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# Dexmedetomidine causes neuroprotection via astrocytic $\alpha_2$-adrenergic receptor stimulation and HB-EGF release

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## Abstract

**Background:** Dexmedetomidine is a specific agonist of the $\alpha_{2A}$-adrenoceptor with sedative, analgesic, neuro-protective, and anti-delirious effects. At clinically relevant concentrations, it stimulates both inhibitory auto-receptors and post-junctional receptors found on astrocytes, but not on neurons. The stimulated pathway releases epidermal growth factor receptor (EGFR) agonist(s) that can act on all brain cell types. Dexmedetomidine's ability to improve treatment and prognosis in critically ill patients in the ICU is clinically relevant.

**Methods:** Dexmedetomidine's neuro-protectant mechanisms of action were tested during oxidative damage, using cultured astrocytes and the very vulnerable glutamatergic cerebellar granule neurons. Primary cultures of cerebellar granule neurons prepared from 7-day-old CD-1 mice were cultured for 8 days, and primary cultures of astrocytes from newborn CD-1 mice for 3 weeks until full maturation. Cell viability during extended incubation with and without $H_2O_2$ was tested by a methylthiazoletetrazolium (MTT) assay, and released heparin-binding epidermal growth factor (HB-EGF) in medium from astrocyte cultures measured by sandwich ELISA.

**Results:** Dexmedetomidine administration directly to $H_2O_2$-exposed neurons had no cyto-protective effect. Conditioned medium from astrocytes treated for 30-120 min with 50 nM dexmedetomidine increased neuronal survival by >50%, provided astrtocytic $\alpha_2$-adrenoceptors were not atipamezole-inhibited. Dexmedetomidine's protective effect was also prevented when neuronal treatment with astrocyte-conditioned medium took place in the presence of AG 1478, inhibiting neuronal EGF receptors.

**Conclusion:** At clinically relevant concentrations dexmedetomidine is neuro-protective against oxidative damage by stimulating astrocytic $\alpha_2$-adrenoceptors, causing release of HB-EGF. HB-EGF in turn activates neuronal EGF receptors. At these concentrations dexmedetomidine has no direct neuronal effect.

**Keywords**: Astrocyte, dexmedetomidine, EGF receptor, $H_2O_2$, neuron, transactivation

## Introduction

Dexmedetomidine is a specific agonist of the $\alpha_2$-adrenergic receptor [1]. Unlike other sedatives it has both sedative and analgesic effects [2]. It can dramatically reduce post-operative requirements for analgesics [3]. Used alone, its combined analgesic/sedative effect makes it a preferred drug for awake craniotomy [4-7]. Recently, it has attracted considerable interest by its ability to improve treatment and prognosis in patients who are critically ill following severe brain trauma [8,9]. Concerns have occasionally been raised about its effects on the cardiovascular system, but at conventional doses all physiological and metabolic parameters are similar in brain-injured patients treated with either dexmedetomidine or propofol [10]. Dexmedetomidine's effects can be inhibited by the highly specific $\alpha_2$-adrenergic antagonist atipamezole [1].

Originally all dexmedetomidine activities were supposed to be caused by inactivation of presynaptic $\alpha_2$-adrenergic receptors inhibiting noradrenergic activity. However, this interpretation is oversimplified. Dexmedetomidine concentrations well below 100 nM exert prominent effects on cultured astrocytes [11,12], and the $\alpha_2$-adrenoceptor is densely expressed in astrocytes freshly isolated from mouse brain by fluorescence-activated cell sorting [13]. Thus, instead of receiving a subtype-mixed noradrenergic signal from locus coeruleus the cells are specifically activated at their $\alpha_2$-adrenoceptor sites by the drug.

