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Endogenous Galanin Protects Mouse Hippocampal Neurons Against Amyloid Toxicity *in vitro* via Activation of Galanin Receptor-2

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Abstract

Expression of the neuropeptide galanin is known to be upregulated in the brain of patients with Alzheimer's disease (AD). We and others have shown that galanin plays a neuroprotective role in a number of excitotoxic injury paradigms, mediated by activation of the second galanin receptor subtype (GAL_2). In the present study, we investigated whether galanin/GAL2 plays a similar protective role against amyloid-β(Aβ) toxicity. Here we report that galanin or the $GAL_{2/3}$-specific peptide agonist Gal2-11, both equally protect primary dispersed mouse wildtype (WT) neonatal hippocampal neurons from 250 nM $A\beta_{1–42}$ toxicity in a dose dependent manner. The amount of $A\beta_{1–42}$ induced cell death was significantly greater in mice with loss-of-function mutations in galanin (Gal-KO) or GAL_2 (GAL_2-MUT) compared to strain-matched WT controls. Conversely, cell death was significantly reduced in galanin over-expressing (Gal-OE) transgenic mice compared to strain-matched WT controls. Exogenous galanin or Gal2-11 rescued the deficits in the Gal-KO but not the GAL2-MUT cultures, confirming that the protective effects of endogenous or exogenous galanin are mediated by activation of GAL_2. Despite the high levels of endogenous galanin in the Gal-OE cultures, the addition of exogenous 100 nM or 50 nM galanin or 100 nM Gal2-11 further significantly reduced cell death, implying that GAL_2-mediated neuroprotection is not at maximum in the Gal-OE mice. These data further support the hypothesis that galanin over-expression in AD is a neuroprotective response and imply that the development of a drug-like GAL_2 agonist might reduce the progression of symptoms in patients with AD.

Keywords

Alzheimer's disease; amyloid toxicity; galanin; GAL_2; neuroprotection; transgenic models

INTRODUCTION

The pathological hallmarks of AD are the presence of neurofibrillary tangles and senile plaques. The amyloid-β (Aβ) peptide, a predominantly 40–42 amino-acid fragment of the

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amyloid-β protein precursor (AβPP) derived from the cleavage of AβPP by β- and γ-secretases [1], is the major component of neuritic plaques [2]. Aβ deposition triggers a long-term neuropathological cascade, which includes neuronal loss and markedly distorted axons and dendrites, which lead to dendritic regression and spine loss [3, 4]. Dystrophic neurites are typically associated with senile plaques and both are correlated with cognitive deficits [5]. Neurites contain a large number of neurotransmitter and neuromodulatory substances, which are for the most part, reduced in the AD brain [6]. By contrast, the expression of the neuropeptide galanin [7, 8] is increased in AD [9, 10]. In AD, thickened galanin-immunoreactive fibres hyper-innervate surviving cholinergic basal forebrain (CBF) neurons, the locus coeruleus, as well as cortical and hippocampal projection neurons [9-12]. Further, galanin peptide levels as well as galanin receptor binding sites are increased in the neocortex, hippocampus, amygdala, and basal forebrain in the AD brain [13-17]. Studies using mice transgenic for AβPP bearing familial AD-related gene mutations exhibit age-dependent increases in hippocampal galanin neurite formation in close opposition to amyloid containing plaques [18, 19]. These hypertrophied galaninergic fibers are thickened with bulbous endings [18], very similar to that observed in AD.

