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RESEARCH PAPER

Signalling pathways for transactivation by dexmedetomidine of epidermal growth factor receptors in astrocytes and its paracrine effect on neurons

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Background and purpose: Stimulation of astrocytes by the α_2-adrenoceptor agonist dexmedetomidine, a neuroprotective drug, transactivates epidermal growth factor (EGF) receptors. The present study investigates signal pathways leading to release of an EGF receptor ligand and those activated during EGF receptor stimulation, and the response of neurons to dexmedetomidine and to astrocyte-conditioned medium.
Experimental approach: Phosphorylation of $ERK_{1/2}$ was determined by western blotting and immunocytochemistry, and phosphorylation of EGF receptors by immunoprecipitation and western blotting. mRNA expression of fos family was measured by RT-PCR.
Key results: *Pertussis* toxin (0.2 $\mu g\,ml^{-1}$) an inhibitor of $\beta\gamma$ subunit dissociation from $G\alpha_i$ protein, and GF 109203X (500 nM), a protein kinase C inhibitor, abolished $ERK_{1/2}$ phosphorylation. PP1 (10 μM), inhibiting Src kinase and GM 6001 (10 μM), an inhibitor of Zn-dependent metalloproteinase, abolished $ERK_{1/2}$ phosphorylation by dexmedetomidine (50 nM), but not that by EGF (10 $ng\,ml^{-1}$), showing Src kinase and metalloproteinase activation during the first stage only; AG 1478 (1 μM), an inhibitor of the EGF receptor tyrosine kinase, abolished $ERK_{1/2}$ phosphorylation. Dexmedetomidine-induced EGF receptor phosphorylation was prevented by AG 1478, GM 6001, PP1 and GF 109203X and its induction of cfos and fosB by AG 1478 and by U0126 (10 μM), an inhibitor of ERK phosphorylation, indicating downstream effects of $ERK_{1/2}$ phosphorylation. EGF and conditioned medium from dexmedetomidine-treated astrocytes, but not dexmedetomidine itself, induced ERK phosphorylation in primary cultures of cerebellar neurons.
Conclusions and implications: Dexmedetomidine-induced transactivation pathways were delineated. Its paracrine effect on neurons may account for its neuroprotective effects.
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Keywords: ERK; PKC; gene expression; $\beta\gamma$ subunits of G_i protein; Src kinase; metalloproteinase; neuroprotection; paracrine effect of EGF receptor ligand

Abbreviations: DAG, diacylglycerol; EGF, epidermal growth factor receptor; $ERK_{1/2}$, extracellular-regulated kinases 1 and 2; EGF, epidermal growth factor; GFAP, glial fibrillary protein; HB-EGF, heparin-binding epidermal growth factor; PKC, protein kinase C; PLC, phospholipase C; PTX, *Pertussis* toxin; RTK, receptor tyrosine kinase; TGF-α, transforming growth factor-α

Introduction

Dexmedetomidine is a potent and highly specific α_2-adrenergic agonist, which in receptor-binding experiments has an α_2/α_1 selectivity ratio of 1600 or several times higher than clonidine (Virtanen, 1989). It potently activates each of the three subtypes of the α_2-adrenoceptor (the $\alpha_{2A/D}$, the α_{2B} and the α_{2C} receptor), which all are linked to *Pertussis* toxin (PTX)-sensitive Gi/o-coupled receptors (Aantaa *et al*., 1995).

The best known action of dexmedetomidine in brain is a presynaptic inhibition of noradrenaline release and cell firing in noradrenergic neurons, but only a minor fraction of α_2-adrenoceptors appears to be presynaptic (for review, see Hertz *et al*., 2004). Accordingly, several pharmacological effects of dexmedetomidine are independent of inhibition of noradrenaline release. This can be seen from the fact that

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dexmedetomidine generates sedation and potentiates the action of anaesthetics in noradrenaline-depleted animals (Segal *et al*., 1988; Horvath *et al*., 1991–1992). Besides hypnotic/sedative and analgesic effects, low concentrations of dexmedetomidine have, with one exception, consistently been found to have neuroprotective properties in experimental cerebral ischaemia and excitotoxic neuronal injury (for review, see Peng, 2004). The mechanism of the neuroprotection is not known, but dexmedetomidine's neuroprotective effect can also be demonstrated under conditions when it does not diminish ischaemia-induced increase in cerebral noradrenaline release (Engelhard *et al*., 2002).

The target cells in the CNS displaying post-junctional α_2-adrenoceptors include astrocytes (Ebersolt *et al*., 1981; Hertz *et al*., 2004), which mainly express the $\alpha_{2A/D}$-adrenoceptor subtype (Enkvist *et al*., 1996). We reported previously that dexmedetomidine within the concentration range 25–100 nM causes phosphorylation of ERK_1 and ERK_2 ($ERK_{1/2}$) in cultured mouse astrocytes; $ERK_{1/2}$ phosphorylation occurred rapidly, reached a maximum at 20 min and declined after 40 min of stimulation (Peng *et al*., 2003). Based upon inhibition of the phosphorylation by tyrphostin AG 1478, an inhibitor of receptor tyrosine kinases (RTKs) (Levitzki and Gazit, 1995), and by heparin, an antagonist of heparin-binding epidermal growth factor (HB-EGF) we suggested that this effect is a result of transactivation of the epidermal growth factor (EGF) receptor.

Transactivation is a pathway connecting activation of some G protein-coupled receptors, including α_2-adrenoceptors, with ERK phosphorylation in two-stages, as has been elegantly shown in transfected COS-7 cells (Pierce *et al*., 2001). In the first stage the $\beta\gamma$ subunits of the activated, heterotrimeric G_i protein lead in these cells, via activation of cytosolic Src tyrosine kinases, to proteolytic, metalloproteinase-mediated 'shedding' of heparin-binding epidermal growth factor (HB-EGF) from its extracellular transmembrane-spanning HB-EGF precursor; in the second stage the free HB-EGF 'transactivates' EGF receptors in the same and adjacent cells in a conventional manner, that is, the RTK of the EGF receptor is phosphorylated and internalized, contributing directly to Src kinase-, Ras- and Raf-dependent ERK phosphorylation. It is unknown whether similar pathways are followed in astrocytes, and which family members are released from mature astrocytes in primary cultures. However, EGF transforming growth factor-α (TGF-α), HB-EGF, and amphiregulin, have been demonstrated in brain tissue (Birecree *et al*., 1991; Nakagawa *et al*., 1998; Falk and Frisén, 2002; Lu *et al*., 2005), and we have found EGF, TGF-α, and HB-EGF expression in cultured astrocytes (Du *et al*., 2007). It is also not known whether $ERK_{1/2}$ phosphorylation induces gene expression in astrocytes, and whether dexmedetomidine activates $ERK_{1/2}$ phosphorylation in cultured neurons.

In the present work, we have (i) examined the pathways operating during the events leading up to release of an EGF receptor ligand (stage 1) and those activated during EGF receptor stimulation (stage 2); (ii) tested whether dexmedetomidine or conditioned medium from dexmedetomidine-treated astrocytes also causes transactivation of $ERK_{1/2}$ in cerebellar granule neurons in primary cultures. For the first purpose we tested (i) whether the transactivation could be inhibited by interference with α_2-adrenoceptor stimulation or transduction by PTX, an inhibitor of the dissociation of $\beta\gamma$ subunits from $G\alpha_i$ protein or by GF 109203X, an inhibitor of proteinkinase C (PKC); (ii) whether Src kinase was involved before and/or after EGF receptor ligand release by studying the effect of PP1, an inhibitor of Src kinase on both dexmedetomidine- and EGF-induced $ERK_{1/2}$ phosphorylation; (iii) whether $ERK_{1/2}$ phosphorylation induced by dexmedetomidine, but not by EGF could be inhibited by GM 6001, an inhibitor of Zn-dependent metalloproteinase; (iv) whether EGF receptor phosphorylation by dexmedetomidine could be inhibited by AG 1478, GM 6001, PP1 and GF 109203X; (v) whether the increase in $ERK_{1/2}$ phosphorylation induced by dexmedetomidine occurred exclusively in the cytosol or p-$ERK_{1/2}$ also entered the nuclei; and (vi) whether early genes (c-fos, fosB, fra-1 and fra-2) were induced by dexmedetomidine and whether their induction could be inhibited by AG 1478, or U0126, an inhibitor of ERK phosphorylation.

Materials and methods

Cell cultures

All animal procedures were in accordance with the NIH guidelines for care and use of animals in research, and the protocols were approved by the Local Animal Ethics Committee of China Medical University.

