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Activation of the galanin receptor 2 (GalR2) protects the hippocampus from neuronal damage

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Abstract

Expression of the neuropeptide galanin is up-regulated in many brain regions following nerve injury and in the basal forebrain of patients with Alzheimer's disease. We have previously demonstrated that galanin modulates hippocampal neuronal survival, although it was unclear which receptor subtype(s) mediates this effect. Here we report that the protective role played by galanin in hippocampal cultures is abolished in animals carrying a loss-of-function mutation in the second galanin receptor subtype (GalR2-MUT). Exogenous galanin stimulates the phosphorylation of the serine/threonine kinase Akt and extracellular signal-regulated kinase (ERK) in wild-type (WT) cultures by 435 ± 5% and 278 ± 2%, respectively. The glutamate-induced activation of Akt was abolished in cultures from galanin knockout animals, and was markedly attenuated in GalR2-MUT animals, compared with WT controls. In contrast, similar levels of glutamate-induced ERK activation were observed in both loss-of-function mutants, but were further increased in galanin over-expressing animals. Using specific inhibitors of either ERK or Akt confirms that a GalR2-dependent modulation in the activation of the Akt and ERK signalling pathways contributes to the protective effects of galanin. These findings imply that the rise in endogenous galanin observed either after brain injury or in various disease states is an adaptive response that reduces apoptosis by the activation of GalR2, and hence Akt and ERK.

Keywords

Akt; extracellular signal-regulated kinase; galanin; galanin receptor 2; hippocampus; neuroprotection

Expression of the neuropeptide galanin (Tatemoto *et al*. 1983) is markedly up-regulated in many brain regions following nerve injury (Cortes *et al*. 1990; Brecht *et al*. 1997). Furthermore, levels of the peptide are elevated after transient focal cerebral ischemia in the rat (Rao *et al*. 2002) and in the basal forebrain of patients with Alzheimer's disease (Chan-Palay 1988). These studies have led a number of investigators to hypothesize that galanin might play a trophic role (Elliott-Hunt *et al*. 2004; Ding *et al*. 2006) in addition to its previously defined neuromodulatory effects (Fisone *et al*. 1987; Pieribone *et al*. 1995). We have previously demonstrated that the neuropeptide acts as a survival and growth-promoting factor to subsets of neurons in the peripheral and central nervous systems (Holmes *et al*. 2000; O'Meara *et al*. 2000; Mahoney *et al*. 2003). More recently, we have demonstrated that galanin modulates hippocampal neuronal survival in a number of *in-vitro* models of excitotoxic injury (Elliott-Hunt *et al*. 2004), consistent with the previous work by Ben-Ari and colleagues (Zini *et al*. 1993). Exposure to either glutamate or staurosporine induced

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significantly more neuronal cell death in galanin knockout (GalKO) organotypic hippocampal cultures than in wild-type (WT) controls. Conversely, less cell death was observed in organotypic cultures from galanin over-expressing (GalOE) transgenic animals after exposure to staurosporine. Furthermore, exogenous galanin protected WT cultures from either glutamate- or staurosporine-induced cell death.

The receptor subtypes that mediate the protective effects of galanin in the central nervous system are unclear. To date, three galanin receptor subtypes have been cloned and designated galanin receptor 1 (GalR1) (Burgevin *et al.* 1995), galanin receptor 2 (GalR2; Fathi *et al.* 1997; Howard *et al.* 1997) and galanin receptor 3 (GalR3; Wang *et al.* 1997). An analysis of the localization of each galanin receptor subtype has been hindered by the lack of specific antisera that may be used for immunohistochemistry. *In-situ* hybridization studies have shown that GalR1 is mainly synthesized in the ventral Cornu Ammonis field-1 (CA1) CA1 and subiculum, but is neither synthesized in the dorsal fields nor in the dentate gyrus (DG) (O'Donnell.D *et al.* 1999). In contrast GalR2 is synthesized in the DG but not in the CA fields (O'Donnell.D *et al.* (1999), although the protein is transported to the terminal fields of the CA1 and Cornu Ammonius field-3 (CA3) and half of all galanin binding in the hippocampus is GalR2-dependent (Lu *et al.* 2005b). No GalR3 synthesis has been detected in either the hippocampus or the DG (Mennicken *et al.* 2002).

Here we report that GalR2 is the predominant receptor subtype that transduces the neuroprotective effects of galanin in the hippocampus. After excitotoxic neuronal injury, a GalR2-dependent increase in the activation of the Akt and extracellular signal-regulated kinase (ERK) signalling pathways contributes to these neuroprotective effects.