The above findings have led to a number of functional studies addressing the role played by galanin in the modulation of acetylcholine (ACh) release, learning, and memory. The injection of galanin into the rat ventral hippocampus inhibits scopolamine-stimulated ACh release and impairs spatial learning acquisition in the water maze [20, 21]. Conversely, application of the peptide into the dorsal hippocampus stimulates the release of ACh and facilitates spatial learning [22, 23]. These site-specific effects are mirrored by the findings that galanin binding sites are present in both the ventral and dorsal hippocampus, but with five-fold higher concentrations in the ventral hippocampus [24]. Transgenic mice with ectopic over-expression of galanin localized to the adrenergic neurons (using the DBH promoter) have deficits in spatial learning and acquisition in the water maze [25], while mice with more widespread ectopic galanin over-expression (using the PDGF promoter) have normal learning and memory retention in the water maze task, compared to wild-type (WT) controls [26]. These studies have led some investigators to postulate that the up-regulation of galanin in AD might be detrimental and would further impair cognition [20, 25]. In contrast, it has also been hypothesized that amyloid-induced galanin fiber over-expression in AD plays a trophic and neuroprotective role to minimize the impact of amyloid toxicity upon neuronal function and cell survival.

To further study the role of galanin in neuronal survival and growth, we have generated transgenic mice bearing loss- or gain-of-function mutations in the galanin gene [27, 28], and demonstrated that the neuropeptide acts as a survival factor to subsets of neurons in the dorsal root ganglia (DRG) and CBF [29, 30]. Further, the peptide is a trophic factor to adult sensory neurons, which are dependent upon galanin for neurite extension after injury [31], mediated by activation of the second galanin receptor subtype (GAL_2) [32]. We have also demonstrated that galanin and Gal2-11 (which has 500-fold selectivity for GAL_2 and GAL_3 compared to GAL_1 [33, 34]) both play a neuroprotective role in the central nervous system (CNS) by reducing cell death in *in vivo* and *in vitro* hippocampal models of excitotoxicity [35]. We have extended these findings using the above injury paradigms of neuronal injury by applying them to the Gal-KO, GAL_2-MUT, and Gal-OE mice. Collectively, these data demonstrate that the neuroprotective role played by galanin in the hippocampus is mediated by activation of GAL_2, and is abolished in GAL_2-MUT mice [36]. Of note, there is no evidence to date that the developmental cell survival role played by galanin in the basal forebrain alters or modulates the neuroprotective role played by galanin/GalR2 in the adult after neuronal injury.

Most recently, several studies have demonstrated that the addition of exogenous galanin or Gal2-11 is neuroprotective against Aβ toxicity in primary rat hippocampal [37] or cholinergic [38] cultures, human fetal brain cultures [39], and in the SN56 cholinergic cell line [40], and maintains or increases the expression of cell survival genes in the AD basal forebrain [41]. Here we show by modulation of endogenous levels of galanin or GAL_2 in transgenic mice, that the neuropeptide has a significant neuroprotective role against Aβ toxicity via activation of GAL_2. These data further support the hypothesis that galanin over-expression in AD is a neuroprotective response and imply that the development of a drug-like GAL_2 agonist might reduce the progression of symptoms in patients with this disease.

MATERIALS AND METHODS

Animals

All animals were fed standard chow and water ad libitum and animal care and procedures were performed within the United Kingdom Home Office protocols and guidelines.

Galanin over-expressing (Gal-OE) mice

Details of the strain and breeding history are as described [27, 42]. In brief, galanin over expressing mice, bred to homozygosity, were generated using a ~25 kb transgene containing the entire murine galanin coding region and 19.9 kb of upstream sequence. The transgene was excised by restriction digest and microinjected into fertilized oocytes. The transgenic line denoted OE2 was then bred and characterized (see Bacon et al. for further details [42]). The line has remained inbred on the CBA × C57BL6 (CBA/Bl6) F1 hybrid background. WT mice that were strain-, age-, and gender-matched were used as controls in all experiments.

Galanin knockout (Gal-KO) mice

Details of the strain and breeding history are as described [28]. 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. Strain-, age-, and gender-matched WT mice were used as controls in all experiments.

