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**Notch/Neurogenin 3 signalling is involved in the neuritogenic actions of oestradiol
in developing hippocampal neurones**

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Abstract

The ovarian hormone oestradiol promotes neuritic outgrowth in different neuronal types, by mechanisms that remain elusive. Recent studies have shown that the Notch-regulated transcription factor neurogenin 3 controls neuritogenesis. In this study we have assessed whether oestradiol regulates neurogenin 3 in primary hippocampal neurones. As expected, neuritogenesis was increased in the cultures treated with oestradiol. However, the neuritogenic action of oestradiol was not prevented by ICI 182.780, an antagonist of classical oestrogen receptors. Oestradiol decreased the expression of Hairy and Enhancer of Split-1, a Notch-regulated gene that negatively controls the expression of neurogenin 3. Furthermore, oestradiol increased the expression of neurogenin 3 and regulated its distribution between the neuronal cell nucleus and the cytoplasm. The effect of oestradiol on neurogenin 3 expression was not blocked by antagonists of classical nuclear oestrogen receptor-mediated transcription and was not imitated by selective agonists of nuclear oestrogen receptors. In contrast, G1, a ligand of G protein receptor 30 / G protein-coupled oestrogen receptor, fully reproduced the effect of oestradiol on neuritogenesis, neurogenin 3 expression and neurogenin 3 subcellular localisation. Moreover, knockdown of neurogenin 3 in neurones by transfection with small interference RNA for neurogenin 3 completely abrogated the neuritogenic actions of oestradiol and G1. These results suggest that oestradiol regulates neurogenin 3 in primary hippocampal neurones by a non-classical steroid signalling mechanism, which involves the downregulation of Notch activity and the activation of G protein receptor 30 / G protein-coupled oestrogen receptor or of other unknown G1 targets. In addition, our findings indicate that neurogenin 3 participates in the neuritogenic mechanisms of oestradiol in hippocampal neurones.

Introduction

Notch receptors (Notch 1 to 4, in vertebrates) are integral membrane proteins that bind to several different ligands, such as Delta 1, 3 and 4 and Jagged 1 and 2 (in mammals) (1). Upon ligand binding, Notch is proteolytically cleaved within its transmembrane domain by presenilin-1, the enzymatic component of the γ -secretase complex. The proteolysis of Notch results in the release of its active intracellular domain, which is translocated into the cell nucleus. Canonical Notch signalling involves the binding of Notch intracellular domain to DNA-binding cofactors and the subsequent activation of the transcription of target genes (1,2).

In developing neurones Notch signalling is involved in the control of neurite extension and remodelling. The activation of Notch inhibits neurite outgrowth or causes their retraction, whereas inhibition of Notch 1 signalling promotes neurite extension (3,4). The activation of Notch in neuronal cultures from mice hippocampus results in a decrease in the number of dendrites and in an increase in their length. These effects are associated with an increase in the expression of the transcription factors Hairy and Enhancer of Split (*Hes*)1 and *Hes*5 (5). In turn, Hes1 negatively controls the expression of a series of proneural genes from the bHLH superfamily, analogous of Achaete scute and Atonal (6). One of these genes is neurogenin 3 (*Ngn3*). The overexpression of *Ngn3* in cultured hippocampal neurones causes an increase in the number of dendrites and a transient decrease in the number of afferent GABAergic synaptic inputs, resulting in an increase in the ratio of excitation/inhibition and in an increase in neuronal activity (7).

In contrast to Notch, oestradiol, as *Ngn3*, promotes axonal and dendritic outgrowth in the CNS (8). In addition, the effects of oestradiol on hippocampal neurones show some additional similarities with the effects of *Ngn3*. Thus, in CA1 pyramidal neurones oestradiol increases the density of both dendritic spines and axospinous synapses and decreases the activity of GABAergic synaptic inputs (9). The mechanism mediating the neuritogenic effects of oestradiol are still not well understood. Nevertheless, IGF-I receptor (10,11), TrkB receptor (12), cAMP/ protein kinase A (PKA) (13), PKC (14), Src/Ras/ERK signalling and cAMP-response element-binding protein (CREB) (14,15) have been shown to be involved in the hormonal neuritogenic effect in different neuronal types. Some of these signalling pathways that are regulated by oestradiol may interact with Notch signalling (16-18). Indeed, a recent study has shown that oestradiol downregulates Notch signalling in developing hippocampal neurones (19). Given the similarities between the neuritogenic effect of *Ngn3* and oestradiol we hypothesise that oestradiol, by inhibition of Notch signalling, may increase the expression of *Ngn3*. Here we show that oestradiol promotes neuritogenesis in hippocampal neurones by an unclassical steroid signalling mechanism that involves the regulation of *Ngn3*.

Materials and Methods

Animals

CD1 mice were raised in the Cajal Institute and used to generate embryos for this study. The day of vaginal plug was defined as E0. All procedures for handling and killing the animals used in this study were in accordance with the European Commission guidelines (86/609/CEE) and were approved by our institutional animal care and use committee.

Hippocampal neuronal cultures and experimental treatments

The hippocampus was dissected out from embryonic day 17 mouse embryos and dissociated to single cells after digestion with 0.5% trypsin (Worthington Biochemicals, Freehold, NJ) and DNase I (Sigma-Aldrich) at 37° C for 15 min and washed in Ca²⁺/Mg²⁺-free Hank's Buffered Salt Solution (20). Neurones were counted and plated on 6-wells plates or glass coverslips coated with poly-L-lysine (Sigma-Aldrich) at a density of 300-700 neurones/mm², and they were cultured in phenol red free Neurobasal supplemented with B-27 and GlutaMAX I (Invitrogen, Crewe, United Kingdom). Under these conditions, glial growth should be reduced to less than 0.5% at 5 days in vitro (DIV) (21). Nevertheless we used cultures at 1 and 3 DIV and at these stages the level of astrocyte contamination was 5% at 1 DIV and 2% at 3 DIV, as judged by immunocytochemistry for glial fibrillary acidic protein (GFAP), neurone-specific β III tubulin and DAPI nuclear staining.

