

UPTEC X 05 028
MAY 2005

ISSN 1401-2138

NORA BUCHT

Galanin induced
activation of MAPK
in primary hippocampal
neuronal cultures

Master's degree project



UPPSALA
UNIVERSITET

Molecular Biotechnology Programme

Uppsala University School of Engineering

UPTEC X 05 028	Date of issue 2005-05	
Author	Nora Bucht	
Title (English)	Galanin induced activation of MAPK in primary hippocampal neuronal cultures	
Title (Swedish)		
Abstract	<p>Galanin is a neuropeptide that is widely expressed in both the CNS and PNS, as in the endocrinal system. Galanin modulates a variety of physiological/pathological processes and has been implicated in disorders such as epilepsy, depression and Alzheimer's disease. Galanin act as a ligand on specific galanin receptors. To use galanin receptors as drug targets, the signaling pathways of each receptor has to be determined. Previous experiments conducted in galanin-receptor expressing cell-lines suggest intracellular signaling due to galanin stimulation via activation of mitogen activated protein kinase (MAPK) pathways. To examine this in a native system of galanin-receptor expressing cells, primary hippocampal neuronal cultures were treated with galanin, and western blots stained for the activation of Erk-proteins known to be involved in MAPK pathways. MAPK activation in primary hippocampal cultures was shown due to stimulation with galanin, an activation that peaked after 10 minutes, and could be blocked using the galanin receptor antagonist M40.</p>	
Keywords	Galanin, galanin receptors, G-proteins, GPCRs, MAPK, Erk, hippocampus	
Supervisors	Professor Tamas Bartfai, M.D. Xiaoying Lu The Scripps Research Institute	
Scientific reviewer	Professor Fred Nyberg Department of pharmaceutical biosciences, Uppsala University	
Project name	Sponsors	
Language	Security	
ISSN 1401-2138	Classification	
Supplementary bibliographical information	Pages 33	
Biology Education Centre Box 592 S-75124 Uppsala	Biomedical Center Tel +46 (0)18 4710000	Husargatan 3 Uppsala Fax +46 (0)18 555217

Galanin induced activation of MAPK in primary hippocampal neuronal cultures

Nora Bucht

Sammanfattning

Galanin är en liten neuropeptid som har visats påverka en mängd olika fysiologiska processer såsom minnesformering, skakningar, mat intag och sexuellt beteende. Man har länkat peptiden till sjukdomar såsom epilepsi, depression och Alzheimers sjukdom. Man har sett att peptiden medierar sin effekt i nervcellerna genom att agera som aktiverande ligand på receptorer på nervcellerna.

Galanin verkar på speciella galaninreceptorer. Idag känner man till tre stycken galaninreceptorer och tror att de, genom att de aktiverar olika svar i cellen samt har skild distribution i olika delar av hjärnan, kan vara svaret på hur galanin kan påverka så många olika system med så varierande resultat på skilda platser i hjärnan. Receptorerna som galanin aktiverar tillhör en receptorfamilj vid namn G-proteinkopplade receptorer p.g.a. att de alla, efter extracellulär aktivering via ligand, aktiverar det intracellulära svaret med hjälp av G-proteiner. Vilka G-proteiner som aktiveras leder till olika svar i cellen och ger således olika effekt.

Tidigare studier i cellinjer som genom transfektion uttrycker galaninreceptorer på ytan påvisar en aktivering av MAPK (mitogen activated protein kinase) kaskader vid stimulering med galanin. Detta behöver nu kontrolleras i ett nativt system av naturligt receptoruttryckande nervceller, då de transfekterade cellerna kanske inte visar vad som egentligen sker i nervcellerna.

I detta experiment dissekerades hippocampus, som är en hjärnstruktur som funnits vara viktig för bl.a. minne och skakningar samt även visat sig innehålla galaninreceptorer, ut ur musfosterhjärnor. Hippocampusnervcellerna odlades på cellplattor och behandlades vid mogen ålder med galanin och M40, en galaninreceptorantagonist. Aktivering av MAPK kaskaden genom behandling med galanin kunde påvisas, den nådde sitt maximum efter 10 minuters behandling, och kunde helt eller delvis blockeras av M40. Således kunde det konfirmeras att den aktivering av MAPK-signalkaskaden som observerats i transfekterade receptoruttryckande cellinjer också gäller för nervceller.

Examensarbete 20 p i Molekylär bioteknikprogrammet

Uppsala universitet Maj 2005

Contents

1. Introduction	5
1.1 Galanin receptors	6
1.2 The physiological/pathological role of the galaninergic system	6
1.2.1 Galanin in pain behaviour	6
1.2.2 Galanin in memory and Alzheimer's disease	7
1.2.3 Galanin in epilepsy and seizures	8
1.2.4 Galanin in depression	8
1.2.5 Galanin in neuroprotection/neurotrophic effects	9
1.2.6 Galanin in feeding behaviour	9
1.3 Background	10
1.3.1 The aim of the project	10
1.3.2 The MAPK signaling pathway	11
1.3.3 G-proteins and G-protein coupled receptors (GPCRs)	12
1.3.4 Previous studies/ background knowledge	14
2. Material and methods	15
2.1 The dissection	15
2.2 Making of the plates	15
2.3 Treating the cell cultures	16
2.3.1 Experimental setup	16
2.3.2 Performing the experiments	16
2.4 Western blot	17
2.5 Quantification	17
3. Results	18
4. Discussion	24
5. Future perspectives	25
6. Acknowledgement	26
7. References	26
8. Appendix	28

1. Introduction

Galanin is a neuropeptide of 29 amino acids (30 in humans) that is widely expressed in both the central and peripheral nervous system, as in the endocrinal system. [1] It was discovered at Karolinska Institutet in 1983 in porcine intestines, [2] and was named galanin after the N-terminal glycine and the C-terminal alanine residues. The peptide was shown to be much conserved between different species, usually 29 amino acids long and C-terminally amidated, human galanin shown to be an exception with 30 amino acids and with a C-terminal serine residue as a free acid. [2, 3]

Galanin modulates a variety of physiological/pathological processes such as cognitive performance, food intake, pain sensation and sexual behaviour. [3] Galanin is highly inducible, showing distinct up-regulation after pathological disturbance in the nervous system. [4] Galanin is strongly neurotrophic during development and subsequent to injury, as well as neuroprotective. [5, 3] It also affects nociception, regulates pain thresholds and seizures when applied intraventricularly. These pathophysiological functions of the galanergic system make it interesting when it comes to several disorders such as Alzheimer's disease, epilepsy and depression. [3]

The fact that galanin, like many other neuropeptides, mediates its effects by binding to G-protein coupled receptors (GPCRs) makes it even more interesting, since this class of proteins represent an relatively easily targeted class by pharmacologists through the use of agonists and antagonists. No remarkable desensitization of the receptors after galanin stimulation has been observed. [6] To date, three different galanin receptors have been cloned, GalR1-3. [7]

Galanin and galanin receptors show an extensive distribution throughout the central nervous system. High immunoreactivity of galanin and galanin receptors are found in the hippocampus, hypothalamus, LC neurons and medulla oblongata. [1] Galanin has been shown to co-localize with several classical neurotransmitters such as acetylcholine, dopamine, glutamate, noradrenaline and serotonin, and acts generally as an inhibitory modulator on neurotransmitter release, having hyperpolarizing properties. [8, 9]

Several galanin receptor antagonists have been constructed chimerically, usually using gal(1-13) as N-terminus and an amid in the C-terminus. The one used in this study, M40, is constructed from gal(1-13)Pro₂(Ala Leu)₂Ala-amide. [10] Also a galanin receptor agonist, a non-peptide ligand called galnon, has been manufactured synthetically, using binding studies of the different amino acid residues in galanin to see which ones are the most important for binding, making the tripeptidomimetic ligand accordingly. Based on the known pharmacophores of galanin and galnon, a combinatorial library were synthesized and screened against the galanin receptors. Galmic was identified as an active compound with affinity for the galanin receptors. Finding non-peptide ligands is important since the peptide ligands are vulnerable to enzymatic degradation and unable to cross the blood-brain barrier. [11, 12] The development of Galanin OE- (overexpressing) and galanin KO- (knockout) as well as GalR1- and GalR2-KO mice strains have also yielded strong tools to further explore the galanergic system. [13, 14]

1.1 Galanin receptors

The effects of galanin are mediated via special galanin receptors. The first binding profiles indicated multiple receptor subtypes; different binding affinities in different tissues for both the full length peptide and subparts of it were observed in native systems with galanin. Interactions with multiple signal transduction pathways have resulted in further speculation about receptor subtype diversity in native systems. To prove this hypothesis, cloning was required. [7]

Molecular cloning initially identified two galanin receptors, GalR1 and GalR2, both belonging to the G protein coupled receptor superfamily, showing the seven transmembrane domains typical for the family. The amino acid sequences of GalR1 and GalR2 share only about 40% homology, and though they both bind to galanin with high affinity, the receptors can be pharmacologically distinguished by gal(2-29), a galanin fragment missing the Gly residue in the highly conserved N-terminal, which binds preferentially to GalR2. Following studies of gal(2-11) showed that this peptide binds to GalR2 with a much greater affinity than gal(1-29), and is nowadays used as a receptor discriminating ligand. [7]

A third receptor, GalR3, have also been cloned with a homology to GalR1 of 38% and GalR2 of 55%. Even though the homology of the different receptors seems to be very low, they are much conserved between species, suggesting an important physiological role of the receptors. All three receptors are expressed throughout the central nervous system, even though they have been shown to have different distributions. The intracellular signaling profiles of the different galanin receptors have been studied through the examination of coupling profiles to G-proteins. This is further described in the 'background knowledge' section. [7]

1.2 The physiological/pathological role of the galaninergic system

1.2.1 Galanin in pain behaviour

Pathological conditions such as nerve injury and inflammation makes the expression of neuropeptides in sensory neurons change dramatically. This indicates that these neuropeptides may be important for survival and regeneration of injured neurons, and may implicate pain processing. Galanin and galanin receptors are highly expressed in sensory neurons, suggesting a role for galanin in nociceptive transmission. [15]

Peripheral nerve injury rapidly induces an extensive up-regulation of galanin-like immunoreactivity in DRG (dorsal root ganglion) neurons of all sizes. [4, 16] DRG neurons in rats show elevated galanin levels already 24h after sciatic nerve transaction. Galanin expression reaches a maximum within a week and is maintained

in the absence of nerve regeneration. [17] Galanin is thought to be required for survival of some of the sensory neurons during development, as Gal KO mice show reduced number of DRG neurons and incapacity to regenerate after injury. [18] Galanin may be released from damaged sensory neurons since nerve injury leads to increased synthesis, central transport and terminal storage of galanin. [15]

Galanin is also thought to inhibit nociception as it has been shown to potentiate the antinociceptive effect of morphine. These effects might be through signaling via GalR1 since opiate withdrawal enhances GalR1 expression. Gal KO mice show hyperalgesia while galanin over-expression elevates nociceptive thresholds and reduces neuropathic pain behaviour. It is thought that galanin may reduce the amount of transmitter released from nociceptive afferents by depolarizing the membrane terminals of sensory afferents. [15]

1.2.2 Galanin in memory and Alzheimer's disease

Alzheimer's disease (AD) is recognized by a severe loss of neurons in the basal forebrain, which leads to reduced levels of neuromodulators including acetylcholine, dopamine and noradrenaline. Galanin has been linked to deficits in memory and learning in AD since galaninergic inter-neurons closely surround the cholinergic cell bodies in humans, and since galanin functions as an inhibitory modulator of acetylcholine in the hippocampus. [19]

Galanin has been linked to cognitive impairments in numerous studies and in several memory tasks including Morris water maze. It is thought that galanin may inhibit learning and memory by inhibiting neurotransmitter release and neuronal firing rate. When most neuromodulators show decreased concentrations with neuronal cell loss, galanin-containing fibers hypertrophy and hyperinnervate the surviving neurons of the basal forebrain in the late stage of AD. [20, 21] The loss of cholinergic neurons in the nucleus basalis shows a correlation with the increase in galanin immunoreactivity. [22, 23] An increased number of galanin receptor binding sites in late stage AD are observed within the cholinergic basal forebrain in nucleus basalis and hippocampal areas, which suggests that receptor plasticity accompanies the hyperinnervation of galanin. [21- 24]

Based on current knowledge it has been suggested that galanin might reduce cholinergic signaling in the basal forebrain by inhibiting acetylcholine release, especially in late stage AD. The fact that galanin has neuroprotective properties has arisen the hypothesis that the increase in galanin expression is a response to neuronal damages, and that galanin in fact tries to rescue the neurons from dying, trying to maintain cholinergic signaling. [19] Impairment of cognitive functions by galanin and inhibition of acetylcholine release shows a different aspect. Reduced acetylcholine release is seen in galanin OE mice, as inhibition of glutamate release. Cognitive impairments characteristic for AD and reduced long-term potentiation (LTP) is also shown. [25, 26] Even more contradictory results are presented; a recent study shows altered cognitive functions and increased acetylcholine release from hippocampus in rats, agreeing with the neuroprotective hypothesis and suggesting that the augmented

levels of galanin reduces cognitive deficits characteristic for AD. [27] Further investigation of how galanin mediate these different effects, via which receptor what response is given, is needed to outline these discrepancies and be able to use the galaninergic system for treatment of AD patients.