GAL_2 mutant (GAL_2-MUT) mice

Details of the strain and breeding history are as described [32]. In brief, mice deficient for the *GALR2* gene were generated and licensed from Lexicon Genetics. The 5.17 kb gene-trap vector VICTR48 (VIral Construct for TRapping) was inserted within the single intron of the murine GAL2 gene in a 129 Sv/EvBrd ES cell line clone [43]. Omnibank clone OST105469 was used to obtain germ-line transmission of the disrupted *GALR2* allele. Heterozygote pairs on the C57BL6 × 129SvEvBrd (Bl6/129 Sv) background were transferred to the University of Bristol and then bred to homozygosity and have been maintained on that background. Strain-, age-, and gender-matched WT mice were used as controls in all experiments.

Preparation of primary neuronal cultures

Hippocampi from 2- to 3-day-old mouse pups were dissected and placed into 4°C collection buffer prepared with Hanks' balanced salt solution (calcium and magnesium free) (GIBCO/BRL), 10% 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid (HEPES, ICN), 10 μg/ml Penstrep (GIBCO/BRL), and 0.5% Bovine Serum Albumin (ICN). Enzymatic digestion, isolation, and culture of hippocampal neurons were performed as previously described [35], with the exception of the substitution of 0.83 U/ml Dispase (Sigma) instead of Trypsin. To inhibit glial cell growth, 5′Fluoro 2′ Deoxyuridine (Sigma), was added (10 μg/ml) to the

cultures after 24 h. Cells were counted and plated at 40,000 cells/well onto DL-polyornithine-coated (Costar) black-wall 96-well plates (Corning, Arlington, UK). Cells were placed in Minimal Essential Media (GIBCO/BRL), with 5% Fetal Bovine Serum (GIBCO/BRL), 5% Horse Serum (GIBCO/BRL), 2 mM L-Glutamine (GIBCO/BRL), 5 μg/ml Insulin (Sigma), 10 ug/ml Penstrep and 5 mM HEPES for 2 h before media was changed to Neurobasal media, as described [35]. Cultures were incubated at 37°C with ambient oxygen and 5% CO_2 for 5 days before experimentation and the media was changed on the fourth days.

Preparation of fibrillar Aβ (fA$\beta_{1–42}$)

A$\beta_{1–42}$ (American Peptide Company Inc., USA) was dissolved in dimethyl sulfoxide (DMSO, Sigma) and then diluted to a concentration of 1 mM in phosphate buffered saline (PBS, Sigma) and incubated at 22°C or 37°C for 24 or 48 h. Electron microscopy was then undertaken at the University of Bristol central imaging facility. A 5 μl drop of the A$\beta_{1–42}$ suspension was placed on a formvar and carbon coated copper grid and left to dry for 5 min. Excess fluid was removed using a filter paper and the grid was placed on top of a 3% Uranyl acetate solution in water. After 1 min excess fluid was removed using filter paper and air dried. The sample was examined using a FEI Tecnai12 Biotwin equipped with a 4*4 k bottom-mounted EAGLE CCD camera. Results demonstrated that incubating the peptide at 37°C for 48 h resulted in material that displayed a predominantly fibrillar form (Supplementary Fig. 1; available online: http://www.j-alz.com/issues/25/vol25-3.html#supplementarydata01). That protocol was then used to generate fA$\beta_{1–42}$ for all future experiments.

Treatments

Dispersed primary hippocampal cultures were cultured with or without the addition of the following: fA$\beta_{1–42}$, L-glutamic acid (Sigma), galanin peptide (Bachem), or Gal2-11 (Astra-Zeneca, Montreal).

A$\beta_{1–42}$ Hippocampal toxicity

Dispersed primary hippocampal cultures were exposed to concentrations varying from 1 μM to 500 μM fA$\beta_{1–42}$ for 48 h or glutamic acid for 3 h with or without the addition of varying concentrations of galanin or Gal2-11. Neuronal injury was measured by the presence of propidium iodide. After membrane injury, the dye enters cells, binds to nucleic acids, and accumulates, rendering the cell brightly fluorescent. The viability of neurons was measured by counting of both live and dead neurons using high content microscopy and analysis using the IN Cell Analyser 1000 microscope as previously described [44, 45]. Image acquisition and quantification of fluorescence intensity and localization was performed using IN Cell Analyzer Workstation 3.5 software (IN Cell Investigator, GE Healthcare, Amersham, UK). Red channel (propidium iodide) and blue channel using 4′,6-diamidino-2-phenylindole (DAPI) images were used to define whole-cell and nuclear regions, respectively. Images were acquired with two single fields of view (0.6 mm^2) and a 10 × objective. A total of 300–500 cells per field were typically analyzed, and up to two fields per well were captured in experiments performed in triplicate, meaning that in each experiment, data were normally derived from at least 1000 individual cells per treatment.