Cell treatments

At 1 or 3 days in vitro (DIV) the culture medium was replaced for 2 h by fresh medium devoid of B27 and GlutaMAX I supplement. Then the cells were incubated for 2 h with one of the following test compounds, alone or in combination: 17 β -oestradiol (10⁻⁸-10⁻¹²M); the ER antagonist ICI 182.780 (10⁻⁸M; Sigma-Aldrich Co., St Louis, MO); the selective ER α agonist propylpyrazole triol (PPT; 10⁻⁸M; Tocris BioScience, Bristol, UK); the selective ER β agonist diarylpropionitrile (DPN; 10⁻⁹M; Tocris); the selective ER α antagonist 1,3 - bis (4-hydroxyphenyl) - 4 - methyl - 5 - [4 - (2 - piperidinyl - ethoxy) phenol] - 1H - pyrazole dihydrochloride (MPP; 10⁻⁸M; Tocris); the selective

ER β antagonist 4 - [2 - phenyl - 5,7 - bis (trifluoromethyl) pyrazolo [1,5-a] pyrimidin - 3 - yl] phenol (PHTPP; 10^{-8} M; Tocris) and the GPR30 agonist G1 (10^{-8} and 10^{-10} M; Calbiochem, San Diego, CA). Test compounds were dissolved at 10^{-2} M in ethanol and then to the final concentration in Neurobasal medium. For immunocytochemistry assays, the treatments were performed during 16 h, and cultures were not deprived of B27 and GlutaMAX I supplement.

Inhibition of Ngn3 expression using small interfering RNAs

Small interfering RNAs (siRNAs) oligonucleotides were purchased from Applied Biosystems/Ambion and the concentration was 25 nM during transfection. The following siRNAs were used: siRNA#1 targeting Ngn3 (sense, AACUACAUCUGGGCACUGAtt; antisense, UCAGUGCCCAGAUGUAGUUgt); siRNA#2 targeting Ngn3 (sense, GCUUCUCAUCGGUACCCUUt; antisense, AAGGGUACCGAUGAGAAGCct). A non targeting siRNA was used as negative control.

Neurones were co-transfected at 3 days in vitro (DIV) using the Effectene Transfection Reagent (Qiagen GmbH, Hilden, Germany), following the manufacturer's instructions with pEGFP-C2 (Clontech, USA) plus one of the siRNA oligonucleotides targeted to Ngn3 or the siRNA negative control. After 16h of expression time the cultures were processed for immunostaining. The same plasmids and siRNAs were nucleofected into cultured neurones using an Amaxa nucleofector with the Mouse Neurone Kit (Amata, Gaithersburg, MD) according to the manufacturer's instructions and after 1 DIV, the neurones were harvested and processed for real time PCR analysis.

Analysis of gene expression by quantitative real-time polymerase chain reaction (q-PCR)

Total RNA was extracted from cultures with illustra RNAspin Mini RNA isolation kit from GE Healthcare (Buckinghamshire, UK). First strand cDNA was prepared from RNA using the First Strand Synthesis kit from Fermentas GMBH (St Leon-Rot, Germany) following the manufacturer instructions. Quantitative real-time PCR was performed using the ABI Prism 7000 Sequence Detector (Applied Biosystems, Weiterstadt, Germany). TaqMan probes and primers for *Ngn3*, *Hes1* and for the control housekeeping gene, *Gapdh*, were Assay-on-Demand gene expression products (Applied Biosystems). Primer sequences for *Gpr30* were designed using Primer Express (AB) and were: forward, 5'- TGCTGCCATCCAGATTCAAG -3' and reverse, 5'- GGGAACGTAGGCTATGGAAAGAA-3'. Real-time PCRs were performed following the suppliers instructions using the TaqMan or Sybr Green Universal PCR Master Mix. All reactions were done in triplicates, from 3 different cultures. *Ngn3*, *Hes1* and *Gpr30* expression was normalised for *Gapdh* expression.

Immunocytochemistry

Cells were fixed for 20 min at room temperature in 4% paraformaldehyde and permeabilised for 4 min with 0.12% Triton-X plus 0.12% gelatine in phosphate buffered saline (PBS). Cells were then washed with PBS/gelatine and incubated for 1 h with anti-GFAP rabbit polyclonal antibody (DakoCytomation, Milno, Italy; diluted 1:500 in PBS/gelatine), with anti- β III tubulin mouse monoclonal antibody (TUJ1; COVANCE, Emeryville, CA; diluted 1:1000 in PBS/gelatine), with anti-Ngn3 mouse monoclonal antibody (F25A1B3; developed by Ole D. Madsen and obtained from the NICHD Developmental Studies Hybridoma Bank, University of Iowa, Iowa City, IA; diluted

1:2000 in PBS/gelatine) or with goat anti-GFP (Abcam, Cambridge, UK; diluted 1:1000 in PBS/gelatine). After washing in the same buffer, cells were incubated for 1h at room temperature with goat anti mouse Cy2 (Jackson ImmunoResearch Europe Ltd, Newmarket, Suffolk, England, diluted 1:1000) for the detection of β III tubulin; with anti-mouse-biotin antibody followed by streptavidin-peroxidase (Jackson), Tyramide (Perkin-Elmer Life Sciences, Boston, MA) and Alexa 568-streptavidin (Molecular Probes, Invitrogen, Barcelona, Spain) for the detection of Ngn3 or Alexa 488-donkey anti goat (Molecular Probes, diluted 1/500) for detection of GFP. Cell nuclei were stained with DAPI.

