

In the cerebellum GalR2-EGFP had a very limited distribution and was only found in Purkinje cell bodies and its dendritic processes in flocculus.

4.2.5 PONS

Many strongly GalR2-EGFP$^+$ cell bodies were observed in the periaqueductal gray, the LC, the medial and lateral parabrachial, and the tegmental nuclei. GalR2-EGFP$^+$ nerve fibers were only detected in the lateral parabrachial nucleus.

4.2.6 MEDULLA OBLONGATA

GalR2-EGFP$^+$ cell bodies were observed in the hypoglossal and solitary tract nucleus; the giganto- and paragigantocellular nuclei; and the intermediate reticular nucleus, ventral and dorsal parts. GalR2-EGFP$^+$ nerve fibers were seen in the caudal superficial spinal trigeminal nucleus.

4.2.7 CIRCUMVENTRICULAR ORGAN/OTHERS

Strongly fluorescent cells were observed in the subcommissural and subfornical organs. However, only a proportion of the cells was immunoreactive. Many fluorescent cell bodies were observed in area postrema. Fluorescent fibrous structures, possibly meninges, were found in the midline between the most rostral parts of cortex and OB, along the ventral aspects of cortex.

4.3 AGE-RELATED FUNCTIONAL PLASTICITY OF GALANIN SIGNALING IN THE HIFO (PAPER III).

When a presynaptic neuron receives two stimuli in rapid succession, the postsynaptic response will commonly be larger for the second than for the first pulse — a phenomenon known as paired-pulse facilitation (PPF) (López, 2001).

PPF of fEPSPs at the LPP-dentate gyrus synapses was elicited in dentate gyrus after stimulation with different interpulse intervals. Slices from young adult WT mice showed significant facilitation of the 2nd EPSP evoked with paired-pulse stimuli. The

average facilitation was 13 ± 6.6% when interpulse interval was 25 ms, and 19.6 ± 3.9% when interpulse interval was 50 ms (n = 15). However, PPF was significantly reduced in slices from old WT mice (>20 months). This is consistent with a study on aged C57BL/6 mice by Froc et al. (2003). In the GalOE mice we found that PPF was reduced, especially in old mice. Thus, PPF was reduced in slices from young adult GalOE mice and were not observed at all in slices from old GalOE animals, when the same stimulus parameters were applied. To further explore the involvement of galanin, the unselective, putative galanin receptor antagonist M35 (Bartfai et al., 1992) was added to the bath. M35 increased PPF in slices from young and old GalOE mice both at interpulse interval 25 and at 50 ms. In slices from old WT mice M35 only increased PPF at 50 ms, but M35 had no effect in slices taken from young WT mice. These findings indicate that the rescuing effect of M35 on PPF in GalOE and old WT mice is due to blocking the inhibitory effect of endogenously released galanin. These data indicate that galanin is involved in aged-related reduction of synaptic plasticity in the HiFo.

Kinney et al. (2009), using in vivo electrophysiology, reported that PPF in rat was not affected by galanin or galanin 2-11, suggesting that galanin has no effect on presynaptic function in the dentate gyrus. In the same model, activation of both GalR1 and GalR2 inhibited LTP in the dentate gyrus (Kinney et al., 2009). This discrepancy from our findings may relate to our use of mice and that the effect in our test was seen in old mice and a GalOE mouse; as well as to the experiment model, our recording done on in vitro slice preparation, whereas Kinney et al. used in vivo recording in rat with i.c.v. drug application; also differences in dose may contribute.

When considering the cognate galanin receptor involved, this would be in agreement with the plasticity in expression of GalR2 in studies on seizure activity (Mazarati and Lu, 2005). The galanin receptor involved in the age-related functional plasticity of galanin shown in the PPF experiments is not known, but GalR2 would be a possible candidate.

4.4 IN VITRO STUDIES ON GALR2 IN ISOLATED CHOLINERGIC BASAL FOREBRAIN NEURONS (PAPER IV)

4.4.1 TSI OF CHOLINERGIC BASAL FOREBRAIN NEURONS

Our target specific isolation (TSI) of cholinergic neurons (Figure 8) at E17-18 generally yielding 20,000-30,000 cells/embryo of isolated neurons predominantly expressing phenotypic markers of cholinergic cells (Figure 8) Consequently, cultures prepared from E17 rat basal forebrains have been used throughout.

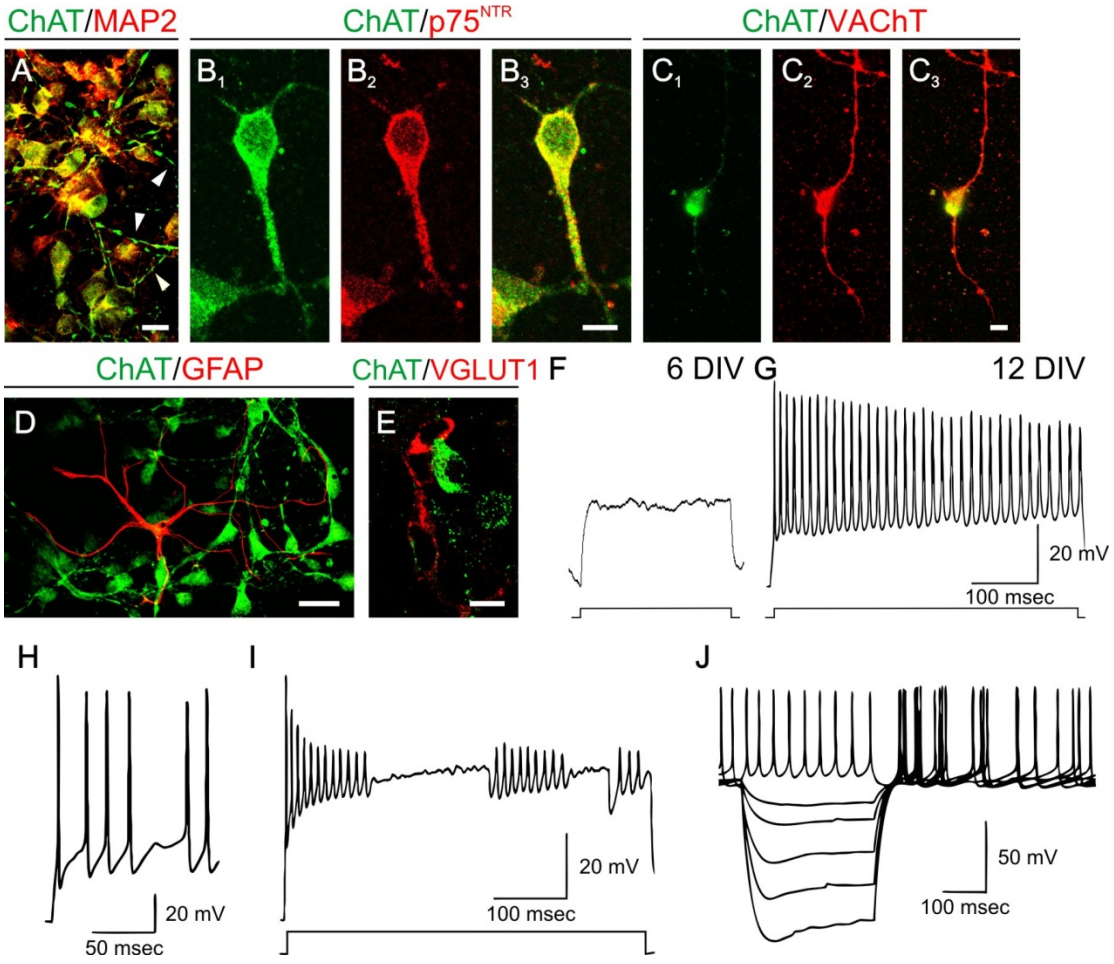

Figure 8. Clarification of target specific isolation of $p75^{NTR}$ expressing basal forebrain cells. After 6 DIV a layer of MAP2-positive neurons harbouring axons and dendrites was formed. Almost all MAP2-positive cells also expressed ChAT (A); note the distribution of ChAT to the MAP2-negative axon, arrowhead. Also the selection marker $p75^{NTR}$ (B) and the cholinergic marker VAChT (C) were expressed in these neurons. Cultures were contaminated with GFAP positive astrocytes (D) and very few VGLUT1 expressing neurons (E) that never colocalized with ChAT immunoreactivty. Current-clamp recording of cholinergic neurons at 6 and 12 DIV. Cells cultured for 6 days showed no spontaneous or depolarization-induced firing behaviour (F). After 10-12 DIV cells became excitable and produced a train of action potentials upon depolarization (G). Cholinergic neurons exhibit slow depolarization underlying action potentials when depolarized (H) and show strong adaptation when further depolarized (I). After the membrane potential is released from -105 mV to resting membrane potential, cholinergic neurons immediately start firing (J). DIV, days in vitro. Scale bars = 20 µM (A-D); 50 µM (E)

Cholinergic neurons are particularly reliant on the support of trophic factors and morphogens during brain development, including fibroblast growth factors (FGFs) (Levi-Montalcini, 1987). Since specific requirements of cholinergic cells to survive and undergo phenotypic differentiation during basal forebrain development are largely unexplored (Schnitzler et al., 2008), we have applied culture media conditioned by astroglia with basal forebrain identity. The use of conditioned medium was required and sufficient to rescue cholinergic neurons in vitro. Replacement of glial condition medium 48 h after isolation did not impact cell survival. Primary neuron cultures enriched in cholinergic neurons remained viable at least up to 20 days under optimal

culture conditions. Long-lasting culture experiments beyond this period have not been performed.

