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Short communication

GalR3 mediates galanin proliferative effects on postnatal hippocampal precursors

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GalR3 mediates galanin proliferative effects on postnatal hippocampal precursors

Abstract

Galanin, a neuropeptide co-released from noradrenergic and serotonergic projection neurons to the dentate gyrus, has recently emerged as an important mediator for signaling neuronal activity to the subgranular neurogenic stem cell niche supporting adult hippocampal neurogenesis. Galanin and its receptors appear to play key roles in depression-like behaviour, and effects on hippocampal neurogenesis are relevant to pharmacological strategies for treating depression, which in part appear to rely on restoring altered neurogenesis. We previously demonstrated that the GalR2/3 receptor agonist Gal 2-11 is proliferative and proneurogenic for postnatal hippocampal progenitor cells; however, the specific receptor mediation remained to be identified.

With the recent availability of M1145 (a specific GalR2 agonist), and SNAP 37889 (GalR3 specific antagonist), we extend our previous studies and show that while M1145 has no proliferative effect, the co-treatment of postnatal rat hippocampal progenitors with Gal 2-11 and SNAP 37889 completely abolished the Gal 2-11 proliferative effects. Taken together, these results clearly demonstrate that GalR3 and not GalR2 is the specific receptor subtype that mediates the proliferative effects of galanin on hippocampal progenitor cells. These results implicate GALR3 in the mediation of galanin neurogenic effects and, potentially, its neurogenic anti-depressant effects.

Introduction

Adult mammalian neurogenesis occurs within discrete regions of the CNS, including the subgranular zone of the hippocampal dentate gyrus (Lie, Song et al. 2004). Hippocampal neurogenesis is important for memory processing, learning and behavioral responses Gould, Beylin et al. (1999), (Shors, Miesegaes et al. 2001, Deng, Aimone et al. 2010). Reduced hippocampal neurogenesis is associated with mood and cognitive impairments observed under various pathological conditions, including epilepsy and Alzheimer’s disease (Malberg, Eisch et al. 2000, Jessberger, Zhao et al. 2007, Rockenstein, Mante et al. 2007, Barkas, Redhead et al. 2012). The mechanisms underlying neurogenic regulation remains poorly understood and the link between neural activity and the stem cell niche remains key to its understanding (Song, Zhong et al. 2012).

GABA-ergic interneurons residing within the stem cell niche of subgranular cell layer of the dentate gyrus signal neuronal activity and co-release important neuropeptides under specific firing conditions (Hinson, Rowell et al. 2015)(Zaben and Gray 2013). Elegant optogenetic experiments have shown that interneuron activation directly modulates subgranular stem cells where neuronal circuitry mechanisms regulate adult quiescent neural stem-cell fate decision (Song, Zhong et al. 2012). A number of co-released neuropeptides from these interneurons have been studied extensively in recent years, with vasoactive intestinal peptide and neuropeptide Y emerging as key modulators of neurogenesis (Howell, Doyle et al. 2005, Howell, Silva et al. 2007, Zaben, Sheward et al. 2009) (see (Zaben and Gray 2013) for a review). Galanin has particularly sparked interest for its potential role in regulating neurogenesis but also in learning and memory (Ogren, Kuteeva et al. 2006) and mood disorders (Kuteeva, Hokfelt et al. 2008, Saar, Lahe et al. 2013). This highly conserved 29 amino acid neuropeptide acts as an inhibitory hyperpolarizing neuromodulator via three G protein coupled receptors: GalR1, GalR2 and GalR3 (Mitsukawa, Lu et al. 2008). The activation of GalR1 and/or GalR3 receptors results in a depression-like phenotype, while activation of the GalR2 receptor attenuates depression-like behavior (Kuteeva, Hokfelt et al. 2008).

We have previously shown that galanin 2-11 (AR-M1896), a GalR2/3 agonist, has a proliferative effect on hippocampal precursor cells. In this study, we specifically defined the receptor medication of this proliferative effect of galanin on postnatal hippocampal precursors using recently available pharmacological agents. We show that GalR3 receptor subtype mediates a proliferative effect on hippocampal progenitor cells and the endogenous cell proliferation of these cells in vitro. These findings together with our previous findings (Abbosh, Lawkowski et al. 2011) strongly implicate GalR3 as an important mediator of progenitor cell proliferation in the hippocampus, and importantly from a therapeutic perspective, implicate GalR3 as a target for drug discovery studies in the treatment of mood disorders and restoring learning and memory impairment.