Analysis of neuritogenesis

To assess the effect of oestradiol and G1 on neuritogenesis the number of primary β III tubulin-immunoreactive neurites was counted at 1 DIV at a magnification of 400X. The number of primary neurites was counted in 50 to 70 cells per experimental condition. In addition, the neuritic arbour was assessed at 3 DIV by the method of SHOLL (22) using a grid of 6 concentric circles with increasing radius of 20 μ m. The grid was superimposed to the images of β III tubulin-immunoreactive neurones at a magnification of 400X, placing the innermost circle over the perikaryon. The number of neurites intersecting each circle was counted in 50 to 70 cells per experimental condition.

Analysis of Ngn3 subcellular distribution

In order to assess Ngn3 subcellular distribution by immunocytochemistry, confocal analysis was performed in a Leica (Bensheim, Germany) microscope. Images were acquired digitally using a 40x oil immersion objective and fluorescence filters. Randomly selected fields containing cells counterstained with DAPI were digitalised,

and the Mean Gray Value for Ngn3 immunostaining in the nuclei and cytoplasm areas was measured using ImageJ 1.37v software (freely available at the National Institutes of Health: rsbweb.nih.gov/ij/). The values were background subtracted using the average Mean Gray Value of the preparation background in each of the experimental conditions and data were represented as relative fluorescence intensity of nucleus versus cytoplasm of each neurone. Subcellular distribution of Ngn3 was assessed in 25 to 52 neurones for each experimental condition.

Statistical analysis

Statistical significance between two groups was assessed by student t-test and between more than two groups by one-way analysis of variance (ANOVA) followed by the Bonferroni correction, using GraphPad Prism 5 (GraphPad Software, Inc., San Diego, CA). The levels of significance were denoted as * # $p < 0.05$, ** # $p < 0.01$, *** # $p < 0.001$. The number used for statistical analysis was the number of independent experiments. Data in the figures are the results of 3 independent experiments (n=3), except for figures 1 and 2 in which n=4. Data are represented as mean + SEM.

Results

Oestradiol promotes neuritogenesis of hippocampal neurones in primary cultures

The first experiment was designed to confirm the neuritogenic effect of oestradiol in our model system. We selected neuronal cultures at 1 and 3 DIV because these developmental stages correspond to neurite outgrowth and dendritic branching, respectively. As shown in Fig. 1, oestradiol significantly increased the number of primary neurites in hippocampal neurones at 1 DIV and the number of intersections of the neurites with the lines of the grid used for Sholl analysis at 3 DIV.

Oestradiol reduces *Hes1* expression and enhances *Ngn3* expression in hippocampal neurones

In a second experiment we assessed whether oestradiol regulates the expression of the neurogenic transcription factor *Ngn3* and of the transcription factor *Hes1*, which mediates the effect of Notch on *Ngn3* expression. Oestradiol, at doses of 10^{-12} to 10^{-8} M, significantly decreased *Hes1* mRNA levels (Fig. 2A) and significantly increased *Ngn3* mRNA levels (Fig. 2B).

Oestradiol regulates the subcellular localisation of Ngn3 in hippocampal neurones

Previous studies have shown that Ngn3 in hippocampal neurones is translocated from the cell nucleus to the cytoplasm at the moment of the initiation of neurite development and then from the cytoplasm back to the cell nucleus at the end of the differentiation process (23). In agreement with these previous findings we observed more Ngn3 immunoreactivity in the cell nucleus of control neurones at 1 DIV, before the initiation of neurite development, than at 3 DIV, when neurite development is initiated (Fig. 3). Oestradiol increased the cytoplasmic localisation of Ngn3 at 1 DIV and the nuclear localisation of Ngn3 at 3 DIV (Fig. 3).

Classical nuclear oestrogen receptor signalling is not involved in the effect of oestradiol on *Ngn3* expression

To determine whether the effect of oestradiol on *Ngn3* expression was mediated by classical transcriptional regulation by oestrogen receptors (ERs), the cultures were treated with PPT and DPN, selective agonists of ER α and ER β mediated transcription,

respectively. Neither PPT nor DPN, tested separately or in combination, were able to significantly affect the mRNA levels of *Ngn3* (Fig. 4A,B).

To further explore the role of ER-mediated transcription on the effect of oestradiol, the cultures were exposed to the hormone in presence of ICI 182,780 (ICI), an antagonist of both ER α and ER β mediated transcription. ICI, tested at a dose of 10^{-8} M, did not prevent the effect of oestradiol on *Ngn3* mRNA levels (Fig. 4C,D). On the contrary, ICI per se significantly increased the mRNA levels of *Ngn3* (Fig. 4C,D). Then, we tested the effect of MPP and PHTPP, selective antagonists of ER α and ER β mediated transcription, respectively. Neither MPP nor PHTPP were able to block the effect of oestradiol on *Ngn3* mRNA levels (Fig. 4E,F). In addition, MPP per se significantly increased the mRNA levels of *Ngn3* at 1 DIV and PHTPP significantly increased the effect of oestradiol on *Ngn3* levels at 3 DIV.

Classical nuclear oestrogen receptor signalling is not involved in the effect of oestradiol on neuritogenesis

The previous findings suggest that oestradiol regulates *Ngn3* by a mechanism independent of classical ER mediated transcription. Thus, we assessed whether classical ERs are involved in the regulation of neuritogenesis by oestradiol in our model. Neuronal cultures were treated for 3 hours with vehicle, 10^{-10} M 17 β -oestradiol or 10^{-10} M 17 β -oestradiol in presence of 10^{-8} M ICI. As shown in figure 5, ICI did not affect the neuritogenic effect of oestradiol.