4.4.2 CHARACTERIZATION OF CHOLINERGIC NEURONS

Cultures were analysed for the presence of cholinergic (ChAT), vesicular ACh transporter (VAChT), p75NTR and non-cholinergic markers: glutamate decarboxylase 65/67 (GAD), vesicular GABA transporter (vGAT), vesicular glutamate transporter 1(vGluT1), pan-neuronal (microtubule associated protein 2 [MAP2], and the non-neuronal marker glia fibrillary acidic protein (GFAP). Primary cultures contained many MAP2 immunoreactive cells ubiquitously expressing ChAT immunoreactivity (Figure 8A), p75NTR and VAChT (Figure 8B, C), indicating enrichment of cholinergic neurons within our cultures (Binder et al., 1985). Surplus cells were GFAP immunoreactive astrocytes (Figure 8D) and occasionally encountered VGLUT1 immunoreactive cells (Figure 8E).

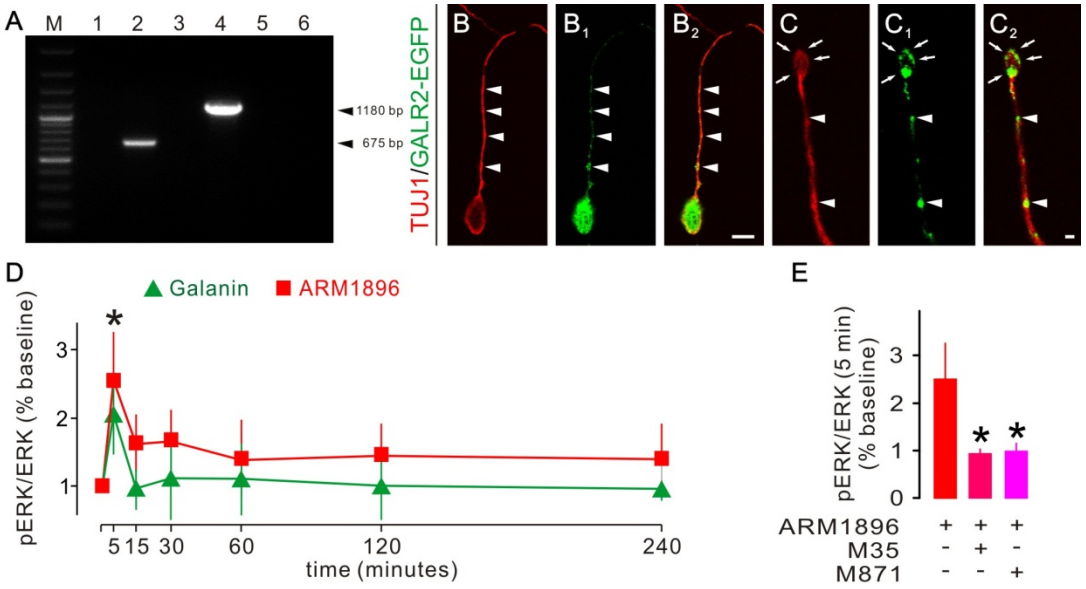

Figure 9. Expression of GalR1 (lane1), GalR2 (lane 2), GalR3 (lane 3) and GAPDH (lane 4) was determined using RT-PCR. The quality of the sample and the specificity of the GalR2 primer set were validated by omitting the sample (lane 5) and omitting the reverse transcriptase step in the sample preparation (lane 6). GalR1 (lane 1) and GalR3 (lane 3) could not by detected by RT-PCR. However, RT-PCR for GalR2 (lane 2) and GAPDH (lane 4) resulted in amplification of a product of predicted sizes (675 bp and 1.18kb respectively; A) To study the subcellular distribution of GalR2 receptors in cholinergic neurons, cells were transfected with GalR2-EGFP. GalR2-EGFP was mainly located in the soma (B), but clusters of GalR2-EGFP could also be identified in the proximal (arrowheads in B) and distal(arrowheads in C) portion of the developing axon, and plenty of GalR2-EGFP could be identified in the central domain of the growth cone (arrows in C). Endogenous expression of functionally active GalR2 in cholinergic basal forebrain neurons was confirmed by GalR2 induced phosphorylation of Erk (D,E). Galanin (0.1µM) and ARM1896 (0.1µM) induced a rapid phosphorylation of ERK that peaked at 5 minutes of stimulation (D). GalR2 induced phosphorylation of Erk could be fully antagonized by applying the non-selective galanin receptor antagonist M35 or the selective GalR2 receptor antagonist ARM871 (E). Scale bar = 10 µM (B,C).

We have tested ´the electrophysiological profiles of cultured cholinergic neurons. The cultured cholinergic cells fired trains of action potentials after 12 or 16 days' culture (Figure 8F, G). Cultured neurons exhibited slow depolarization underlying action potentials (Figure 8H) reminiscent of cholinergic neurons sampled in acute brain slice preparations (Griffith and Matthews, 1986; Markram and Segal, 1990). Spike trains appeared to accommodate (Figure 8I), and could also be evoked when pre-applying hyperpolarizing currents to test rebound activity (Figure 8J).

4.4.3 GALR2 IN THE CBF NEURON CULTURE

In the adult rodent basal forebrain brain many neurons express galanin receptors (Burazin et al., 2000a; Miller et al., 1997; Mitchell et al., 1999; O'Donnell et al., 1999). Therefore, we analysed the expression of galanin receptor 1-3 in our cultures by RT-PCR (n = 4 independent experiments). We only found detectable levels of GalR2 mRNA in our cholinergic neuron preparations. In contrast, neither GalR1 nor GalR3 mRNA expression was detected (Figure 9A).

We have examined the subcellular localization of GalR2 in cultured cholinergic neurons. Given persisting shortcomings with GalR2 immunolocalization (Lu and Bartfai, 2009a), we have adopted a molecular biological approach by transfecting cholinergic cultures with a GalR2-EGFP construct (Xia et al., 2004). GalR2-EGFP was targeted towards the axon, concentrated in varicose structures in the initial segment of the axon (Figure 9B), and also ventured into the distal axon segment and the motile growth cone (Figure 9C). Analysis with the high-resolution laser-scanning microscope suggests that GalR2-EGFP can be inserted into the plasmalemma of cholinergic neurons.

We addressed whether endogenous expressed GalR2 induces downstream signalling in our cholinergic cultures by testing the activation of the MAPK pathway, a known signal transducer of activated GalR2s (Wittau et al., 2000). Both galanin itself as well as ARM1896, a mixed GalR2/GalR3 agonist (Liu et al., 2001; Lu et al., 2005b), induced rapid and significant phosphorylation of the 42 and 44 kDa isoforms of the extracellular signal-regulated kinase (Erk1/2) in cholinergic neuron-rich cultures. Erk1/2 phophorylation peaked after 5 min of stimulation after which pERK levels were quickly restored to baseline values (Figure 9D). Both M35, a non-selective GalR antagonist, and M871, a GalR2-selective antagonist (Gregersen et al., 1993; Jimenez-Andrade et al., 2006), blocked ARM1896-induced Erk1/2 phosphorylation in cholinergic neurons (Figure 9E).

Cumulatively, our data suggest that cholinergic neurons harbour functional GalR2s, whose axonal recruitment and signalling through the MAPK pathway could impact fundamental processes of neuronal differentiation including growth cone motility (Doherty et al., 2000) and axonal elongation (Karasewski and Ferreira, 2003).