1.2.3 Galanin in epilepsy and seizures

Galanin innervates many structures in the brain, one of them is the hippocampus. Hippocampus is known to be important in memory and learning, but it has also been shown to be of importance for modulating seizure activity. Regulation of initiation and maintenance of seizures is seen in the dentate gyrus of the hippocampus. [28] Presumably, galanin alters hippocampal excitability and modulates seizure activity by inhibiting presynaptic glutamate release during epileptic discharge, and in developing long-term potentiation (LTP). [29]

Several experiments indicate an anticonvulsant effect of galanin; by administration of galanin into the lateral ventricle of rats, [30] and in the use of Gal KO mice which show lower thresholds for developing seizures [25] while Gal OE mice show increased resistance to induced seizures. [31] Galanin receptor agonists prevent induction of seizure activity, [12] while seizure activity is facilitated by galanin receptor antagonists. [32]

Studies of galanin content in rat models of status epilepticus (SE) show less galanin and galanin immunoreactive fibers in the hippocampus. This indicates that seizure activity and seizure progression deplete galanin in the hippocampus. [32] In three different models of SE augmented susceptibility to seizures were shown in GalR1 KO mice, while it was the severity of the seizures that was increased in GalR2 knockdown rat models. [33] These findings summoned bring us the present hypothesis about how galanin acts in seizure development, through GalR1 in the initiation phase and GalR2 during the maintenance.

1.2.4 Galanin in depression

Galanin co-localizes with monoamines known to be involved in mood disorders, which indicates a link between galanin and depressive-like behaviours. [8, 34] It has been shown that Gal OE mice have an increased release of serotonin and noradrenaline upon stressful stimulation. An increased floating in the forced swim test compared to control was also observed, suggesting more depressive-like behaviour and lower tolerance for stress. [35, 36, 37] Indications of galanin having the opposite effect, and being an antidepressant, are presented in a recent study below.

Among the most common drugs used for treatment of major depression today are the selective serotonin reuptake inhibitors (SSRI). Although the elevation of synaptic serotonin concentrations is fast, the clinical antidepressant effects requires 14-21 days

of treatment, indicating cooperation with other systems in the brain to make functional rearrangements in the CNS. Functional changes in signaling within the major serotonergic nucleus DRN (dorsal raphe nucleus) and its projection areas, due to transcriptional and translational events, may be required for the therapeutic effects. [38]

In a recent study with the SSRI fluoxetine (FLX) the expression of galanin m-RNA were up-regulated 100% and the GalR2-binding sites 50% in the rat DRN, suggesting galanin as one potential player, besides serotonin, in mediating the long-term effects of FLX. Further studies showed that the galanin receptor antagonist M40 attenuated the antidepressant-like effect of FLX in the forced swim test, and that the galanin receptor agonist galnon, by direct activation of the galanin receptors, produced an antidepressant-like effect in the same task. Other indications that galanin is involved in depression includes decreased galanin expression in DRN, hippocampus, and hypothalamus in rat models of depression, as well as a recent clinical study in which preliminary evidence for an antidepressant effect of galanin in depressed patients have been reported. [38]

1.2.5 Galanin in neuroprotection/neurotrophic effects

Galanin has been suggested to have a trophic role in the injured nervous system since it shows an extensive up-regulation after nerve injury. [4] Galanin also shows elevated levels prenatally compared to postnatally which implies importance during neuronal development. [5] In a Gal KO model it was showed that absence of galanin during embryonic development resulted in the reduction of small neurons by 13% in L4 and L5 DRG neurons and loss of a third of the cholinergic neurons in the basal forebrain. [39]

DRG neurons from Gal KO mice showed a decreased number of neurite outgrowths and shorter neurite lengths when cultured *in vitro*, [18] which were obstructed by the addition of galanin. In accordance with this, increased neurite outgrowth was shown after addition of the galanin receptor agonist gal(2-11), but reduced after addition of M35, a galanin receptor antagonist. [40] A further evidence for the developmental role of galanin is the expression of galanin mRNA, as well as GalR1 and GalR2 transcripts, in embryonic stem cells. [41]

1.2.6 Galanin in feeding behaviour

Galanin's possible role in feeding behaviour has been investigated since the discovery of galanin and its receptors in brain regions that regulate ingestive behaviours. Findings that galanin, when administered centrally, increase ingestion in rats has now been replicated in many laboratories. In rats that were held on ad libitum access to food and water intraventricularly administered galanin produced a significant and dose-dependent increase in consumption. Consistent evidence of galanin-induced feeding across species and at different ventricular injection locations has also been shown in several studies. [42]

The greatest effect of galanin on consumption was found in the paraventricular nucleus of the hypothalamus. Since many neurotransmitters in the hypothalamus are known to influence feeding and galanin is known to coexist with several other neurotransmitters, it is possible that the effects seen by galanin could be due to stimulation or inhibition of another major neurotransmitter. Elucidating this, some experiments indicate that galanin induces feeding by releasing hypothalamic norepinephrine. Other studies suggest that galanin may activate neurons in the hypothalamus that project to regions of the nucleus accumbens, in which the rewarding system is activated due to food intake. It is also shown that the opiate receptor antagonist, naloxone, reduces galanin-stimulated feeding in a dose dependent manner, proposing a possible interaction of galanin with endogenous opioid peptides that promote feeding. [42]

The galanin and galanin mRNA concentrations in rats who consumed high quantities of fat were significantly increased in the paraventricular nucleus of the hypothalamus, while food deprivation did not show a significant change of galanin mRNA levels, nor did lifelong moderate caloric restriction. These findings suggest that galanin is synthesized in the hypothalamus in response to ingestion, but not in response to starvation. Mixed nutrient diets given to galanin treated rats showed large increases in consumption of both high-fat and high-carbohydrate diets, but with a natural preference for fat. The findings that endogenous galanin seems to play a minor role in normal feeding in rats but a significant one in abnormal feeding suggest a role for galanin in human appetite disorders. Clinical studies in humans have assayed endogenous galanin levels in eating disorders, showing higher plasma concentration of galanin in obese women, while anorectic patients had normal levels. To be able to come to any conclusions regarding this, further studies are needed in this field. [42]

1.3 Background

1.3.1 The aim of the project

The diverse physiological functions exerted by galanin may be regulated by multiple G protein-coupled receptor subtypes and intracellular signalling pathways. To be able to use the galaninergic system to create drugs, one has to know exactly how the system works. My project was to look at neuronal cultures created from foetal mice hippocampi and determine if galanin, via its receptors, activates the Erk1 (extracellular signal-regulated kinase) /Erk2 MAPK signaling cascade. This has been suggested from experiments in receptor-expressing cell-lines, but verification in the native environment of naturally receptor-expressing neurons is needed.

1.3.2 The MAPK signaling pathway

Sequential activation of kinases (protein kinase cascades) is a common mechanism of cellular transduction in many cellular processes. There are several related intracellular signaling cascades collectively known as mitogen-activated protein kinase (MAPK) signaling cascades. These MAP kinases are a conserved class of proline-directed serine/threonine kinases that are activated in response to a variety of extracellular stimuli, and mediate signal transduction from the cell surface to the nucleus. In combination with several other signaling pathways, they can differentially alter the phosphorylation status of transcription factors, thus initiating cellular processes such as proliferation, differentiation, development, stress response and apoptosis. Each of these cascades, including Erk1/2, p38 Hog, JNK/SAPK, Erk3 and Erk5, consist of three to six tiers of protein kinases that sequentially activate each other by phosphorylation, making them part of a superfamily of protein kinases. [43, 44]

The most widely studied cascade is that of Erk1/Erk2 MAP kinases. These two proteins are 85% identical and are ubiquitously expressed in the cell. These MAP kinases are activated as a response to stimulation by serum, growth factors, cytokines, some GPCR ligands and certain stresses, among other things. In a general activation scheme (see Fig.1), eventually the active Raf kinase phosphorylates a dual specificity kinase, MEK, and activates it. MEK1 then binds to Erk and phosphorylates either a threonine or a tyrosine residue and then dissociates. The monophosphorylated Erk then rebinds to an active MEK1 for dual phosphorylation and complete activation. The activated MEK1 phosphorylates Erk1 on threonine¹⁸³ and tyrosine¹⁸⁵ and Erk2 on threonine²⁰² and tyrosine²⁰⁴. The dually phosphorylated forms of Erk1 and Erk2 have an activity over a 1000-fold greater than the singly or unphosphorylated forms. At one time, there is usually only one active form of Erk1 or Erk2 in the cell but three low active forms, unphosphorylated or singly phosphorylated at either the threonine or tyrosine residue. [44, 45]