Statistical analysis

Data are presented as the mean±SEM. Student *T*-test or one-way ANOVA with appropriate post-hoc comparison tests were used to analyze difference between genotypes and the different ligands. Level of significance was set at $p < 0.05$.

RESULTS

Exogenous galanin and Gal2-11 protect WT hippocampal cultures from fA$\beta_{1–42}$-induced cell death

We first studied fA$\beta_{1–42}$-induced cell death in dispersed neonatal hippocampal cultures from the three WT strains. In all strains 48 h exposure to fA$\beta_{1–42}$-induced a concentration-dependent increase in cell death with little or no consistent cell death at less than 25 nM, whereas at concentrations above 500 nM virtually all cells died and detached from the culture plate and therefore, could not be accurately counted (data not shown). 250 nM fA$\beta_{1–42}$ reproducibly induced cell death in all three WT strains (varying between 31.6% and 36.5%, Figs. 1 and 2 and Supplementary Fig. 2; available online: http://www.j-alz.com/issues/25/vol25-3.html#supplementarydata01) and this concentration was used for all future experiments. As a positive control 5–6 mM glutamate was used to induce a similar amount of apoptosis (varying between 28.9% and 34.9%) to that observed with 250 nM fA$\beta_{1–42}$ (Figs. 1 and 2).

Galanin and the $GAL_{2/3}$ peptide agonist Gal2-11 both demonstrated similar degrees of significant dose-dependent neuroprotection in dispersed hippocampal neurons from all three wildtype strains when exposed to 250 nM fA$\beta_{1–42}$ (Figs. 1 and 2 and Supplementary Fig. 2). Maximum neuroprotection was obtained with 100 nM galanin varying between 52% and 81% and 51% and 84% for 100 nM Gal2-11. Similarly, 100 nM galanin was neuroprotective after exposure to glutamate, and significantly reduced cell death between 51% and 76%, similar to our previously findings [35, 36]. Treatment with galanin or Gal2-11 alone had no effect on cell death in any of the WT strains (Figs. 1 and 2). Of note, galanin and Gal2-11 both demonstrated greater neuroprotection in the Bl6/129 Sv mice, especially at lower concentrations, than in the two other strains studied (Figs. 1 and 2).

Transgenic manipulation of endogenous galanin and GAL_2 levels modulate fA$\beta_{1–42}$-induced hippocampal cell death and responses to exogenous galanin and Gal2-11

Cell death in Gal-KO and GAL_2-MUT mice after exposure to fA$\beta_{1–42}$ was significantly increased by 22% and 23% respectively, and by 23% and 38% respectively with glutamate, when compared to strainmatched WT controls (Fig. 1). Conversely, cell death was significantly reduced in Gal-OE mice by 51% and 38% after exposure to fA$\beta_{1–42}$ or glutamate, respectively when compared to strain-matched WT controls (Fig. 2).

Exogenous galanin and Gal2-11 both fully and equally rescued the above deficits in the Gal-KO mice after exposure to fA$\beta_{1–42}$ or glutamate (Fig. 1A). In contrast, neither peptide had any effect in the GAL_2-MUT cultures (Fig. 1B). Despite the high levels of endogenous galanin in the Gal-OE cultures and the significant reduction in cell death, the addition of either exogenous 100 nM or 50 nM galanin or 100 nM Gal2-11 further significantly reduced cell death in these mice (Fig. 2).