The $\alpha_2$-adrenergic signaling pathway has been studied in cultured astrocytes [12,14]. It connects activation of $\alpha_2$-adrenoceptors with ERK phosphorylation in two-stages, separated by trans-activation of the epidermal growth factor (EGF) receptor. This receptor is highly expressed in both neurons and astrocytes, but in freshly isolated brain cells its mRNA expression is highest in astrocytes (B. Li, L. Hertz and L. Peng, unpublished experiments). In the first stage, the βγ subunits of the activated heterotrimeric $G_i$ protein lead, via activation of

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cytosolic Src tyrosine kinases, to metalloproteinase-mediated 'shedding' of heparin-binding epidermal growth factor (HB-EGF) from its transmembrane-spanning HB-EGF precursor. In the second stage, released HB-EGF 'transactivates' EGF receptors in the same and adjacent cells (including neurons) by phosphorylating EGF receptors, leading to Ras- and Raf-dependent ERK phosphorylation [12,14]. The astrocytic effects may contribute to dexmedetomidine's analgesic effects, at least in the spinal cord [15,16]. Dexmedetomidine has repeatedly been found to have neuro-protective effects against ischemia in experimental models [17]. This has not been confirmed clinically. It is able to protect against trauma in hippocampal organotypic cultures [18].

The present study was carried out to show if the neuro-protective effects of dexmedetomidine are secondary to its stimulation of astrocytic $\alpha_2$-adrenergic receptors. Based on knowledge that oxidative damage contributes greatly to post-traumatic brain injury [19,20], oxidative neuronal injury was induced with $H_2O_2$ in the glutamatergic cerebellar granule neurons. The hypothesis was tested that 'conditioned' medium from dexmedetomidine-treated astrocyte cultures would enhance neuronal viability due to release of an EGF receptor agonist, whereas direct administration of dexmedetomidine to neurons or treatment with non-conditioned medium would have no effect. Furthermore, it was examined if the protection found after addition of medium from dexmedetomidine-treated astrocytes was abolished by treatment of the astrocytes with the specific $\alpha_2$-adrenergic antagonist atipamezole. This was confirmed, but atipamezole addition directly to $H_2O_2$-exposed neurons treated with dexmedetomidine had no effect.

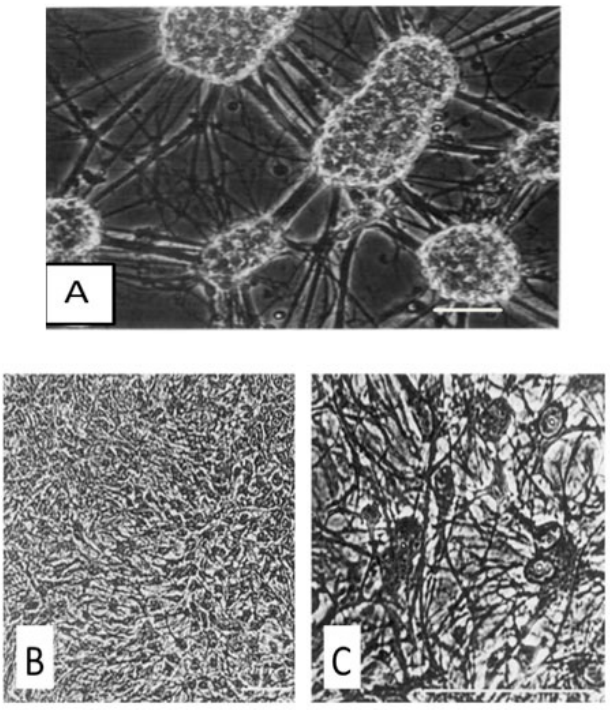


**Figure 1. Photomicrographs of the culture types used.** **A**. Phase-contrast micrograph of living primary culture of 8-day-old granule cell neurons [from 22]. Bar = 50 μm. **B** and **C**. Phase-contrast micrographs of 4-week-old primary cultures of astrocytes from neonatal mouse brain grown in the presence of 0.25 mM dBcAMP from the age of 2 weeks shown at low magnifications (**B**) and higher magnifications (**C**) [from 24]. At each magnification the bar equals 200 μm.