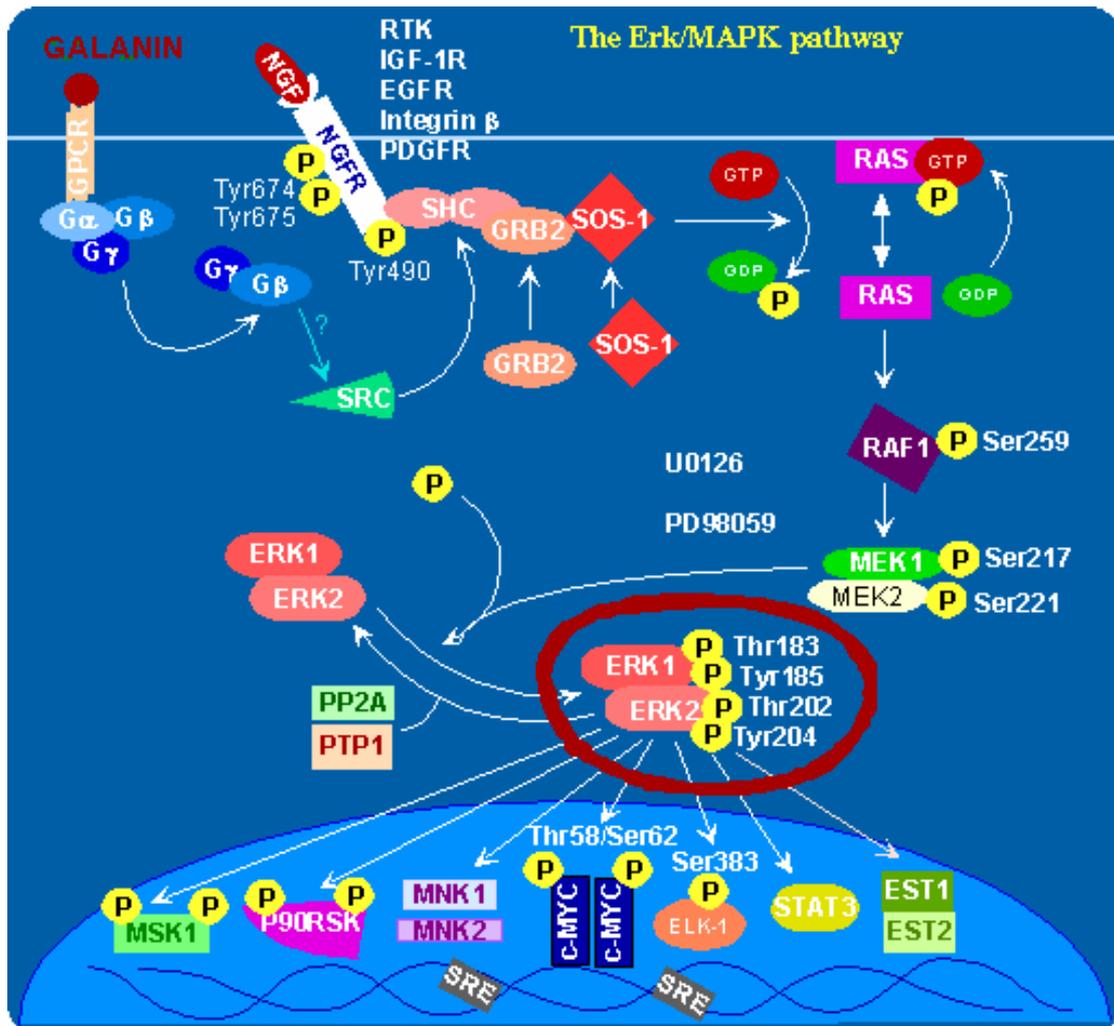


Fig. 1. The MAPK-Erk signaling pathway. Illustration adapted from EMD Biosciences homepage [47].

1.3.3 G-proteins and G-protein coupled receptors (GPCRs)

G-proteins are so called because they bind the **g**uanine nucleotides **GDP** and **GTP**. They are heterotrimers associated with the inner surface of the plasma membrane and transmembrane receptors, GPCRs. The three subunits of the G-protein are $G\alpha$, $G\beta$ and $G\gamma$. The basic activation mechanism of the GPCR starts with the receptor in its inactive state, with the associated G-protein subunit $G\alpha$ containing GDP in its binding site. When a ligand binds to the GPCR, an allosteric change takes place in the receptor triggering a following allosteric change in $G\alpha$, causing GDP to leave and be replaced by GTP. This change activates $G\alpha$, which dissociates from the remaining dimer of $G\beta\gamma$. Activated $G\alpha$ then activates an effector molecule. Other mechanisms exist, with $G\beta\gamma$ as the activating part, but these are less common. [44]

Which effect this activation is going to have is depending on which effector molecule that is being activated, which in turn is depending on the $G\alpha$ subunit. At least 20 different kinds of $G\alpha$ molecules are found in mammalian cells. These are divided into different classes, such as G_s , G_i , G_q , G_o and G_t (G_o and G_t really belonging to the G_i class). [44]

The G_s class activates the effector molecule adenylyl cyclase, the *s* standing for stimulatory (see Fig. 2). Adenylyl cyclase is an enzyme in the inner face of the plasma membrane which in its active form catalyzes the conversion of ATP into the second messenger cyclic AMP (cAMP). cAMP then activates protein kinase A (PKA) which phosphorylates proteins at specific Serine or Threonine side chains. cAMP can also activate the transcription factor CREB (cAMP response element binding protein) which, when bound to its response element in the promoters of regulated genes, turns on gene transcription. [44]

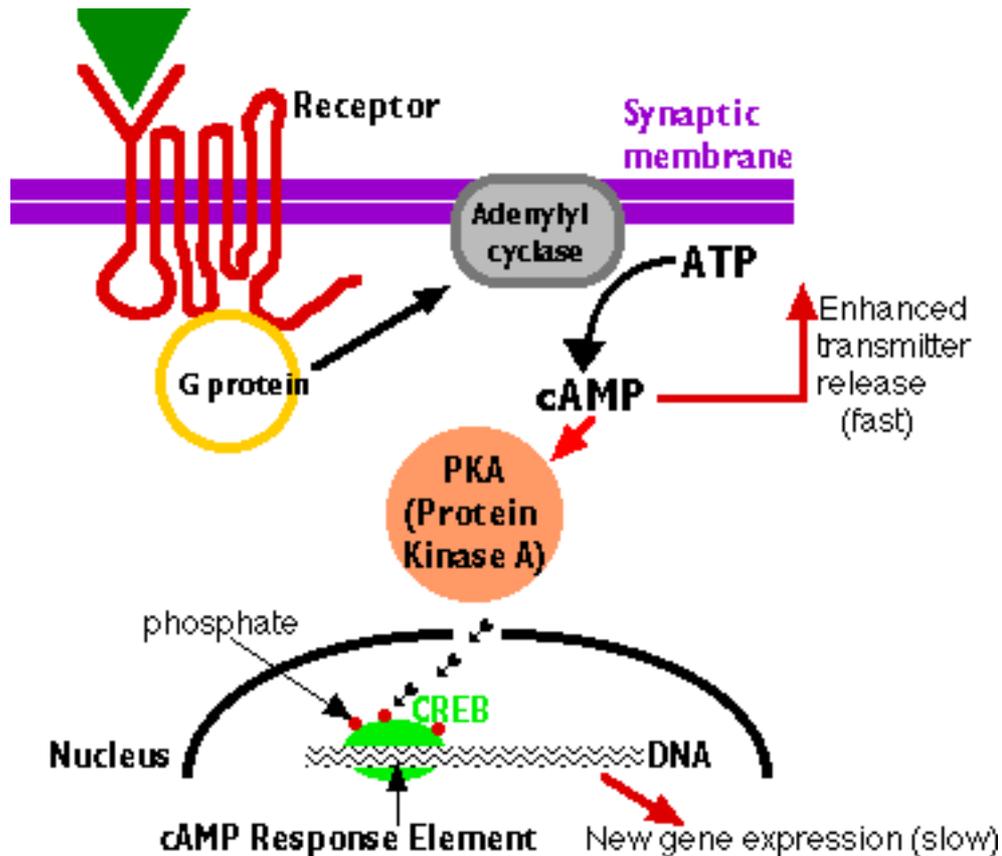


Fig. 2. The activation of PKA via G_s proteins. With permission from Karl Loren [48].

The G_i class has the opposite effect of the G_s , inhibiting adenylyl cyclase and lowering the levels of cAMP in the cell, *i* standing for inhibitory. A subgroup of G_i , G_t , contains the molecule responsible for generating a signal in the rods of the retina in response to light, *t* standing for transducin. G_{α_t} triggers the breakdown of cyclic GMP (cGMP). G_o reduces cAMP levels, open G-IRK (G-protein coupled inward rectifying K^+) channels and stimulate MAPK. [44]

G_q activates the enzyme PLC (phospholipase C) which cleaves PIP_2 (phosphatidyl inositol-4,5-bisphosphate) into two second messengers, diacylglycerol (DAG) and inositol triphosphate (IP_3). DAG binds to and activates protein kinase C (PKC). IP_3 activates calcium ion influx into the cell which, among other things, also activates PKC (see Fig. 3). [44]

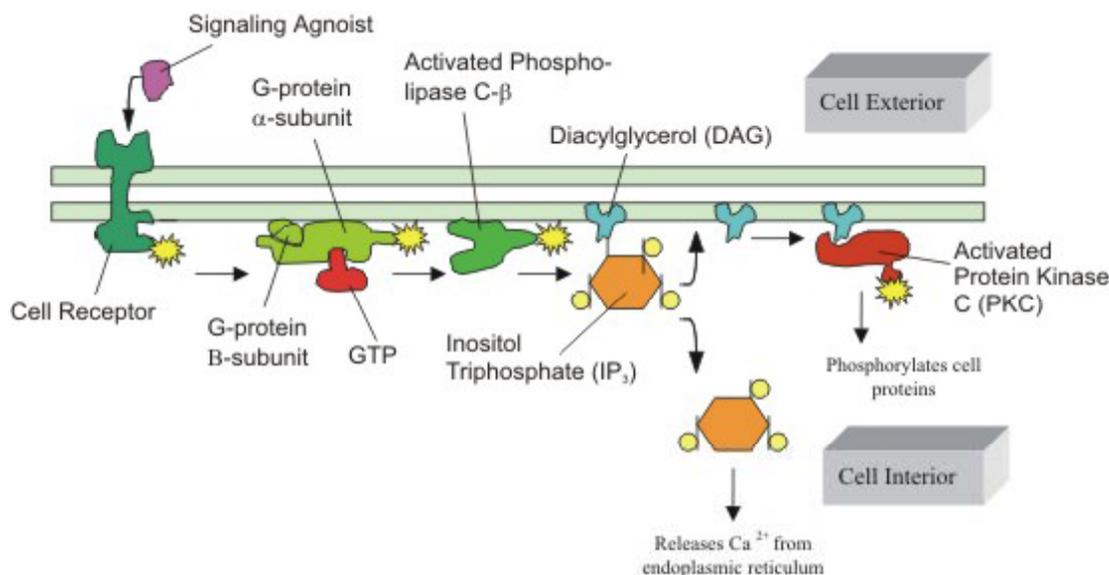


Fig 3. Activation of PKC via G-proteins. With permission from Ashok Kumar [49].

1.3.4 Previous studies/ background knowledge

To study the potential for the galanin receptors to couple to different G-proteins, GalR1- and GalR2-expressing Chinese hamster ovary (CHO) cell lines were generated and examined for their ability to couple to the Gs, Gi, Go and Gq proteins. It was found that galanin was unable to increase cAMP concentrations in GalR1- and GalR2-expressing cells, indicating inability for the receptors to couple to the Gs protein. On the contrary, galanin inhibited forskolin-stimulated cAMP production in GalR1/CHO cells by 70% and in GalR2/CHO cells by 30%, indicating a strong coupling between GalR1 and Gi and a more moderate one between GalR2 and Gi. [46]

Both receptors mediated MAPK activity. The stimulation mediated by GalR1 was inhibited by expression of the C-terminus of the C-adrenergic receptor kinase, which inhibits Gβγ signalling specifically, but was unaffected by the PKC inhibitor or cellular depletion of PKC. On the contrary, GalR2-mediated MAPK activation was unaffected by βARKct expression, but abolished by inhibition of PKC activity. This suggests that GalR1 is coupled to a Giβγ signaling pathway to mediate MAPK activation while GalR2 uses a distinct one, consistent with Go-mediated MAPK activation. [46]

Galanin was unable to stimulate inositol phosphate (IP) accumulation in cells expressing GalR1, but stimulated a 7-fold increase in IP production in cells expressing GalR2, not affected by pertussis toxin, suggesting linkage with Gq/G11. Thus, GalR1 seems to activate only the Gi pathway while GalR2 seems to activate Gi, Go, and Gq/G11 pathways. These different signalling profiles, the ability to discriminate between the receptors with different parts of the ligand and different distribution patterns of the receptors might be the answer to how galanin can regulate so many diverse physiological functions. [46]

2. Material and methods

2.1 The dissection

15-16 day pregnant Swiss-Webster mice were sacrificed, using an anaesthetic overdose of halothane in a jar. The belly were carefully sterilised with 70% alcohol, and thereafter cut open for removal of the foetuses. The foetuses were beheaded and placed in 1X dissection media (25 g glucose, 35 g sucrose, 1.75 g sodium bicarbonate and 500 ml 10X HBSS for 10X). Under a microscope in an air ventilated hood, the brains were removed and put in another Petri dish with dissection media on ice. Thereafter the hippocampi from each brain were removed very carefully using special dissection forceps and stored in yet another Petri dish on ice. Removal of the meninges from the brain tissue is very important since otherwise macrophages will occur in the neuronal cultures, destroying the neurons.