Primary cultures of astrocytes, from newborn CD-1 mice of either sex, were prepared as previously described (Hertz *et al*., 1998) with minor modifications. The neopallia of the cerebral hemispheres, which roughly corresponds to the forebrains, were aseptically isolated (eliminating basal ganglia, olfactory lobes and cerebellum), vortexed to dissociate the tissue, filtered through nylon meshes with pore sizes of 80 and subsequently 10 μm, diluted in culture medium and planted in Falcon Primaria culture dishes. The culture medium was a Dulbecco's medium with 7.5 mM glucose, initially containing 20% horse serum and the cultures were incubated at 37 °C in a humidified atmosphere of CO_2/air (5:95%). The culturing medium was exchanged with fresh medium of similar composition on day 3, and subsequently every 3–4 days. From day 3, the serum concentration was reduced to 10%, and after the age of 2 weeks, 0.25 mM dibutyryl cyclic AMP (dBcAMP) was included in the medium. Such cultures are known to be highly enriched (>95 purity) in glial fibrillary protein- (GFAP-) and glutamine synthetase-expressing astrocytes (Hertz *et al*., 1985). The cultures were used after at least 3 weeks of culturing.

Cerebellar granule neurons were cultured as described by Peng *et al*. (1991) with minor modifications. Briefly, 7-day-old mouse pups (either sex) were rapidly decapitated and the brains taken out. The cerebella were aseptically separated from the remainder of the brain, and after removal of the meninges, the cerebellar tissue was cut into cubes of ~0.4 mm side dimensions, exposed to trypsin in a calcium–magnesium-free salt solution, reintroduced into tissue culture medium, passed through nylon sieves and seeded into polylysine-coated standard 35-mm tissue culture dishes

(Wuzhou Medical Plastic Factory, Zhejiang, China), using one cerebellum per culture dish. The cultures were grown in a modified Dulbecco's medium, in which the glucose concentration was increased to 30 mM and the K^+ concentration to 24.5 mM, the glutamine concentration was decreased to 0.8 mM and 7% horse serum was added. The elevation of the K^+ concentration is necessary for normal development of the cells (Peng *et al*., 1991), better cell survival is found with 0.8 than with 2.0 mM glutamine in the medium, and the increase in glucose concentration allows culturing without medium change, which is poorly tolerated by the cells. After 2 days, cytosine arabinoside was added to the medium to a final concentration of 40 μM to curtail the number of astrocytes that develop in the cultures.

Drug treatment

For determination of $ERK_{1/2}$ phosphorylation and EGF receptor phosphorylation, the culturing medium was gently removed and the cells were incubated in corresponding medium without serum at 37 °C for certain time periods in the absence or presence of dexmedetomidine or/and specific inhibitors. The reaction was stopped by washing with ice-cold phosphate-buffered saline (PBS) containing 7.5 mM glucose, and the cells were scraped off the dishes.

Astrocyte-conditioned medium

Astrocytes were incubated for 10 min in culturing medium without serum in the absence (control) and presence of dexmedetomine at 37 °C. Thereafter, the medium was collected and transferred to neuronal cultures. In some samples, 300 nM atipamezole, an antagonist of the α_2-adrenoceptor was added. Cerebellar granule cells were incubated with astrocyte-conditioned medium for 20 min at 37 °C.

Immunocytochemistry

After drug treatment, the cells were fixed with 100% methanol for 6 min at −20 °C. They were washed with PBS and left at 4 °C until use. Cells were permeabilized by incubation in PBS containing 0.3% Triton X-100 and 5% goat serum for 30 min as previously described (Peng *et al*., 1997). Monoclonal antibody against p-$ERK_{1/2}$ was used at 1:100 dilution, and secondary antibody TRITC-conjugated goat anti-mouse was used at 1:100 dilution. Incubation time for the first antibody was overnight at 4 °C and for the second antibody 2 h at room temperature. Hematoxylin at 0.2% was used for nucleus staining. Images were captured with an Olympus DP 71 camera (Tokyo, Japan) using the Image Pro Plus 4.5 software (Media Cybernetics Inc., Silver Spring, MD, USA) coupled to an Olympus BX51 microscope. The magnification level was ×400. The densitometry of p-ERK staining was quantified by the Image Pro Plus 6.0 software based on the staining intensity and area across the cells. The average value was taken from three areas (330 × 437 μm) in each cover slip.

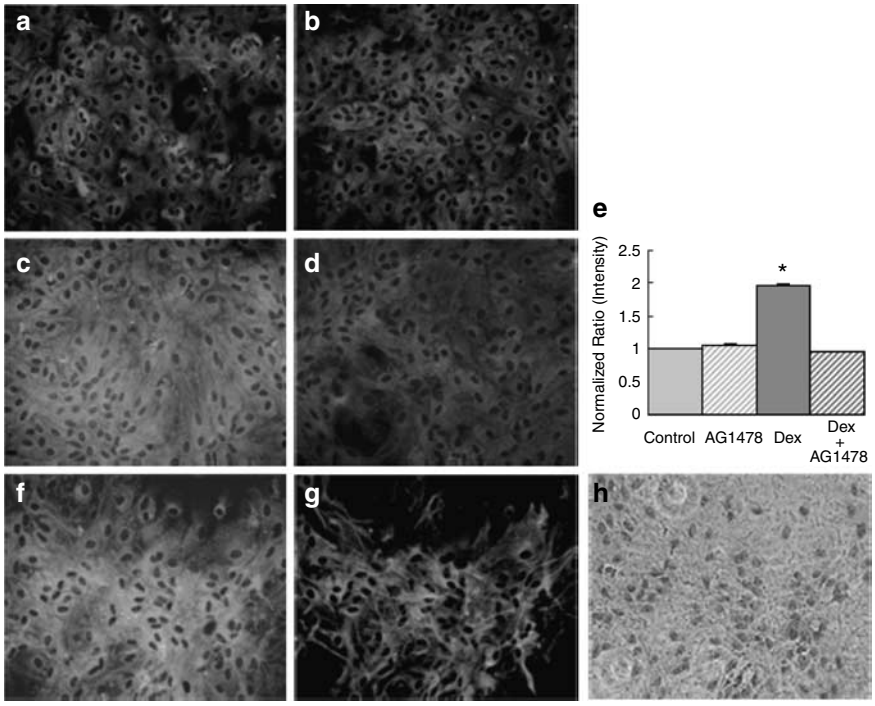

Figure 1 Immunofluorescence staining of phosphorylated $ERK_{1/2}$ in astrocyte cultures. After 20 min of incubation without any drug (**a**), with 1 μM AG 1478 (**b**), with 50 nM of dexmedetomidine (**c**) or with dexmedetomidine plus AG 1478 (**d**), cells were labelled with monoclonal antibody to phosphorylated ERK. Images were quantified with Image Pro Plus 6.0 software (**e**). Average values of p-ERK were obtained from three individual areas in each slice. s.e.m. values are indicated by vertical bars. *Indicates statistically significant ($P<0.05$) difference from control, AG 1478 or dexmedetomidine plus AG 1478 groups for p-ERK analysed by one-way ANOVA followed by Fisher's LSD test. Lack of nucleus translocation of p-ERK was determined by triple-staining with p-ERK (**f**), GFAP (**g**) and hematoxylin (**h**).

Western blotting for ERK and Fos family

Cells were harvested in 0.5 ml of ice-cold buffer (0.25 M sucrose, 10 mM HEPES, the phosphatase inhibitors α-mercaptoethanol (10 mM) and phenylmethyl sulphonyl fluoride (1 mM), and 1 mM sodium orthovanadate, pH 7.4). A whole cell lysate was prepared by homogenization. Protein content was determined by the Bradford method (Bradford, 1976), using bovine serum albumin as the standard. Samples containing 50 μg protein were applied on slab gels of 12% polyacrylamide. After transfer to nitrocellulose membranes, the samples were blocked by 5% skimmed milk powder in TBS-T (30 mM Tris–HCl, 125 mM NaCl, 0.1% Tween 20) for 2 h, and the nitrocellulose membranes were incubated with the first antibody, specific to either p-ERK, ERK, or Fos proteins for 1.5 h at room temperature. After washing, specific binding was detected by goat-anti-mouse or goat-anti-rabbit horseradish peroxidase-conjugated secondary antibody.

Staining was visualized by ECL detection reagents (Amersham Biosciences, Buckinghamshire, UK), followed by exposure to film (FuJi Photo Film Co., Ltd, Tokyo, Japan). The results were collected by Flurchem imaging system.