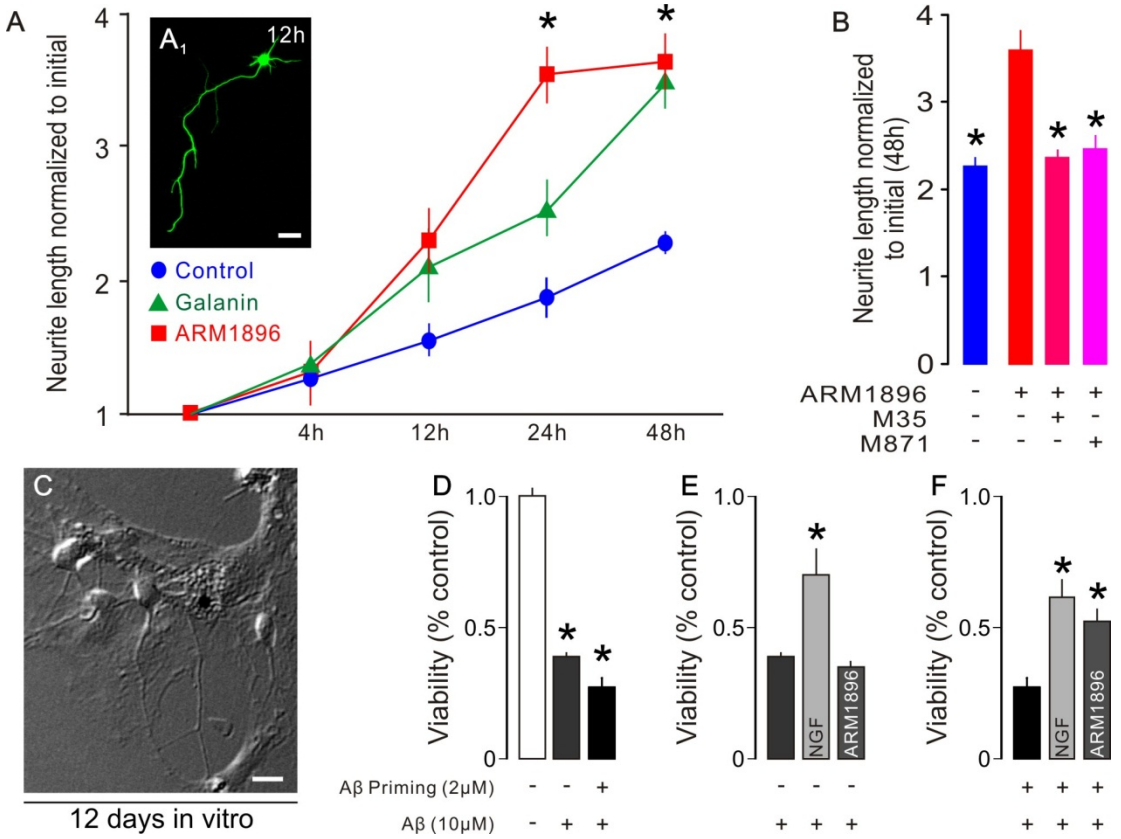

Figure 10. Galanin induces trophic effect on CBF neuron culture. Neurite length of CBF neuron culture was quantified after different treatments (control, 0.1 µM GAL, 0.1 µM ARM1896) time periods. Values represent average + SEM of neurite length normalized to initial point from three independent experiments (A); one individual CBF neuron in neuron culture after 12 hours (A1). GalR2 activity-induced enhancement of neurite outgrowth could be fully antagonized by applying the non-selective galanin receptor antagonist M35 or the selective GalR2 receptor antagonist ARM871 (B). Protection of GalR2 activity on cholinergic neurons againt A β 1-42 toxicity. Microphotograph shows the morphology of CBF neurons after culturing for 12 days (A1). CBF neuron viability is measured by MTT test; the cultures were treated with 10 µM A β (D, E, F). 100ng NGF or 100nM ARM1896 was added with A β (E). In F 2 µM of A β was added as priming before 100 ng NGF or 100 nM ARM1896. Viability of CBF neuron culture was presented as percentage of control.Scale bar = 20 µM (A1,C).

4.4.4 GALANIN INDUCES TROPHIC EFFECT ON CBF NEURON IN VITRO

Cholinergic neurons increase their axonal complexity and can form synapses in culture. Based on previous studies on the role of GalR2 on axon growth, we hypothesized that galanin can impact cholinergic axonal growth responses through GalR2s. Therefore, we have applied galanin or ARM1896 (both 100 nM) for 48 h and measured the total neurite length after 4, 12, 24 and 48 h of incubation (Figure 10A). We found that cholinergic neurons under control conditions (48h) had a total neurite length of 309.78 ± 18.91 µ m/cell (n = 84 cells). Galanin-treated cholinergic neurons had a total neurite length of 421.49 ± 9.14 µ m/cell (n = 57 cells, p = 0.038 vs. control; Figure 10), and AM1896 induced significant neurite elongation during 48h (373.99 ± 34.93 µ m/cell; n

= 114 cells, p = 0.019 vs. control; Figure 10A). Both M35 and M871 (both at 100 nM), the latter being a GalR2-selective antagonist (Jimenez-Andrade et al., 2006), abolished the ARM1896-induced growth response (Figure 10B). Thus, we conclude that a novel function of galanin signalling in developing cholinergic neurons is the organization of axonal and dendritic complexity.

To investigate the trophic effects of galanin on cholinergic neurons during a toxic insult, cholinergic cultures kept in vitro for 12 days (Figure 10C) were exposed to a toxic concentration of Aβ 1-42 (Aβ). Treatment of cholinergic neurons with 10 µM Aβ dramatically reduced cell viability (Figure 10D). Treatment of cultures with NGF during Aβ exposure significantly increased cell viability (Figure 10E). Stimulation of GalR2 could not protect cholinergic neurons against Aβ toxicity (Figure 10E). The lack of GalR2 mediated protection could be due to low availability of GalR2 at the cell membrane. Interestingly, it is known that GalR binding sites within the cholinergic basal forebrain are increased in AD (Mufson et al., 2000). Therefore we pre-treated our cholinergic cultures with a sublethal dose of Aβ (2 µM) that did not directly affect cell viability and slightly increased vulnerability to toxic levels of Aβ (10 µM; Figure 10D). In this experimental paradigms both NGF and ARM1896 significantly increased cell viability (Figure 10F).

5 DISCUSSION

There is evidence that a common feature of neuropeptides is their ability to react/adapt to various challenges, such as stress, traumatic events, neuron degeneration, mental disorders and drug abuse, one function being to modulate activity of co-expressed neurotransmitters (Hokfelt et al., 2003). Galanin shares this profile, and it is likely that GalR2 signaling is a component of such a dynamic system participating in homeostatic and pathological processes.

In agreement with its wide distribution, manifold roles have been attributed to galanin in brain functions. Here we would like to focus on some related examples, where GalR2, and the other galanin receptors, may be involved. They include cognition/ learning/memory (Crawley, 1996; Crawley, 2008; Kinney et al., 2002; McDonald et al., 1998; Ogren et al., 1998; Robinson, 2004), emotion-related behaviour, such as anxiety and depression (Fuxe et al., 1991; Karlsson and Holmes, 2006; Lu et al., 2007; Lundstrom et al., 2005a; Morilak et al., 2003; Ogren et al., 2006; Weiss et al., 1998), seizure activity (Lerner et al., 2008) and addiction (Picciotto, 2008).

5.1 ASPECTS ON METHODOLOGY

5.1.1 SENSITIVITY AND SPECIFICITY OF THE GFP IMMUNOREACTIVITY VISUALIZED IN GALR2-EGFP MICE

The present study shows that the GalR2-EGFP construct is widely distributed in the mouse brain, both in cell bodies and nerve terminals. Still, GalR2 is probably expressed in more systems than shown here. Expression levels are in general low, a view supported by the limited distribution of GalR2 transcripts reported in the ABA throughout the mouse brain (Lein et al., 2007). Moreover, it cannot be excluded that false positives are included in our mapping, for example the genetic manipulation involved in creating the GalR2-EGFP mouse may have resulted in ectopic expression patterns. This is the case in a mouse overexpressing galanin under the dopamine ß-hydroxylase (DBH) promoter (He et al., 2005). However, overall our results agree with previous in situ studies on the distribution of GalR2 mRNA in the rat brain (Bouret et al., 2000; Burazin et al., 2000a; Mitchell et al., 1999; O'Donnell et al., 1999). Nevertheless, differences exist, also when comparing with the ABA (see below).

It is in general assumed that the sensitivity of the in situ hybridization method is higher than immunohistochemistry for visualizing cell bodies. This is particularly apparent when working with proteins, e.g. neuropeptides that are released and thus have to be replaced by de novo synthesis and rapid transport into extensive terminal networks. Also, with the in situ approach cell bodies can be seen without colchicine treatment, which by itself may have unexpected side effects (see below). Receptor molecules, on the other hand, are less abundant than peptides and have a slow turnover (as they are not released) and consequently low mRNA levels. Here in situ hybridization may in fact be less sensitive than immunohistochemistry (Kopp et al., 2002). Since GalR2 antibodies are not available, we cannot test this hypothesis which at least seems to be true for the Y1 receptor (Kopp et al., 2002).

5.1.2 COLCHICINE TREATMENT

Colchicine, an inhibitor of meiosis and axonal transport, has been used to visualize neuropeptides in cell bodies for many decades. For example, in an early mapping study it was shown that colchicine treatment increases the number of substance P^+ cell groups

to >30 versus a single group seen in the non-treated rats (Ljungdahl et al., 1978). Thus, preventing centrifugal transport causes a dramatic accumulation of various molecules in the soma, as first shown by Dahlström (1968) and Kreutzberg (1969). This approach has been extensively used in other and our laboratories, recently also for receptors. Thus, while mapping the neuropeptide Y (NPY) Y2 receptor, mainly considered a presynaptic receptor (Wahlestedt et al., 1986) and thus centrifugally transported, we observed a markedly increased number of labeled cell bodies after staining brain sections of a colchicine-treated mouse with a Y2-specific antibody (Stanic et al., 2006). There is evidence that also GalR2 is a presynaptic receptor, at least in some systems (Ma et al., 2001). It may therefore be anticipated that GalR2 is centrifugally transported from the cell body to the nerve terminals. In agreement, colchicine treatment resulted in a distinctly increased number of fluorescent cell bodies as compared to untreated mice.

It is important to note that colchicine does not only block transport but also can increase mRNA levels, that is upregulates synthesis of peptides, including galanin, in some brain systems (Cortes et al., 1990a). In some cases the opposite occurs, e.g. the transcript for the neuropeptide cholecystokinin in rat cortex is decreased (Cortes et al., 1990a). This may, for example, be due to interuption of retrograde transport of growth factors, necessary for stimulation of protein/peptide synthesis. Also, importantly, colchicine early on was shown to have toxic effects on hippopcampal neurons, the granule cells in the dentate gyrus being particularly sensitive (Goldschmidt and Steward, 1980; Goldschmidt and Steward, 1982). This may be one reason for some distinct discrepancies between the ABA and our own results. For example, GalR2 transcript was present in both granule and pyramidal cells in the mouse HiFo (Lein et al., 2007), whereas these cells were negative in our study, these possibly due to cell injury/death. So expression of GalR2 transcript in granule cells of (untreated) rats (Burazin et al., 2000a; O'Donnell et al., 1999; Xu et al., 1998b) is certainly also true for mouse (Lein et al., 2007), even if not detected in our GalR2-EGFP mouse.