2.2 Making of the plates

The plates were incubated with poly-D-lysine (300 μ l/well) overnight in 37°C to make the cells attach better. After the incubation, the wells were washed three times with sterile water before the plating of the neurons. The dissection media containing the removed hippocampi (approx. 2 ml) were then transferred into a sterile falcon tube and spinned in a centrifuge, 1000 rpm for one minute, to gather the hippocampi at the bottom of the tube. The dissection media were removed, replaced by cell culturing media (5 ml Fetal Bovine Serum, 5 ml Donor Horse Serum, 1 ml glutamine and 89 ml media stock (900 ml Gibco Water, 100 ml 10X Minimum Essential Medium and 90 ml glucose bicarbonate, the glucose bicarbonate made from 1 L Gibco Water, 29.66 g sodium bicarbonate and 44.44 g glucose)), and the solution homogenised. One mouse often yielded at least 10 foetuses (i.e. 20 hippocampi) which was just enough for two plates, but sometimes just one was made for greater concentrations of neurons. The cells were to be plated on 24 well plates, containing 0,5 ml of the homogenised cell solution in each. The cell plates were placed in an incubator for primary neurons, 37 °C and CO₂ level of 7 %.

After about 7-10 days the cell cultures needs to be treated with a mitotic inhibitor, AraC (cytosine arabinofuranoside) to a concentration of 5 μ M, to stop further glial expansion. The glia cells are essential for neuronal survival, taking care of the excess glutamate in the media and helping the neurons to grow by exchanging nutrients between them. But one layer of glial cells is enough, after 7-10 days micro-glias are starting to form which kills the neurons. Hence, one has to check the proliferation of the glia from day six and see when the perfect time is to inhibit (when this is varies from one cell-culture to another). The timing is very important since if it is too late, the micro-glias will kill the neurons, and too early the neurons will not have the chance to develop properly, they will retract in growth and ultimately die. About 21 days from the day of the plating, the neurons are forming a nice web of fully grown axons and dendrites and the plate is ready for use.

2.3 Treating the cell cultures

2.3.1 Experimental setup

The different treatments used in the experiments were:

Ctrl

gal(1-29) (5 μ M)

gal(2-11) (5 μ M)

M40 (5 μ M)

gal(1-29) (5 μ M) + M40 (5 μ M)

gal(2-11) (5 μ M) + M40 (5 μ M)

Samples were both untreated and treated with 1 μ M TTX and a time course of both gal(1-29) and gal(2-11) were made. A single experiment in DRN neurons was also conducted.

2.3.2 Performing the experiments

The first step in treating the neurons is to wash them, exchanging 2/3 of the cell culturing media for a media without serum (1X HCSS) three times, to calm the cells down. The cells are left in the incubator like this for about 90 minutes, which could kill them, so their condition has to be checked before proceeding with the next step. In the experiments where TTX (tetrodo-toxin) was used, a sodium channel blocker that prevents action potential firing in the neurons, it was added to the media at this state. TTX is used to see the effect induced by galanin on the individual neurons, and not the effect triggered by other neurons in the network. It could also be useful in determining if the effect is pre or post synaptic. In the wells where the galanin antagonist M40 were used, it was also added at this time. Then the cells were left in the incubator again for another 30 minutes. After that, galanin was added, and the plates put back in the incubator. They were left there for ten minutes (which is suggested from the timecourses to be the ultimate incubation time), except for in the making of the timecourses, of course.

The next step was to harvest the cells, starting with washing the cells on ice with cold PBS (phosphate-buffered saline) three times. All PBS were removed and 70 μ l lysis-buffer with phosphatase inhibitor were added to each well. The cells were lysed for about 15 minutes on ice, rubbed loosed from the plate using a plastic spatula and removed from the plate into eppendorf tubes. The tubes were centrifuged at 10 000 rpm in 4°C for 15 minutes whereupon the supernatant was transferred to another tube. The protein concentration was measured using a BCA protein assay.

2.4 Western blot

The gels made for Tris-glycine SDS-Polyacrylamide Gel Electrophoresis consisted of a 10% resolving gel and a 5% stacking gel. One tenth of 1M DTT and loading buffer were added to the samples, whereafter the samples were boiled for 10 minutes at 100°C to denaturize the protein and loaded onto the gel with 10 µg/well as where 10 µl of the Bench Mark prestained protein ladder (10748-010) which were used for size comparison of the fragments. They were run in 1X running buffer (tris/glycine with 10% SDS) for about an hour at a constant current of 50 mA for two gels.

The proteins in the gels were then transferred onto a membrane using transfer buffer (running buffer with 10% methanol to make the proteins move from the gel to the membrane). The membranes were then put in 1% acetic acid to lower the pH whereafter they were stained with Ponceau to see if the running of the gel and the transfer had succeeded. After this check the membranes were washed with water, and finally TBS-tween (0,5% tween), until all Ponceau was gone. After this the membranes were blocked for at least one hour at RT in 6 % non fat dry milk solution (milk powder + TBS-tween) to prevent unspecific binding of the Ab to the membrane.

Primary antibody, Phospho-p44/42 MAPK (E10) mouse antibody from cell signaling, which detects dually phosphorylated p44 (Erk1) and p42 (Erk2) proteins (i.e. the activated form), was put 1:2000 in milk solution. The membranes were incubated with primary Ab at 4°C overnight. After this they were washed 3 times for 10 minutes in TBS-tween, and put in secondary Ab (1:5000). The membrane were incubated at RT for at least an hour, then washed 3 times for 20 minutes, in TBS-tween the first two times and just TBS the last. After this the membranes were developed. After development the membranes were stripped and reprobed with primary Ab p44/42 (cell signaling) in milk solution 1:2000. This Ab detects all Erk1 and Erk2 proteins, even the unphosphorylated forms, and is used for correct comparison between wells.

2.5 Quantification

The pictures yielded from the development were scanned using Mac program Magic Scan. The scanned pictures were then quantified for the peak and average intensity of each well using Quantity-One. These results are summoned in Table 6 in the appendix. The ratio between phospho-Erk/total Erk was calculated for every well in every blot. To be able to compare the blots with each other, the ratios were normalized setting the background (untreated control wells) to 1 in every blot. Since there is no activation in these wells, the wells in the two blot pictures (phospho-Erk and Erk) should have the same intensity if exposed alike (supposing the Ab binds to the exact same level). Since this is almost impossible, pretending it is not; setting the ratio in the control well to 1, and compensating for the changes necessary to do so in the other wells, removes the errors from the exposure. This makes it possible to compare the different blots with each other. The normalized values and the calculated averages, standard deviations and standard errors are seen in table 1-5 in the result section.

3. Results

The normalized results are seen in Table 1-5 below, the raw data and normalization calculations are seen in Table 6 in the appendix. The most important is the peak intensity, but the average intensity is also measured as a control since it is supposed to correlate with the peak intensity. The graphs are made from activation of Erk1 the Ab presumably detects Erk1 levels more accurately than Erk2. In Table 1 below the results from the timecourse using gal(1-29) is presented.

Treatment	ERK1 (p44) Peak Intensity	ERK1(p44) Average Intensity	ERK2 (p42) Peak Intensity	ERK2 (p42) Average Intensity
ctrl	1	1	1	1
gal(1-29) 1min.	0.86	0.97	1.36	1.36
gal(1-29) 5min.	1.71	1.15	0.92	0.81
gal(1-29) 10min.	6.62	3.26	2.34	3.07
gal(1-29) 15min.	5.62	2.59	1.66	2.67
gal(1-29) 30min.	2.1	0.82	0.93	1.37
gal(1-29) 60min.	2.38	1.53	1.15	2.25

Table 1. Timecourse of Erk activation due to stimulation with gal(1-29) during 60 minutes.

A graph of the peak intensity of gal(1-29) activated Erk1 during 60 minutes (column 1) is seen in Figure 4 below. The timecourse shows a maximal neuronal stimulation due to galanin after approximately 10 minutes.

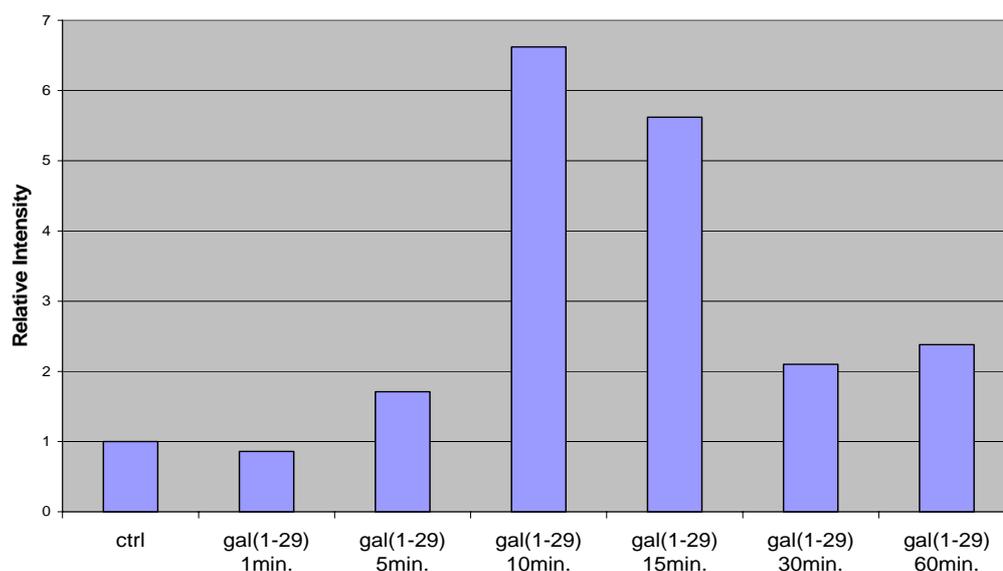


Fig. 4. The peak intensity of activated Erk1 during 60 minutes stimulation with gal(1-29).

The next experiment, a timecourse using gal(2-11) is presented in Table 2 below. Even though the result is not as clear as with the full length peptide a maximum is seen after 10 minutes even here.

Treatment	ERK1 (p44) Peak Intensity	ERK1(p44) Average Intensity	ERK2 (p42) Peak Intensity	ERK2 (p42) Average Intensity
ctrl	1	1	1	1
gal(2-11) 1min.	0.71	0.64	0.14	0.2
gal(2-11) 5min.	1.35	1.25	0.13	0.33
gal(2-11) 10min.	1.95	1.6	0.17	0.42
gal(2-11) 15min.	0.95	0.82	0.1	0.2
gal(2-11) 30min.	1.22	1	0.13	0.34

Table 2. Timecourse of Erk activation due to stimulation with gal(2-11) during 30 minutes

A graph of the peak intensity of gal(2-11) activated Erk1 during 30 minutes is seen in Figure 5 below (column 1 from Table 2 above). Activation is not as pronounced as with gal(1-29) but seems to peak at the same time.