Materials and methods:

Postnatal rat hippocampal cell culture

Animal experimentation was conducted in compliance with the United Kingdom Animals (Scientific Procedures) Act, 1986. Every effort was made to minimize the number of animals used and their suffering. Rat hippocampal progenitor cell cultures were generated from postnatal Sprague Dawley rats (P7-10) as described previously (Howell, Scharfman et al. 2003). Briefly, hippocampi were dissected under sterile conditions and cut using a McIlwain tissue chopper. Tissue slices were digested with papain (2mg/mL, 22.0 U/mg, Sigma) in pre-warmed culture medium (Neurobasal A, Invitrogen; 2% B-27, Life Technologies; 0.5mM Glutamine, Sigma) for 30 mins at 37ºC. Following cell release by trituration, progenitor cells were purified on a two step Optiprep gradient by centrifugation for 15 mins at 400g. Viable cells were plated directly on pre-coated poly-L-lysine (50µg/mL, Sigma) 24 well plates at a density of 100, 000 cells per mL in culture medium. Cells were washed and replaced with fresh culture medium 2 hours post plating. All culture medium contained 1% antibiotic/antimycotic (Penicillin/Streptomycin and Fungizone, Life Technologies). Cells were grown under control conditions for 3DIV in a humidify incubator (5%CO2, 95% air, 37ºC).

Pharmacology

To examine the specific galanin receptor subtype mediating the proliferative effects on NSPCs (Abbosh, Lawkowski et al. 2011), we used three different peptides: Galanin 2-11 (AR-M1896) binds to GalR2 and GalR3 with similar affinity (Lu, Lundstrom et al. 2005). SNAP 37889 is a selective GalR3 antagonist (Swanson, Blackburn et al. 2005) whilst the novel peptide M1145 [(RG)2-N-galnin(2-13)-VL-(P)3-(AL)2-A-amide] is a GalR2-specific agonist that has more than 90- and 76-fold higher affinity for GalR2 over GalR1 and GalR3, respectively (Runesson, Saar et al. 2009).

Cells were then either maintained under control conditions or exposed to 10nM of the GalR2 specific agonist M1145 (a concentration at which it does not interact with GALR3 (Runesson, Saar et al. 2009)). Cultures in separate wells were treated with the GalR2/3 agonist GAL 2-11 as a positive control, and to see whether we could replicate our previous findings (Abbosh, Lawkowski et al. 2011). To quantify proliferation, cells under different conditions were simultaneously pulsed with the S-phase marker BrdU for the terminal 6hrs prior to fixation.

Assessment of cell proliferation:

The thymidine analogue bromodeoxyuridine (BrdU) was used to measure cell proliferation. BrdU is incorporated into the DNA of proliferating cells during the S phase of the cell cycle (Kuhn and Cooper-Kuhn 2007). BrdU (Sigma) was added directly to cells in culture for the terminal 6 hours to a final concentration of 20µM. Experimental conditions were added at the same time. Cells were then fixed in 4% paraformaldehdye (PFA, Sigma) for 30 mins at 4ºC.

Immunohistochemistry:

PFA fixed cells were washed with phosphate buffered saline (PBS) and then treated with 2M HCl for 30 mins at 37ºC for antigenic retrieval of BrdU. Non-specific binding sites were blocked with 5% donkey blocking serum (DBS) in PBS with 0.1% Triton X (PBS-T) for 1 hour at 20ºC. Primary antibodies were diluted in 5% DBS in 0.1% PBS-T overnight at 4ºC. The following primary antibodies were used: rat anti-BrdU (1:200, AbD Serotec), mouse anti-rat nestin (1:200, BD Biosciences). Cells were washed with PBS. Secondary antibodies diluted in 0.1% PBS-T were then applied for 2 hours at 20ºC in darkness. Species specific Alexa Fluor 488 and 555 conjugates (Invitrogen) were used at a dilution of 1:1000. Cells were washed with PBS and counterstained with the nuclear stain 4',6-diamidino-2-phenylindole DAPI

(5µg/mL, Sigma) diluted in ddH2O for 6 mins at 20ºC in darkness. Cells were then washed three times with PBS. To ensure specific fluorescence, negative controls were generated by omitting primary antibodies.

Imagine, cell counting and statistical analysis:

Images were taken on a DM RBE microscope (Leica Microsystems Limited) at 20x magnification. Six systemically randomized fields per well were taken using the Leica Application Suite image-capturing system version 3.8.0. Data was averaged per well and expressed in cells/mm2 or as a percentage, based on a sample of 4 wells per condition per experiment. All experiments were repeated at least three times. Graph Pad prism data analysis software (GraphPad inc, San Diego, CA, USA) was used to plot data points. For statistical comparisons, Student's t test or a one-way ANOVA was used with Neuman-Keuls post hoc tests ($p < 0.05$ considered significant).