G1, a ligand of GPR30/GPER, mimics the effect of oestradiol on neuritogenesis, *Ngn3* expression and *Ngn3* subcellular localisation

Since the previous results suggested that oestradiol is regulating *Ngn3* expression and neuritogenesis in hippocampal neurons by a mechanism independent of classical ER mediated transcription, we examined the effect of G1, a ligand of GPR30/GPER, which is a putative membrane oestrogen receptor. First, we confirmed that *Gpr30/Gper* mRNA is expressed in the cultures of hippocampal neurones (Fig. 6A). No significant differences were observed in *Gpr30/Gper* mRNA levels between 1 DIV and 3 DIV. The treatment of the cultures with G1 increased the mRNA levels of *Ngn3* in a dose-dependent manner at 1 and 3 DIV (Fig. 6B). In addition, G1 increased the number of primary neurites at 1 DIV and the number of intersections of the neurites with the lines of the grid used for Sholl analysis at 3 DIV (Fig. 6C,D). Finally, G1 also reproduced the effect of oestradiol on *Ngn3* subcellular localisation at 1 and 3 DIV (Fig. 7).

Downregulation of *Ngn3* with specific siRNAs blocks the effects of oestradiol and G1 on neuritogenesis of cultured hippocampal neurones

To assess whether *Ngn3* mediates the effects of oestradiol and G1 on neuronal morphology, two different double-stranded siRNA oligonucleotides targeting *Ngn3* (siRNA#1, siRNA#2) were electroporated in hippocampal neuronal cultures. A non targeting siRNA was used as control. Fig. 8A shows that both siRNAs targeting *Ngn3* significantly reduced *Ngn3* mRNA expression in comparison to the control siRNA. Next we added oestradiol and G1 to cultures co-transfected with pEGFP-C2 plus either control siRNA, siRNA#1 or siRNA#2 and we analyzed the morphology of hippocampal neurones. Fig. 8B,C shows that *Ngn3*-specific siRNA oligonucleotides, per se, induced a decrease in dendritic tree complexity as evaluated by Sholl method. In addition,

oestradiol and G1 induced an increase in dendritic tree complexity in cultures transfected with the control siRNA, but not in the cultures transfected with *Ngn3*-specific siRNA oligonucleotides.

Discussion

In agreement with previous findings in different *in vitro* and *in vivo* experimental models and different neuronal cell types (8,10-15,24), our present results confirm that oestradiol promotes neuritogenesis in primary hippocampal neurones from mice. To further explore the mechanisms involved in the neuritogenic effect of oestradiol, we have assessed in this study the effect of the hormone on the expression of *Ngn3*, a proneural gene that is regulated by Notch activity and that is involved in the control of dendrite morphology and synaptic plasticity of cultured hippocampal neurones (5,7,23).

The findings of the present study indicate that oestradiol increases the expression of *Ngn3* in developing hippocampal cultures. Since the vast majority of cells in these cultures are neurones, the effect of oestradiol is most probably on neuronal *Ngn3*. The effect of oestradiol on *Ngn3* expression was more pronounced at 3 DIV than at 1 DIV. This may be due to the fact that basal *Ngn3* expression levels are higher at 1 DIV than at 3 DIV (23). Thus, under conditions of high basal *Ngn3* expression levels, the effect of oestradiol may be less evident than under conditions of low *Ngn3* levels. However, other alternatives, including possible developmental changes in the expression or activity of oestradiol-regulated intracellular signalling molecules, can not be excluded.

In parallel to the effect of oestradiol on the expression of *Ngn3*, the hormone also regulates its subcellular localisation in hippocampal neurones, promoting its

translocation from the cell nucleus to the cytoplasm at 1 DIV and from the cytoplasm to the cell nucleus at 3 DIV. These modifications in the subcellular localisation of Ngn3 are observed during the normal differentiation of hippocampal neurones and are associated with the initiation of neurite development (movement from the nucleus to the cytoplasm) and with the end of the differentiation process (movement from the cytoplasm back to the nucleus) (23). Therefore, the effect of oestradiol on Ngn3 subcellular localisation is compatible with a hormonally-induced acceleration of neuronal differentiation.

Since *Ngn3* expression and subcellular localisation in developing hippocampal neurones is regulated by Notch signalling (5,7,23), we assessed the effect of oestradiol on *Hes1*, one of the primary effectors of Notch in developing hippocampal neurones (5). Our findings, showing that oestradiol decreases *Hes1* expression, are in agreement with previous results showing that oestradiol downregulates Notch signalling in developing hippocampal neurones (19).

To further analyse the mechanisms involved in the regulation of Ngn3 by oestradiol, we assessed several compounds that act as promoters or inhibitors of ER α and ER β mediated transcription. ICI 182,780 (ICI), an antagonist of both ER α and ER β mediated transcription was unable to block the effect of oestradiol on *Ngn3* expression. Interestingly, ICI was also unable to block the neuritogenic effect of oestradiol. Although ICI has been shown to block the neuritogenic effect of oestradiol in several neuronal types, such as hypothalamic neurones, dorsal root ganglion cells and PC12 cells (10,11,25), other studies have shown that ICI does not block oestrogenic effects on neuritogenesis in mouse midbrain dopaminergic neurones (13) and in male rat

hypothalamic neurones incubated with oestradiol added to medium conditioned by astroglia derived from ventral mesencephalon (26,27). This suggests that oestradiol may activate different signalling mechanisms to induce neuritogenesis depending on the neuronal type or the culture conditions. Glial support of oestradiol-induced neurite outgrowth (26,27) exerted by the 2-5% of astrocytes present in our cultures could explain why ICI failed to block oestradiol effects. Nevertheless, our findings are in agreement with previous results showing that ICI does not block oestrogen induced neuritogenesis in hippocampal neurones in vitro (28). In addition, ICI increased *Ngn3* expression as oestradiol did. This result is in agreement with previous findings showing that ICI has some estrogenic effects on hippocampal neurones and regulates the phosphorylation of ERK1, ERK2 and Akt and the expression of spinophilin and Bcl-2 in a similar manner than oestradiol (29). In contrast, PPT and DPN, selective agonists of ER α and ER β mediated transcription, respectively, were not able to imitate the effect of oestradiol on *Ngn3* expression. Furthermore, MPP and PHTPP, selective antagonists of ER α and ER β mediated transcription, respectively, were not able to antagonise the effect of oestradiol on *Ngn3* expression and even imitated (MPP) or potentiated (PHTPP) the effect of oestradiol. All these findings suggest that the hormone is regulating *Ngn3* expression by a non-canonical mechanism, which probably is independent of classical nuclear ER mediated transcription.