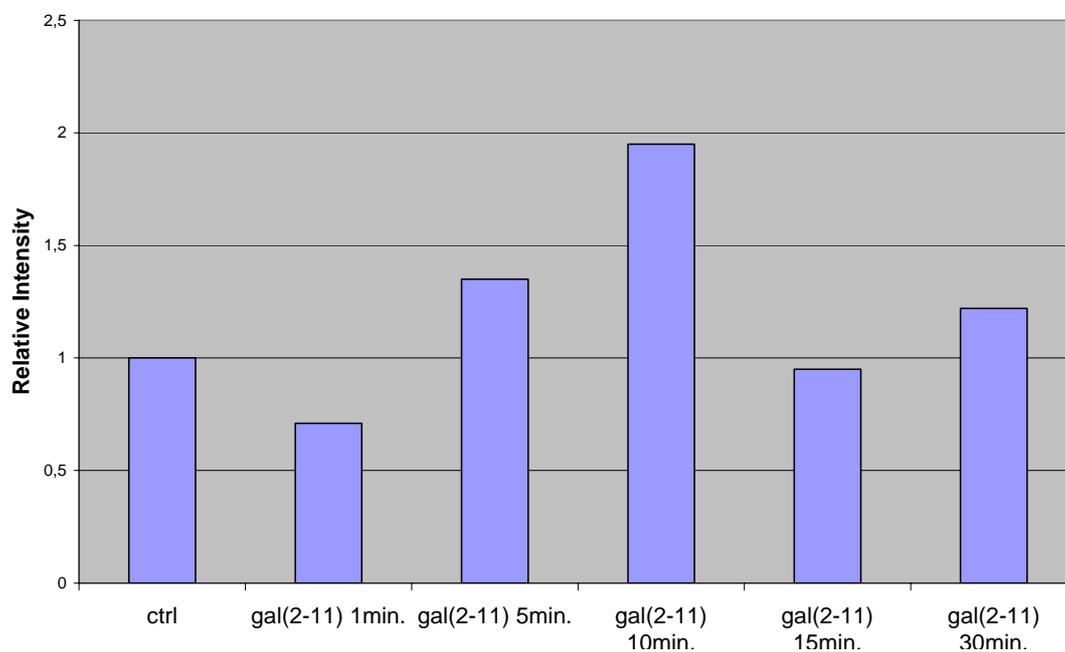


Fig. 5. The peak intensity of activated Erk1 during 30 minutes stimulation with gal(2-11).

In Table 3 below, the results from neuronal stimulation by incubation with gal(1-29), gal(2-11) and M40 from the TTX omitted wells are presented. Calculated averages, standard deviations and standard errors for each treatment are also presented.

Treatment	ERK1 (p44) Peak Intensity	ERK1(p44) Average Intensity	ERK2 (p42) Peak Intensity	ERK2 (p42) Average Intensity
Ctrl	1	1	1	1
gal(1-29)	3.3	8.4	0.46	1.87
	2.57	2.46	0.89	1.12
Average	2.935	5.43	0.675	1.495
Standard dev.	0.516	4.200	0.304	0.530
Standard error	0.37	2.97	0.21	0.11
gal(2-11)	0.71	0.89	0.96	1.76
M40	0.93	1.2	0.68	1.95
	1.14	1.15	1.01	1.75
Average	1.035	1.175	0.845	1.85
Standard dev.	0.148	0.035	0.233	0.141
Standard error	0.1	0.02	0.16	0.1
gal(1-29)+M40	1.23	1.22	0.71	1.5
	0.57	1.23	0.77	0.79
Average	0.9	1.225	0.74	1.145
Standard dev.	0.467	0.007	0.042	0.502
Standard error	0.33	0.005	0.03	0.35
gal(2-11)+M40	0.33	0.54	1.29	1.52

Table 3. Normalized values and averages, standard deviations and standard errors for the experiments not using TTX.

A graph of the peak intensity of activated Erk1 based on experiments summoned in column 1 Table 3 is seen in Figure 6 on the next page. The average from every treatment is presented, as is the standard errors as error bars. The results indicate stimulation of the Erk/MAPK pathway due to gal(1-29) stimulation, but not by gal(2-11). This stimulation is blocked by the receptor antagonist M40.

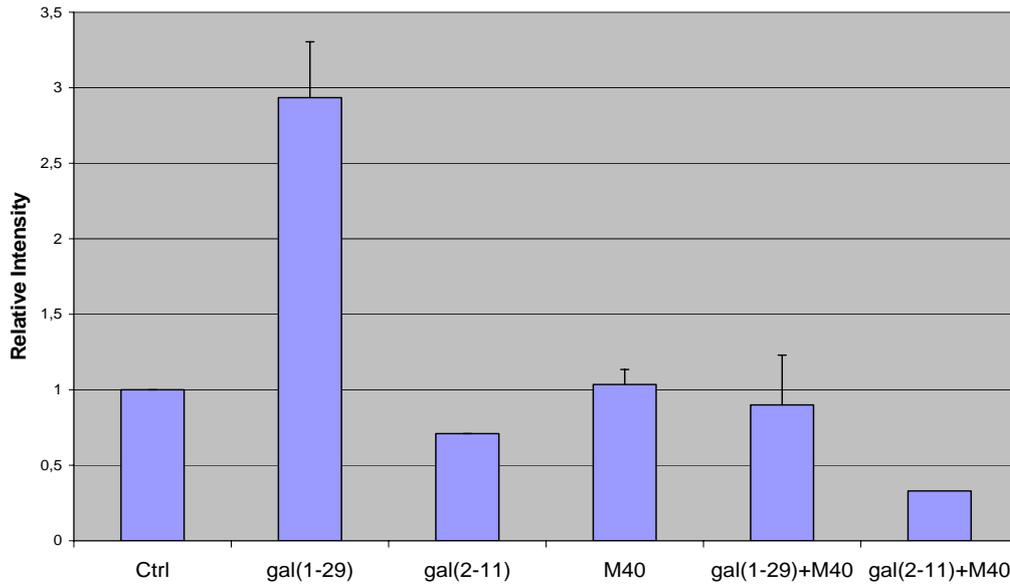


Fig. 6. The peak intensity of activated Erk1 for the different treatments in table 3.

In Table 4a and 4b below the results from the different treatments using the sodium channel blocker TTX are presented. These results are in agreement with the ones not using TTX (Table 3). This indicates that the effect seen by galanin is pre-synaptic since blocking the firing of action-potentials seems to make no difference.

Treatment	ERK1 (p44) Peak Intensity	ERK1(p44) Average Intensity	ERK2 (p42) Peak Intensity	ERK2 (p42) Average Intensity
ctrl+TTX	1	1	1	1
gal(1-29)+TTX	3.55	3.89	1.17	2.17
	7.77	18.5	1.4	3.15
	10.35	16.76	0.4	1.06
	9.11	6.9	4.48	11.68
	1.53	3.38	1.11	2.59
	1.17	1.69	1.04	1.69
	0.33	1.18	0.56	0.71
	Average	4.83	7.471	1.451
Standard dev.	4.155	7.197	1.381	3.794
Standard error	1.57	2.72	0.52	1.43
gal(2-11)+TTX	1.14	1.4	0.96	1.88
	0.69	1.45	1.76	4.15
	1.13	2.57	1.06	2.2
	0.81	0.94	1.15	1.87
	Average	0.943	1.59	1.233
Standard dev.	0.228	0.692	0.360	1.094
Standard error	0.11	0.35	0.18	0.55

Table 4a. Normalized values and averages, standard deviations and standard errors for the experiments using TTX, part one.

Treatment	ERK1 (p44) Peak Intensity	ERK1(p44) Average Intensity	ERK2 (p42) Peak Intensity	ERK2 (p42) Average Intensity
M40+TTX	3.32	2.82	1.42	2.65
	1.85	2.62	1.22	1.57
	1.3	1.57	0.83	0.7
	0.93	1.52	1.25	2.46
	1.14	1.59	1.23	1.84
	0.95	0.91	0.79	0.65
	Average	1.582	1.838	1.123
Standard dev.	0.916	0.731	0.254	0.849
Standard error	0.37	0.3	0.1	0.35
gal(1-29)+M40+TTX	3.23	3.89	1.01	1.41
	2.08	1.75	1.07	0.79
	0.62	1.03	0.98	1.12
	0.46	0.65	0.9	0.55
	0.45	1.04	1.5	3.75
	0.86	1.02	1.09	1.52
	5.39	5.36	1.09	2.69
Average	1.87	2.106	1.091	1.69
Standard dev.	1.865	1.802	0.193	1.139
Standard error	0.7	0.68	0.07	0.43
gal(2-11)+M40+TTX	0.39	1.2	0.73	1.19
	0.71	1.07	1.07	2.13
	1.25	1.5	2.33	4.09
	1.57	1.63	1.78	2.4
	Average	0.98	1.35	1.478
Standard dev.	0.53	0.259	0.717	1.209
Standard error	0.26	0.13	0.36	0.6

Table 4b. Normalized values and averages, standard deviations and standard errors for the experiments using TTX, part two.

A graph of the peak intensity of activated Erk1 based on experiments summoned in Table 4a and 4b (column 1) is seen in Figure 7 below. As with the experiments not using TTX, an activation of the Erk/MAPK pathway is seen by gal(1-29) and furthermore blocked by M40.

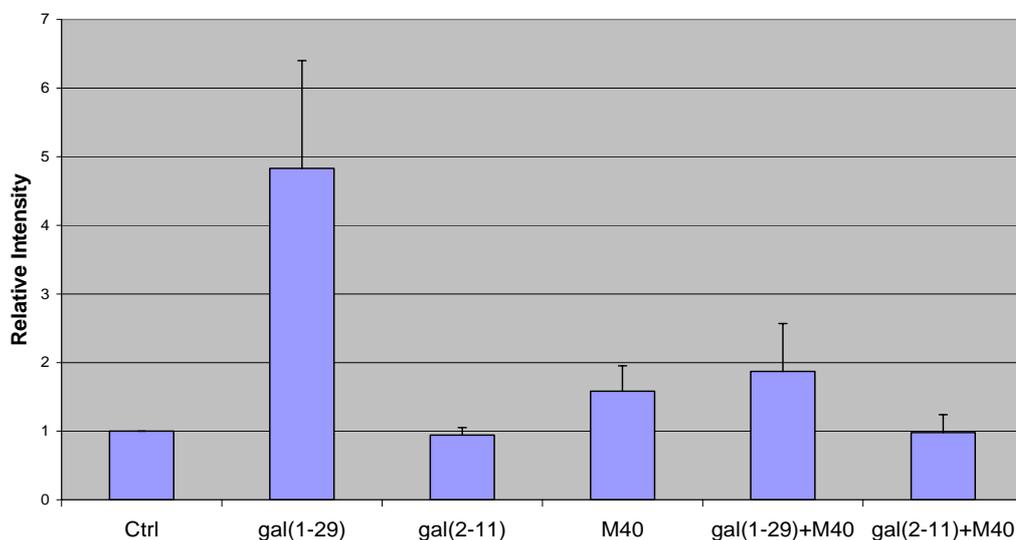


Fig. 7. The peak intensity of activated Erk1 for the different treatments in table 4a and 4b.

In Table 5 below, the results of a single experiment in DRN neurons are presented. In these experiment only activation by gal(1-29) and gal(2-11), with and without TTX, is investigated. No antagonist is used in this experiment.