Materials and methods

Animals

All animals were fed standard chow and water *ad libitum*. Animal care and procedures were performed in accordance with the United Kingdom Home Office protocols and guidelines.

GalKO mice—Details of the strain and breeding history have been previously described (Wynick et al. 1998). In brief, mice homozygous for a targeted mutation in the galanin gene were generated using the E14 cell line. A PGK-Neo cassette in reverse orientation was used to replace exons 1–5, and the mutation was bred to homozygosity and has remained inbred on the 129OlaHsd strain. Age- and sex-matched WT littermates were used as controls in all experiments.

GalOE mice—Details of the strain and breeding history have been previously described (Bacon *et al.* 2002). In brief, GalOE mice, bred to homozygosity, were generated using a ~25-kb transgene containing the entire murine galanin coding region and 19.9 kb of the upstream sequence. The transgene was excised by restriction digest and microinjected into fertilized oocytes. The transgene has remained inbred on the CBA/B6 F1 hybrid background. Age- and sex-matched WT littermates were used as controls in all experiments.

GalR2-MUT mice—Details of the strain and breeding history have been previously described (Hobson *et al.* 2006). In brief, mice deficient for the *GalR2* gene were generated and licensed from Lexicon Genetics (The Woodlands, TX, USA). The 5.17-kb gene-trap vector VICTR48 (VIral Construct for TRapping) was inserted within the single intron of the murine *GalR2* gene in a 129Sv/EvBrd ES cell-line clone (Zambrowicz *et al.* 2003). An omnibank clone OST105469 was used to obtain germ-line transmission of the disrupted *GalR2* allele. Heterozygote pairs on the C57BL/6J × 129/SvEvBrd background were transferred to the University of Bristol and then bred to homozygosity, and have been

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maintained on that background. Age- and sex-matched WT littermates were used as controls in all experiments.

Organotypic hippocampal cultures

Organotypic cultures were prepared as previously described (Elliott-Hunt *et al.* 2002). In brief, the hippocampi from 5–6-day-old male pups were rapidly removed under a dissection microscope and sectioned transversely at 400 μm using a McIlwain tissue chopper (Mickle Laboratory Engineering, Guildford, Surrey, UK). $N = 5$ animals were used for each experiment. The slices were culturedin 95% air and 5% CO_2 at 37°C on a microporous transmembrane biopore membrane (Millipore, Poole, Dorset, UK), in a six-well plate in 50% minimal essential medium with Earle's Salts, without L-glutamine, 50% Hank's Balanced Salt Solution (Sigma Chemical Company Ltd, Poole, Dorset, UK), 25% heat-inactivated Horse Serum (Harlan Sera Laboratory, Loughborough, Leicestershire, UK), 5 mg/mL glucose (Sigma Chemical Company Ltd) and 1 mL glutamine (Gibco BRL, Paisley, UK).

Glutamate-induced hippocampal damage

Organotypic hippocampal cultures (14 day) from either WT or GalR2-MUT animals were placed in 0.1% bovine serum albumin (BSA) with serum-free media for 16 h before incubation for 3 h with glutamic acid (Sigma Chemical Company Ltd), either with or without the addition of the following chemicals: galanin peptide (Bachem, Weil am Rhein, Germany), AR-M1896 [Gal(2-11)Trp-Thr-Leu-Asn-Ser-Ala-Gly-Tyr-Leu-Leu-NH_2] (AstraZeneca, Montreal, Quebec, Canada), PD98059 (an ERK 1/2 inhibitor; Calbiochem, San Diego, CA, USA) or LY294002 [a phosphatidylinositol (PI3K) inhibitor; Calbiochem).

Cultures were then washed with serum-free medium and incubated for a further 24 h before imaging. Regional patterns of neuronal injury in the organotypic cultures were observed by performing experiments in the presence of propidium iodide. After membrane injury, the dye enters cells, binds to nucleic acids, and accumulates, rendering the cell brightly fluorescent. The CA1/CA3 and DG neuronal subfields were clearly visible in a bright-field image. The area encompassing the neuronal cell bodies of these regions was measured and neuronal damage was assessed using the density slice function in Scion IMAGE software (http://www.scioncorp.com) to establish the signal above the background. The area of the subfields expressing the exclusion dye propidium iodide was measured and expressed as a percentage of the total area of the subfields, as assessed in the bright-field image. Furthermore, for consistency in setting the parameters accurately when using the density slice function, the threshold was set against a positive control set of cultures exposed to 10 mM glutamate.