GPR30, also known as G protein-coupled oestrogen receptor (GPER), is a putative membrane associated ER. Although there is considerable debate on whether GPR30 is or not acting as an ER (30-33), there is evidence that G1, a ligand of GPR30, imitate the effects of oestradiol in different cell types and tissues (34,35). Our results are compatible with an action of oestradiol through GPR30. Indeed, the oestradiol

concentration range used in our experiments fits with its reported affinity for GPR30, with an EC₅₀ value of approximately 0.5 nM (36). Besides, it has been recently shown that in breast cancer SK-BR-3 cells, G1 may bind to the ER α 36 variant of ER α , whose expression is increased by GPR30 (37). Whatever the mechanism of action of G1 is, our findings indicate that this compound is able to fully reproduce the effect of oestradiol on neuritogenesis, *Ngn3* expression and Ngn3 subcellular localisation. Moreover the neuritogenic actions of oestradiol and G1 are blocked when *Ngn3* expression is downregulated by specific siRNA. These results suggest that oestradiol and G1 may act through common mechanisms to regulate Ngn3 expression. In addition, our findings also suggest that Ngn3 mediates the effect of oestradiol and G1 on the development of hippocampal neurones.

In conclusion, our findings suggest that oestradiol regulates neuritogenesis in primary hippocampal neurones by a non-classical steroid signalling mechanism that involves the activation of G1 molecular targets, the downregulation of Notch activity and the upregulation of Ngn3 expression.

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Figure legends

Figure 1, Effect of 17 β -oestradiol on the morphology of hippocampal neurones in culture. Neurones from E17 mouse hippocampi were plated at an initial cell density of 300/mm². After 1 or 3 days in vitro (DIV), 17 β -oestradiol (17 β E, 10⁻¹⁰ M) or vehicle were added for 16 h and cultures were prepared for immunofluorescence analysis. (A) Representative fluorescence images of hippocampal neurones immunostained for β III-tubulin (green) with cell nuclei stained with DAPI (blue). (B) Results of morphometric evaluation. The graphs show the number of primary neurites at 1 DIV and the number of intersections of the neurites with the lines of the grid used for Sholl analysis at 3 DIV. A total of 50 to 70 cells were assessed from each experimental group. Data are mean + SEM. The n used for statistical analysis was the number of independent experiments (n=4). Significance levels were determined using an unpaired *t*-test. *** p<0.001 versus control values.

Figure 2, *HES1* and *Ngn3* mRNA levels are significantly modified in hippocampal neuronal cultures treated with 17 β -oestradiol. Neurones from E17 mouse hippocampi were plated at an initial cell density of 300/mm². After 1 day in vitro (DIV), 17 β -oestradiol (17 β E, 10⁻¹² M to 10⁻⁸ M) or vehicle were added for 2h and cells were harvested for RNA extraction. The mRNA levels of *Hes1* (A) and *Ngn3* (B) were measured on cell lysates by real time RT-PCR. Data represent the mean+SEM and are expressed as percentage of control values. Asterisks indicate statistical differences in comparison to control values (*, p<0.05; **, p<0.01; ***, p<0.001) as determined using one-way analysis of variance (ANOVA) followed by the Bonferroni correction.

Figure 3, Oestradiol regulates the subcellular distribution of Ngn3 during the development of hippocampal neurones. Cultured hippocampal neurones untreated or treated with 17 β -oestradiol (17 β E, 10⁻¹⁰ M) were fixed at 1 and 3 DIV and fluorescence was analysed for Ngn3 immunostaining (red) and the nuclear marker DAPI (blue). (A) Representative micrographs of control and oestradiol treated neurones. Magenta colour in the cell nucleus is due to colocalisation of Ngn3 and DAPI. (B) Quantification of the subcellular localisation of Ngn3 in the cell nucleus and the cytoplasm. Randomly selected fields containing cells counterstained with DAPI were digitalised and the Mean Gray Value for Ngn3 immunostaining was measured in the cell nucleus and the cytoplasm using ImageJ 1.37v software. The graphs show the mean + SEM of the relative fluorescence intensity in nuclei versus cytoplasm. A total of 25 to 52 cells were assessed for each experimental group. The n used for statistical analysis was the number of independent experiments (n=3). Significance levels were determined using two way ANOVA followed by the Bonferroni correction. ***, **, statistical differences for the data sets connected by horizontal lines (***p<0.001, **p<0.01); ###, statistical differences in comparison to control cultures at 1 DIV (p < 0.001); ^^, statistical differences in comparison to control cultures at 3 DIV (p < 0.01).

Figure 4, Effect of agonists and antagonists of classical oestrogen receptors on Ngn3 mRNA levels. Neuronal cultures were treated for 3 hours with vehicle (Control), 10⁻¹⁰ M 17 β -oestradiol (17 β E), 10⁻⁸ M PPT (ER α agonist), 10⁻⁹ M DPN (ER β agonist), 10⁻⁸ M ICI 182,780 (ICI; ER α and ER β antagonist), 10⁻⁷ M MPP (ER α antagonist) or 10⁻⁷ M PHTPP (ER β antagonist) at 1 DIV (A,C,E) and 3 DIV (B,D,F) . Graphs show Ngn3 mRNA levels in cell lysates. Data represent the mean+SEM. *,**,*** statistical differences (* p < 0.05, ** p < 0.01, *** p<0.001) in comparison to the values of control

cultures; ###, statistical differences ($p < 0.001$) in comparison to cultures treated with 17β -oestradiol.