There are also other reasons that not all GalR2 cell bodies in the mouse brain may have been revealed. Thus, colchicine treatment does not appear to be equally distributed throughout the brain after i.c.v. injection, but most robustly in areas close to the lateral ventricle. Also, we observed an apparently stronger effect of colchicine in ventral brain areas, possibly due to gravity causing a ventral deposition of the drug. Finally, colchicine influences the animals in many ways, e.g. by reducing food and water intake, which will affect the metabolism and general status, which in turn can influence signaling molecules.

5.1.3 COMPARISON WITH THE ALLEN BRAIN ATLAS (ABA)

Our results indicate that, as said, the expression levels of GalR2 are in general low in the mouse brain. This is supported by data in the ABA (Lein et al., 2007), a monumental cartographing of some 20,000 transcripts throughout the mouse brain. The online in situ results from ABA show that GalR2 mRNA is expressed in a limited number of brain regions; and sections at many levels in fact lack signal and/or show a unilateral signal, indicating that the sensitivity of the method used in the ABA is close to detection level. Our study in fact reveals a considerably wider distribution of GalR2 than seen in ABA. On the other hand, it cannot be excluded that this reflects false positives, the genetic manipulation involved may create ectopic expression in the GalR2-EGFP mouse. However, our results do largely agree with previous in situ studies on the distribution of GalR2 mRNA in the rat brain (Bouret et al., 2000; Burazin et al., 2000a; Mitchell et al., 1999; O'Donnell et al., 1999). One obvious discrepancy in addition to granule and pyramidal cells in the HiFo, is our lack of signal

in mitral cells in the olfactory bulb, versus a strong signal in these cells in the ABA. Again, colchicine treatment may be responsible. The ABA also reports a very strong signal from cells apparently in the meninges at anterior levels, which we cannot observe. Conversely, we see a clear signal in medial septum, diagonal band of Broca, thalamic nuclei and hypothalamic nuclei, not reported in the ABA.

5.2 ASCENDING BRAIN STEM SYSTEMS AND GALANIN SIGNALING

There are several ascending systems that coexpress galanin, and where GalR2 signaling probably is important. They include cell bodies in: (i) NA neurons in the LC; (ii) 5-HT neurons in the DRN; (iii) ACh neurons in the basal forebrain and (iv) histamine neurons in the magnocellular tubero-mammillary nucleus. We will here deal with the three former systems and their projections to cortex and/or the HiFo. Lastly we will discuss galanin and GalR2 with some disease states.

5.2.1 GALANIN AND NORADRENERGIC CELL BODIES IN THE LC

NA in LC neurons has a prominent role in organizing the behavioral state of mammalian organisms such as vigilance, arousal, stress responses and modulation of memory systems (Aston-Jones and Cohen, 2005). At the cellular level NA seems to change the "signal-to-noise" ratio of both excitatory and inhibitory activity, making synaptic transmission in target neurons (circuits) more effective (Aston-Jones and Cohen, 2005). It has been shown that the functional state of LC noradrenergic neurons is modulated by neuropeptides such as galanin (Pieribone et al., 1998) and tuberoinfundibular peptide of 39 residues (TIP39) (Usdin et al., 1999). Thus, TIP39 and galanin are both involved in emotions and stress, and affect cognition and memory through regulating NA signaling (Coutellier et al., 2010; Ogren et al., 2010).

The noradrenergic cell group in LC has a robust expression of galanin even under normal circumstances, and at least 80% of the noradrenergic LC neurons produce galanin (Holets et al., 1988; Melander et al., 1986b; Xu et al., 1998b). Also in the human brain galanin is expressed in the LC (Chan-Palay, 1990; Miller et al., 1999).

125I-galanin binding sites are present over the LC region (Pieribone et al., 1995), and mRNAs for all three galanin receptor subtypes (GalR1-R3) seem to be expressed in the LC, although GalR3 mRNA levels are very low (Burazin et al., 2000a; Hawes et al., 2005; Mennicken et al., 2002; O'Donnell et al., 1999; O'Donnell et al., 2003; Parker et al., 1995). Earlier electrophysiological studies have shown that galanin reduces firing rate and induces hyperpolarization/outward current, which persists under conditions in which synaptic input is blocked by tetrodotoxin (TTX), plus low Ca^{2+} medium, presumably via an increase in K^+ conductance (Pieribone et al., 1995; Seutin et al., 1989; Sevcik et al., 1993). This effect is attenuated by the non-selective galanin receptor antagonist M15, but neither by M35 nor M40 (Xu et al., 1998a). These compounds are chimeric peptides developed by Bartfai and colleagues (1992). Beside this direct effect, an indirect, modulating action of galanin on LC neurons has been demonstrated (Xu et al., 2001). Thus, galanin prolonged the NA-induced outward current occurring at low galanin concentrations (0.05–0.1 nM), that had no or very little effect on the membrane potential/current by themselves. Idazoxan, a α_2 antagonist, blocked the NA-, but not the galanin-induced outward current, which indicates that the receptors mediating these responses are different for the two ligands (Aghajanian and Wang, 1987; Svensson, 1987).

Ma et al. (2001) have shown that the selective GalR2 agonist Gal(2-11) (AR-M1896) (Liu et al., 2001) causes inhibition of spike discharge and a slight hyperpolarization only at very high concentrations. Moreover, AR-M1896 is, on a molar basis, much weaker than galanin or AR-M961, an agonist both at GalR1 and GalR2 receptors. This suggests that it is mainly the GalR1 receptor that mediates hyperpolarization of LC neurons at soma/dendrites. This has been further supported by a study showing that M617, a newly developed galanin analogue and selective GalR1 agonist, induces an outward current in LC neurons (Lundstrom et al., 2005b). In contrast, GalR2 may represent a presynaptic receptor transported into the noradrenergic nerve terminals in the forebrain and other LC projection areas. Presence and a possible role of GalR3 in LC neurons remain to be analyzed.

No similar electrophysiological studies have been performed on mouse, but in **Paper II** many GalR2-EGFP+ cell bodies were found in LC and subcoeruleus, providing evidence for GalR2 protein in LC neurons, in agreement with the demonstration of GalR2 transcript by Lein et al (2007), and with rat results (Burazin et al., 2000a; O'Donnell et al., 1999). To what extent NA neurons in mouse LC also express GalR1 (and GalR3) remains uncertain - inspection of the ABA does not provide convincing evidence for this receptor in LC (Lein et al., 2007).

The origin of endogenous galanin responsible for these effects has been discussed. It was early noted that relatively few galanin-immunoreactive (-ir) fibres surround and make contact with NA neurons in the rat LC (Pieribone et al., 1995), leading to the proposal that galanin may also be released from the soma/dendrites of the NA neurons, and perhaps less so from the afferent fibers (Pieribone et al., 1995). This hypothesis was based on studies of the hypothalamic magnocellular neurons showing somatic/dendritic release of vasopressin and oxytocin (Chapman et al., 1986; Ludwig et al., 2005; Ludwig et al., 2002). There is in fact recent histochemical support that this also occurs for galanin in the LC (Vila-Porcile et al., 2009). It is well established that NA has autoinhibitory effects on LC neurons, via the alpha2A adrenoceptor (Aghajanian and Wang, 1987; Svensson, 1987), and somatic/dendritic release of NA from LC neurons has been unequivocally demonstrated using combined amperometry and patch clamp methodology (Huang et al., 2007).

5.2.2 GALANIN AND SEROTONERGIC CELL BODIES IN THE DRN

Serotonin-containing fibers in the brain mainly originate from nine cell clusters (B1-9) in the pontine raphe region and lower brainstem (Dahlstrom and Fuxe, 1964; Steinbusch, 1981), the rostral groups (raphe dorsalis, raphe medianus, and centralis superior, or B7-B9) innervating the telencephalon and diencephalon. The raphe medianus (B8) provides extensive 5-HT innervation on the limbic system; and the DRN (B7) projects to the neostriatum, cerebral and cerebellar cortices, and thalamus (Azmitia and Segal, 1978; Pineyro and Blier, 1999; Tork, 1990).

Serotonin is involved in numerous physiological processes including sleep, appetite, memory/cognitive functions, impulsivity, sexual behavior, and motor function, as well as modulation of limbic/affective responsiveness, such as fear, anxiety and depression (Müller et al., 2009). 5-HT had also been proposed to play a role in neuronal homeostasis and trophic mechanisms, and in neuronal growth and differentiation (Berger et al., 2009; Daubert and Condron, 2010; Lucki, 1998; Ressler and Nemeroff, 2000).

The galanin levels in the 5-HT neurons are normally relatively low, but in contrast to the LC the 5-HT neurons in the raphe nuclei are surrounded by a dense galanin-positive neuropil (5-HT-negative), some nerve endings making synaptic contact with 5-HT neurons (Xu et al., 1998c). Thus, putative galanin receptors on 5-HT neurons may not represent autoreceptors to the same extent as in the NA neurons in LC.

In vitro electrophysiological studies on brain slices containing DRN neurons have shown that galanin applied at relatively high concentrations (10^{-6} mol/L) inhibits the firing rate of 5-HT neurons, probably related to the activation of potassium channels (Xu et al., 1998c). This effect seems to be post-synaptic, i.e. tetrodotoxin (TTX)-insensitive.

It was assumed that this inhibition of DRN 5-HT neurons is mediated via GalR1, even if GalRl expression has not been shown in these neurons (see below). But recent electrophysiological data suggest involvement of the GalR3 receptor (Swanson et al., 2005b). Similar to the situation in LC, low concentrations (10^{-9} mol/L) of galanin enhances the inhibitory action of 5-HT on the DRN neurons via the 5-HT1A receptor (Xu et al., 1998c).