## Methods

### Reagents

Chemicals for medium preparation and most other chemicals were purchased from Sigma (St. Louis, MO, USA). Tyrphostin AG 1478 (N-[(2R)-2-(hydroxamidocarbonymethyl)-4-methylpentanoyl]-L-tryptophanmethylamide) (an inhibitor of the EGF receptor-tyrosine kinase [21]) was obtained from Calbiochem (La Jolla, CA, USA). Dexmedetomidine (D-[4-[(1S)-1-(2,3-dimethylphenyl)ethyl]-1H-imidazole]) was purchased from Chenchuang Pharmaceutical Company (Jinan, China), $H_2O_2$ from Yongqiang Medical Pharmaceutical Company (Shenyang, China), and HB-EGF ELISA kit from Uscnlife Science & Technology Co., Ltd. (Wuhan, China). Atipamezole was purchased from Sigma (St. Louis, MO, USA).

### Cell cultures

Primary cultures of neurons were prepared from 7-day-old CD-1 mice [12,22]. These cultures were chosen because they are the only virtually homogenous glutamatergic cultures and more sensitive to damage than GABA-ergic cultures. A phase contrast micrograph is shown in **Figure 1A**. As can be seen, the cultures contain virtually no astrocytes (less than 5 % of all cells [22]). These cultures were prepared by rapid decapitation of mouse pups and subsequent removal of their brains. The cerebella were aseptically separated from the remainder of the brain. 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 24 well plates. The cultures were grown for 7 days in Sigma's DMEM culturing medium (catalog number D5030), modified by increase of the glucose concentration to 30 mM and of the $K^+$ concentration to 24.5 mM and decrease of the glutamine concentration to 0.8 mM. Seven percent horse serum was added. The elevation of the $K^+$ concentration is necessary for normal development of the cells [22,23], and cell viability is better with 0.8 mM glutamine than with the standard concentration of 2 mM. The increased glucose concentration enables culturing without medium change, which is not well tolerated. 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.

Primary cultures of astrocytes were obtained from newborn CD-1 mice. The neopallia of the cerebral hemispheres were aseptically isolated, vortexed to dissociate the mainly

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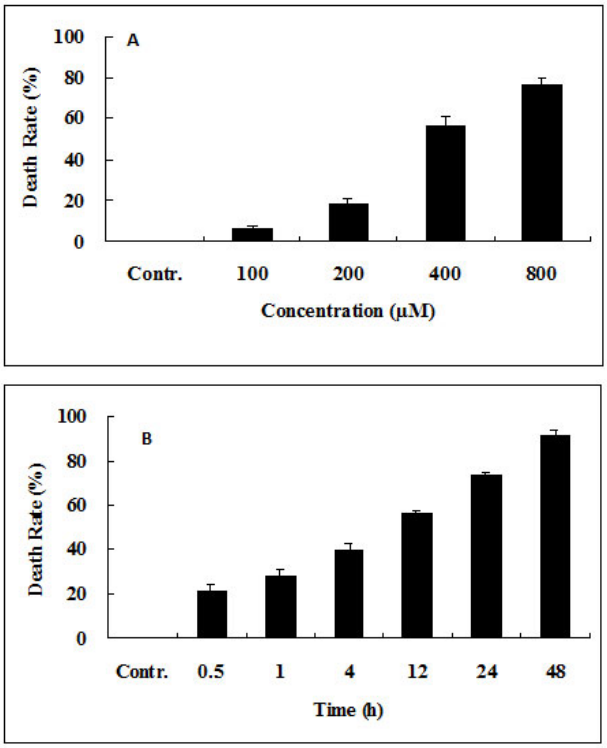


**Figure 2. Concentration and time dependency of $H_2O_2$ neurotoxicity.**
Cerebellar granule neurons in primary cultures were treated with different concentrations of $H_2O_2$ for 12 hrs (**A**) or with 400 μM $H_2O_2$ for different time periods (**B**). Cell death was monitored with an MTT assay. Each column represents percentage cell death relative to that in neurons not exposed to $H_2O_2$. It shows the mean ± SEM of 3 individual experiments, each performed in triplicate using 3 cultures from the same 24-well microplate. Except for 0.5 h with a $p<0.05$, all values in Figures. **A** and **B** differ from the controls with $p<0.01$ or better.