Figure 5, The oestrogen receptor antagonist ICI 182,780 does not block the effect of oestradiol on neuritogenesis. Neuronal cultures at 1 DIV were treated for 16 hours with vehicle (Control), 17β -oestradiol (17β E) or 17β E and ICI 182,780 (ICI). The graph shows the number of primary neurites. Data represent the mean+SEM. A total of 60 to 80 cells were assessed from each experimental group. The n used for statistical analysis was the number of independent experiments (n=3). Significance levels were determined using two way ANOVA followed by the Bonferroni correction. *** statistical difference (** $p < 0.001$) in comparison to the values of control cultures.

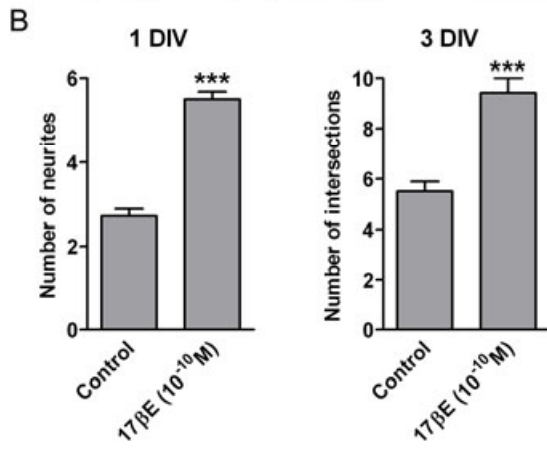
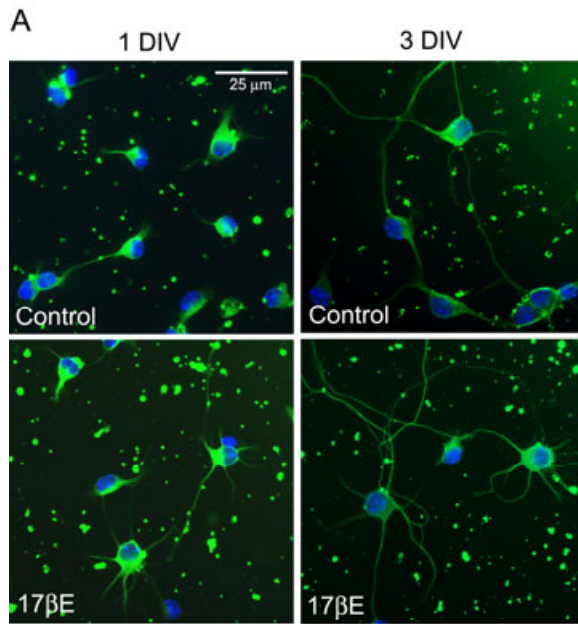
Figure 6, Effect of G1 on hippocampal neurones in culture. Neurones from E17 mouse hippocampi were plated at an initial cell density of $300/\text{mm}^2$. After 1 or 3 DIV, G1 (10^{-10} M or 10^{-8} M) or vehicle were added for 2h or 16 h and cells were harvested for RNA extraction or prepared for immunofluorescence analysis, respectively. (A) *GPR30* mRNA expression in control cultures at 1 and 3 DIV. (B) Effect of G1 treatment on *Ngn3* mRNA levels in hippocampal neuronal cultures at 1 and 3 DIV. Data represent the mean+SEM and are expressed as percentage of control values. Asterisks indicate statistical differences in comparison to control values (*, $p < 0.05$; **, $p < 0.01$; ***, $p < 0.001$) as determined using one-way analysis of variance (ANOVA) followed by the Bonferroni correction. (C) Representative fluorescence images of hippocampal neurones immunostained for β III-tubulin (green) and the nuclear marker DAPI (blue). (D) Results of the morphometric evaluation of the number of primary neurites at 1 DIV and the number of intersections of the neurites with the lines of the grid used for Sholl

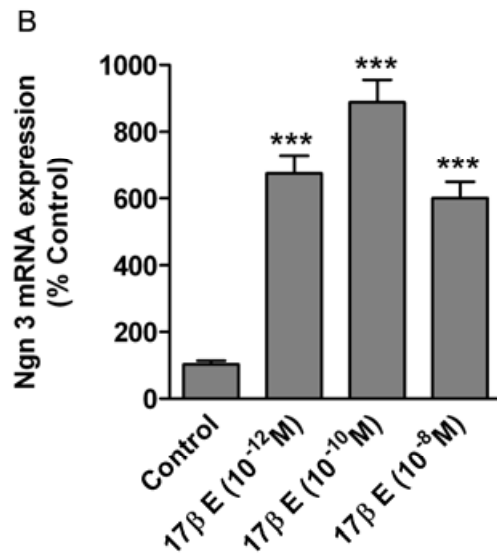
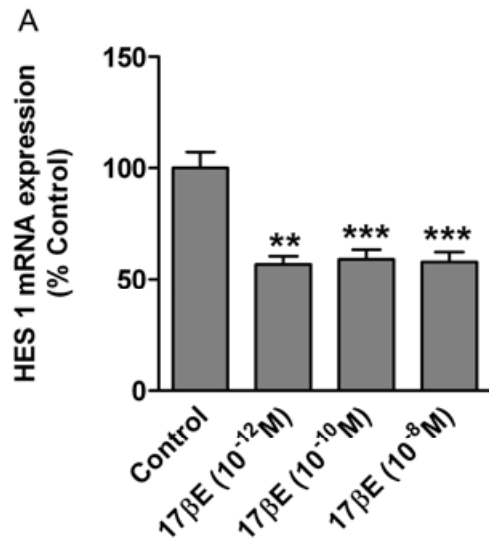
analysis at 3 DIV. A total of 50 to 70 cells were assessed for each experimental group. The n used for statistical analysis was the number of independent experiments. Significance levels were determined using an unpaired *t*-test. * $p < 0.05$, *** $p < 0.001$ versus control values.