Western blotting

Organotypic hippocampal cultures (14 day) from WT, GalOE, GalKO or GalR2-MUT animals were placed in 0.1% BSA with serum-free media for 16 h before incubation with either glutamic acid (Sigma Chemical Company Ltd) or galanin peptide (Bachem) for up to 15 min. Cultures were then lysed in 100 μL sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) sample buffer containing 62.5 mM Tris-HCl (pH 6.8), 2% (w/v) SDS, 10% glycerol and 50 mM mercaptoethanol (Sigma Chemical Company Ltd). Extracts were heated to 95–100°C for 5 min and cooled on ice, then sonicated for 5 min before being subjected to centrifugation at 4000 rpm (1252 *g*) for 5 min to remove any insoluble material. Aliquots were taken for protein determination using a Non-Interfering Protein Assay kit (Genotech, Web Scientific Ltd, Crewe, UK). Samples were subjected to gel electrophoresis using discontinuous SDS-PAGE slab gels (12% running; 5% stacking) in 25 mM Tris buffer containing 250 mM glycine and 0.1% SDS. Molecular weight markers

dissolved in solubilization buffer were also electrophoresed. The resolved proteins were transferred to a nitrocellulose membrane using a wet transfer system (Biorad Trans Blot, Hemel Hempstead, Herts, UK) at 100 mA for 90 min. The membrane was then blocked with Tris-buffered saline (TBS) containing 5% non-fat dry milk and 0.1% Tween 20 at room temperature (22°C) for 1 h, and was subsequently incubated overnight at 4°C in primary antibody solutions for phospho-ERK 1 : 1000, total ERK 1 : 1000, phospho-Akt 1 : 1000 and total Akt 1 : 1000 (all antisera were obtained from Cell Signalling Technology, Hertfordshire, UK); each dissolved in TBS containing 5% non-fat dry milk, 0.1% Tween 20. Nitrocellulose membranes (Amersham, Buckinghamshire, UK) were washed three times with TBS-Tween 20 (0.1%), incubated with goat anti-rabbit antibody conjugated with horseradish peroxidase (1 : 2000 in blocking solution) (Cell Signaling Technology) for 1 h at room temperature. Following washing three times with TBS-Tween 20 (0.1%), immunoreactivity was revealed by an enhanced chemiluminescence kit (ECL; Cell Signaling Technology) and detected using X-ray films. The immunoblot films were scanned and the digitalized images analysed with Scion IMAGE software. Data are expressed as a percentage of phosphorylated protein, corrected for differences in loading assessed by the quantities of total ERK and Akt. Of note, a concentration of 6 mM glutamate was necessary to induce a robust up-regulation in phosphorylated Akt (pAkt) and ERK (pERK) in the animals on the CBA/B6 background (GalOE and WT controls), whereas a concentration of only 2 mM glutamate was needed to induce a similar degree of up-regulation in pAkt and pERK, in the transgenic animals on the 129 background (GalKO, GalR2-MUT and WT controls).

Statistical analysis

Data are presented as the means ± SEM. Non-parametric Mann–Whitney U *post-hoc* tests were used to analyse the difference between genotypes and different ligands and/or glutamate points. A level of significance of $p < 0.05$ was selected.

Results

Effects of exogenous galanin and Gal2-11 on glutamate-induced cell death in WT and GalR2-MUT organotypic hippocampal cultures

We first studied the neuroprotective effects of galanin and Gal2-11 on GalR2-MUT and WT organotypic hippocampal cultures (Fig. 1). Glutamate at 2 mM caused significantly greater levels of neurotoxicity in the GalR2-MUT than in WT cultures in the CA1 (45.6 ± 5.6 vs. 31 ± 7.7%, respectively) and CA3 (32.2 ± 5.3 vs. 10 ± 1.4%, respectively) regions of the hippocampus, similar to that previously observed in GalKO animals (Elliott-Hunt *et al.* 2004). The co-administration of ether galanin or Gal2-11 with glutamate provided significant neuroprotection in WT organotypic cultures (Fig. 1), consistent with our previous data and the study by Pirondi and colleagues (Elliott-Hunt *et al.* 2004; Pirondi *et al.* 2005). Conversely, the addition of either galanin or Gal2-11 had no protective effects in the GalR2-MUT cultures in the presence of glutamate (Fig. 1). There was no effect of either galanin or Gal2-11 on cell death in either the WT or GalR2-MUT organotypic hippocampal cultures in the absence of glutamate (data not shown). These findings imply that GalR2 is the principal subtype that transduces galanin-mediated hippocampal neuroprotection.

Galanin stimulates Akt and ERK activation

As GalR2 activation appears to be key to the neuroprotective role played by galanin in the hippocampus, we asked which down-stream intracellular signalling pathways might transduce these effects. Western blotting, run in triplicate, was used to study whether exogenous galanin could increase the phosphorylation of Akt and/or ERK in hippocampal organotypic cultures from WT animals. Results demonstrate that Akt and ERK are both

rapidly activated within 2 min by the addition of 1 μM galanin by 435 ± 5% and 278 ± 2%, respectively (Fig. 2).