Results

Consistent with our previous findings, GAL 2-11 as a GALR2/3 agonist enhanced the rate of hippocampal progenitor cell proliferation as measured by the increased mitotic index of nestin-expressing cells (0.21 ± 0.01 vs. 0.16 ± 0.006) (**Figure 1**) (Abbosh, Lawkowski et al. 2011). However, the GalR2 specific agonist M1145 had no effect on the rate of proliferation of this cell subpopulation (0.16 ± 0.007 vs. 0.16 ± 0.006) (**Figure 1**) suggesting a possibly pure GALR3 mediation. To examine this hypothesis, hippocampal cell cultures were again grown for 3DIV and treated with the GalR3-specific antagonist SNAP 37889 and pulsed with BrdU for the terminal 6 hours prior to fixation. Interestingly, SNAP 37889 (2.5uM) not only completely abolished the proliferative effect of Galanin 2-11 (0.09 ± 0.006 vs. 0.19 ± 0.004), but also significantly reduced the baseline rate of proliferation of hippocampal nestin expressing progenitor cells (0.10 ± 0.008 vs. 0.16 ± 0.006) (**Figure 2**). Taken together, these results clearly demonstrate that GalR3 mediates the proliferative effect of galanin on hippocampal progenitor cells and is implicated in endogenous cell proliferation of hippocampal progenitors cells in an *in vitro* culture system.

Discussion

Under physiological conditions, hippocampal neurogenesis is implicated in hippocampal-dependent learning and spatial memory (Kempermann 2002). Its reduction and altered quality in many diseases particularly chronic temporal lobe epilepsy (Hattiangady and Shetty 2010) may be potentially responsible for these patients learning and memory deficits (Barkas, Redhead et al. 2012) and depression-like behavior (Jessberger, Nakashima et al. 2007). Neuropeptides including galanin have emerged as important modulators of hippocampal neurogenesis (Zaben and Gray 2013). In this regard, we have previously demonstrated a proliferative effect of the GalR2/3 agonist galanin 2–11 on hippocampal progenitor cultures generated from postnatal Wistar rats (Abbosh, Lawkowski et al. 2011); suggesting an important role for this peptide in promoting hippocampal neurogenesis. We, herein, utilized the recent availability of the GalR2 specific agonist M1145 and the GalR3-specific antagonist SNAP 37889 to further delineate the receptor mediation of the proliferative effect of galanin on hippocampal progenitor cells *in vitro*. Our data demonstrated that while M1145 had no proliferative effects, SNAP37889 completely abolished the galanin 2-11 proliferative effect (**Figure 1**). SNAP37889 has also reduced endogenous cell proliferation in cultures. These results, taken together with our previous finding in which GalR1 involvement was excluded and transcripts for both GalR2 and GalR3 receptors were identified in culture, identify GalR3 as the receptor mediator of galanin proliferative effects on postnatal hippocampal progenitor cells (**Figure 2**). Since the mechanisms governing neurogenesis in the late postnatal period are highly conserved in adulthood (Pleasure, Collins et al. 2000), our findings may implicate GalR3 in the altered hippocampal neurogenesis in many adulthood diseases; particularly in depression where the behavioural effects of some antidepressants require hippocampal neurogenesis (Santarelli, Saxe et al. 2003). Indeed, galanin and its receptors play key roles in depression and its treatment (McLaughlin and Robinson 2002, Lu, Barr et al. 2005, Lu, Sharkey et al. 2007, Mitsukawa, Lu et al. 2008). While galanin receptor GalR1/2 agonists exhibit antidepressant-like effects in the rat forced swim test (Mitsukawa, Lu et al. 2008), GalR3 antagonists exhibit anxiolytic and antidepressant-like activity (Lu, Sharkey et al. 2007). Galanin is co-expressed in acetylcholinergic, serotonergic and noradrenergic projection neurons to the hippocampus, and can inhibit co-transmitter release (Fisone, Wu et al. 1987). It is, therefore, important to distinguish between direct actions on hippocampal precursor cells and indirect actions either on synaptic transmission of other neurotransmitters or actions on other neuronal systems in vivo. This may explain the paradoxical pro-neurogenic findings we report here and the antidepressant effects of GalR3 antagonists *in-vivo*, and clearly the elucidation of these interactions will require further study. The value of this study is its demonstration of a direct role of galanin on hippocampal precursors mediated via the GalR3 receptor, which may serve as a target for development of novel antidepressant drugs through modulation of hippocampal neurogenesis.