In vivo microdialysis provides further evidence that galanin has an inhibitory effect in the DRN. Thus, given i.c.v. galanin caused a potent, dose-dependent reduction of 5-HT release in the HiFo in awake rats (Kehr et al., 2002), probably mediated by activation of the galanin receptors at the DRN cell body level, since intrahippocampal administration of galanin did not affect 5-HT release (Kehr et al., 2002).

The use of galanin receptor-selective compounds has indicated a role of GalR2 on 5-HT release. Thus, activation of the GalR2 receptor in the DRN, using the GalR2 agonist M1896, increased 5-HT release in the HiFo (Mazarati et al., 2005). A more recent study, also on rat, reports that this effect may be indirect, via activation of local inhibitory GABA neurons, mediated via GalR2 (Sharkey et al., 2008).

The firing rate of the 5-HT neurons (and therefore 5-HT release) is controlled by the somatodendritic 5- HT_{1A} autoreceptor. A number of studies have indicated a close interaction between galanin and the 5-HT_{1A} receptor in the DRN, both at the cell body and nerve terminal level. Under basal, non-stressful conditions, i.c.v. galanin administration causes a time-dependent reduction in affinity (kd values) and an increase in number of the 5-HT_{1A} autoreceptors (Razani et al., 2000), as well as a decrease of 5-HT_{1A} mRNA levels in the DRN (Razani et al., 2000). Galanin/5-HT_{1A} receptor interactions are also supported by the finding that the reduction in 5-HT release induced by i.c.v. galanin is partially counteracted by pre-treatment with the 5-HT_{1A} antagonist WAY-100635 (Yoshitake et al., 2003). The galanin receptor involved in the interaction with 5-HT_{1A} is probably GalR1, since these receptors have been shown to heteromerize (Borroto-Escuela et al., 2010).

The localization and distribution of galanin receptors in the DRN are, as indicated above, less clear than in the LC. Both GalR1 and GalR2 mRNA expression is low in rat, while GalR3 signal has not been detected (Burazin et al., 2000a; Mennicken et al., 2002; O'Donnell et al., 1999; O'Donnell et al., 2003). The ABA provides little information on this issue (Lein et al., 2007). **Paper II** shows GalR2-$EGFP^+$ cell bodies in the mouse PAG, mainly in the ventral part but, importantly, apparently not showing the characteristic distribution of the 5-HT neurons. In fact, our double-labeling provided no evidence for GalR2-serotonin coexistence, nor was there a clear coexistence with GAD, although the distribution pattern resembled that of GABA neurons in mouse (Fu et al., 2010; Lein et al., 2007). It should be noted that the number of GAD^+ cell bodies

detected in our mice was considerably lower than seen in the ABA (Lein et al., 2007). Thus, at this stage coexistence of GalR2 and GABA cannot be excluded and, clearly, the transmitter phenotype of the GalR2^{+} neuron population still needs further investigation. Nevertheless, data suggest that the inhibitory action of galanin on 5-HT neurons may be mediated via GalR3/R1, while the GalR2 activation can enhance 5-HT release. Also, how galanin regulates the 5-HT neurons, if directly (Xu et al., 1998c) or through GalR2 (Lu et al., 2007) thus remains to be analyzed.

5.2.3 GALANIN AND THE CHOLINERGIC BASAL FOREBRAIN (CBF) SYSTEM

The CBF neurons are parts of the ascending reticular activating system, forming a complex encompassing the medial septum, the horizontal and vertical diagonal band of Broca, and nucleus basalis of Meynert (in primates). Together they provide the major cholinergic afferents for the cerebral cortex and HiFo, each division having its distinct target (Figure10). Thus, neurons located in the medial septum innervate predominantly the HiFo; while those of the vertical and horizontal diagonal band project to the anterior cingulate cortex and olfactory bulb and ventral HiFo; neurons in the nucleus basalis of Meynert project to the amygdala and throughout the rest of the cortical mantle (Arendt et al., 1990; Gaykema et al., 1990; Jones et al., 1976; Kievit and Kuypers, 1975; Lamour et al., 1982; Mesulam et al., 1983; Pearson et al., 1983; Saper, 1984)(Figure 11).

The CBF complex is involved in numerous physiological processes in the brain, such as controlling cerebral blood flow (Barbelivien et al., 1999; Biesold et al., 1989; Sato et al., 2004), sleepwake cycle (Lee et al., 2005), higher cognitive functions as learning and memory, as well as emotions and fear and stress (Arendt and Bigl, 1986; Bigl and Schliebs, 1998; Jakab and Leranth, 1995; McKinney and Jacksonville, 2005), and attention (Bentley et al., 2004; Sarter et al., 2003).

Galanin is closely associated with the CBF neurons. This was first recognized when galanin was co-localized with ChAT in a subpopulation of basal forebrain neurons in rat, but only after colchicine treatment (Melander et al., 1985b; Senut et al., 1989). Also electrocoagulation of the ventral HiFo causes increased galanin levels in CBF neurons (Cortes et al., 1990b). Under normal circumstances, galanin transcript levels in the CBF neurons are, however, very low (Miller et al., 1998).

Interestingly, there is a distinct species difference in the distribution of galanin within the basal forebrain neuron. In apes and humans, like in rat, galanin-IR intrinsic neurons are few as are fibers innervating CBF neurons (Benzing et al., 1993; Mufson et al., 1993), whereas monkeys have high galanin levels in the entire neuron (Benzing et al., 1993; Melander et al., 1986c). This species difference suggests that galanin may be of more importance in modulating the CBF system in certain monkey species, at least in adults (Benzing et al., 1993).

In fact, galanin in the basal forebrain may have important functions during the prenatal period. Thus, galanin is critical for the basal forebrain cholinergic neurons during the early postnatal development , galanin-KO mice having 40% fewer neurons of this type (O'Meara et al., 2000). There is, however, no information on a possible developmental role of galanin in the three other ascending systems (NA, 5-HT, histamine).

Transcripts for all three galanin receptor subtypes have been detected in the rat (Mennicken et al., 2002; Miller et al., 1997; O'Donnell et al., 1999; O'Donnell et al., 2003) and mouse (He et al., 2005) basal forebrain. GalR1 mRNA was only found in a few septal cholinergic neurons but often in GABAergic neurons in rat (Miller et al., 1997). In **Paper II** many GalR2^{+} neurons are shown in the basal forebrain of mouse. However, GalR2 transcript is not reported in this area in the ABA (Lein et al., 2007). Our double-labeling experiments suggest that the EGFP-GalR2-positive neurons are not cholinergic, but some may be GABAergic and/or express a calcium-binding protein.

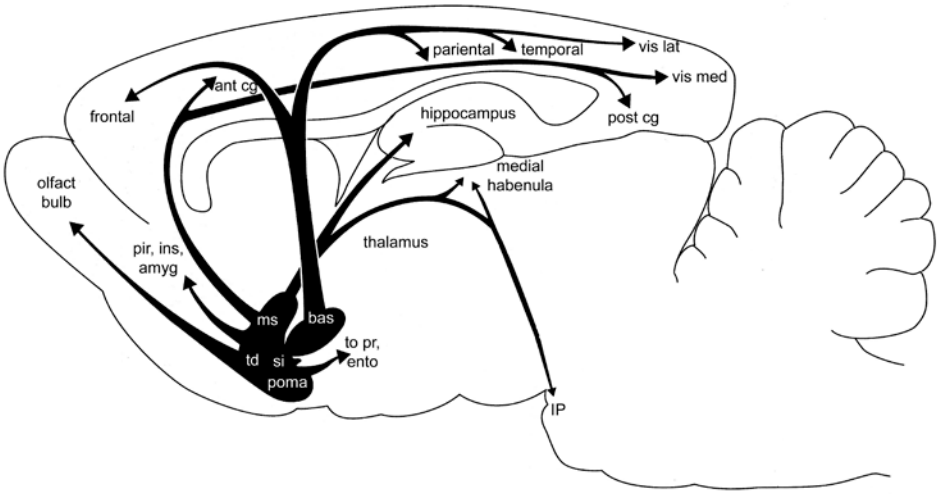

Figure 11. Cholinergic innervation of the cortical mantle. The basal forebrain cholinergic complex comprising medial septum (ms), horizontal and vertical limbic diagonal band of Broca (td), and nucleus basalis of Meynert (bas) provides the mayor cholinergic projections to the cerebral cortex and the HiFo. Neurons located in the medial septum innervate predominantly the hippocampus, while those of the vertical and horizontal diagonal band project to the anterior cingulate cortex and olfactory bulb (limbic cortex), respectively. Cholinergic neurons of the nucleus basalis of Meynert provide efferents to the amygdala and throughout the rest of the cortical mantle. Reproduced from Iversen et al. (2008), with permission from the authors and Oxford University Press.

There are several functional studies on galanin related to the basal forebrain cholinergic complex. By using whole cell patch-clamp electrophysiology recording together with single-cell reverse transcription-polymerase chain reaction, Jhamandas et al. (2002) found that galanin in the rat has excitatory effects on cholinergic, but not GABAergic neurons. Thus, galanin caused a decrease in whole cell potassium currents, without effect on calcium or sodium currents in neurons acutely dissociated from the ventral forebrain (Jhamandas et al., 2002). These effects of galanin were mediated through decreasing conductance of the delayed rectifier (IK), calcium-activated potassium (IC) and transient outward potassium (IA) channels. Thus galanin reduced IK- and IA-evoked conductence by depolarizing pulses and did not have any significant effect on tetra-ethylammonium (TEA) (blocker of IK and IC)-resistant current.