Galanin modulation of glutamate-induced Akt and ERK activation

We then went on to look for differences in the levels of pAkt and pERK in hippocampal organotypic cultures treated with glutamate from GalKO, GalR2-MUT and GalOE animals, each compared with their respective strain-matched WT controls. Acute treatment with glutamate induced a potent increase in pAkt and pERK in hippocampal cultures from all three WT strains, run in triplicate (Figs 3 and 4). In GalKO cultures the glutamate-induced activation of Akt was abolished (Fig. 3, top panel), and markedly attenuated in GalR2-MUT animals (Fig. 3, middle panel), compared with strain-matched WT controls. The glutamate-induced increase in pAkt observed in GalOE cultures was equal to strain-matched WT controls (Fig. 3, bottom panel). In contrast, the glutamate-induced activation of ERK1 was similar in GalKO and GalR2-MUT animals compared with strain-matched WT controls (Fig. 4, top and middle panels), whereas the levels of pERK1 after treatment with glutamate was substantially elevated in GalOE cultures compared with strain-matched WT controls (Fig. 4, bottom panel).

Effect of inhibitors of Akt and ERK on galanin-mediated hippocampal neuroprotection

The above findings using GalKO and GalR2-MUT animals imply that the glutamate-induced rise in pAkt is galanin and GalR2 dependent, and the increase in hippocampal cell-death observed in the GalKO and GalR2-MUT animals may therefore be the consequence of a reduction in the activation of the Akt, rather than the ERK, signalling pathway. In contrast, high levels of endogenous galanin in the GalOE animals increases the levels of pERK, but not pAkt, beyond that observed with glutamate alone, and may thus explain the observed reduction in apoptotic cell death in the GalOE animals (Elliott-Hunt *et al.* 2004), despite unchanged levels of Akt activation. In order to further investigate the relative contributions of the Akt and ERK signalling pathways to the protective effects of galanin following the addition of glutamate, we used specific inhibitors of the PI3K (and hence Akt) and mitogen-activated protein kinase (MAPK, and hence ERK) pathways, using LY294002 (Vlahos *et al.* 1994) and PD98059 (Alessi *et al.* 1995), respectively. We reasoned that at maximal galanin stimulation (1 μM) both Akt and ERK should play major roles in the survival effects of galanin, whereas at lower concentrations of galanin (10 nM) the Akt pathways should predominate. Figure 5 demonstrates that both inhibitors increase the level of glutamate-induced cell death in the CA1 region of the hippocampus (similar findings were observed in the CA3 region, data not shown), consistent with previous reports (Mize *et al.* 2003; Zheng and Quirion 2004), and had no effect on cell death in the absence of glutamate (data not shown). Galanin at 1 μM and 10 nM significantly reduced the level of cell death induced by glutamate, although the degree of protection was greater at the higher dose of galanin, and was similar to that previously observed (Elliott-Hunt *et al.* 2004). In the presence of the Akt inhibitor, the neuroprotective effects of both concentrations of galanin were completely abrogated (Fig. 5). The protective effects of 1 μM galanin were also completely abrogated by the inhibition of ERK, whereas the inhibitor had no significant effect on neuroprotection at 10 nM galanin (Fig. 5).

Discussion

We have previously shown that both the transgenic manipulation of endogenous levels of galanin and the addition of exogenous galanin modulates the survival of hippocampal neurons under excitotoxic conditions, where apoptotic cell death occurs (Elliott-Hunt *et al.* 2004). We and others have shown that the galanin fragment Gal2-11 (also known as AR-M1896), which acts as an agonist with 500-fold selectivity for GalR2 compared with GalR1

(Liu *et al.* 2001), is equipotent to galanin in terms of its neuroprotective effects to the mouse and rat hippocampus, and the rat forebrain (Elliott-Hunt *et al.* 2004; Pirondi *et al.* 2005; Ding *et al.* 2006). More recently, Gal2-11 has also been shown to activate GalR3 *in vitro* with an affinity similar to GalR2 (Lu *et al.* 2005a).

We have now extended these findings using a recently characterized transgenic mouse with a loss-of-function mutation in GalR2 (Hobson *et al.* 2006) generated by the insertion of a gene-trap vector within the single intron of the murine *GalR2* gene. In these animals no change in galanin or GalR1 expression was detected either in the brain or in a range of other tissues in the GalR2-MUT mice, compared with WT controls. Here we demonstrate that the neuroprotective effects of galanin are abolished in the absence of a functional GalR2. As the activation of GalR1 and GalR3 by galanin, and GalR3 by Gal2-11, had no protective effects in the GalR2-MUT animals, these findings add further weight to our hypothesis that GalR2 is the principal subtype that transduces galanin-mediated hippocampal neuroprotection.