DISCUSSION

We and others have shown that transgenic manipulation of the endogenous levels of galanin or GAL_2, or the addition of exogenous galanin or Gal2-11 modulate the survival of cultured hippocampal neurons under excitotoxic conditions where apoptotic cell death occurs [35, 36, 46]. More recently these studies have been extended to Aβ-induced neuronal apoptosis. Treatment of cultured human embryonic cortical neurons with galanin significantly inhibited Aβ-induced cell death [39]. Similarly, galanin and Gal2-11 both significantly reduced Aβ toxicity in primary rat hippocampal [37] or cholinergic [38] neuronal cultures, and in cholinergic septal neuron 56 cells (a hybrid of mouse septal neurons and N18TG2 neuroblastoma cells) [40].

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In the present study, we provide additional data demonstrating that exogenous galanin or Gal2-11 both provide significant neuroprotection against Aβ or glutamate toxicity in three different WT strains, confirming the above data sets. Moreover, we have now extended these findings using a panel of previously characterized transgenic mice with loss-of-function mutations in galanin or GAL_2 and over-expression of galanin in the CNS using the previously described 20 kb galanin promoter region [35]. Aβ-induced cell death is significantly increased in Gal-KO and GAL_2-MUT mice and reduced in Gal-OE transgenic mice, each compared to strain-matched WT controls. Importantly, exogenous galanin or Gal2-11 fully rescue the deficits in the Gal-KO but have no effect in the GAL_2-MUT mice lacking a functional GAL_2. Since activation of GAL_1 and GAL_3 by galanin, and GAL_3 by Gal2-11 had no protective effects in the GAL_2-MUT mice, these findings add further weight to the hypothesis that the galanin over-expression seen in AD is a neuroprotective response, mediated by activation of GAL_2. We are currently extending these *in vitro* findings into an *in vivo* model of Aβ-induced neuronal dysfunction by crossing the Gal-OE mice to an AβPP/PS1 expressing line and then studying whether the over-expression of galanin modulates the previously described cognitive deficits in that line.

The intracellular signaling pathways that mediate the neuroprotective effects of GAL_2 activation have yet to be fully defined. GAL_2 couples to both $G_{i/o}$ and inhibits adenylyl cyclase [47] and also signals via $G_{q/11}$ to activate phospholipase C (PLC) and protein kinase C (PKC) [47, 48]. Our previous studies using cultured hippocampal or sensory neurons have shown that the addition of galanin rapidly and potently stimulates phosphorylation of the serine/threonine kinase Akt and extracellular signal-regulated kinases (ERK) [32, 36]. These findings are also consistent with previous publications that hippocampal protection after excitotoxic damage is dependent in part upon activation of ERK [49, 50] and/or Akt [51, 52]. Recent data demonstrates that the neuroprotective effects of galanin against Aβ-induced apoptosis, are mediated by: (a) reversal of the Aβ-induced reduction in pERK and pAkt [38], (b) down-regulation of Bax levels [37, 39], and (c) attenuation in the cleavage of caspase 3 [37, 38, 40].

Irrespective of which signaling pathways mediate the neuroprotective effects of GAL_2 activation, our findings that the addition of exogenous galanin or Gal2-11 in Gal-OE mice further reduces apoptosis, is of considerable interest in the context of the raised levels of galanin reported in AD [41]. This result implies that GAL_2-mediated neuroprotection is not yet at maximum in situations where endogenous galanin expression is raised. This further supports the premise that the development of drug-like GAL_2 agonists or positive allosteric modulators (as recently described [53]) might reduce the progression of symptoms in patients with AD.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