cerebrocortical tissue, and filtered through nylon meshes with pore sizes of 80 μm and subsequently 10 μm, diluted in culture medium. All cultures were planted in Falcon Primaria culture dishes. The culture medium was DMEM with 7.5 mM glucose, initially containing 20% horse serum. The cultures were incubated at 37°C in a humidified atmosphere of $CO_2$/air (5:95%). The medium was replaced 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 2 weeks, 0.25 mM dibutyryl cyclic AMP (dBcAMP) was added to the medium. Such cultures are known to be highly enriched (>95% purity) in glial fibrillary acidic protein (GFAP-) and glutamine synthetase expressing astrocytes [24]. Results obtained with these cultures replicate those in freshly isolated astrocytes [25]. They were used after at least 3 weeks of culturing. A phase contrast micrograph of such cultures [24] at high and low magnification shows the absence of neurons (**Figure 1B and C**).

## Cell viability assay

Cell viability was tested by a methylthiazoletetrazolium (MTT) colorimetric assay [26]. This assay measures the metabolic activity of mitochondria. Initially neuronal sensitivity to MTT was tested in the 7-day-old cultures by i) incubation with different concentrations of $H_2O_2$ for 12 hrs (**Figure 2A**); and ii) incubation for different lengths of time at 400 μM $H_2O_2$ (**Figure 2B**). The incubation was carried out in tissue culture medium without serum after addition of 1/10 volume of MTT solution (5 mg/mL in phosphate-buffered saline [PBS]) at 37°C. The reaction was stopped with the addition of a DMSO solution. The absorbance (A) was measured with a microplate reader at a test and reference wavelength of 570 and 630 nm, respectively. Percentage cell death was determined as $(1-[A_{treated}/A_{control}]) \times 100$, and survival as $[A_{treated}/A_{control}]) \times 100$.

## Drug treatment

When the effect of dexmedetomidine was tested on neuronal cultures, 50 nM was added to cultures incubated in 1 ml DMEM at 37°C 30 min before the addition of $H_2O_2$. In some cases a relevant concentration (300 μM) of the specific $\alpha_2$-adrenergic antagonist atipamezole [1] was also present from the start of the incubation. The dexmedetomidine concentration of 50 nM was chosen in order to use a concentration within the range of those determined in studies of its neuro-protective effect [27,28]. It is also consistent with the ability of 25-50 nM dexmedetomidine to cause maximum ERK phosphorylation and $[Ca^{2+}]_i$ in astrocytes [12,29] and inability of dexmedetomidine concentrations up to 10 μM to increase $[Ca^{2+}]_i$ in pure cultures of neurons [30]. Astrocytes also respond to higher concentrations of dexmedetomidine with an increase in $[Ca^{2+}]_i$. However, this results from stimulation of imidazoline receptors, which are irrelevant at therapeutic levels of dexmedetomidine [30]. The effect of astrocyte-conditioned medium (ACM), with or without dexmedetomidine (and in some cases atipamezole), was tested in a similar manner (see also below). To test the involvement of EGF receptor pathway, an inhibitor of the EGF receptor-tyrosine kinase (1 μM AG 1478), was added to the neuronal cultures. This occurred 15 min before ACM obtained from astrocyte cultures treated with dexmedetomidine was added. When astrocyte-conditioned medium was tested, the astrocytes were incubated at 37°C for a specific length of time in DMEM without serum in the absence (control), or presence, of 50 nM dexmedetomidine or 50 nM dexmedetomidine plus AG1478 or atipamezole. Thereafter, 1 ml ACM was collected and transferred to neuronal cultures.

## Determination of HB-EGF

After astrocyte cultures had been incubated for 30 min at 37°C in DMEM without serum in the absence (control) or presence of dexmedetomidine the medium was collected. HB-EGF measurement in the supernatants was performed by sandwich ELISA using matched antibody pairs and an ELISA kit containing all wells and solutions mentioned below. All