All three galanin receptor subtypes couple to $G_{i/o}$ and inhibit adenylyl cyclase (Habert Ortoli *et al.* 1994; Kolakowski *et al.* 1998; Wang *et al.* 1998), in addition GalR2 also signals via $G_{q/11}$ to activate phospholipase C (PLC) and protein kinase C (PKC) (Wang *et al.* 1998; Wittau *et al.* 2000). The down-stream signalling pathways that are activated by GalR2 and may mediate the protective effects of galanin have yet to be fully elucidated. However, previous work using Chinese hamster ovary (CHO) cell lines that stably express either GalR1 or GalR2 showed both receptors can activate MAPK 2–3-fold(Wang *et al.* 1998). Furthermore, studies on small-cell lung cancer clonal cell lines that express GalR2 and galanin demonstrated that GalR2 activation increases MAPK phosphorylation by 1.6-fold, in a PKC-dependent manner (Wang *et al.* 1998). Here we show that the addition of galanin rapidly and potently stimulates the levels of pAkt and pERK in WT hippocampal organotypic cultures. These findings are consistent with previous studies that have demonstrated that hippocampal protection after excitotoxic damage is dependent in part upon the activation of ERK (Ozawa *et al.* 1999; Maher 2001) and/or Akt (Culmsee *et al.* 2002; Gary *et al.* 2003), and is analogous to work by Almeida and colleagues (Almeida *et al.* 2005) who showed that brain-derived neurotrophic factor (BDNF) also plays a neuroprotective role in primary hippocampal neurons after excitotoxic damage, via the activation of Akt and ERK.

We next asked whether the transgenic manipulation of the endogenous levels of galanin or the abolition of GalR2 activation would result in changes in the levels of glutamate-induced pAkt and/or pERK. The glutamate-induced increase in pAkt, but not pERK, is abolished in the GalKO animals, implying that activation of the kinase is galanin dependent. Similarly, the glutamate-induced rise in pAkt in the GalR2-MUT animals is greatly attenuated, indicating that the effect is principally mediated via the activation of GalR2. Furthermore, it is possible that in the GalR2-MUT animals, galanin may activate GalR1 and/or GalR3 in the absence of a functional GalR2, which may contribute to the attenuation in pAkt levels. These data imply that the increased hippocampal cell death observed in the GalKO and GalR2-MUT animals may be the consequence of a reduction in the activation of the Akt, rather than the ERK, signalling pathway. In contrast, high levels of endogenous galanin (in the GalOE animals) increase the levels of pERK, but not pAkt, beyond that observed with glutamate alone. These findings would explain the observed reduction in apoptotic cell-death in the GalOE animals (Elliott-Hunt *et al.* 2004), despite unchanged levels of Akt activation. At present, which galanin receptor subtype(s) mediate(s) the increase in the levels of pERK observed in the GalOE animals is unkonwn. As stated above, either GalR1 or GalR2 can activate MAPK/ERK (Wang *et al.* 1998), and although we are unaware of any published data demonstrating that GalR3 directly activates ERK, this subtype may also play a role in the observed up-regulation of pERK in the GalOE mice.

Results using selective inhibitors for Akt and ERK are consistent with the above results. At low levels of galanin, Akt is the predominant signalling pathway that mediates the protective effects of the peptide, whereas at high levels of galanin (that occur after neuronal injury), both the PI3K/Akt and MAPK/ERK pathways contribute to the neuroprotective effects of galanin and GalR2 activation. There are at least two explanations for these findings: firstly, there may be cross-talk between these two signalling pathways, consistent with two previous studies that have shown such interactions in the hippocampus (Almeida *et al.* 2005) and in the 293T human embryonic kidney clonal cell line (Sato *et al.* 2004); secondly, there may be a common mechanism acting downstream of the two signalling pathways, e.g. stimulation of the serum response factor (SRF), which plays a role in cortical neuronal survival (Chang *et al.* 2004), and/or an induction in the expression of a number of anti-apoptotic genes (e.g. either *Bcl-2* or *Bcl-x*). Irrespective of whether one or both of these possibilities are correct, these data imply that a GalR2-specific agonist would have utility in various forms of brain injury or damage, either reducing or minimising neuronal cell death.