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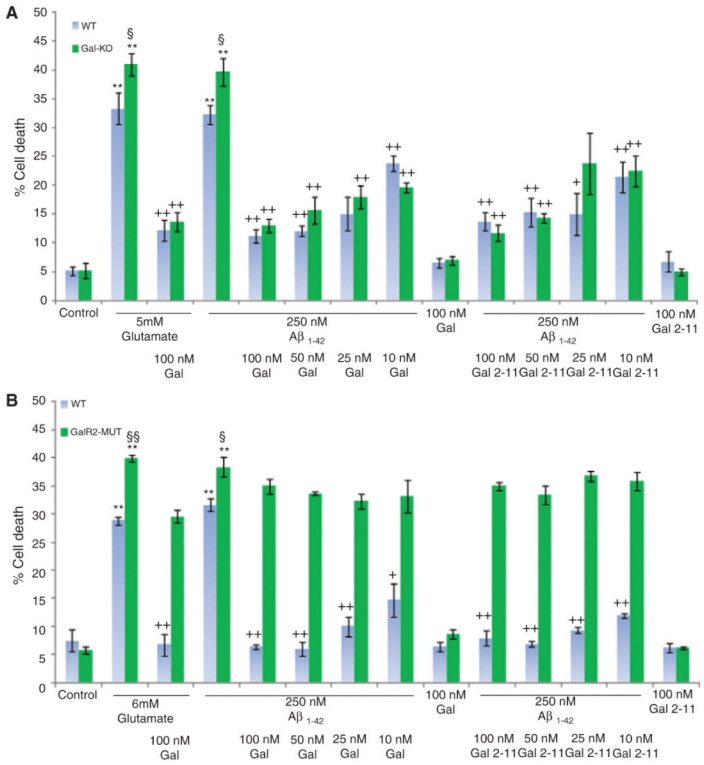

Fig. 1.

Percentage cell death in (A) Gal-KO and (B) GAL2-MUT both compared to strain matched WT hippocampal cultures, treated with 250 nM fA_1-42 in the presence of varying concentrations of galanin or Gal2-11. In both loss-of-function mutations there is a significant increase in fA_1–42 and glutamate-induced cell death compared to strain-matched WT cultures. Addition of galanin or Gal2-11 significantly rescues the deficits in the Gal-KO but not the GAL2-MUT cultures. $^{**}p < 0.01$ treatment versus control. $\S p < 0.05$ Gal-KO or GAL2-MUT versus WT. $\S\S p < 0.01$ GAL2-MUT versus WT. $+p < 0.05$ treatment different to fA_1–42 or glutamate alone. $++p < 0.01$ treatment different to fA_1–42 or glutamate alone.

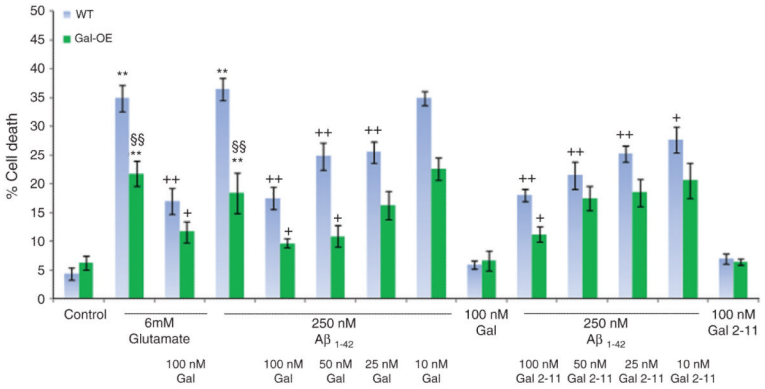

Fig. 2.

Percentage cell death in Gal-OE and strain matched WT hippocampal cultures treated with 250 nM fA_1–42 in the presence of varying concentrations of galanin or Gal2-11. In the Gal-OE mice there is a significant decrease in fA_1–42 and glutamate-induced cell death compared to strain-matched WT cultures. Addition of 100 nM and 50 nM galanin or 100 nM Gal2-11 further and significantly rescues the cell death. $^{**}p < 0.01$ treatment versus control. §§$p < 0.01$ Gal-OE versus WT. $+p < 0.05$ treatment different to fA_1–42 or glutamate alone. $++p < 0.01$ treatment different to fA_1–42 or glutamate alone.

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