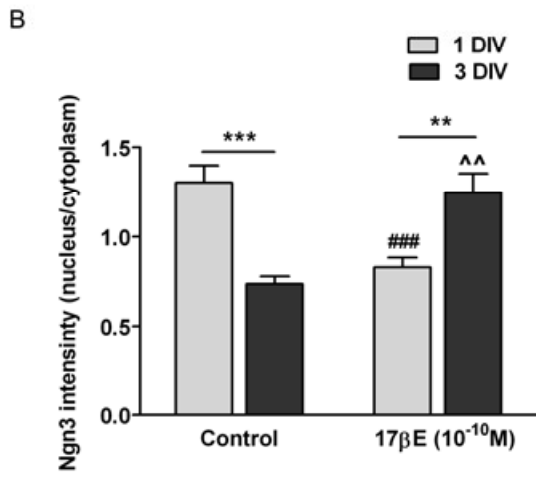
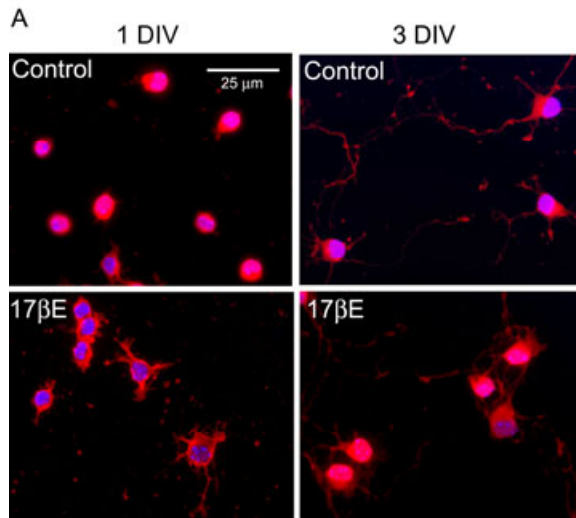
Figure 7, G1 regulates the subcellular distribution of Ngn3 during the development of hippocampal neurones. Cultured hippocampal neurones untreated or treated with G1 (10^{-10} M) were fixed at 1 and 3 DIV and fluorescence was analysed for Ngn3 immunostaining (red) and the nuclear marker DAPI (blue). (A) Representative micrographs of control and G1 treated neurones. (B) Quantification of the subcellular localisation of Ngn3 in nuclei and cytoplasm of neurones. The graphs show the mean + SEM of the relative fluorescence intensity in nuclei versus cytoplasm. A total of 52 cells were counted for each experimental group. The n used for statistical analysis was the number of independent experiments (n=3). Significance levels were determined using two way ANOVA followed by the Bonferroni correction. ***, statistical differences for the data sets connected by horizontal lines ($p < 0.001$); ###, statistical differences in comparison to control cultures at 1 DIV ($p < 0.001$); ^^^, statistical differences in comparison to control cultures at 3 DIV ($p < 0.001$).

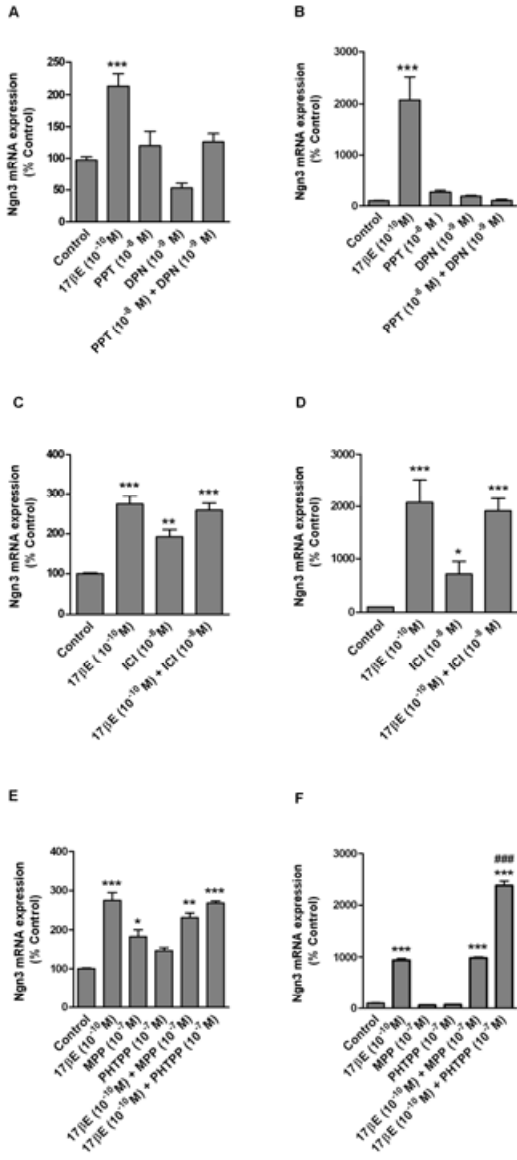
Figure 8, Downregulation of Ngn3 expression impairs the effects of 17 β -oestradiol and G1 on the morphology of hippocampal neurones in culture. (A) Effects of siRNAs on the levels of Ngn3 mRNA expression. Dissociated E17 hippocampal cells were nucleofected with siRNAs targeting Ngn3 (siRNA#1, siRNA#2) and non targeting siRNA (negative control, NC) using an Amaxa nucleofector with the Mouse Neuron Kit. After 1 DIV, cells were harvested and processed to real time PCR analysis. Data are