In summary, we have demonstrated that GalR2 is the principal receptor subtype that mediates the protective effects of galanin in the hippocampus. At low levels of the peptide, Akt would appear to underlie, at least in part, the protective effects of galanin. As endogenous levels of the peptide rise after neuronal injury, then both the Akt and ERK signalling pathways contribute to the neuroprotective effects of galanin, either by cross-talk and/or by a common down-stream mechanism. Our findings are consistent with the hypothesis that the rise in endogenous galanin expression observed in various brain injury or disease states, is an adaptive response that reduces apoptosis by the activation of GalR2, and hence Akt and ERK. As further novel galanin receptor-specific ligands (e.g. the recently published GalR3-antagonists; Swanson *et al.* 2005) become available, they too should be tested in models of excitotoxic brain injury.

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Abbreviations used

BSA	bovine serum albumin
DG	dentate gyrus
ERK	extracellular signal-regulated kinase
GalKO	galanin knockout
GalOE	galanin over-expressing
GalR2	galanin receptor 2
GalR2-MUT	loss-of-function mutation in GalR2
MAPK	mitogen-activated protein kinase
PI3K	phosphatidylinositol
PKC	protein kinase C
SDS-PAGE	sodium dodecyl sulfate-polyacrylamide gel electrophoresis
TBS	Tris-buffered saline
WT	wild-type

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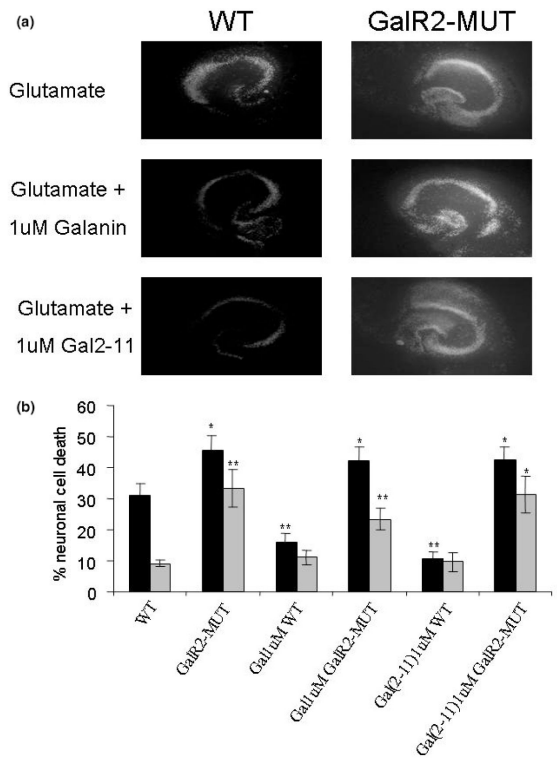

Fig. 1.

Effect of treatment with 2 mM glutamate on GalR2-MUT (loss-of-function mutation in the galanin receptor 2) and wild-type (WT) hippocampal organotypic cultures, in either the presence or the absence of 1 μM of either galanin or Gal2-11. Cultures were then placed in serum-free media for a 24-h recovery period and imaged to measure the percentage of cell death. Results and representative images demonstrate significantly greater cell death in the CA1 and CA3 regions of the hippocampus in GalR2-MUT than WT cultures. The protective effects of either galanin or Gal2-11 are abolished in GalR2-MUT cultures. Bars represent mean ± SEM, $n = 3$. *Significant difference ($p < 0.05$) from WT glutamate-treated cultures, **significant difference ($p < 0.01$) from WT glutamate-treated cultures.

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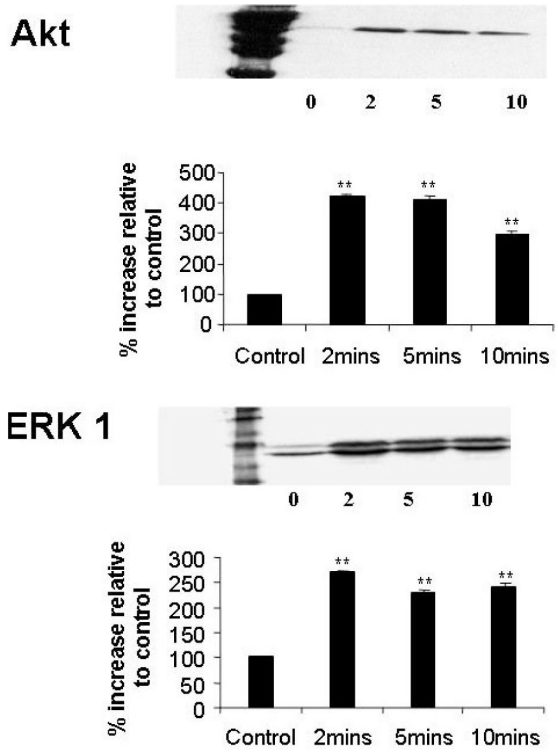

Fig. 2.