Figures:

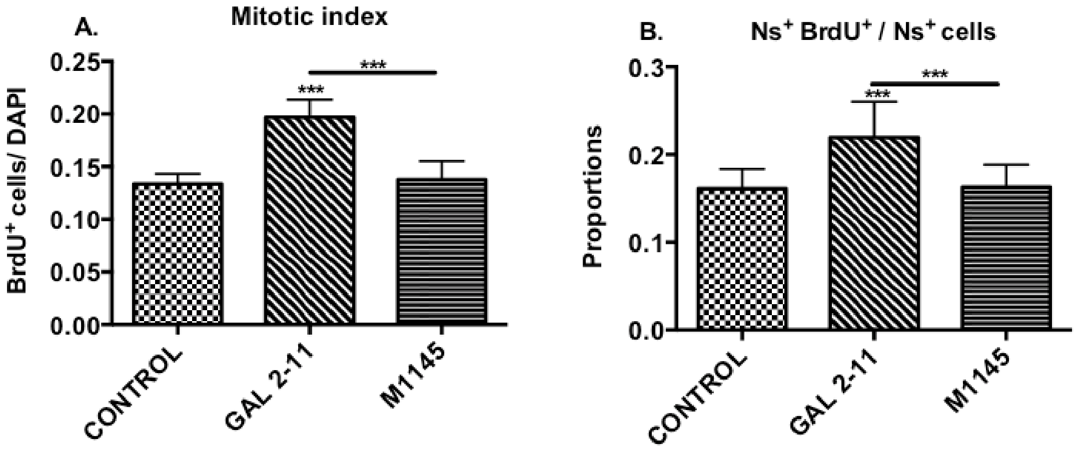

Figure 1: GalR3 mediates the proliferative effects on hippocampal progenitor cells. Cultures were grown under control conditions for 3days in vitro and given a terminal 6 hour exposure to BrdU and experimental conditions (100nM Galanin 2-11 (Gal 2-11), 10nM M1145). Proportion of BrdU incorportating cells expressing nestin, with respect to the total number of BrdU incorporating cells. Data represents mean ± SE based on a sample that represents 12 wells per condition from 3 different experiments. Comparisons between control and treatment conditions are a one-way ANOVA with Neuman-Keul's multiple comparison test (**, $p < 0.01$, ***, $p < 0.001$).

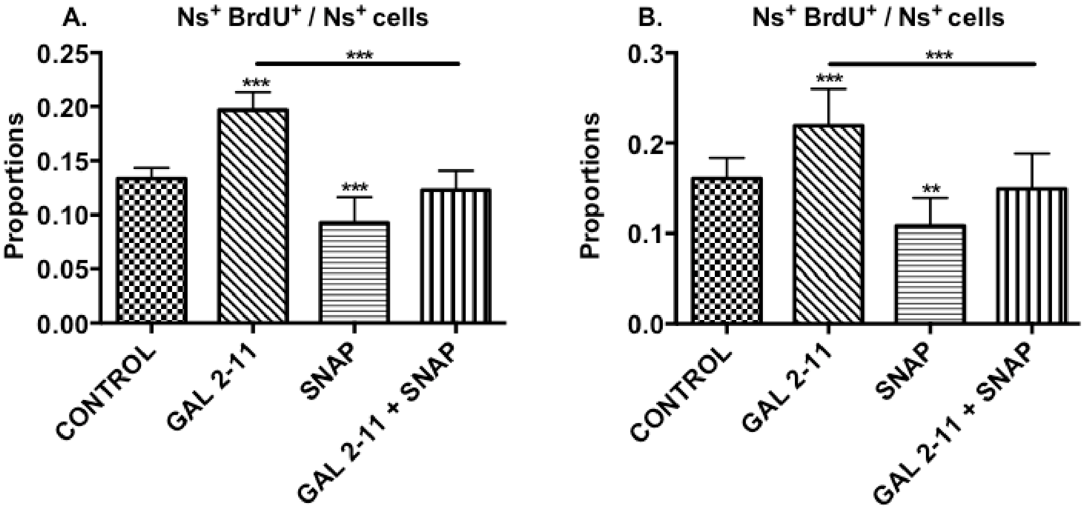

Figure 2: GalR3 antagonist SNAP decreases the proliferative effects on nestin expressing hippocampal progenitor cells. Cultures were grown under control conditions for 3days in vitro and given a terminal 6 hour exposure to BrdU and experimental conditions (100nM Galanin 2-11 (Gal 2-11), 2.5uM SNAP 37889 (SNAP), combination of Galanin 2-11 and SNAP 37889). Proportion of BrdU incorportating cells expressing nestin, with respect to the total number of BrdU incorporating cells. Data represents mean ± SE based on a sample that represents 12 wells per condition from 3 different experiments. Comparisons between control and treatment conditions are a one-way ANOVA with Neuman-Keul’s multiple comparison test (**, $p < 0.01$, ***, $p < 0.001$).

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