Iberiotoxin, a selective blocker of calcium-activated potassium channels, or blockade of calcium influx reduced the galanin effects. This is in agreement with the results in **Paper IV** showing GalR2 mRNA in isolated rat CFB neurons. And there are several studies also indicate the presence of GalR2 in neurons of this type in rat (for review, see Counts et al., 2003). On the other hand, our GalR2 in vivo mapping in mouse detected no GalR2 in cholinergic but only in GABAergic CFB neurons. If correct, species differences exist.

5.3 GALANIN IN THE HIPPOCAMPAL FORMATION

Galanin is under normal circumstances found only in a small number of neuronal cell bodies in the HiFo (Melander et al., 1986a; Skofitsch and Jacobowitz, 1985). After colchicine treatment (Calza et al., 1998a; Calza et al., 1998b; Xu et al., 1992) and during spreading depression (Shen et al., 2003) galanin$^+$ glial cells appear.

However, as discussed above, galanin is coexisting in NA, 5-HT, ACh and histamine cell bodies projecting to the HiFo (and other regions). Early histochemical (Melander et al., 1986a) and biochemical (Gabriel et al., 1988) studies indicated that galanin also was present in the nerve terminals of these neurons. However, only with more sensitive histochemical techniques was it possible to more exactly describe these systems. Thus, high levels of galanin are found in the noradrenergic terminals in the cortex and HiFo (Xu et al., 1998b), suggesting that this is the major origin of endogenously released galanin. In addition, there is a galanin$^+$, non-noradenergic network in the anterior, ventral HiFo (Xu et al., 1998b). In contrast, there is so far meager histochemical evidence that galanin under normal circumstances is present in serotonergic, histaminergic or cholinergic nerve terminals in the HiFo (but see Melander et al., 1986a), although this may be a question of method sensitivity.

Galanin can affect NA transmission, e.g. by inhibiting NA release in hypothalamic slices, partially mediated by activation of α_2-adrenoreceptors (Tsuda et al., 1989). The decrease in NA release in the HiFo produced by i.c.v. galanin infusion is probably mediated at the cell body level (Yoshitake et al., 2003), as is the case also for 5-HT.

Transcript for the GalR1 and GalR2 has been found in the rat HiFo. GalR1 mRNA was found in the ventral subiculum, CA3 and CA1, whereas GalR2 mRNA was mainly seen in granule cells in the dorsal and ventral dentate gyrus and to a lesser extent in the ventral CA3 and CA1 (Burazin et al., 2000a; O'Donnell et al., 1999; O'Donnell et al., 2003). Hohmann et al. (2003) reported GalR1$^+$ cells in the HiFo in the mouse. Thus, there are local targets for galanin in afferent systems both in mouse and rat.

In the ABA a few sections with successful hybridization show GalR2 mRNA both in granule and pyramidal cells. In contrast, as discussed above, our GalR2-EGFP mouse only show few positive neurons in the HiFo, possibly due to the toxic effect of colchicine.

With regard to presynaptic receptors on afferents to the HiFo, there is some evidence that GalR2 is a presynaptic receptor in rat LC neuron (see above); the GalR2 presumably is centrifugally transported from the cell bodies to the nerve terminals in the HiFo. To what extent also other afferents have presynaptic GalR2s is unclear (Mazarati et al., 2005). However, galanin inhibits ACh release in rodent (Fisone et al., 1987) and monkey (Fisone et al., 1991) hippocampal slice preparations.

Early electrophysiology studies, showed that galanin presynaptically inhibits excitatory glutamatergic neurotransmission when bath applied to the hippocampal slice

(Zini et al., 1993). Postsynaptically, it was reported that galanin causes only a slight hyperpolarization without obvious change in membrane resistance in ventral CA1 (Dutar et al., 1989). This is consistent with autoradiographic studies showing a distinct 125I-galanin (1–29) binding only in the ventral hippocampus and not in dorsal cortical and hippocampal areas of rat brain (Melander et al., 1988; Skofitsch et al., 1986). However, a binding site for 125I-galanin (1–15ol) has been demonstrated in the dorsal HiFo and cortex and striatum by Hedlund et al. (1994). Moreover, Xu et al. (1999) found that in the dorsal CA3 field galanin (1–15) causes a dose-dependent, TTX-resistant hyperpolarization accompanied by increases in membrane conductance in the pyramidal neurons. Most of the galanin (1–15)-sensitive neurons did not respond to galanin (1–29), galanin (1–16), or d-tryptophan2-galanin (1–29) (Xu et al., 1999). In contrast, galanin (1–29) and galanin (1–16) affected all tested LC neurons, and galanin(1–15) had the weakest effect among the tested peptides causing hyperpolarization in 31 of 36 tested neurons (Xu et al., 1999). These results may indicate presence of a yet-to-be identified galanin (1–15)-selective binding site on CA3 neurons in the dorsal hippocampus. Binding studies on galanin receptors expressed in cell lines have shown that all three subtypes bind galanin (1–29) with higher affinity than galanin (1–15) (Pang et al., 1998; Smith et al., 1998).

5.4 GALANIN AND ITS RECEPTORS IN ASCENDING SYSTEMS: RELATION TO DISEASE

5.4.1 DEPRESSION

The neurochemical, histological and electrophysiological profiles of galanin discussed above strongly indicate involvement of galanin in the regulation of monoaminergic systems, which are dysfunctional in depression. In the context of the relation between galanin and monoamines, it is relevant to study how galanin might affect depression-like behavior. Many efforts have already been put in studies on a possible involvement of galanin in regulation of depression-like behavior (Table 4).

Treatment/mutation	*Species*	*Behavioral paradigm*	*Behavioral effect*	*References*
Galanin i.c.v.	Rat	FST	Non	(Weiss et al., 1998)
Galanin intra-VTA	Rat	FST	Prodepressive	(Weiss et al., 1998)
Galanin antagonist (M15) intra-VTA	Rat	FST	Antidepressant-like	(Weiss et al., 1998)
Galanin intra-hypothalamus	Rat	FST	None	(Weiss et al., 1998)
Galanin i.c.v.	Mouse	FST	None	(Holmes et al., 2005b)
Galanin agonist (galmic)	Rat	FST	Antidepressant-like	(Bartfai et al., 2004)
Galanin agonist (galnon)	Rat	FST	Antidepressant-like	(Lu et al., 2005a)
GalR3 antagonist (SNAP 37889)	Rat	FST	Antidepressant-like	(Swanson et al., 2005a)
Galanin overexpression (D β H promoter)	Mouse	FST	None	(Holmes et al., 2005b)
Galanin overexpression (PDGF-B promoter)	Mouse	FST	Prodepressive	(Kuteeva et al., 2005a)
GalR1 knockout	Mouse	TST	None	(Holmes et al., 2005b)
GalR2 knockout	Mouse	TST	None	(Gottsch et al., 2005)

Table 4. Effects of galaninergic treatment or genetic mutations on depression-like behaviour. i.c.v.- intracerebroventricular, i.v.- intravenous, FST- forced swim test, TST- tail suspension test, VTA- ventral tegmental area. From Kuteeva (2007) (PhD thesis)

Fuxe and collaborators (Fuxe et al., 1991) first raised the possibility of a galanin involvement in depression-like behavior. Bing et al. (1993) showed that galanin given i.c.v. exerts an anxiolytic-like action. Weiss and colleagues showed in rat that the immobility time in the forced swim test (FST) was increased (pro-depressive) after infusion of galanin into the ventral tegmental area, and that the nonselective galanin receptor antagonist M15 decreased immobility time in the FST (antidepressive) (Weiss et al., 1998; Weiss et al., 2005). In contrast, systemic injection of the non-peptide GalR3 antagonist SNAP 37889 produced antidepressant-like effects in different models of depression/anxiety (Barr et al., 2006; Swanson et al., 2005b). Interestingly, an increased density of galanin binding sites was found in the DRN in the Flinders sensitive rat line, a putative rat model of depression selected for high FST immobility, compared to controls (Bellido et al., 2002). The anxiogenic-like action of intra-amygdala administered galanin (Moller et al., 1999) together with the fact that exposure to chronic stress in rat increased galanin gene expression in the amygdala (Sweerts et al., 1999) and LC (Holmes et al., 1995) also indicates involvement of the galanin system in stress reactivity.

Some other recent studies have, however, suggested that increased galanin signaling may have an antidepressant-like effect. The systemically active, non-peptide galanin agonists galmic and galnon, administered intraperitoneally (i.p.) prior to the FST, were shown to decrease immobility time (Bartfai et al., 2004; Lu et al., 2005a). Moreover, both chronic treatment with the SSRI fluoxetine and electroconvulsive treatment were shown to increase galanin mRNA levels in the DRN and LC accompanied by an increase in GalR2 (but not GalR1) receptor binding sites (Lu et al., 2005a). Thus, GalR2 seems to mediate antidepressive effects. The putative galanin receptor antagonist M40 attenuated the antidepressant-like effect of fluoxetine in the FST (Lu et al., 2005a). It has also been reported that galanin given intravenously (i.v.) exerts an antidepressant-like effect with suppression of rapid eye movement (REM) sleep in humans (Murck et al., 2004; Toppila et al., 1995). The diversity of galanin's effect on monoamine function and depression-related behaviors might be explained by effects exerted via different receptor subtypes (GalR1-GalR3) (Branchek et al., 2000) under different circumstances.

The complexity of the role of galanin in depression-related behaviors is also reflected in the studies using genetically modified mice. Mice overexpressing galanin (GalOE) under the DBH promoter as well as mutant mice lacking the GalR1 receptor (GalR1-KO) failed to show the expected signs of increased depression-like behaviour in the tail suspension test, an animal model of depression-like behaviour (Holmes et al., 2005a). Also, GalR2-KO mice exhibited normal baseline behaviour in the same type of test (Gottsch et al., 2005). Whereas transgenic mice overexpressing galanin under the platelet-derived growth factor B (PDGF-B) promoter (Holmberg et al., 2005) exhibit increased depression-like behavior and altered monoaminergic response in the FST (Kuteeva et al., 2005b).