Effect of acute treatment with 1 μM galanin on the percentage of phosphorylated Akt and extracellular signal-regulated kinase (ERK) in wild-type (WT) hippocampal organotypic cultures for the indicated time points. Histograms represent quantitative western blot analysis of phosphorylated Akt (top) and ERK1 (bottom). Data are expressed as a ratio of the normalized percentages of ether phospho- and total-Akt or ERK. Representative blots for each phospho-protein are shown above each histogram. Results demonstrate a robust stimulation of pAkt and pERK1 within 2 min of the addition of 1 μM galanin. Bars represent the mean ± SEM, $n = 3$. *Significantly different ($p < 0.05$) from control cultures; **significantly different ($p < 0.01$) from control cultures.

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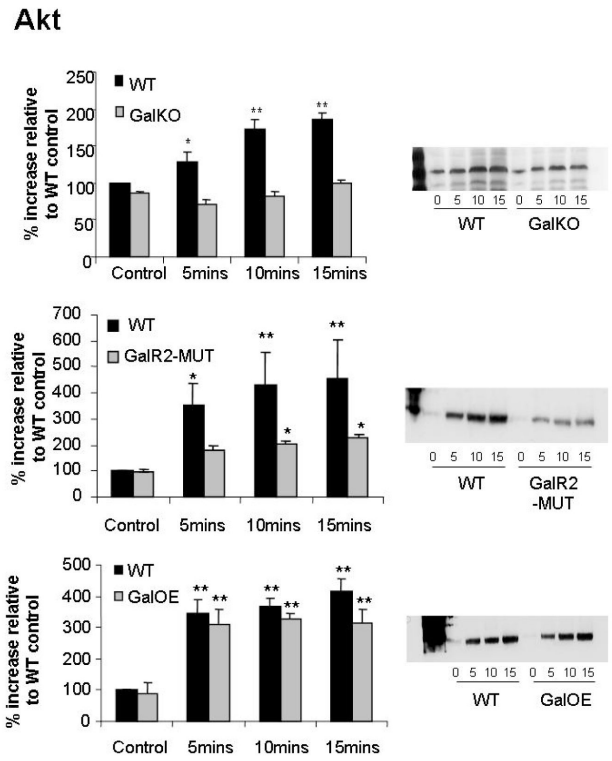

Fig. 3.

Modulation of Akt activation in response to 2 m_M glutamate toxicity in galanin knockout (GalKO) and GalR2-MUT (mutation in the galanin receptor 2) hippocampal organotypic cultures, and 6 m_M glutamate toxicity in galanin over-expressing (GalOE) hippocampal organotypic cultures, for the indicated time points, each compared with their respective strain-matched wild-type (WT) controls. Histograms represent quantitative western blot analysis of phosphorylated Akt in GalKO and WT cultures (top left), GalR2-MUT and WT cultures (middle left), and GalOE and WT cultures (bottom left). Data are expressed as a ratio of the normalized percentages of phospho- and total-Akt. Representative blots for each genotype are shown on the right. Results demonstrate a significant stimulation of pAkt within 5 min of the addition of glutamate in all three WT strains. The increase in pAkt levels is abolished in the GalKO cultures, greatly attenuated in the GalR2-MUT cultures, and is unchanged in the GalOE cultures (compared with the WT cultures treated with glutamate). Bars represent the mean ± SEM, $n = 3$. *Significantly different ($p < 0.05$) from WT control cultures; **Significantly different ($p < 0.01$) from WT control cultures.

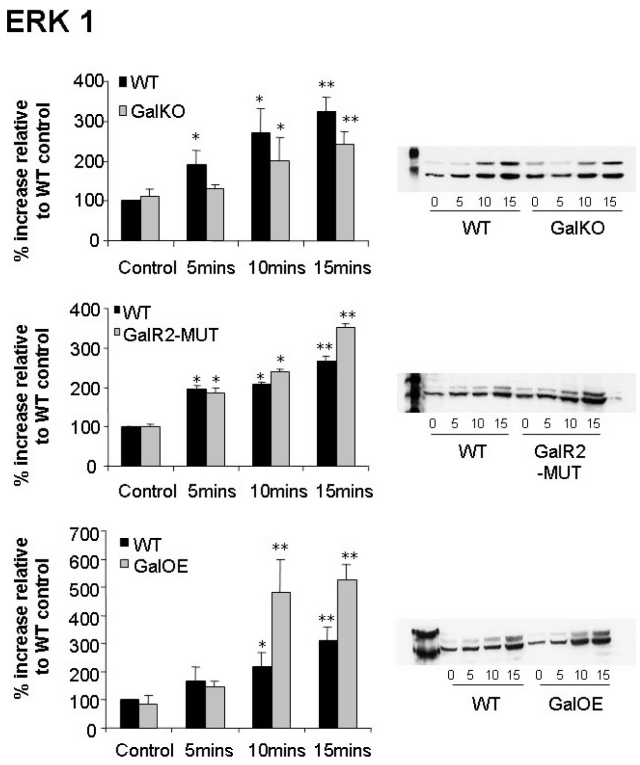

Fig. 4.