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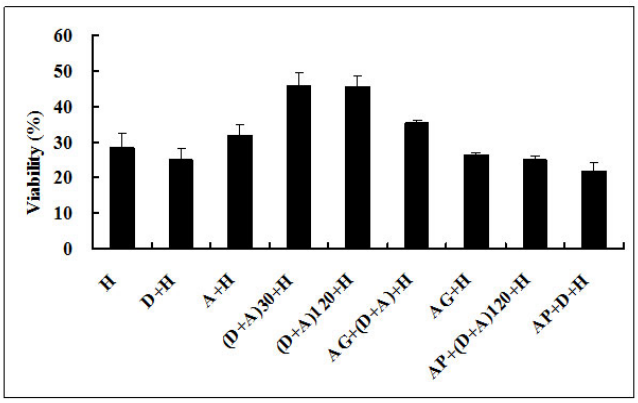


**Figure 3. Neuroprotection by dexmedetomidine against $H_2O_2$ cytotoxicity requires conditioned medium from dexmedetomidine-treated astrocytes with functioning $\alpha_2$-adrenergic receptors as well as neuronal EGF receptor signaling.**
Cerebellar granule neurons in primary cultures were treated with 400 μM $H_2O_2$ for 12 hrs in the presence or absence of additional drug(s). Cell viability was monitored with an MTT assay, with each column representing percentage cell survival, not cell death as in **Figure 2.** When dexmedetomidine was added the concentration was 50 nM, and with the exception of AG1478 and atipamezole, all drug or medium additions were made 30 min before exposure to $H_2O_2$. Astrocyte-conditioned medium was 1 ml. H: $H_2O_2$ exposure for 12 hrs. (n=7). D+H: Direct addition of dexmedetomidine (n=7). A+H: Addition of 30-min-astrocyte-conditioned medium (n=7). (D+A)30+H: Addition of 30-min-dexmedetomidine-treated astrocyte-conditioned medium (n=7). (D+A)120+H: Addition of 120-min-dexmedetomidine-treated astrocyte-conditioned medium (n=3). AG+(D+A)+H: Addition of 30-or-120-min-dexmedetomidine-treated astrocyte-conditioned medium 15 min after addition of 1 μM AG 1478 (n=4). AG+H: Addition of 1 μM AG 1478 15 min before addition of $H_2O_2$ (n=3). AP+(D+A)120+H: Addition of 300 nM atipamezole to 120-min-dexmedetomidine-treated astrocyte-conditioned medium 15 min before the beginning of the incubation of the astrocytes (n=4). AP+D+H: Addition of 300 nM atipamezole to neuronal cultures 15 min before addition of $H_2O_2$ (n=4). All relevant differences are at P<0.05 or less: (D+A)30+H and (D+A)120+H are significantly different from all other conditions, except AG+(D+A)+H at P<0.002 or P<0.001, with the only exceptions that the significance level between (D+A)120+H and H is at P<0.032 and those between AG+(D+A)+H and (D+A)120+H and (D+A)30+H are at P<0.047 and P<0.012, respectively. Results after dexmedetomidine treatment for 30 and 120 min were not significantly different from each other (P=0.93).

standards, controls, and samples were run in triplicate in each assay. Initially, 100 μl of experimental sample, or of different dilutions of the standard or blank was dispended into ELISA kit wells precoated with appropriate antibodies. The wells were incubated at 37°C for 2 hrs to allow completion of antigen/antibody reaction. All liquids were removed, and 100 μl of 'Detection Reagent A working solution' was added to each well followed by incubation for 1 h at 37°C. After aspiration of this solution and 3 times wash with 'Wash Solution', 100 μl of 'Detection Reagent B working solution' was added at to each well. This was followed by incubation for 30 min at 37°C, aspiration of the solution and 5 additional washes with 'Wash Solution'. After addition of 90 μl of 'Substrate Solution' to each well and incubation for 15-25 min at 37°C, 50 μl of 'Stop Solution' was added. Measurements were immediately carried out at 450 nm on the microplate reader.

### Statistical analysis
All values were expressed as mean ± SEM. Comparison between two data sets was analyzed by student's t-test, whereas differences between multiple groups were analyzed by analysis of variance (ANOVA) followed by Fisher's LSD test. *P* values of less than 0.05 were considered statistically significant.