5.4.2 ALZHEIMER'S DISEASE

The cholinergic component of the basal forebrain complex became of particular interest when a relationship between the loss of cholinergic neurons/corticopetal cholinergic afferents and memory deficit in Alzheimer's disease (AD) was discovered

(Coyle et al., 1983; Davies and Maloney, 1976; Perry et al., 1977; White et al., 1977). Molecular underpinnings of the 'selective' vulnerability of cholinergic basal forebrain neurons in AD are not completely understood, an altered responsiveness to glutamate (Harkany et al., 2000) or deficiency in trophic factors (Salehi et al., 2000) being possible culprits. Also, neuropeptides have been of interest, including cholecystokinin, vasoactive intestinal peptide, substance P, somatostatin, dynorphin B, neurotensin and galanin, which all are expressed in the rat basal forebrain (Lamour et al., 1988), and can undergo activity modulated expression plasticity (Agoston et al., 1994). These are possibly involved in modulation of cholinergic signaling.

It has been observed that galaninergic fibers within the human CBF hypertrophy and apparently hyperinnervate surviving cholinergic neurons in late stage AD patients with a severe CBF neuronal loss (Bowser et al., 1997; Chan-Palay, 1988; Mufson et al., 1993). Galanin levels are increased throughout cortex in AD (Bierer et al., 1995; Gabriel et al., 1994). There are also increases in galanin receptor binding in the cortex, basal forebrain, hippocampus, entorhinal cortex, and amygdala (McMillan et al., 2004; Mufson et al., 2000; Perez et al., 2002; Rodriguez-Puertas et al., 1997). Detailed studies show a significant increase in the density of galanin receptor binding sites within the anterior nucleus basalis subfield of late AD patients compared to controls and early AD patients (Mufson et al., 2000), as well as galanin and galanin receptor overexpression during the different stages of CBF neuron degeneration in AD (Counts et al., 2006; Petersen, 2004). It has been proposed that the overexpression of galanin and galanin receptors is caused by the neurodegenerative lesions in AD, triggered by fibrillar β-amyloid deposits (Kowall and Beal, 1989). There is also a dramatic upregulation of galanin in the HiFo in some AD mouse models (Diez et al., 2000, 2003).

Single-cell gene expression studies have shown that the levels of mRNAs encoding select subclasses of protein phosphatase subunits (PP1a and PP1g) are stable in galanin hyperinnervated neurons but downregulated in non-hyperinnervated CBF neurons in AD (Counts et al., 2003). The reduction of PP1 and PP2A subunits activity is implicated in tau hyperphosphorylation, which precipitates neurofibrillary tangles (NFT) pathology and subsequent cytoskeletal destabilization in vulnerable neurons (Trojanowski et al., 1995). These observations indicate that galanin hyperinnervation may delay NFT pathology in CBF neurons in AD and exert a protective effect.

These results suggest that the overexpression of galanin in the late stage of AD is a protective response counteracting degeneration, serving as differentiation/survival signal (Tarasov et al., 2002). An interesting question is then which receptor is involved in the protective effect of galanin in Alzheimer's disease. There are several lines of evidence indicating that the GalR2 receptor is an important player mediating the protective and trophic effects of galanin in basal forebrain. Thus, the GalR2 agonist AR-M1896 can protect, both in mouse (Elliott-Hunt et al., 2004);Elliott-Hunt et al., 2007) and rat (Pirondi et al., 2005; Pirondi et al., 2010) primary neuronal hippocampal cultures from glutamate-, staurosporine- or ß-amyloid-induced toxicity. In agreement galanin failed to prevent glutamate-induced cell death in hippocampal primary cultures from GalR2 knockout mice (Elliott-Hunt et al., 2007). Furthermore, the galanin-induced, neuroprotective activation of Akt and Erk in hippocampal cultures from WT mice was significantly attenuated in cultures from GalR2 knockout mice (Elliott-Hunt et al., 2007). Moreover, galanin, by activating GalR2 receptor, can attenuate the β-amyloid toxicity in rat CBF neuron primary cultures (Ding et al., 2006). Finally, studies in studies on PC12 cells galanin can induce neurite outgrowth in an Erk-dependent

manner through the GalR2 receptor, further supporting a neurotrophic effect of GalR2 activation (Hawes et al., 2006).

In our studies (**Paper IV)**, direct stimulation of GalR2 could not protect cholinergic neurons against Aβ toxicity. The lack of GalR2 mediated protection could be due to low availability of GalR2 at the cell membrane. Interestingly, it is known that GalR binding sites within the cholinergic basal forebrain are increased in AD (Mufson et al., 2000). Therefore we pre-treated our cholinergic cultures with a sublethal dose of Aβ (2 μM) that did not directly affect cell viability and slightly increased vulnerability to toxic levels of Aβ (10 μM). In this experimental paradigm ARM1896 significantly increased cell viability. This may indicate that GalR2 expression and/or subcellular localization are changed during the progressing pathogenesis of AD.

5.4.3 EPILEPSY

The galaninergic input to the HiFo, including the dentate gyrus is mainly associated with the noradrenergic innervation (Xu et al., 1998b). The dentate gyrus represents a major gateway in the propagation of seizure activity (Heinemann et al., 1992). An anti-convulsant action of galanin was first shown by Mazarati et al. (1992). Induction of status epilepticus (SE) leads to a profound depletion of galanin in the dentate gyrus already after 30 min of self-sustaining seizures (Mazarati, 2004; Mazarati et al., 1998). A *de novo* expression of galanin in the hippocampal interneurons caused by seizures themselves was found and proposed to be possible compensative mechanism for the fatigue of galanin (Arabadzisz et al., 2005; Fetissov et al., 2003; Mazarati et al., 1998; Wilson et al., 2005). Furthermore, intrahippocampal administration of GalR agonists during this transition time rapidly and irreversibly aborted seizures (Mazarati et al., 2000; Mazarati et al., 1998). Taken together these data suggest that galanin may indeed act as an anticonvulsant peptide. However, the depletion of galanin of the HiFo may contribute to the progression of seizures (Lerner et al., 2008). Further evidence for an anti-epileptic effect of endogenous galanin comes from studies on transgenic mice. For example mice overexpressing galanin under PDGF B promoter have higher after-discharge threshold upon hippocampal stimulation and higher resistance to the progression of kindled seizures (Kokaia et al., 2001b; Schlifke et al., 2006).

The roles of galanin receptor subtypes in the seizure activity process have been elucidated using genetically modified mice, including GalR1 knockout mice (Jacoby et al., 2001; Jacoby et al., 2002; Schauwecker, 2010). These mice show spontaneous seizure activity (Jacoby et al., 2002), and exhibit impressive regulations of multiple peptides (Fetissov et al., 2003), collectively termed ‘SE peptidergic phenotype’ by Wasterlain et al. (2000). The GalR1 knockout mice have a reduced frequency of miniature inhibitory postsynaptic currents (mIPSCs) in hippocampal CA1 pyramidal neurons, thus implicating a presynaptic impairment of the synaptic inhibition in seizure phenotype (McColl et al., 2006). Mazarati et al. (2004b) studied another line of GalR1 knockout mice, not exhibiting spontaneous seizures, and they developed a more severe and longer-lasting seizure activity, and a more profound seizure-induced hippocampal neuronal injury under conditions of pilocarpine- or perforant path stimulation (PPS)-induced SE.

Mazarati et al. (2005) showed that activation of GalR1, but not of GalR2, can attenuate seizures induced by pertussis toxin (PTX) combined with PPS. In agreement, SE could be elicited by the same duration of PPS in controls and GalR2-downregulated

mice treated with GalR2 peptide nucleic acid (PNA). However, once established, seizures were significantly more severe in rats with GalR2 downregulation than in control rats. This result supported the idea, that GalR2 inhibited SE during its maintenance phase (Mazarati et al., 2004a) (Figure 12). It may be hypothesized that the involvement of GalR2 is a result of hyperactivity of the neuronal network during the initial phase of SE, increasing expression of the GalR2 gene in certain neuron populations, most likely the granule cells. In fact, a transgenic mouse over-expressing galanin in granule cells shows a certain degree of resistance to kindling-induced seizure activity (Kokaia et al., 2001a). GalR2 coupled to G_i in these cells could hypothetically act as a presynaptic inhibitory autoreceptor decreasing cAMP levels. In support of this idea an increase in GalR2 mRNA has recently been reported in amygdala after seizures (Christiansen and Woldbye, 2010). Similar dramatic seizure-induced increases have been reported for the NPY Y2 receptor transcript (Kopp et al., 1999; Schwarzer et al., 1998; Vezzani et al., 2000). As said, in our study we were not able to detect GalR2 in principal neurons of the HIFo, neither in granule nor pyramidal cells. It would have been interesting to expose our GalOE mouse to epileptic seizures. If the GalR2-EGFP construct is strongly upregulated, we could perhaps detect it with our methodology.