Normalized values	ERK1 (p44) Peak Intensity	ERK1(p44) Average Intensity	ERK2 (p42) Peak Intensity	ERK2 (p42) Average Intensity
DRN				
ctrl	1	1	1	1
gal(1-29)	1.56	1.25	1.19	1.71
gal(2-11)	2.11	1.5	1.14	1.36
ctrl+TTX	1	1	1	1
gal(1-29)+TTX	1.5	1.09	1.46	1.52
gal(2-11)+TTX	1	0.55	1.4	1.15

Table 5. Values from a single experiment in DRN neurons.

A graph of the peak intensity of activated Erk1 based on a single experiment in DRN neurons presented in Table 5 is seen in Figure 8 below. This indicates a somewhat stronger activation of Erk1 by gal(2-11) than gal(1-29).

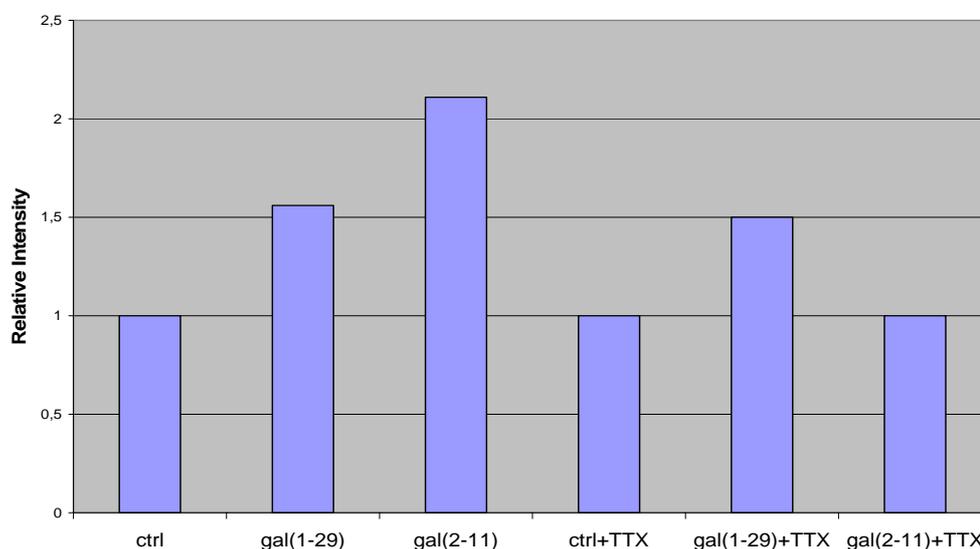


Fig. 8. The peak intensity for activated Erk1 in a single experiment in DRN neurons.

The interpretations of the results from the different experiments are presented in the discussion.

4. Discussion

Previous experiments in galanin receptor expressing CHO cell-lines showed MAPK activation in response to galanin treatment. Coupling of different G-proteins to the galanin receptors outlined a base for this experiment. In these cell-lines, expressing either GalR1 or GalR2, a basic hypothesis regarding the intracellular signaling pathways due to galanin stimulation of galanin receptors was formed.

In these previous experiments it was shown that both receptors mediated MAPK signaling cascades when exposed to galanin. Since this experiment was performed in transfected cell-lines, the question remains; does the signaling follow the same pattern in the native system, in a neuron naturally expressing the receptors. To determine if this was in fact so, primary hippocampal neuronal cultures, known to express galanin receptors, were altered from dissection of foetal mouse brains and subsequently treated with galanin and galanin antagonists.

Western blot analysis of the experiments, staining for Erk activation, showed a significant increase of activated Erk in gal(1-29) treated neurons also treated with TTX (Table 4a and 4b). In neurons not treated with TTX the activation was not as profound but followed the same pattern suggesting the effect to be pre-synaptic, since blocking of action-potential firing by TTX seemed to make no difference (Table 3). The timecourse showed that the activation induced by gal(1-29) peaked after approximately 10 minutes (Table 1), which was the incubation time used for following experiments. The activation due to gal(1-29) was also blocked, or partially blocked by the galanin receptor antagonist M40 (Table 4a and 4b/ Figure 7).

The timecourse of gal(2-11) also showed maximal activation of Erk proteins after 10 minutes (Figure 5), but the activation was not as legible as with gal(1-29). In fact, this experiment was unable to show consistent gal(2-11) stimulation in hippocampal neurons. This might be due to signaling via different receptor subtypes since gal(2-11) is used as a selective ligand for GalR2, and the previous experiments indicated different pathways to mediate MAPK activation via GalR1 and GalR2, and that stimulation of GalR2 activates MAPK to a lesser extent since it also can activate other pathways. This may also depend on different distributions of the receptors since a single experiment in DRN neurons indicated that gal(2-11), and to a lesser extent gal(1-29), activated MAPK (Table 5).

5. Future perspectives

As a direct continuation of this experiment, to further investigate the signaling pathways of galanin, is to continue this experiment in a larger scale and with other neuronal tissues. To check for the difference between gal(1-29) and gal(2-11) in PKC inhibition/depletion and to determine if the C-terminus of β ARK can act as an inhibitor, which of course might be hard in neurons. Try to investigate the differences between the receptors, maybe using tissue from receptor knock-out mice. Check for CREB activation using CREB Ab in western blot to further analyze the pathways.

When it comes to the future of galanin, there is almost nowhere to start in determining what should or could be done in the future, it is too much. The new strains of GalR1- and GalR2 KO mice, together with the Gal KO and Gal OE mouse strains and the galanin receptor antagonists and agonists, provide powerful tools for further characterization of the galaninergic system. The making of GalR3 KO mice, as well as double and triple KO is yet ahead of us. To further characterize the functional coupling of the receptors, to determine exactly which receptor that mediates which response, and to construct receptor selective ligands is one way to go, to ultimately be able to use the galaninergic system for treatment of many disorders such as eating disorders, epilepsy and AD.

6. Acknowledgement

I would like to thank Professor Tamas Bartfai for giving me the opportunity to perform this work at his lab at The Scripps Research Institute, San Diego. I would also like to thank Xiaoying Lu for supervision and Svetlana, Jacinta and Marga for helping with the dissection and culturing of the neurons. I would also like to thank everyone else in the Bartfai lab for answering my questions, helping out and making me feel welcome in the lab!

7. References

1. Jacobowitz D. M. *et al.* (2004) Galanin in the brain: chemoarchitectonics and brain cartography – a historical review. *Peptides* 25, 433-464
2. Tatemoto K. *et al.* (1983) Galanin - a novel biologically active peptide from porcine intestine. *FEBS Lett* 164, 124-128
3. Vrontakis M. E. (2002) Galanin: a biologically active peptide. *Curr Drug Targets CNS Neurol Disord* 1, 531-541
4. Hökfelt T. *et al.* (1987) Increase of galanin-like immunoreactivity in rat dorsal root ganglion cells after peripheral axotomy. *Neurosci Lett* 83, 217-220
5. Gabriel S. M. *et al.* (1989) Tissue specific sex differences in galanin-like immunoreactivity and galanin mRNA during development in the rat. *Peptides* 10, 369-374
6. Lang R. *et al.* (2001) Biphasic response to human galanin of extracellular acidification in human Bowes melanoma cells. *Eur J Pharmacol* 423, 135-141
7. Branchek T.A. *et al.* (2000) Galanin receptor subtypes. *Trends Pharmacol Sci* 21, 109-117
8. Melander T. *et al.* (1986) Coexistence of galanin-like immunoreactivity with catecholamines, 5-hydroxytryptamine, GABA and neuropeptides in the rat CNS. *J Neurosci* 6, 3640-3654
9. Hökfelt T. *et al.* (1987) Coexistence of peptides with classical neurotransmitters. *Experientia* 43, 768-780
10. Bartfai T. *et al.* (1993) Galanin receptor ligand M40 peptide distinguishes between putative galanin-receptor subtypes. *Proc Natl Acad Sci U S A* 90, 11287-11291
11. Ceide S. C. *et al.* (2004) Synthesis of galmic: A nonpeptide galanin receptor agonist. *PNAS* 101, 16727-16732
12. Saar K. *et al.* (2002) Anticonvulsant activity of a nonpeptide galanin receptor antagonist. *Proc Natl Acad Sci U S A* 99, 7136-7141
13. Crawley J. N. *et al.* (2002) Galanin overexpressing transgenic mice. *Neuropeptides* 36, 145-156
14. Wynick D. *et al.* (2002) Targeted disruption of galanin. New insights from knock-out studies. *Neuropeptides* 36, 132-144
15. Wiesenfeld-Hallin Z. *et al.* (2001) Neuropeptides in neuropathic and inflammatory pain with special emphasis on cholecystokinin and galanin *Eur J Pharmacol* 429, 49-59
16. Ma X. *et al.* (2001) Effects of galanin receptor agonists on locus coeruleus neurons. *Brain Res* 919, 169-174
17. Villar M. J. *et al.* (1989) Neuropeptide expression in rat dorsal root ganglion cells and spinal cord after peripheral nerve injury with special reference to galanin. *Neuroscience* 33, 587-604
18. Holmes F. E. *et al.* (2000) Targeted disruption of the galanin gene reduces the number of sensory neurons and their regenerative capacity. *Proc Natl Acad Sci USA* 97, 11563-11568
19. Counts S. E. *et al.* (2001) Galanin: neurobiologic mechanisms and therapeutic potential for Alzheimer's disease. *CNS Drug Rev* 7, 445-470
20. Chan-Palay V. (1988) Galanin hyperinnervates surviving neurons of the human basal nucleus of Meynert in dementias of Alzheimer's and Parkinson's disease: a hypothesis for the role of galanin in accentuating cholinergic dysfunction in dementia. *J Comp Neurol* 273, 543-557
21. Chan-Palay V. (1998) Neurons with galanin innervate cholinergic cells in the human basal forebrain and galanin and acetylcholine coexist. *Brain Res Bull* 21, 465-472