Modulation of extracellular signal-regulated kinase 1 (ERK1) activation in response to 2 mM glutamate toxicity in galanin knockout (GalKO) and GalR2-MUT (mutation in the galanin receptor 2) hippocampal organotypic cultures, and 6 mM glutamate toxicity in galanin over-expressing (GalOE) hippocampal organotypic cultures, for the indicated time points, each compared with their respective strain-matched wild-type (WT) controls. Histograms represent quantitative western blot analysis of phosphorylated ERK1 in GalKO and WT cultures (top left), GalR2-MUT and WT cultures (middle left), and GalOE and WT cultures (bottom left). Data are expressed as a ratio of the normalized percentages of phospho- and total-ERK1. Representative blots for each genotype are shown on the right. Results demonstrate a significant stimulation of pERK1 within 10 min of the addition of glutamate in all three WT strains. The increase in pERK1 levels was not statistically different in the GalKO and GalR2-MUT cultures, compared with the WT cultures treated with glutamate. A significantly greater increase was observed in the GalOE cultures, 10 and 15 min after the addition of glutamate, compared with the WT cultures. Bars represent the mean ± SEM, $n = 3$. *Significantly different ($p < 0.05$) from WT control cultures, **significantly different ($p < 0.01$) from WT control cultures.

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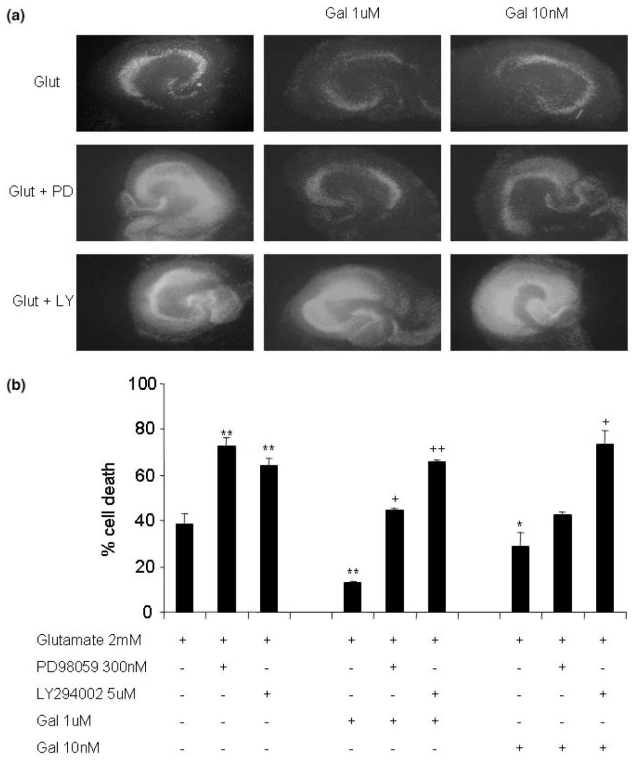

Fig. 5.

Effect of treatment with 2 mM glutamate on wild-type (WT) hippocampal organotypic cultures in either the presence or the absence of 1 μM or 10 nM galanin, and/or 5 μM LY294009, and/or 300 nM PD98059. Cultures were then placed in serum-free media for a 24-h recovery period and imaged to measure the percentage of cell death in the CA1 region (solid bars) of the hippocampus. Results and representative images demonstrate an increase in cell death with either inhibitor after glutamate treatment. The protective effects of 1 μM galanin are abrogated with inhibitors of either the ERK or the Akt signalling pathways, whereas the protective effects of 10 nM galanin is abrogated by the Akt, but not the ERK, inhibitor. Bars represent mean ± SEM, $n = 3$. *Significantly different ($p < 0.05$) from glutamate-treated cultures; **significantly different ($p < 0.01$) from glutamate-treated cultures, $^{+}$significantly different ($p < 0.05$) from galanin- and glutamate-treated cultures, $^{++}$significantly different ($p < 0.01$) from galanin- and glutamate-treated cultures.