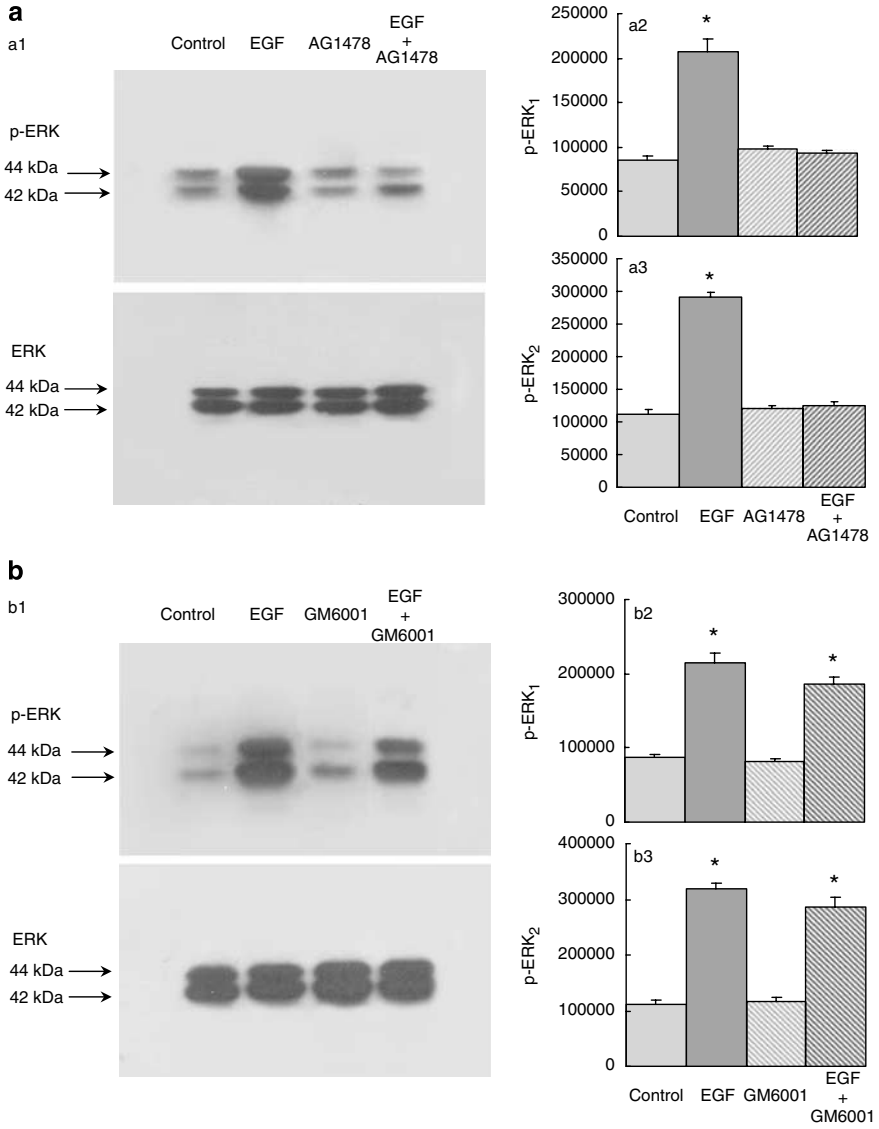

Figure 2 EGF-induced $ERK_{1/2}$ phosphorylation requires EGF receptor, but not Zn-dependent metalloproteinase, activation in astrocytes. Bands of 44 and 42 kDa represent phosphorylated ERK_1 (p-ERK_1) or ERK_1 and phosphorylated ERK_2 (p-ERK_2) or ERK_2, respectively. (**a**) After pretreatment with AG 1478 for 15 min, cells were incubated for 20 min in the absence of any drug (Control) or in the presence of 10 ng ml^{-1} of EGF, of 1 μM of AG 1478, an inhibitor of EGFR or of EGF plus AG 1478. (a1) Immunoblot from a representative experiment. Similar results were obtained from three independent experiments. All results are means ± s.e.m. of scanned band intensity of p-ERK_1 (a2) and p-ERK_2 (a3). *Indicates statistically significant ($P<0.05$) difference from control, AG 1478 or EGF plus AG 1478 groups for p-ERK_1 and p-ERK_2 analysed by one-way ANOVA followed by Fisher's LSD test. (**b**) After pretreatment with GM 6001 for 15 min, cells were incubated for 20 min in the absence of any drug (Control) or in the presence of 10 ng ml^{-1} of EGF, of 10 μM of GM 6001, an inhibitor of metalloproteinase or of EGF plus GM 6001. (b1) Immunoblot from a representative experiment. Similar results were obtained from three independent experiments. All results are means ± s.e.m. of scanned band intensity of p-ERK_1 (b2) and p-ERK_2 (b3). *Indicates statistically significant ($P<0.05$) difference from control or GM 6001 groups for p-ERK_1 and p-ERK_2 analysed by one-way ANOVA followed by Fisher's LSD test.

Band density was measured with Window AlphaEaseTM FC 32-bit software.

Immunoprecipitation and western blotting for EGFR

After homogenization, whole cell lysates (1000 μg) were incubated with 8 μg of anti-EGFR antibody (Upstate Biotechnology) for 12 h at 4 °C. Thereafter 200 μl of washed Protein G agarose bead slurry was added, and the mixture was incubated for another 2 h at 4 °C. The agarose beads were collected by pulsing centrifuge (5 s in the microcentrifuge at 14 000 *g*), the supernatant drained off and the beads boiled for 5 min. Thereafter, the supernatant was collected by pulsing centrifuge and the entire immunoprecipitates were subjected to 10% SDS-polyacrylamide gel electrophoresis (PAGE). After transfer to nitrocellulose membranes, the membranes were incubated with the first antibody, specific to either phosphotyrosine at 1 × 800 dilution or rabbit anti-EGFR antibody (Cell Signaling Technology) at 1 × 1000 dilution for 2 h at room temperature.

RT-PCR

For determination of mRNA expression of cfos and fosB by reverse transcription-PCR (RT-PCR), a cell suspension was prepared by discarding the culturing medium,

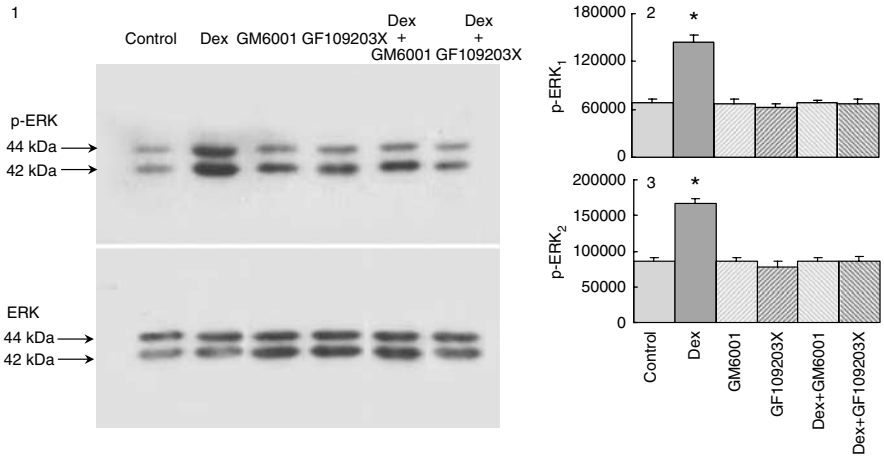

Figure 3 $ERK_{1/2}$ phosphorylation induced by dexmedetomidine requires Zn-dependent metalloproteinase and PKC activation in astrocytes. Bands of 44 and 42 kDa represent p-ERK_1 or ERK_1 and p-ERK_2 or ERK_2, respectively. After pretreatment with GM 6001 or GF 109203X for 15 min, cells were incubated for 20 min in the absence of any drug (Control) or in the presence of 50 nM of dexmedetomidine, of 10 μM of GM 6001, an inhibitor of metalloproteinase, of 500 nM of GF 109203X, an inhibitor of PKC, of dexmedetomidine plus GM 6001, or of dexmedetomidine plus GF 109203X. (**1**) Immunoblot from a representative experiment. Similar results were obtained from three independent experiments. All results are means ± s.e.m. of scanned band intensity of p-ERK_1 (**2**) and p-ERK_2 (**3**). *Indicates statistically significant ($P<0.05$) difference from control, GM 6001, GF 109203X, dexmedetomidine plus GM 6001 or dexmedetomidine plus GF 109203X groups for p-ERK_1 and p-ERK_2 analysed by one-way ANOVA followed by Fisher's LSD test.

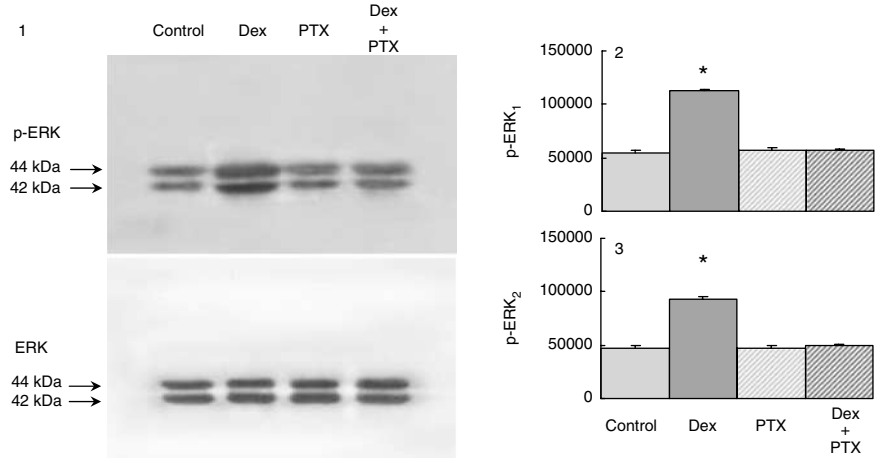

Figure 4 $ERK_{1/2}$ phosphorylation induced by dexmedetomidine requires function of βγ subunits of G_i protein in astrocytes. Bands of 44 and 42 kDa represent p-ERK_1 or ERK_1 and p-ERK_2 or ERK_2, respectively. After pretreatment with PTX for 15 min, cells were incubated for 20 min in the absence of any drug (Control) or in the presence of 50 nM of dexmedetomidine, of 0.2 μg ml^{-1} of PTX, an inhibitor of βγ subunits of G_i protein, or of dexmedetomidine plus PTX. (**1**) Immunoblot from a representative experiment. Similar results were obtained from four independent experiments. All results are means ± s.e.m. of scanned band intensity of p-ERK_1 (**2**) and p-ERK_2 (**3**). *Indicates statistically significant ($P<0.05$) difference from control, PTX, or dexmedetomidine plus PTX groups for p-ERK_1 and p-ERK_2 analysed by one-way ANOVA followed by Fisher's LSD test.

adding Trizol to cultures on ice and scraping the cells off the culture dish. The RNA pellet was precipitated with isopropanol, washed with 70% ethanol and dissolved in 10 μl sterile, distilled water and an aliquot was used for determination of the amount of RNA (Kong *et al*., 2002).