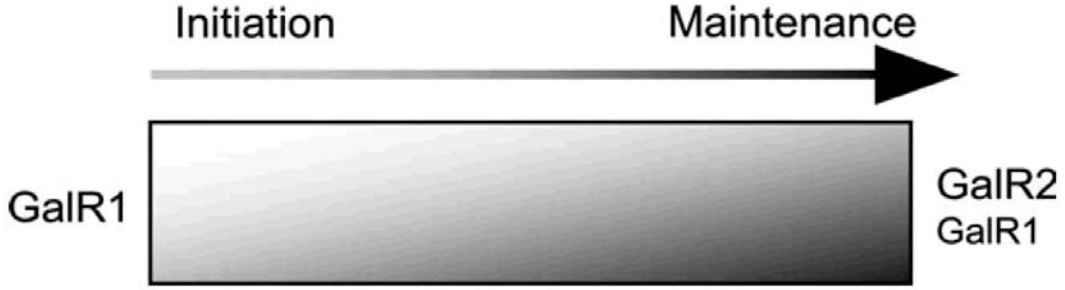

Figure 12. Regulation of early and advances stages of SE by GalR1 and GalR2. While during the early phase of SE galanin controls seizures through activation of GalR1, GalR2 becomes involved in mediating of anticonvulsant effects of galanin as seizures progress (Mazarati and Lu, 2005).

Taken together, both GalR1 and GalR2 have anticonvulsant effects in limbic SE. However, there are phase-specific differences in GalR1 and GalR2 regulation of seizures, GalR1 activation being more important during the initial phase, and GalR2 activation more profound during the maintenance phase, as summarized in fig 11. These phase-specific differences are important for the future development of new antiepileptic drugs. In addition to anti-seizure effects, activation of galanin receptor subtypes can also protect against seizure-caused neuronal injury. This provides a further perspective for the application of galanin or its analogues to rescue from seizure-associated injury and chronic epilepsy resulting from SE.

5.4.4 ADDICTION

There is evidence from studies on rat that LC is a brain area that contributes to expression of the physical signs of withdrawal from psychostimulants, a situation when the NA neuron is hyperactive (Maldonado and Koob, 1993). Picciotto and colleagues

have provided strong evidence that galanin may play an important role under these circumstances (Picciotto, 2008). Thus, this group has, for example, shown that (i) local administration of galanin into the cerebral ventricle attenuates the morphine conditioned place preference by shifting the dose response curve and increasing the threshold dose to a no longer rewarding level in mice (Zachariou et al., 1999); (ii) galanin and galnon, a blood-brain barrier-penetrating galanin receptor agonist, can attenuate the hyperactivation of LC noradrenergic neuron induced by opiate withdrawal in transgenic mice (Zachariou et al., 2003); (iii) galnon reverses or normalizes morphine-induced locomotor activation in galanin KO mice (Hawes et al., 2008); and (iv) galanin KO mice are more sensitive to morphine application in the place preference paradigm compared to their WT littermates (Hawes et al., 2008). The intracellular signaling pathway regulated by opiate withdrawal are related to GalR1, but not GalR2 or GalR3 signaling (Zachariou et al., 2001; Zachariou et al., 2000).

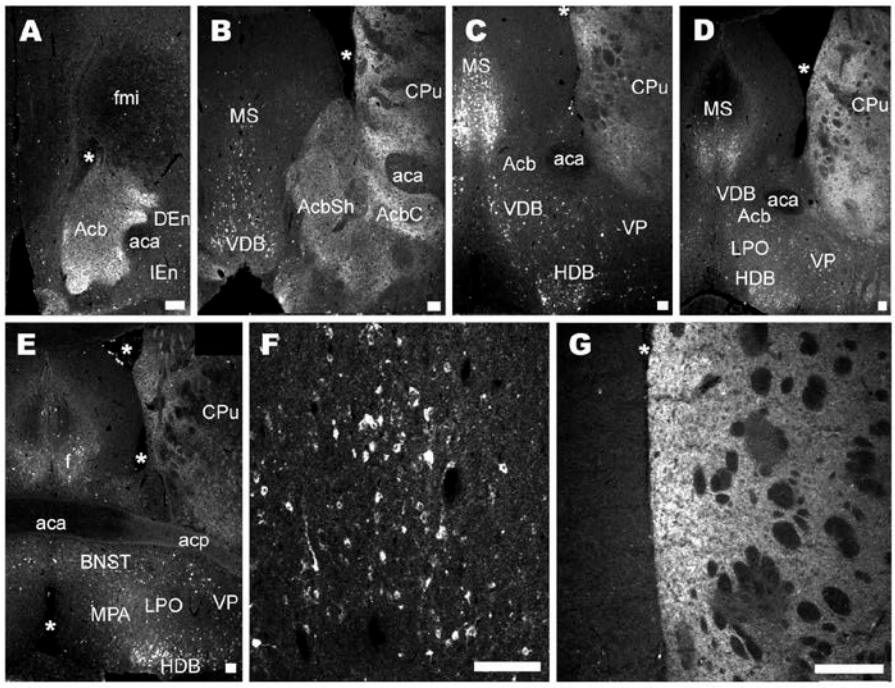

Figure 13. Immunofluorescence photomicrographs showing the distribution of GFP-LI in coronal sections at different rostrocaudal levels of basal forebrain area of GalR2-EGFP transgenic mice. A-E: Low magnification images showing the overview of GFP^+ cell bodies and fibers in the basal forebrain area. F: GFP^+ cell bodies in the medial septal nucleus (MS). G: Strong GFP-LI fibers in the periventricular part of caudate putamen (striatum). Scale bar is 100 µM.

The dose range for galanin affecting morphine's effect in locomotion and on conditioned place preference test is very different. the threshold dose for galanin in the latter test being 0.25 mg/kg, but at this dose locomotion is not affected (Hawes et al., 2008). The difference between these two behavioral test paradigms may be due to activation of different galanin receptor subtypes in different CNS neuron circuits (Picciotto, 2010). As said, GalR1 may be the critical receptor in LC (even if this subtype has not yet been demonstrated in the mouse LC).

It has been proposed that drug reinforcement, leading to compulsive drug abuse and addiction, is mediated by activation of the mesolimbic DA system (Koob, 1992). Our mapping results show, in addition to GalR2 in LC, a strong GalR2-EGFP^{+} fiber staining in a projection area of the A10 DA group, that is in the medial and ventral striatum and the nucleus accumbens (Figure 13), suggesting that galanin via GalR2 signaling is part of the forebrain reward circuit. Unfortunately, only limited studies have been done to test direct effects of galanin on the mesolimbic DA system, but it has been reported that local infusion of galanin into the rat VTA, but not into the nucleus accumbens, decreases locomotor activity and modulates DA synthesis (Ericson and Ahlenius, 1999). How, and if, this is related to the presumably presynaptic GalR2-positive nerve terminals in the mouse nucleus accumbens/medial ventral striatum, remains to be elucidated.

Taken together, by regulating the excitably of LC NAergic neuron via GalR1 in rat, galanin can attenuate psychostimulant withdrawal, making galanin signaling a possible target for treatment of addiction. In addition, the present results on mouse suggest that galanin can influence drug reward directly or indirectly through the mesolimbic DA system, but more work is needed to identify possible underlying mechanisms.

6 CONCLUSIONS

The genetic fusion of GalR2 with EGFP made it possible for us to study the trafficking of GalR2. After expression in PC12 cells, the fusion protein was predominantly localized on the plasma membrane with some intracellular fluorescent structures (vesicles) in the resting state, mainly in the perinuclear region. It could be activated by galanin which resulted in an increase in intracellular Ca^{2+} levels. The GalR2 undergoes constitutive endocytosis and recycling, as well as both ligand-dependent and -independent GalR2 internalization using the clathrin-dependent, endocytic recycling pathway. These results support the view that the GalR2-EGFP conjugate is functional.

A transgenic mouse was generated which expresses the GalR2-EGFP construct under the GalR2 promoter. With this transgenic mouse, combined with a sensitive immunohistochemical technique, we have studied the putative distribution of GalR2 in the mouse brain. The results show that GalR2 is widely expressed in the mouse brain. The need for colchicine treatment to visualize most of the cell bodies supports the idea that GalR2 primarily is a presynaptic receptor. It is shown that GalR2 is present in areas harboring some major classic neurotransmitters systems, but coexistence was so far only established with NA neurons in the LC and GABA neurons in the basal forebrain. Nevertheless, generation of specific GalR2 antibodies or at least sensitive riboprobes complementary to mouse GalR2 mRNA are needed to confirm the present results. In particular, antibodies are needed to show the full extent of the localization of GalR2 in the entire neuron, that is cell bodies, dendrites, axons and nerve terminals.

There are various functional alterations in hippocampal networks in aged animals and humans, which may contribute to the age-related, impaired synaptic plasticity occurring in the HiFo and cortex and may underlie memory/cognition impairments. The lateral perforant path, the input gateway to the dentate gyrus, is functionally altered by the ageing process and may be correlated to changes in neuropeptide systems, for example in galanin binding in hippocampal subregions. The present findings, showing an age- and galanin-dependent reduction in synaptic plasticity in the HiFo, support this idea. It may be speculated that galaninergic mechanisms could represent therapeutic targets for treatment of age-related memory deficits and AD.

The overexpression of galanin together with its receptors in the degenerating AD brain has been interpreted to represent a region-specific innate neuroprotection. Gene expression profiling studies have shown that CBF neurons from AD tissue with galanin hyperinnervation have a higher concentration of transcripts that promote CFB neuronal function and survival. Also, the overexpressed galanin may preserve neuron functionality, as it has been shown that galanin inhibits cholinergic transmission in HiFo. These data indicate existence of function-preserving and protective effects mediated by strengthened galanin signaling in AD. Using a newly developed method based on target-specific isolation (TSI) we have generated highly purified CFB neuron cultures. Our data obtained in this model further support a trophic effect of galanin activity through the GalR2 receptor. This piece of evidence may therapeutic potential of GalR analogues to treat AD.

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