## Results
### $H_2O_2$ cytotoxicity
Exposure to $H_2O_2$ induced neuronal death, the extent of which, depended upon concentration of $H_2O_2$ (**Figure 2A**) and length of incubation time (**Figure 2B**). Cell death reached ~80% (~20% survival) during incubation with 800 μM $H_2O_2$ for 12 hrs (**Figure 2A**) and ~60% with 400 μM $H_2O_2$ for 12 hrs, (**Figure 2B**). Based on these results incubation with 400 μM $H_2O_2$ for 12 hrs was chosen to study the effect of dexmedetomidine on cell survival.

### Dexmedetomidine effects
Addition of the pharmacologically relevant 50 nM dexmedetomidine concentration directly to the neuronal cultures did not enhance cell survival (**Figure 3**). Astrocyte-conditioned medium alone had also no significant effect on neuronal survival. However, conditioned medium from astrocytes treated for either 30 or 120 min (**Figure 3**) with 50 nM dexmedetomidine significantly improved neuronal survival. Conditioned astrocyte medium harvested after 15 min of incubation had no neuro-protective effect (results not shown). Addition of atipamezole [**1**] 15 min before the beginning of the incubation of the astrocytes abolished the neuro-protective effect of treatment with medium harvested from astrocytes treated with dexmedetomidine. In contrast, atipamezole caused no change when added directly to $H_2O_2$/dexmedetomidine-treated neuronal cultures.

The protective effect on neurons by dexmedetomidine was inhibited if the astrocyte-conditioned medium contained AG 1478, an inhibitor of EGF receptors (**Figure 3**). We therefore investigated whether the astrocyte-conditioned medium contained an EGF receptor agonist, focusing on HB-EGF, based on a previously shown inhibition of dexmedetomidine-induced ERK phosphorylation by heparin [**11**,**12**]. Under control conditions astrocyte-conditioned medium contained 20±8.1 (n=3) pmol/ml HB-EGF after 30 min of incubation in serum free medium. In the presence of dexmedetomidine

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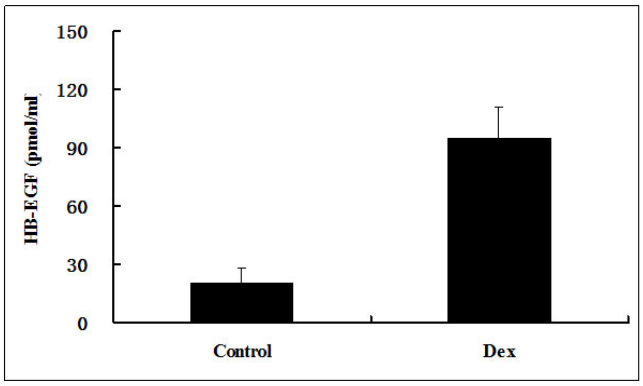


**Figure 4. Dexmedetomidine induces HB-EGF release in astrocytes culture**.
Astrocytes were treated with 50 nM dexmedetomidine and incubated at 37 $^0$C for 30 min. The culture medium was collected and HB-EGF was measured with an ELISA assay, using matched antibody pairs according to the manufacturer's protocol. All standards, controls, and (DEX) experimental samples were run in triplicate. Dexmedetomidine is significantly (P<0.05) different from control group.

(50 nM) the content of HB-EGF increased to 95±16.0 (n=3) pmol/ml (P<0.006) (**Figure 4**).

## Discussion

$H_2O_2$ caused time and dose-dependent death in primary cultures of neurons. Cell death was inhibited by conditioned medium from astrocytes treated with 50 nM dexmedetomidine. This concentration stimulates only $\alpha_2$-adrenergic receptors [29]. The neuro-protective effect was prevented by the presence of atipamezole during the incubation of the astrocytic cultures. Addition of 50 nM dexmedetomidine directly to the neuronal cultures had no protective effect, and additional exposure to atipamezole did not alter this outcome. This is in agreement with previous findings that dexmedetomidine has also no effect on phosphorylation of $ERK_{1/2}$ in similar neuronal cultures [12]. The inhibition of the neuro-protective effect by administration of the EGF receptor inhibitor AG1478 to the neuronal cultures indicates that the beneficial effect of dexmedetomidine-conditioned astrocytic medium depends upon the release of an EGF receptor agonist and its subsequent effect on neurons. As mentioned in 'Introduction' the EGF receptor is also highly expressed in astrocytes, where its phosphorylation by dexmedetomidine can be inhibited by the $\alpha_2$-adrenoceptor antagonist atipamezole [11]. In contrast, addition of atipamezole directly to cerebellar granule neurons does not prevent the EGF receptor activation exerted after addition of medium from dexmedetomidine-treated astocytes [12]. These observations further eliminate any possibility that the presence of dexmedetomidine in the astrocyte-conditioned medium could play any role in the neuro-protection.