22. Mufson E. J. *et al.* (1993) Galaninergetic innervation of the cholinergic vertical limb of the diagonal band (Ch2) and bed nucleus of the stria terminalis in aging, Alzheimer's disease and Down's syndrome. *Dementia* 4, 237-250
23. Mufson E. J. *et al.* (2000) Galanin receptor plasticity within the nucleus basalis in early and late Alzheimer's disease: an in vitro autoradiographic analysis. *Neuropharmacology* 39, 1404-1412
24. Rodriguez-Puertas R. *et al.* (1997) 125I-galanin binding sites in Alzheimer's disease: increases in hippocampal subfields and a decrease in the caudate nucleus. *J Neurochem* 68, 1106-1113
25. Mazarati A. *et al.* (2000) Modulation of hippocampal excitability and seizures by galanin. *J Neurosci* 20, 6276-6281
26. Steiner R. A. *et al.* (2001) Galanin transgenic mice display cognitive and neurochemical deficits characteristic of Alzheimer's disease. *Proc Natl Acad Sci U S A* 98, 4184-4189
27. Elvander E. *et al.* (2004) Intraseptal muscarinic ligands and galanin: influence on hippocampal acetylcholine and cognition. *Neuroscience* 126, 541-557
28. Lamour Y. *et al.* (1988) Neuropeptides and septohippocampal neurons: electrophysiological effects and distributions of immunoreactivity. *Peptides* 9, 1351-1359
29. Mazarati A. *et al.* (2004) Galanin type 2 receptors regulate neuronal survival, susceptibility to seizures and seizure-induced neurogenesis in the dentate gyrus. *Eur J Neurosci* 19, 3235-3244
30. Mazarati A. *et al.* (1992) Anticonvulsive effects of galanin administered into the central nervous system upon the picrotoxin-kindled seizure syndrome in rats. *Brain Res* 589, 164-166
31. Kokaia M. *et al.* (2001) Suppressed kindling epileptogenesis in mice with ectopic overexpression of galanin. *Proc Natl Acad Sci U S A* 98, 14006-14011
32. Mazarati A. *et al.* (1998) Galanin modulation of seizures and seizure modulation of hippocampal galanin in animal models of status epilepticus. *J Neurosci* 18, 10070-10077
33. Mazarati A. *et al.* (2004) Patterns of seizures, hippocampal injury and neurogenesis in three models of status epilepticus in galanin receptor type 1 (GalR1) knockout mice. *Neuroscience* 128, 431-441
34. Hökfelt T. *et al.* (1998) Galanin in ascending systems. Focus on coexistence with 5-hydroxytryptamine and noradrenaline. *Ann N Y Acad Sci* 863, 252-263
35. Xu Z. Q. *et al.* (1998) Galanin-5-hydroxytryptamine interactions: electrophysiological, immunohistochemical and in situ hybridization studies on rat dorsal raphe neurons with note on galanin R1 and R2 receptors. *Neuroscience* 87, 79-94
36. Seutin V. *et al.* (1989) Galanin decreases the activity of locus coeruleus neurons in vitro. *Eur J Pharmacol* 164, 373-376
37. Yoshitake T. *et al.* (2004) Enhanced hippocampal noradrenaline and serotonin release in galanin-overexpressing mice after repeated forced swimming test. *Proc Natl Acad Sci U S A* 101, 354-359
38. Lu X. *et al.* (2005) A role for galanin in antidepressant actions with a focus on the dorsal raphe nucleus. *PNAS* 102, 874-879
39. O'Meara G. *et al.* (2000) Galanin regulates the postnatal survival of a subset of basal forebrain cholinergic neurons. *Proc Natl Acad Sci U S A* 97, 11569-11574
40. Mahoney S. A. *et al.* (2003) The second galanin receptor GalR2 plays a key role in neurite outgrowth from adult sensory neurons. *J Neurosci* 23, 416-421
41. Anisimov S. V. *et al.* (2002) SAGE identification of gene transcripts with profiles unique to pluripotent mouse R1 embryonic stem cells. *Genomics* 79, 169-176
42. Crawley J. N. (1999) The role of galanin in feeding behavior. *Neuropeptides* 33, 369-375
43. Cobb M. H. (1999) MAP kinase pathways. *Prog Biophys Mol Biol* 71, 479-500
44. Lodish H. *et al.* Molecular cell biology, fourth edition, NYC (1999) W.H. Freeman and Company, 852-855, 878-894
45. Gudermann T. (2001) Multiple pathways of ERK activation by G protein coupled receptors. *Novartis Found Symp* 239, 68-79; 80-84; 150-159
46. Wang S. *et al.* Differential intracellular signaling of the GalR1 and GalR2 receptor subtypes. *Biochemistry* 37, 6711-6717
47. http://www.emdbiosciences.com/html/EMD/ip_erkmapk.html
48. Karl Loren website: Pain is a subjective experience
<http://www.painstudy.com/NonDrugRemedies/Pain/>
49. Dr. Ashok Kumar: Baylor College of Medicine, The role of mechanical stress in skeletal myocytes: MAPK SIGNAL TRANSDUCTION PATHWAYS, *Reviews in Undergraduate Research* - Issue 1

8. Appendix

Raw data and calculated ratios	ERK1 (p44) Peak Intensity	ERK1(p44) Average Intensity	ERK2 (p42) Peak Intensity	ERK2 (p42) Average Intensity
1-4 11/5				
Ctrl+TTX ppERK	80,11	52,59	254,56	148,48
Ctrl+TTX ERK	255	193,06	255	216,48
Ctrl+TTX (ppERK/ERK)	0,31	0,27	1	0,69
Normalized value	1	1	1	1
gal(1-29) +TTX ppERK	255	166,92	255	239,56
gal(1-29) +TTX ERK	232,78	159,04	218,33	159,59
gal(1-29) +TTX (ppERK/ERK)	1,1	1,05	1,17	1,5
Normalized value	3,55	3,89	1,17	2,17
M40+TTX ppERK	229,33	117	250,78	231,33
M40+TTX ERK	222,67	153,44	176,56	126,19
M40+TTX (ppERK/ERK)	1,03	0,76	1,42	1,83
Normalized value	3,32	2,82	1,42	2,65
gal(1-29)+M40+TTX ppERK	255	172,63	255	168,52
gal(1-29)+M40+TTX ERK	255	163,73	253,78	173,04
gal(1-29)+M40+TTX (ppERK/ERK)	1	1,05	1,01	0,97
Normalized value	3,23	3,89	1,01	1,41
1-8 11/01				
Ctrl ppERK	93,33	27,09	255	130,61
Ctrl ERK	219,22	110,58	254,33	120,31
Ctrl (ppERK/ERK)	0,43	0,24	1	1,09
Ctrl+TTX ppERK	28,67	14,27	182,56	97,94
Ctrl+TTX ERK	224,78	170,89	255	159,48
Ctrl+TTX (ppERK/ERK)	0,13	0,08	0,72	0,61
Normalized value	1	1	1	1
gal(1-29) +TTX ppERK	255	118,58	255	201,39
gal(1-29) +TTX ERK	251,78	80,3	253,11	105,16
gal(1-29) +TTX (ppERK/ERK)	1,01	1,48	1,01	1,92
Normalized value	7,77	18,5	1,4	3,15
gal(1-29) ppERK	14,78	10,04	182,11	78,22
gal(1-29) ERK	228	79,52	238,78	132,33
gal(1-29) (ppERK/ERK)	0,06	0,13	0,76	0,59
gal(1-29)+M40 ppERK	5,67	4,76	94,11	60,04
gal(1-29)+M40 ERK	203,56	103,96	168,89	122,14
gal(1-29)+M40 (ppERK/ERK)	0,03	0,05	0,56	0,49
M40 ppERK	25,33	14,64	246,67	146,53
M40 ERK	138,11	95,17	160,56	71,93
M40 (ppERK/ERK)	0,18	0,15	1,54	2,04
M40+TTX ppERK	46,33	19,87	203,89	137
M40+TTX ERK	189,89	96,06	234,89	142,11
M40+TTX (ppERK/ERK)	0,24	0,21	0,87	0,96
Normalized value	1,85	2,62	1,22	1,57
gal(1-29)+M40+TTX ppERK	55,44	18,81	152	60,69
gal(1-29)+M40+TTX ERK	205,11	134,22	198,67	127,31
gal(1-29)+M40+TTX (ppERK/ERK)	0,27	0,14	0,77	0,48
Normalized value	2,08	1,75	1,07	0,79
1-8 11/11				
Ctrl ppERK	26,67	19,53	204,22	90

Ctrl ERK	67,11	53,59	52,22	35,67
Ctrl (ppERK/ERK)	0,4	0,36	3,91	2,52
Normalized value	1	1	1	1
Ctrl+TTX ppERK	67,33	42,67	240	202,08
Ctrl+TTX ERK	181,33	114,28	95,11	61,89
Ctrl+TTX (ppERK/ERK)	0,37	0,37	2,52	3,26
Normalized value	1	1	1	1
gal(1-29) ppERK	228,44	214,26	241,89	240,22
gal(1-29) ERK	172,89	70,83	137,33	51
gal(1-29) (ppERK/ERK)	1,32	3,02	1,76	4,71
Normalized value	3,3	8,4	0,46	1,87
gal(1-29) +TTX ppERK	243,11	161,5	255	242,83
gal(1-29) +TTX ERK	63,44	26	254,44	70,71
gal(1-29) +TTX (ppERK/ERK)	3,83	6,2	1	3,43
Normalized value	10,35	16,76	0,4	1,06
M40 ppERK	62	39,5	242,11	238,7
M40 ERK	167,56	92,39	92,11	48,94
M40 (ppERK/ERK)	0,37	0,43	2,63	4,88
Normalized value	0,93	1,2	0,68	1,95
M40+TTX ppERK	66	39,06	251,33	187,58
M40+TTX ERK	138,33	70,09	121	83,04
M40+TTX (ppERK/ERK)	0,48	0,58	2,08	2,26
Normalized value	1,3	1,57	0,83	0,7
gal(1-29)+M40 ppERK	75,33	43,11	254,89	188,94
gal(1-29)+M40 ERK	154,44	97,25	93,89	50,52
gal(1-29)+M40 (ppERK/ERK)	0,49	0,44	2,72	3,74
Normalized value	1,23	1,22	0,71	1,5
gal(1-29)+M40+TTX ppERK	28,11	19,78	248,67	173,13
gal(1-29)+M40+TTX ERK	123,44	51,8	101,56	48
gal(1-29)+M40+TTX (ppERK/ERK)	0,23	0,38	2,45	3,61
Normalized value	0,62	1,03	0,98	1,12
1-5 11/19				
Ctrl+TTX ppERK	54,11	23,07	255	102,44
Ctrl+TTX ERK	190,56	114,04	242,56	126,17
Ctrl+TTX (ppERK/ERK)	0,28	0,2	1,05	0,81
Normalized value	1	1	1	1
gal(1-29) +TTX ppERK	109,33	41,4	253,78	237,93
gal(1-29) +TTX ERK	42,89	30,04	54	25,14
gal(1-29) +TTX (ppERK/ERK)	2,55	1,38	4,7	9,46
Normalized value	9,11	6,9	4,48	11,68
gal(2-11) +TTX ppERK	81,33	41,11	255	248,74
gal(2-11) +TTX ERK	254,22	149,04	253,56	162,22
gal(2-11) +TTX (ppERK/ERK)	0,32	0,28	1,01	1,53
Normalized value	1,14	1,4	0,96	1,88
gal(1-29)+M40+TTX ppERK	33,33	21,19	236,78	82,37
gal(1-29)+M40+TTX ERK	252,11	157,59	249,56	184,96
gal(1-29)+M40+TTX (ppERK/ERK)	0,13	0,13	0,95	0,45
Normalized value	0,46	0,65	0,9	0,55
gal(2-11)+M40+TTX ppERK	19,11	17,97	155,22	102,41
gal(2-11)+M40+TTX ERK	177,78	73,97	202,78	105,59
gal(2-11)+M40+TTX (ppERK/ERK)	0,11	0,24	0,77	0,97
Normalized value	0,39	1,2	0,73	1,19
1-7 11/19				