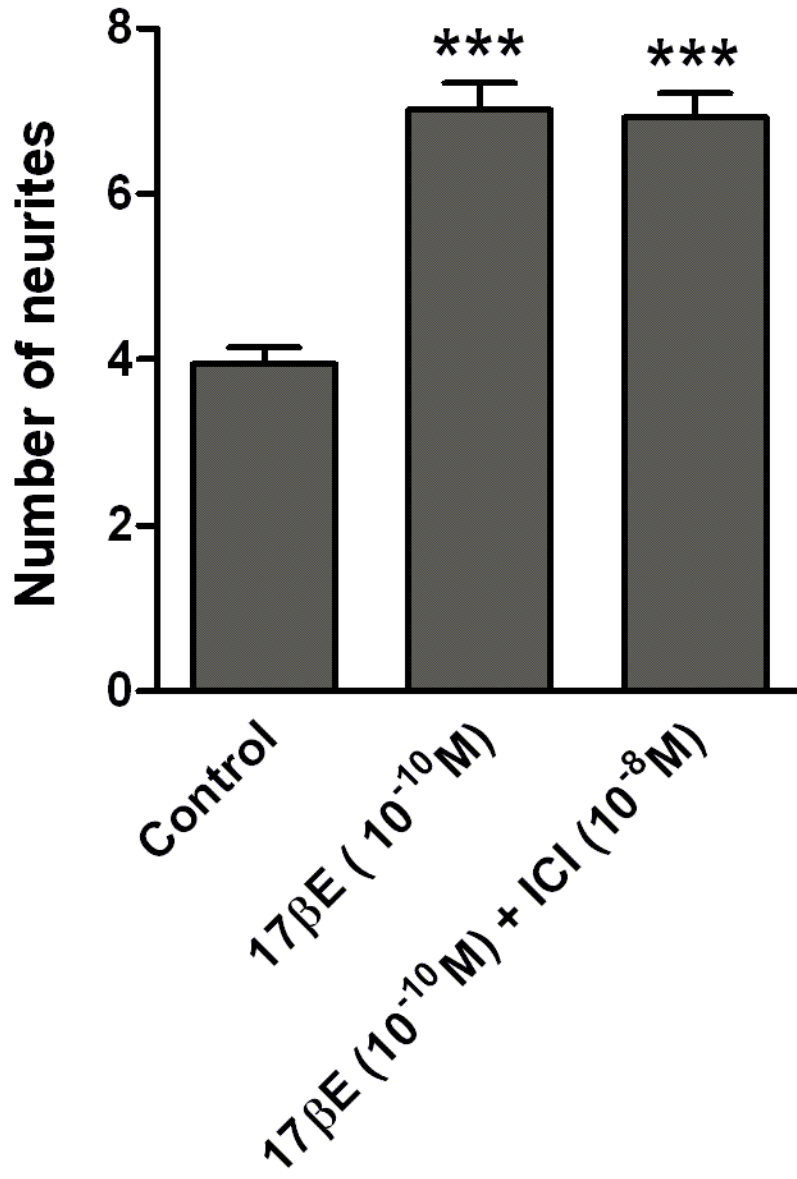
mean \pm SEM. Significance levels were determined using two way ANOVA followed by the Bonferroni correction. *** $p < 0.001$ versus negative control. (B) Hippocampal neuronal cultures were co-transfected at 3 DIV with pEGFP-C2 plus non targeting siRNA or one of the siRNA oligonucleotides targeted to Ngn3 and were untreated or treated with 17β -oestradiol and G1. After 16 hours cultures were fixed and processed for GFP immunostaining (green); nuclei were marked with DAPI (blue). (C) Results of the morphometric evaluation. The graphs show the number of intersections of the neurites with the lines of the grid used for Sholl analysis at 3 DIV. A total of 30 to 50 cells were assessed from each experimental group. Data are mean + SEM. The n used for statistical analysis was the number of independent experiments (n=4). Significance levels were determined using an unpaired t-test. *** $p < 0.001$ versus untreated negative control value; nd, no differences between bars connected by horizontal lines.

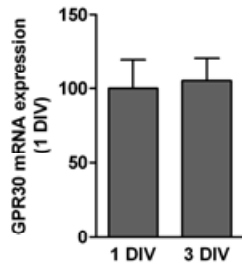
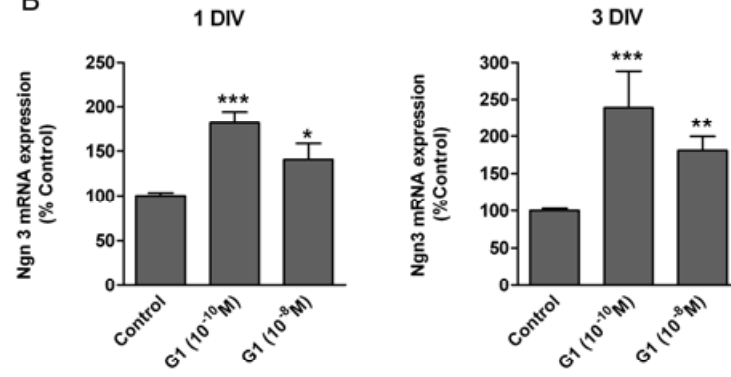
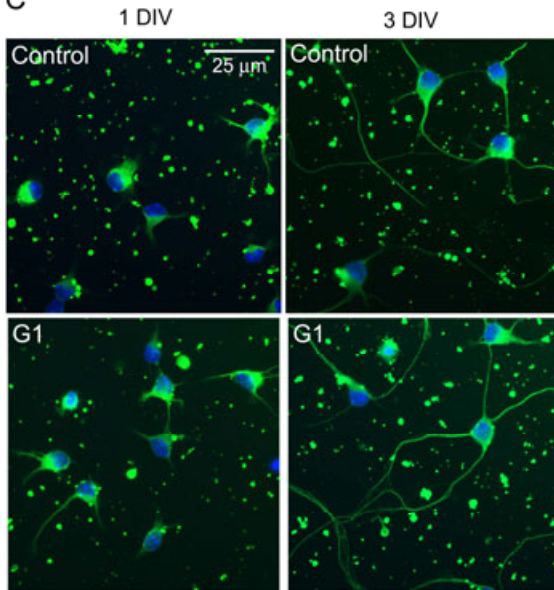










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