RT was initiated by a 5 min-incubation at 65 °C of 1 μg RNA extract with Random Hexamer at a final concentration of 12.5 ng l^{-1} deoxy-ribonucleoside triphosphates (dNTPs) (TaKaRa Biotechnology Co., Ltd., Dalian, China) at a final concentration of 0.5 mM. The mixture was rapidly chilled on ice and briefly spun, and 4 μl 5X first-strand buffer, 2 μl 0.1 M dithiotreitol and 1 μl RNaseOUT recombinant RNase inhibitor (40 U μl^{-1}) were added. After the mixture had been incubated at 42 °C for 2 min, 1 μl (200 U) of Superscript II (Gibco Life Technology Invitrogen, Grand Island, NY USA) was added, and the incubation at 42 °C continued for another 50 min. Subsequently the reaction was inactivated by heating to 70 °C for 15 min, and the mixture was chilled and briefly centrifuged. PCR amplification was performed in a Robocycler thermocycler with sense (5′-GCTGACAGATACACTCCAAGCGG-3′) and antisense (5′-AGGAAGACGTGTAAGTAGTGCAG-3′) for c-fos (Elkeles *et al*., 1999), with sense (5′-AAAAGGCAGAGCTGGAGTCGG-3′)

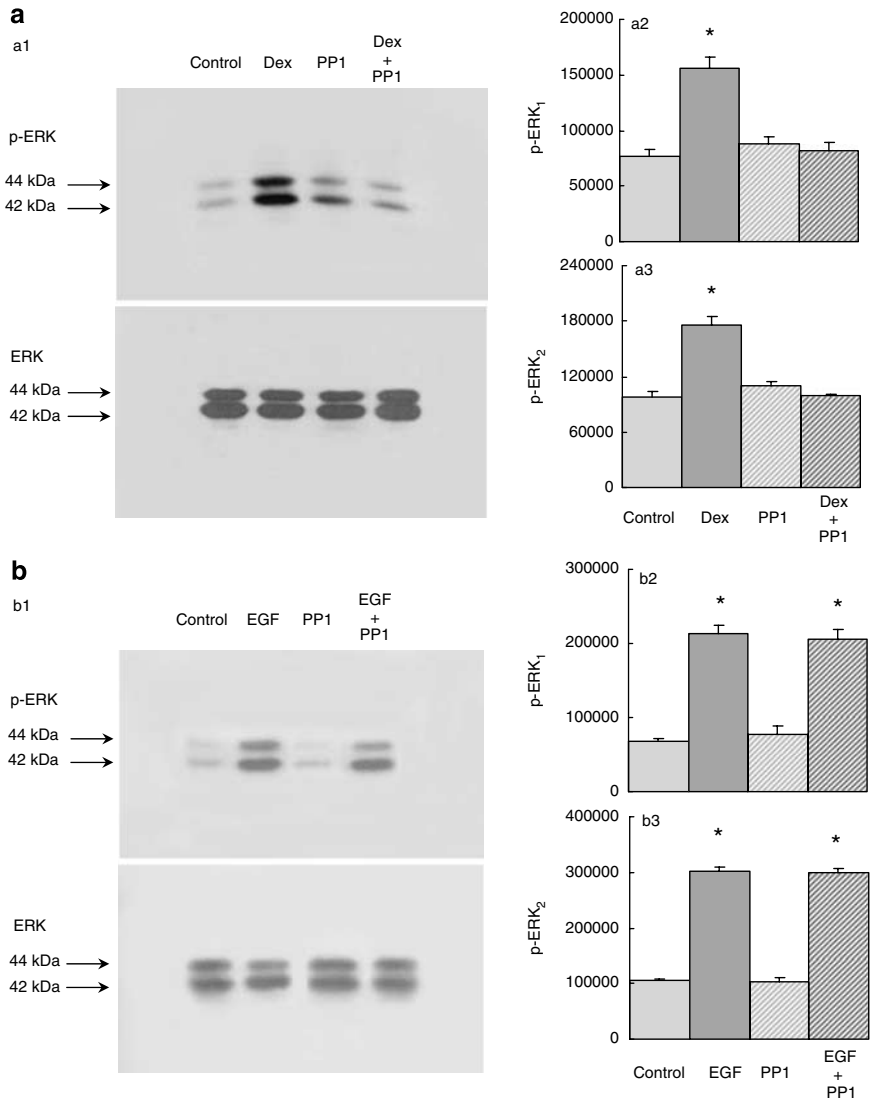

Figure 5 Src kinase is involved in dexmedetomidine-induced, but not EGF-induced $ERK_{1/2}$ phosphorylation in astrocytes. Bands of 44 and 42 kDa represent p-ERK_1 or ERK_1 and p-ERK_2 or ERK_2, respectively. (**a**) After pretreatment with PP1 for 15 min, cells were incubated for 20 min in the absence of any drug (Control) or in the presence of 50 nM of dexmedetomidine, of 10 μM of PP1, an inhibitor of Src kinase or of dexmedetomidine plus PP1. (a1) Immunoblot from a representative experiment. Similar results were obtained from three independent experiments. All results are means ± s.e.m. of scanned band intensity of p-ERK_1 (a2) and p-ERK_2 (a3). *Indicates statistically significant ($P<0.05$) difference from control, PP1, or dexmedetomidine plus PP1 groups for p-ERK_1 and p-ERK_2 analysed by one-way ANOVA followed by Fisher's LSD test. (**b**) After pretreatment with PP1 for 15 min, cells were incubated for 20 min in the absence of any drug (Control) or in the presence of 10 ng ml^{-1} of EGF, of 10 μM of PP1, an inhibitor of Src kinase or of EGF plus PP1. (b1) Immunoblot from a representative experiment. Similar results were obtained from three independent experiments. All results are means ± s.e.m. of scanned band intensity of p-ERK_1 (b2) and p-ERK_2 (b3). *Indicates statistically significant ($P<0.05$) difference from control or PP1 groups for p-ERK_1 and p-ERK_2 analysed by one-way ANOVA followed by Fisher's LSD test.

and antisense (5′-TGTACGAAGGGCTAACAACGG-3′) for fos B (Inoue *et al*., 2004), and with sense (5′-CCACGGACAACTG CGTTGAT-3′) and antisense (5′-GGCTCATAGCTACTGAAC TG-3′) for TATA-binding protein (TBP) (el-Marjou *et al*., 2000), used as a housekeeping gene. Initially the template was denatured by heating to 94 °C for 2 min, followed by thirty amplification cycles for c-fos and TBP, or by 35 cycles for fosB, each consisting of three periods, the first at 94 °C, the second at 60.8 °C for c-fos, at 59 °C for fosB or at 55 °C for TBP, and the third at 72 °C. The final step was extension at 72 °C for 10 min. The PCR products were separated by 1% agarose gel electrophoresis, and captured by Fluorchem 5500 (Alpha Innotech Corporation, San Leandro, CA, USA). The PCR products were confirmed by sequencing, performed by TaKaRa Biotechnology Co., Ltd., Dalian, China.

Statistics

The differences between individual groups were analysed by one-way ANOVA followed by Fisher's LSD test. The level of significance was set at $P<0.05$.