The agonist present in the astrocyte-conditioned medium is probably mainly or exclusively HB-EGF, which was recovered from this medium. There is precedent for a two-cell-type co-operation in neuro-protection. Another high-molecular growth factor, midkine, is neuro-protective in cultured human neurons. When co-injected in mouse brain together with kainic acid it protects against kainate-induced neuronal death and epileptic activity. In cultures it is only released from astrocytes and in kainate-treated brain it is induced only in astrocytes [31,32].

HB-EGF is mitogenic for many cell types, mediated through activation of the EGF receptor [33]. It is markedly expressed in developing brain and induces cortical neuronal migration during brain development [35,36]. It also increases neurogenesis in adult and aged animals [37,38]. Its expression is increased in many cell types in response to tissue damage [37,39], hypoxia [36], oxidative stress [40,41], and experimental stroke [42]. A neuro-protective effect has been demonstrated experimentally during brain injury in adult animals [36,43,44], but dexmedetomidine-induced neuro-protection has presently not been confirmed clinically. A high spontaneous plasma level after ischemic stroke seems to be associated with a poorer prognosis [45], possibly because it might be caused by greater initial damage. Microglia freshly obtained from mouse brain by FACS also expresses $\alpha_2$-adrenergic receptors [13], and stimulation of cultured microglial cells (with a phospholipase) , leads to release of HB-EGF from a membrane-bound precursor [46]. This response is more complicated than that of astrocytes to dexmedetomidine stimulation, reflecting either cell type differences or differences between the stimulating agents (or both). Thus, down-stream responses could be inhibited not only by the usual inhibitors of the EGF/ERK phosphorylation pathways, but also by TAPI-1, an inhibitor of the metalloproteinase ADAM 17. They included an increased expression of the cytokine TNF-α [46], a suggestion of inflammation. The expression of ADAM17 is increased by ischemia in rat brain slices and in mixed cortical cultures, where its stimulation accounts for most of the release of TNF-α [47,48]. In the hypoxic cultures TNF-α was mainly localized in microglia, although a few astrocytes were included [48]. Dexmedetomidine signaling in cultured astrocytes does not involve ADAM17 [14]. Furthermore, dexmedetomidine at the presently used concentration causes only a slight reduction of TNF-α release in microglia activated by lipopolysaccharide (LPS) [49]. Dexmedetomidine's ability to counteract up-regulation of inflammatory molecules in murine macrophages is even less potent [50]. It is therefore not likely that a reported especially pronounced beneficial effect of dexmedetomidine on a subgroup of delirious, septic and mechanically ventilated patients [51] should be due to an effect on microglia. This concept is further supported by an observation in a rat model of monoarthritis, where dexmedetomidine blocks thermal hyperalgesia and very significantly reduces activation of astrocytes, but not of microglia [16]. Since this is an *in vivo* observation it should be noted that key points of the dexmedetomidine-activated $\alpha_2$-adrenergic pathway determined in astrocytes, have been

qualitatively and quantitatively confirmed in the brain *in vivo* and in brain slices [52].

## Conclusion

Dexmedetomidine shows a neuro-protective effect, which is due to $\alpha_2$-adrenoceptor-stimulated release of HB-EGF from astrocytes, and subsequent activation of neuronal EGF receptors. A neuro-protective effect has not been confirmed clinically, but in certain situations dexmedetomidine is an anaesthetic of choice. Further clarification is needed of the role(s) of astrocytes under these conditions.

### Competing interests

The authors declare that they have no competing interests.

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