Ctrl ppERK	33,67	22,08	255	157,78
Ctrl ERK	164,22	64,4	238,56	110,42
Ctrl (ppERK/ERK)	0,21	0,34	1,07	1,43
Normalized value	1	1	1	1
gal(1-29) 1min. ppERK	30,11	19,53	253,11	139,72
gal(1-29) 1min. ERK	166,67	59,91	174	71,94
gal(1-29) 1min. (ppERK/ERK)	0,18	0,33	1,45	1,94
Normalized value	0,86	0,97	1,36	1,36
gal(1-29) 5min. ppERK	86,22	47,33	247,78	155,96
gal(1-29) 5min. ERK	242,56	122,56	249,11	135,41
gal(1-29) 5min. (ppERK/ERK)	0,36	0,39	0,99	1,15
Normalized value	1,71	1,15	0,92	0,81
gal(1-29) 10min. ppERK	169	45,82	255	246,53
gal(1-29) 10min. ERK	121,67	41,22	101,11	56,3
gal(1-29) 10min. (ppERK/ERK)	1,39	1,11	2,52	4,38
Normalized value	6,62	3,26	2,34	3,07
gal(1-29) 15min. ppERK	198,11	92,85	255	219,47
gal(1-29) 15min. ERK	167,56	105,67	143	57,87
gal(1-29) 15min. (ppERK/ERK)	1,18	0,88	1,78	3,8
Normalized value	5,62	2,59	1,66	2,67
gal(1-29) 30min. ppERK	106,67	29,22	255	191,89
gal(1-29) 30min. ERK	242,11	105,98	254,56	98,07
gal(1-29) 30min. (ppERK/ERK)	0,44	0,28	1	1,96
Normalized value	2,1	0,82	0,93	1,37
gal(1-29) 60min. ppERK	89,11	38,95	255	245,52
gal(1-29) 60min. ERK	179,44	74,27	205	76,31
gal(1-29) 60min. (ppERK/ERK)	0,5	0,52	1,24	3,22
Normalized value	2,38	1,53	1,15	2,25
1-6red 12/10				
Ctrl+TTX ppERK	147	62,63	159,44	80,64
Ctrl+TTX ERK	252,78	218,89	157,44	101,37
Ctrl+TTX (ppERK/ERK)	0,58	0,29	1,01	0,8
Normalized value	1	1	1	1
gal(1-29) +TTX ppERK	185,89	100,33	255	226,03
gal(1-29) +TTX ERK	209,56	101,93	226,67	109,44
gal(1-29) +TTX (ppERK/ERK)	0,89	0,98	1,12	2,07
Normalized value	1,53	3,38	1,11	2,59
gal(2-11) +TTX ppERK	94,67	53,17	253	240,82
gal(2-11) +TTX ERK	234,22	126	142,44	72,61
gal(2-11) +TTX (ppERK/ERK)	0,4	0,42	1,78	3,32
Normalized value	0,69	1,45	1,76	4,15
gal(1-29)+M40+TTX ppERK	51,67	40,15	248,33	237,37
gal(1-29)+M40+TTX ERK	195,44	133,7	163,67	78,89
gal(1-29)+M40+TTX (ppERK/ERK)	0,26	0,3	1,52	3
Normalized value	0,45	1,04	1,5	3,75
gal(2-11)+M40+TTX ppERK	89,78	44,47	249,11	239,78
gal(2-11)+M40+TTX ERK	221,56	145,41	231,67	141,44
gal(2-11)+M40+TTX (ppERK/ERK)	0,41	0,31	1,08	1,7
Normalized value	0,71	1,07	1,07	2,13
M40+TTX ppERK	65,22	39,4	234,56	205,64
M40+TTX ERK	120,33	90,33	186,67	104,56
M40+TTX (ppERK/ERK)	0,54	0,44	1,26	1,97
Normalized value	0,93	1,52	1,25	2,46

1-6blue 12/10				
Ctrl+TTX ppERK	211,31	105,82	244,69	184,28
Ctrl+TTX ERK	241,41	151,76	246,65	233,41
Ctrl+TTX (ppERK/ERK)	0,88	0,7	0,99	0,79
Normalized value	1	1	1	1
gal(1-29) +TTX ppERK	226,62	153,39	249,38	234,27
gal(1-29) +TTX ERK	220,96	129,54	242,14	176,55
gal(1-29) +TTX (ppERK/ERK)	1,03	1,18	1,03	1,33
Normalized value	1,17	1,69	1,04	1,69
gal(2-11) +TTX ppERK	208,89	173,79	245,48	209,4
gal(2-11) +TTX ERK	211,31	96,31	233,17	121,16
gal(2-11) +TTX (ppERK/ERK)	0,99	1,8	1,05	1,73
Normalized value	1,13	2,57	1,06	2,2
gal(1-29)+M40+TTX ppERK	168,76	94,59	244,65	171,31
gal(1-29)+M40+TTX ERK	224,41	133,37	227	143,14
gal(1-29)+M40+TTX (ppERK/ERK)	0,75	0,71	1,08	1,2
Normalized value	0,86	1,02	1,09	1,52
gal(2-11)+M40+TTX ppERK	121,79	84,11	247,55	211,69
gal(2-11)+M40+TTX ERK	110,28	80,19	107,11	65,8
gal(2-11)+M40+TTX (ppERK/ERK)	1,1	1,05	2,31	3,22
Normalized value	1,25	1,5	2,33	4,09
M40+TTX ppERK	73,55	54,66	242,45	153,58
M40+TTX ERK	73,55	49,38	198,69	105,73
M40+TTX (ppERK/ERK)	1	1,11	1,22	1,45
Normalized value	1,14	1,59	1,23	1,84
1-6 12/14 DRH				
Ctrl ppERK	19,72	14,82	193	92,03
Ctrl ERK	224,65	122,97	219,59	140,82
Ctrl (ppERK/ERK)	0,09	0,12	0,88	0,65
Normalized value	1	1	1	1
gal(1-29) ppERK	32,38	19,31	252,69	198,87
gal(1-29) ERK	236,35	129,36	243,17	179,64
gal(1-29) (ppERK/ERK)	0,14	0,15	1,04	1,11
Normalized value	1,56	1,25	1,19	1,71
gal(2-11) ppERK	35,38	24,85	242,07	164,83
gal(2-11) ERK	189,38	139,77	241,07	188,4
gal(2-11) (ppERK/ERK)	0,19	0,18	1	0,88
Normalized value	2,11	1,5	1,14	1,36
Ctrl+TTX ppERK	13,24	12,92	150,83	65,12
Ctrl+TTX ERK	229,76	118,86	230,48	116,46
Ctrl+TTX (ppERK/ERK)	0,06	0,11	0,65	0,56
Normalized value	1	1	1	1
gal(1-29) +TTX ppERK	20,72	17,75	223,72	161,72
gal(1-29) +TTX ERK	221,07	143,93	235,55	190,07
gal(1-29) +TTX (ppERK/ERK)	0,09	0,12	0,95	0,85
Normalized value	1,5	1,09	1,46	1,52
gal(2-11) +TTX ppERK	13,86	13,03	219,62	146,42
gal(2-11) +TTX ERK	238,83	232,76	241,76	228,63
gal(2-11) +TTX (ppERK/ERK)	0,06	0,06	0,91	0,64
Normalized value	1	0,55	1,4	1,15
1-6 12/20				
Ctrl ppERK	58,33	30,2	247,56	116,78
Ctrl ERK	120,89	68,07	236,78	113,48

Ctrl (ppERK/ERK)	0,48	0,44	1,05	1,03
Normalized value	1	1	1	1
gal(2-11) ppERK	42,33	22,53	255	219,89
gal(2-11) ERK	125,56	57,56	251,44	121,64
gal(2-11) (ppERK/ERK)	0,34	0,39	1,01	1,81
Normalized value	0,71	0,89	0,96	1,76
gal(2-11)+M40 ppERK	20,56	15,73	255	138,94
gal(2-11)+M40 ERK	132,11	65,22	187,11	88,36
gal(2-11)+M40 (ppERK/ERK)	0,16	0,24	1,36	1,57
Normalized value	0,33	0,54	1,29	1,52
Ctrl+TTX ppERK	28,67	17,89	253,56	164,59
Ctrl+TTX ERK	184,67	109,89	255	163,14
Ctrl+TTX (ppERK/ERK)	0,16	0,16	0,99	1,01
Normalized value	1	1	1	1
gal(2-11)+TTX ppERK	26	21,28	255	184,3
gal(2-11)+TTX ERK	204,33	139,15	223,67	97,44
gal(2-11)+TTX (ppERK/ERK)	0,13	0,15	1,14	1,89
Normalized value	0,81	0,94	1,15	1,87
gal(2-11)+M40+TTX ppERK	26,44	18,15	250,89	168,41
gal(2-11)+M40+TTX ERK	107,56	70	142,67	69,67
gal(2-11)+M40+TTX (ppERK/ERK)	0,25	0,26	1,76	2,42
Normalized value	1,57	1,63	1,78	2,4
1-8 12/20				
Ctrl ppERK	32,78	19,56	255	122,85
Ctrl ERK	228,11	151,72	220,56	94,48
Ctrl (ppERK/ERK)	0,14	0,13	1,16	1,3
Normalized value	1	1	1	1
gal(1-29) ppERK	87,89	45,14	253,67	170,33
gal(1-29) ERK	242,89	143,3	243,11	117,67
gal(1-29) (ppERK/ERK)	0,36	0,32	1,04	1,45
Normalized value	2,57	2,46	0,89	1,12
M40 ppERK	39,11	24,37	250,67	190,58
M40 ERK	249,11	157,75	214,56	83,81
M40 (ppERK/ERK)	0,16	0,15	1,17	2,27
Normalized value	1,14	1,15	1,01	1,75
gal(1-29)+M40 ppERK	19,89	15,97	221,22	100,71
gal(1-29)+M40 ERK	243,78	99,33	244,78	98,07
gal(1-29)+M40 (ppERK/ERK)	0,08	0,16	0,9	1,03
Normalized value	0,57	1,23	0,77	0,79
Ctrl+TTX ppERK	45,22	19,03	248,33	127,39
Ctrl+TTX ERK	254	181,14	169,78	93,89
Ctrl+TTX (ppERK/ERK)	0,18	0,11	1,46	1,36
Normalized value	1	1	1	1
gal(1-29)+TTX ppERK	14,22	13,78	197,67	120,74
gal(1-29)+TTX ERK	246,22	105,64	242,22	125,72
gal(1-29)+TTX (ppERK/ERK)	0,06	0,13	0,82	0,96
Normalized value	0,33	1,18	0,56	0,71
M40+TTX ppERK	37,67	21,02	238,67	114,08
M40+TTX ERK	225,11	201,06	205,89	130,07
M40+TTX (ppERK/ERK)	0,17	0,1	1,16	0,88
Normalized value	0,95	0,91	0,79	0,65
gal(1-29)+M40+TTX ppERK	207,11	85,11	240,56	191,52
gal(1-29)+M40+TTX ERK	213,11	144,71	149,44	52,69

gal(1-29)+M40+TTX (ppERK/ERK)	0,97	0,59	1,61	3,63
Normalized value	5,39	5,36	1,09	2,69
1-6 01/10				
Ctrl ppERK	21,11	16,22	192,96	119,62
Ctrl ERK	34,14	21,68	21,41	17,41
Ctrl (ppERK/ERK)	0,62	0,75	9,01	6,87
Normalized value	1	1	1	1
gal(2-11) 1 min. ppERK	65,31	41,53	229,14	190,49
gal(2-11) 1 min. ERK	148,72	86,22	177,51	146,73
gal(2-11) 1min. (ppERK/ERK)	0,44	0,48	1,29	1,3
Normalized value	0,71	0,64	0,14	0,2
gal(2-11) 5 min. ppERK	87,55	57,89	235,83	203,68
gal(2-11) 5 min. ERK	104,48	61,26	206,04	92,64
gal(2-11) 5min. (ppERK/ERK)	0,84	0,94	1,14	2,2
Normalized value	1,35	1,25	0,13	0,33
gal(2-11) 10 min. ppERK	178,96	107,28	246,24	206,94
gal(2-11) 10 min. ERK	147,41	89,39	159,65	74,32
gal(2-11) 10min. (ppERK/ERK)	1,21	1,2	1,54	2,78
Normalized value	1,95	1,6	0,17	0,42
gal(2-11) 15 min. ppERK	114,55	66,83	223,14	200,58
gal(2-11) 15 min. ERK	193,89	107,05	240,76	149,82
gal(2-11) 15min. (ppERK/ERK)	0,59	0,62	0,93	1,34
Normalized value	0,95	0,82	0,1	0,2
gal(2-11) 30 min. ppERK	103,11	56,05	179,11	147,84
gal(2-11) 30 min. ERK	135,41	74,34	153,45	65,58
gal(2-11) 30 min. (ppERK/ERK)	0,76	0,75	1,17	2,25
Normalized value	1,22	1	0,13	0,34

Table 6. Raw data from all western blots and calculated ratio between phospho Erk and total Erk. The ratios has been normalized to be able to compare the different blots with each other, letting control ratio always be 1.