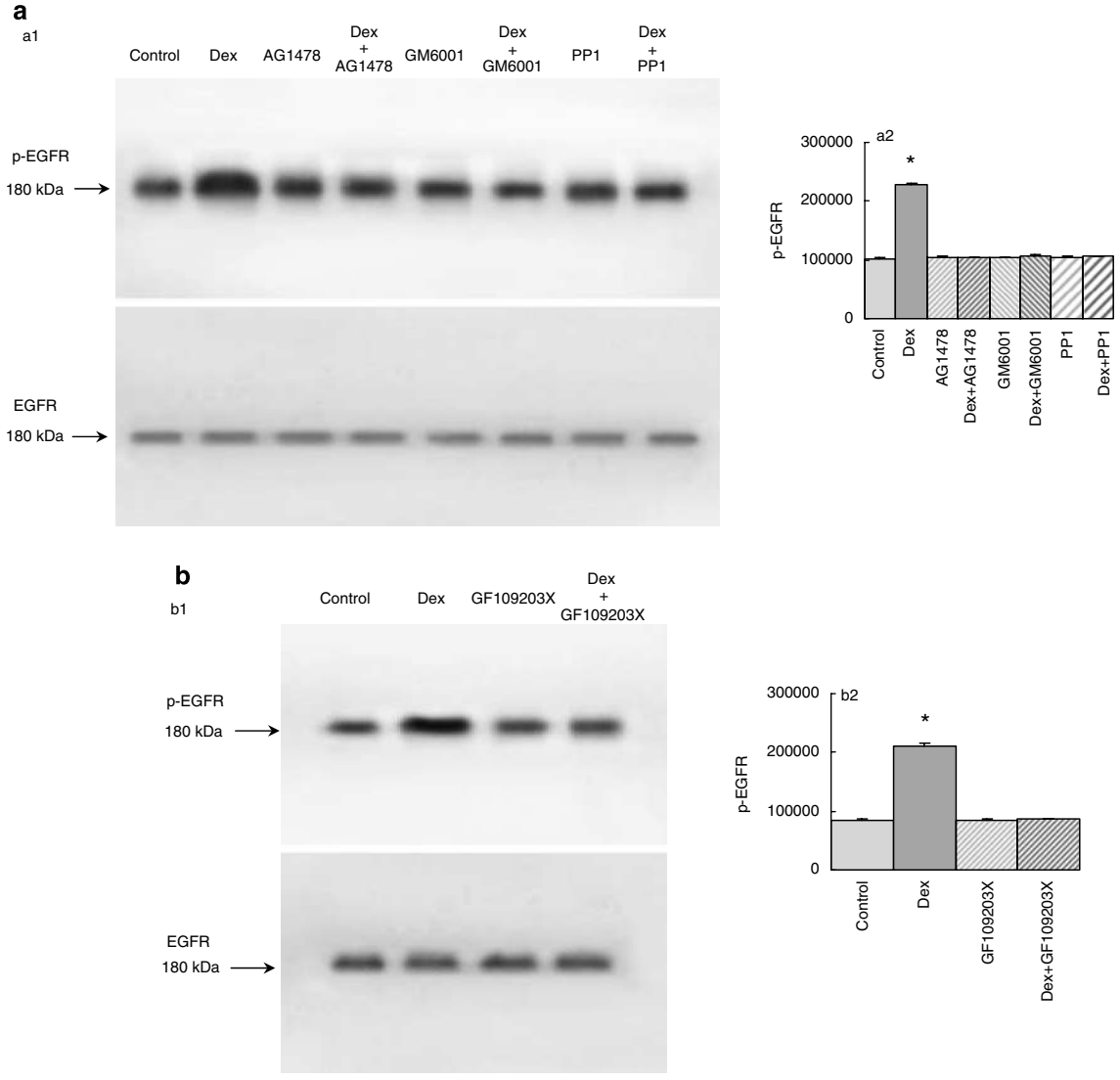

Figure 6 EGF receptor phosphorylation induced by dexmedetomidine in astrocytes. Bands of 180 kDa represent phosphorylated EGF receptor (p-EGFR) or EGF receptor (EGFR), respectively, in primary cultures of astrocytes. (**a**) After pretreatment with AG 1478, GM 6001 or PP1 for 15 min, cells were incubated for 10 min in the absence of any drug (Control) or in the presence of 50 nM dexmedetomidine, of 1 μM AG 1478, an inhibitor of RTK, of dexmedetomidine plus AG 1478, of 10 μM of GM 6001, an inhibitor of metalloproteinase, of dexmedetomidine plus GM 6001, of 10 μM of PP1, an inhibitor of Src kinase or of dexmedetomidine plus PP1. (a1) Immunoblot from a representative experiment. Similar results were obtained from three independent experiments. All results are means ± s.e.m. of scanned band intensity of p-EGFR (a2). *Indicates statistically significant ($P<0.05$) difference from control, AG 1478, dexmedetomidine plus AG 1478, GM 6001, dexmedetomidine plus GM 6001, PP1 or dexmedetomidine plus PP1 groups analysed by one-way ANOVA followed by Fisher's LSD test. (b) After pretreatment with GF 109203X for 15 min, cells were incubated for 10 min in the absence of any drug (Control) or in the presence of 50 nM of dexmedetomidine, of 500 nM of GF 109203X, an inhibitor of PKC, or of dexmedetomidine plus GF 109203X. (b1) Immunoblot from a representative experiment. Similar results were obtained from three independent experiments. All results are means ± s.e.m. of scanned band intensity of p-EGFR (b2). *Indicates statistically significant ($P<0.05$) difference from control, GF 109203X, dexmedetomidine plus GF 109203X groups analysed by one-way ANOVA followed by Fisher's LSD test.

Materials

Dulbecco's medium and horse serum were from Sigma (St Louis, MO, USA) and Gibco BRL (Grand Island, NY, USA), respectively. Chemicals for addition to the medium and most other chemicals, including PTX were purchased from Sigma. Tyrphostin AG 1478, GM 6001, GF 109203X and PP1 were obtained from Calbiochem (La Jolla, CA, USA). Santa Cruz Biotechnology (Santa Cruz, CA, USA) supplied first antibodies, raised against ERK (K-23):sc-94, against phosphorylated ERK (E-4):sc-7383 and against Fos proteins (H-237):sc-28213, the second antibody goat anti-rabbit IgG HRP conjugate, as well as secondary antibody TRITC-conjugated goat anti-mouse. Sigma (St Louis, MO, USA) supplied first antibody, raised against β-actin. For immunoprecipitation, first antibodies against EGF receptors (06–129) and against phosphotyrosine (PY20, 05–947), as well as Protein G agarose bead slurry (16–266) were purchased from Upstate Biotechnology (Lake Placid, NY, USA). The first antibody against EGF receptors (2232) used for western blotting was purchased from Cell Signaling Technology (Danvers, MA, USA). U0126 and the second antibody goat anti-mouse IgG HRP conjugate from Promega (Madison, WI, USA). Dexmedetomidine and atipamezole were kindly donated by Orion Pharma, Turku, Finland.

Results

Cytochemistry

In agreement with our previous findings using western blotting (Peng *et al*, 2003), staining intensity of phosphorylated $ERK_{1/2}$ after 20 min of drug treatment was much higher in cells treated with 50 nM dexmedetomidine than in control cells (Figure 1), as confirmed by quantification of staining intensity of p-ERK (Figure 1e) ($P<0.05$ by one-way ANOVA followed by Fisher's LSD test). There was no significant difference between control cells, cells treated with the EGF receptor RTK inhibitor AG 1478 at 1 μM and cells treated with dexmedetomidine plus AG 1478. Phosphorylated ERK showed cytoplasmic staining, that surrounded, but did not include, the nucleus (Figures 1f–h). Similar results were

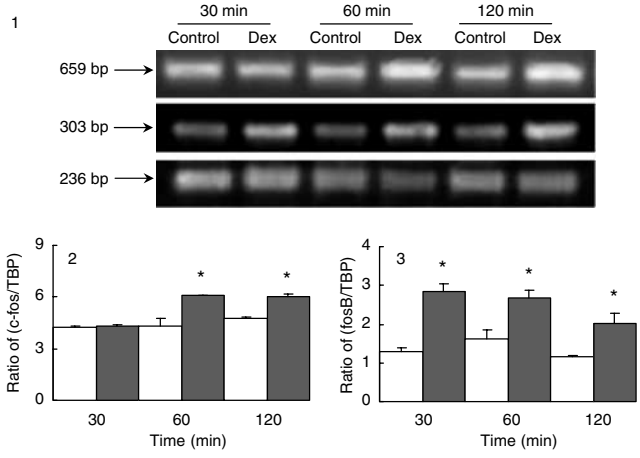

Figure 7 Dexmedetomidine increases mRNA expression of cfos and fosB in astrocytes. Cells were incubated for 30, 60 or 120 min in the absence of any drug (Control) or in the presence of 50 nM of dexmedetomidine (Dex). The size of PCR product of cfos is 659 bp, of fosB 303 bp and of TBP 236 bp. (**1**) Southern blot from a representative experiment. Similar results were obtained from three independent experiments. Average mRNA expression was quantitated as ratios between c-fos and TBP (**2**) and between fosB and TBP (**3**). s.e.m. values are indicated by vertical bars. *Indicates statistically significant ($P<0.05$) difference from control group for cfos and fosB analysed by one-way ANOVA followed by Fisher's LSD test.

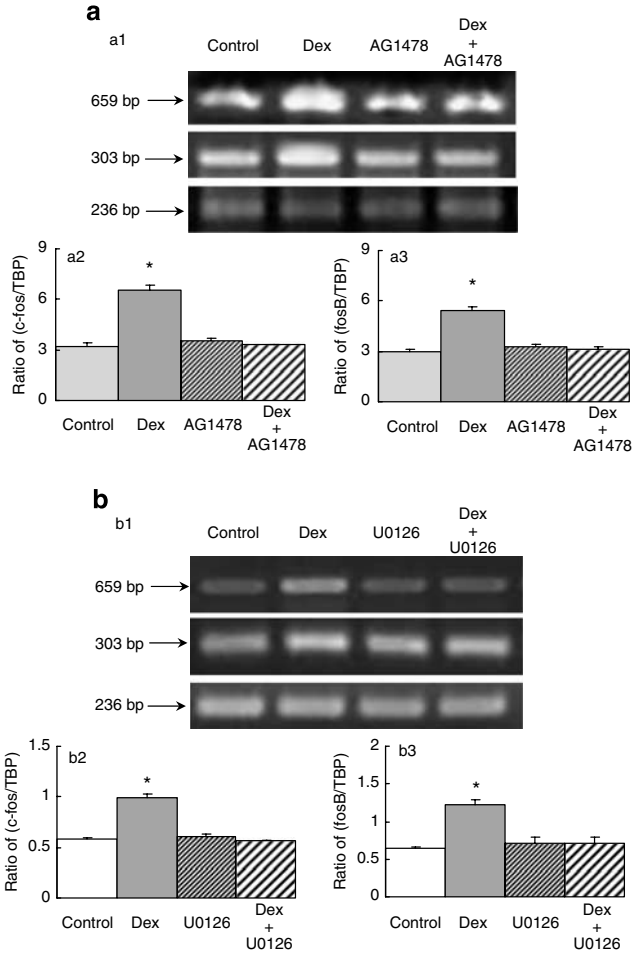

Figure 8 Dexmedetomidine-induced mRNA expression of cfos and fosB requires EGF receptor and ERK action in astrocytes. The size of PCR product of cfos is 659 bp, of fosB 303 bp and of TBP 236 bp. (**a**) Cells were incubated for 60 min in the absence of any drug (Control), in the presence of 50 nM of dexmedetomidine (Dex), of 1 μM of AG 1478, or of dexmedetomidine plus AG 1478. (a1) Southern blot from a representative experiment. Similar results were obtained from three independent experiments. Average mRNA expression was quantitated as ratios between cfos and TBP (a2) and between fosB and TBP (a3). s.e.m. values are indicated by vertical bars. *Indicates statistically significant ($P<0.05$) difference from control, AG 1478 or dexmedetomidine plus AG 1478 groups for cfos and fosB analysed by one-way ANOVA followed by Fisher's LSD test. (**b**) Cells were incubated for 60 min in the absence of any drug (Control), in the presence of 50 nM of dexmedetomidine (Dex), of 10 μM of U0126, an inhibitor of $ERK_{1/2}$ phosphorylation, or of dexmedetomidine plus U0126. (b1) Southern blot from a representative experiment. Similar results were obtained from three independent experiments. Average mRNA expression was quantitated as ratios between cfos and TBP (b2) and between fosB and TBP (b3). s.e.m. values are indicated by vertical bars. *Indicates statistically significant ($P<0.05$) difference from control, U0126 or dexmedetomidine plus U0126 groups for c-fos and fosB.

observed after 10 min, 1 and 2 h of incubation (results not shown).

EGF-induced $ERK_{1/2}$ phosphorylation
Western blots showed that 10 ng ml^{-1} of EGF caused a large increase of $ERK_{1/2}$ phosphorylation ($P<0.05$) in astrocytes after 20 min of exposure (Figure 2). A 44 kDa band represents ERK_1 and a 42 kDa band ERK_2. The stimulation by EGF was sensitive to 1 μM AG 1478, (Figure 2a), but not to 10 μM GM 6001, an inhibitor of Zn-dependent metalloproteinase (Figure 2b). This contrasts with the effect of 50 nM dexmedetomidine, which was abolished not only by AG 1478 (Figure 1) but also by GM 6001 (Figure 3).

Signalling pathways for dexmedetomidine
Figure 3 shows that 20 min of incubation with 50 nM dexmedetomidine induced a significant increase ($P<0.05$) of phosphorylation of $ERK_{1/2}$, which was inhibited by 10 μM GM 6001. A similar inhibition was evoked by 500 nM GF 109203X, an inhibitor of PKC. In contrast neither of these drugs had any effect in the absence of dexmedetomidine.

The inhibition by GF 109203X is consistent with evidence that dexmedetomidine activates the phosphatidylinositide second messenger system (Enkvist *et al*., 1996). It was therefore investigated whether blockade of the initial response to α_2-adrenergic stimulation, activation of G_i protein function, would also inhibit phosphorylation of $ERK_{1/2}$ induced by dexmedetomidine. We found that PTX (0.2 μg ml^{-1}) abolished this dexmedetomidine-induced phosphorylation, but had no effect under control conditions (Figure 4).

As Pierce *et al*. (2001) found Src kinase to be involved both prior to EGF receptor ligand release (stage 1) and during the response to the released ligand (stage 2) the effect of 10 μM PP1, an inhibitor of Src kinase, was studied during both dexmedetomidine- and EGF-induced $ERK_{1/2}$ phosphorylation. This inhibitor blocked dexmedetomidine-induced stimulation almost completely (Figure 5a), but had no effect on EGF-induced $ERK_{1/2}$ phosphorylation (Figure 5b).

Dexmedetomidine-induced EGF receptor phosphorylation
In agreement with the findings presented above regarding ERK phosphorylation, 50 nM dexmedetomidine induced EGF receptor phosphorylation ($P<0.05$), which could be inhibited by AG 1478, GM 6001, PP1 (Figure 6a) and GF 109203X (Figure 6b).

Effects of dexmedetomidine on expression of early genes
To evaluate downstream effects of $ERK_{1/2}$ phosphorylation, the expression of early genes was studied. mRNA expression of cfos and fosB are shown in Figures 7 and 8. The size of PCR product of cfos is 659 bp, of fosB 303 bp and of TBP, used as housekeeping gene, 236 bp. After 30, 60 and 120 min of treatment, dexmedetomidine at a concentration of 50 nM caused a significant increase of fosB mRNA expression (Figure 7) ($P<0.05$), whereas the expression of cfos mRNA showed no change until after 60 min of incubation. Both 1 μM AG 1478, an inhibitor of EGF receptor RTK (Figure 8a) and 10 μM U0126 (Figure 8b), an inhibitor of $ERK_{1/2}$ phosphorylation abolished the stimulation of c-fos and fosB gene expression after 120 min of drug treatment. In contrast, dexmedetomidine had no effect on mRNA expression of fra-1 and fra-2 (results not shown). Protein expression of cFos and FosB is shown in Figures 9 and 10. A 62 kDa band

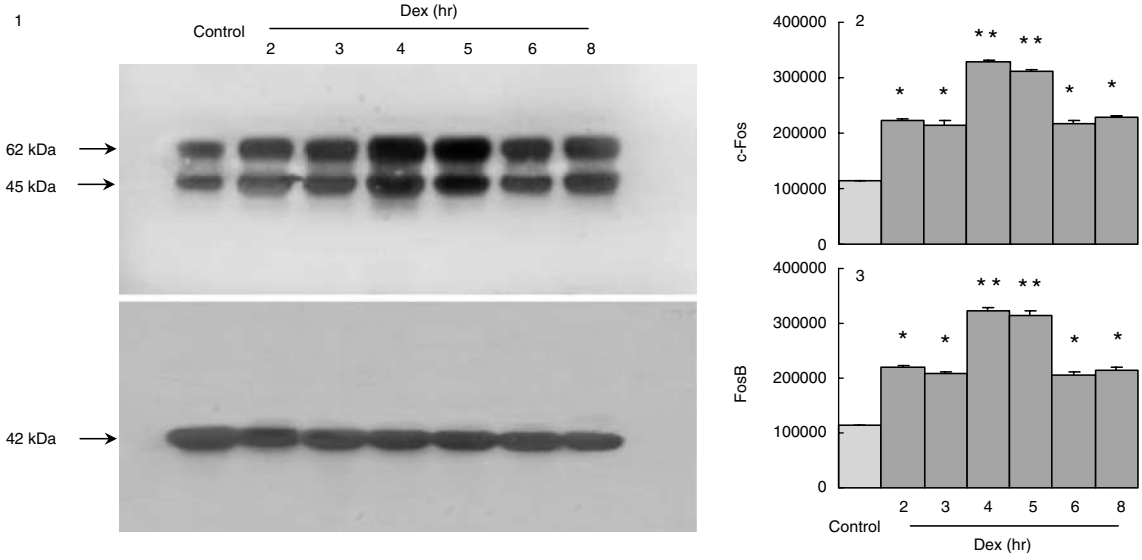

Figure 9 Dexmedetomidine stimulates protein expression of cFos and FosB in astrocytes. Bands of 62, 45 and 42 kDa represent cFos or FosB and β-actin, a housekeeping protein respectively. Cells were incubated for 4 h in the absence of any drug (Control) or for 2, 3, 4, 5, 6 or 8 h in the presence of 50 nM of dexmedetomidine (Dex). (**1**) Immunoblot from representative experiment. Similar results were obtained from three independent experiments. All results are means ± s.e.m. of scanned band intensity of cFos (**2**) and FosB (**3**). *Indicates statistically significant ($P<0.05$) difference from control group for cFos and FosB; **indicates statistically significant ($P<0.05$) difference from control, 2, 3, 6 and 8 h groups for cFos and FosB analysed by one-way ANOVA followed by Fisher's LSD test.

represents FosB, a 45 kDa band cFos and a 42 kDa band β-actin, a house-keeping gene (Figure 9). Both proteins were increased by dexmedetomidine at all times tested (2–8 h) ($P<0.05$). Again both AG 1478 and U0126 prevented the increased expression in the presence of dexmedetomidine (Figure 10).

Lack of dexmedetomidine-induced $ERK_{1/2}$ phosphorylation in neurons

In contrast to the findings in cultured astrocytes, 50 nM dexmedetomidine did not induce $ERK_{1/2}$ phosphorylation in cultured cerebellar granule neurons, a glutamatergic preparation, (Figure 11), whereas EGF at 10 ng ml^{-1} did induce significant ERK phosphorylation in these neuronal cells (Figure 12a) ($P<0.05$).

Induction of ERK phosphorylation in neurons by conditioned medium from dexmedetomidine-treated astrocytes

In contrast to conditioned medium from control astrocytes (in the absence of any drug treatment), conditioned medium from astrocytes treated with 50 nM dexmedetomidine during 10 min caused an increase of ERK phosphorylation ($P<0.05$) in cerebellar granule cells. This effect could not be inhibited by 300 nM atipamezole, a specific α_2-adrenoceptor antagonist (Peng *et al*., 2003) (Figure 12b).

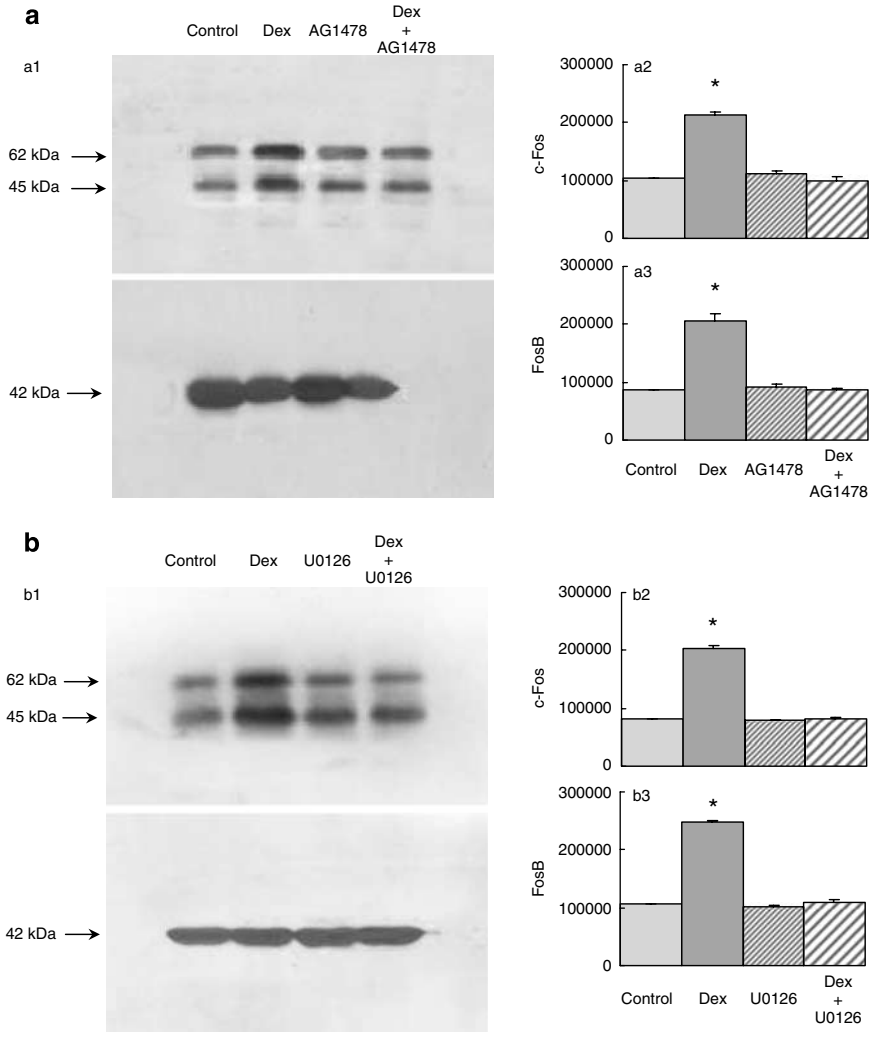

Figure 10 Dexmedetomidine-induced protein expression of cFos and FosB requires EGF receptor and ERK action in astrocytes. Bands of 62, 45 and 42 kDa represent cFos or FosB and β-actin respectively. (**a**) Cells were incubated for 4 h in the absence of any drug (Control), in the presence of 50 nM of dexmedetomidine (Dex), of 1 μM of AG 1478, or of dexmedetomidine plus AG 1478. (a1) Immunoblot from a representative experiment. Similar results were obtained from four independent experiments. All results are means ± s.e.m. of scanned band intensity of cFos (a2) and FosB (a3). *Indicates statistically significant ($P<0.05$) difference from control, AG 1478 or dexmedetomidine plus AG 1478 groups for c-Fos and FosB analysed by one-way ANOVA followed by Fisher's LSD test. (**b**) Cells were incubated for 60 min in the absence of any drug (Control), in the presence of 50 nM of dexmedetomidine (Dex), of 10 μM of U0126, an inhibitor of $ERK_{1/2}$ phosphorylation, or of dexmedetomidine plus U0126. (b1) Immunoblot from a representative experiment. Similar results were obtained from three independent experiments. All results are means ± s.e.m. of scanned band intensity of cFos (b2) and FosB (b3). *Indicates statistically significant ($P<0.05$) difference from control, U0126 or dexmedetomidine plus U0126 groups for cFos and FosB analysed by one-way ANOVA followed by Fisher's LSD test.

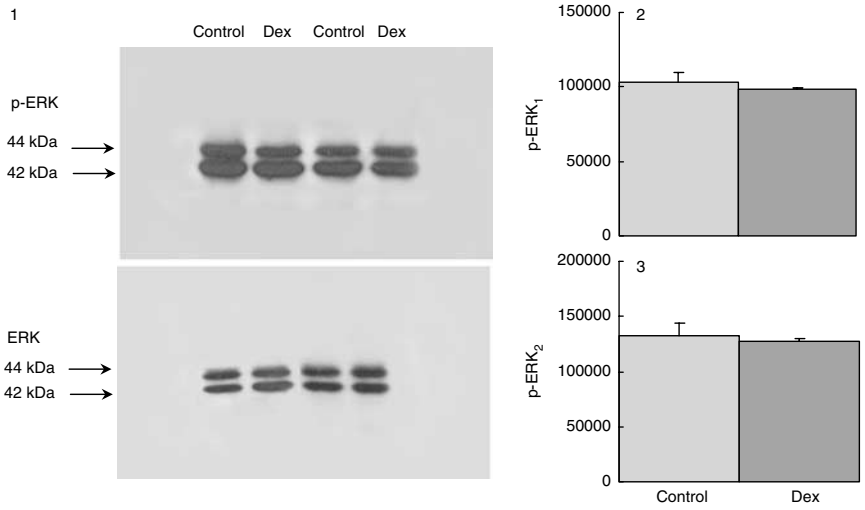

Figure 11 Dexmedetomidine fails to trigger $ERK_{1/2}$ phosphorylation in primary cultures of cerebellar neurons. Bands of 44 and 42 kDa represent p-ERK_1 or ERK_1 and p-ERK_2 or ERK_2, respectively. In (**1**), primary cultures of mouse cerebellar granule cells were incubated for 20 min in the absence of any drug (Control) or in the presence of 50 nM of dexmedetomidine (Dex). Immunoblots are two independent experiments. All results are means ± s.e.m. of scanned band intensity of p-ERK_1 (**2**) and p-ERK_2 (**3**).

Discussion

Signalling pathways leading to $ERK_{1/2}$ phosphorylation

The involvement of EGF receptors in $ERK_{1/2}$ phosphorylation caused by dexmedetomidine is in agreement with our previous findings (Peng *et al*., 2003) and with recent studies (V Prevot, personal communication) using different antibodies (the monoclonal antibody No 9101 and the polyclonal antibody No 9102 from Cell Signaling Technology Inc., Danvers, MA, USA) to recognize p-$ERK_{1/2}$, and $ERK_{1/2}$, and showing that both the TRK inhibitor tyrphostin AG 1478 and metalloproteinase inhibitor GM 6001 blocks the stimulation. As could be expected, $ERK_{1/2}$ phosphorylation by direct exposure to EGF was, in contrast only inhibited by AG 1478, not by GM 6001.

The inhibitory effect of PTX, an inhibitor of disassociation of $\beta\gamma$ subunits from $G_i\alpha$, indicates operation of G_i-coupled receptors via G_i-associated $\beta\gamma$ subunits, and it is in agreement with the findings of PTX-sensitive Ca^{2+} release from intracellular stores by α_{2A}-adrenorecptor stimulation in different cell types expressing this receptor spontaneously or after transfection (Dorn *et al*., 1997). This response is inhibited by U73122, an inhibitor of phospholipase C (PLC). The inhibitory effects of the PKC inhibitor, GF 109203X, is consistent with the concept that PLC activity is involved in dexmedetomidine-induced EGF receptor transactivation, because PLC activity is required for production of diacylglycerol (DAG), the endogenous activator of PKC. Phorbol esters, which activate all known PKC isoforms, have also been reported to cause 'shedding' of HB-EGF from cultured kidney cells (Izumi *et al*., 1998). In contrast, 'shedding' induced in prostate epithelial cells by Ca^{2+} ionophore, that is, further downstream, is not dependent on PKC activity (Dethlefsen *et al*., 1998). Although it has been reported that GF 109203X also had inhibitory effects on MAPKAP kinase-1b (Rsk-2), a substrate of ERK and p70 S6 kinase, a signal pathway in parallel with or regulated by MAP pathway (Alessi, 1997), inhibition of GF 109203X on dexmedetomidine-induced EGF receptor phosphorylation further indicates the involvement of PKC on 'shedding' of growth factors.

The complete inhibition by GM 6001 of dexmedetomidine-induced $ERK_{1/2}$ phosphorylation in astrocytes indicates that metalloproteinase-dependent 'shedding' of growth factors quantitatively accounts for the phosphorylation of $ERK_{1/2}$. This represents a difference from transfected COS-7 cells, which display both transactivation-dependent and transactivation-independent $ERK_{1/2}$ phosphorylation (Pierce *et al*., 2001). Another difference between COS-7 cells and astrocytes is that Src kinase activity in the COS-7 cells is required both for growth factor 'shedding' (stage 1) and during the response to the growth factor (stage 2). However, in astrocytes, the Src kinase inhibitor PP1 inhibited $ERK_{1/2}$ phosphorylation induced by dexmedetomidine, but not that induced by EGF, indicating that the response to the growth factor is Src kinase-independent.

Signalling pathway downstream of $ERK_{1/2}$ phosphorylation

The exclusively cytoplasmic staining of p-$ERK_{1/2}$ shows that there was no translocation of p-$ERK_{1/2}$ into the nucleus, in spite of the observations that mRNA and protein expression of cfos and fosB were upregulated by dexmedetomidine. Similar phenomena have been observed in immortalized GT1-7 cells during transactivation of their EGF receptors by gonadotropin-releasing hormone, when p90 ribosomal S6 kinase (RSK), a substrate of $ERK_{1/2}$, but not $ERK_{1/2}$ itself, was translocated into nucleus (Shah *et al*., 2003).

cfos and fosB were upregulated by dexmedetomidine at both mRNA and protein levels, whereas there was no change in gene expression of fra-1 and fra-2. The upregulation of cfos and fosB could be abolished by AG 1478 and by the inhibitor of $ERK_{1/2}$ phosphorylation U0126, indicating the requirement for both EGF receptor and ERK. Induction of cfos mRNA in retinal Müller cells by EGF has also been observed by Sagar *et al*. (1991). These findings indicate the potential role of dexmedetomidine in regulation of gene

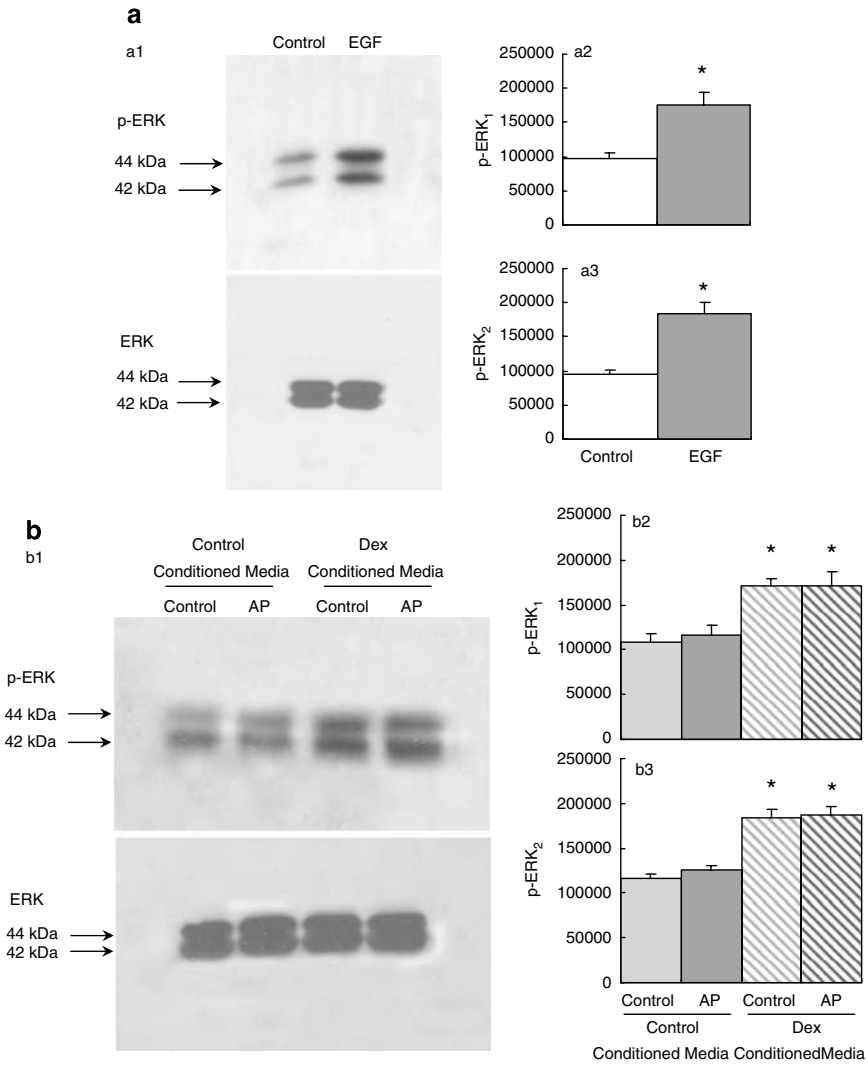

Figure 12 ERK phosphorylation induced by EGF or conditioned medium from astrocytes treated with dexmedetomidine in cerebellar neurons. Bands of 44 and 42 kDa represent p-ERK$_1$ or ERK$_1$ and p-ERK$_2$ or ERK$_2$, respectively. In (**a**), primary cultures of mouse cerebellar granule cells were incubated for 10 min in the absence of any drug (Control) or in the presence of 10 ng ml^{-1} of EGF. (a1) Immunoblot from a representative experiment. Similar results were obtained from four independent experiments. All results are means ± s.e.m. of scanned band intensity of p-ERK$_1$ (a2) and p-ERK$_2$ (a3). *Indicates statistically significant ($P<0.05$) difference from control group for p-ERK$_1$ and p-ERK$_2$ analysed by one-way ANOVA followed by Fisher's LSD test. In (**b**), cells were incubated in conditioned medium from astrocytes treated without any drug (Control) or with 50 nM dexmedetomidine (Dex) for 10 min in the absence or presence of 300 nM atipamezole, an antagonist of α_2 adrenoreceptors. (b1) Immunoblot from a representative experiment. Similar results were obtained from four independent experiments. All results are means ± s.e.m. of scanned band intensity of p-ERK$_1$ (b2) and p-ERK$_2$ (b3). *Indicates statistically significant ($P<0.05$) difference from control or atipamezole groups for p-ERK$_1$ and p-ERK$_2$ analysed by one-way ANOVA followed by Fisher's LSD test.

expression. It will be important to know the types of regulated genes and their functions, as they may represent the underlying mechanisms of neuronal protection.

Lack of dexmedetomidine response in cultured neurons

As cerebellar granule cells in primary cultures express both HB-EGF and TGF-α and respond to glutamatergic stimulation with transactivation (Gu *et al*., 2007) the absence of dexmedetomidine-promoted ERK phosphorylation in cultured cerebellar granule neurons may indicate an absence of postsynaptic α_2-adrenoceptors in these cells. This conclusion is supported by the observation that they also show no increase in free cytosolic Ca^{2+} concentration ($[Ca^{2+}]_I$) in response to dexmedetomidine (Zhao *et al*., 1992). Nevertheless, *in situ* hybridization has shown mRNA for α_2-adrenoceptors in human cerebellar granule cells *in situ* (Schambra *et al*., 2005), and α_2-adrenoceptor activation enhances dendrite growth and reduces the phosphorylation of microtubule-associated protein in cultured cerebral cortical neurons obtained from 15-day-old mouse embryos (1 week before term) and grown in culture for a very short time (1–4 days) (Song *et al*., 2004). However, conditioned medium from astrocytes treated with dexmedetomidine did cause ERK phosphorylation in these neurons, and this effect could not be inhibited by the α_2-adrenergic inhibitor atipamezole, indicating that neuroprotection by dexmedetomidine *in vivo* may be mediated by members of the EGF family released

from astrocytes, that is, EGF, HB-EGF or TGF-α, which are expressed in astrocytes (Du *et al*., 2007) and could thus be involved. Further studies of possible dexmedetomidine effects, mediated by the drug itself or by an astrocytically released EGF agonist, on neurons of different types at different developmental stages and under different conditions are therefore warranted to further determine direct or indirect effects on neurons.

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Conflict of interest

The authors state no conflict of interest.

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