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Dexmedetomidine produces its neuroprotective effect via the α_{2A}-adrenoceptor subtype

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Abstract

Which of the three α_2-adrenoceptor subtypes of α_{2A}, α_{2B}, or α_{2C} mediates the neuroprotective effect of dexmedetomidine was examined in cell culture as well as in an in vivo model of neonatal asphyxia. Dexmedetomidine dose-dependently attenuated neuronal injury (IC_{50}=83±1 nM) in neuronal-glial co-cultures derived from wild-type mice; contrastingly, dexmedetomidine did not exert neuroprotection in injured cells from transgenic mice (D79N) expressing dysfunctional α_{2A}-adrenoceptors. An α_{2A}-adrenoceptor subtype-preferring antagonist 2-[(4,5-Dihydro-1*H*-imidazol-2-yl)methyl]-2,3-dihydro-1-methyl-1*H*-isoindole maleate (BRL44408) completely reversed dexmedetomidine-induced neuroprotection, while other subtype-preferring antagonists 2-[2-(4-(2-Methoxyphenyl)piperazin-1-yl)ethyl]-4,4-dimethyl-1,3-(2H,4H)-isoquinolindione dihydrochloride (ARC239) (α_{2B}) and rauwolscine (α_{2C}) had no significant effect on the neuroprotective effect of dexmedetomidine in neuronal-glial co-cultures. Dexmedetomidine also protected against exogenous glutamate induced cell death in pure cortical neuron cultures assessed by flow cytometry and reduced both apoptotic and necrotic types of cell death. Likewise this neuroprotective effect was antagonised by BRL44408 but not ARC239 or rauwolscine. Dexmedetomidine exhibited dose-dependent protection against brain matter loss in vivo (IC_{50}=40.3±6.1 μg/kg) and improved the neurologic functional deficit induced by the hypoxic-ischemic insult. Protection by dexmedetomidine against hypoxic-ischemic-induced brain matter loss was reversed by the α_{2A}-adrenoceptor subtype-preferring antagonist BRL44408; neither ARC239 nor rauwolscine reversed the neuroprotective effect of dexmedetomidine in vivo. Our data suggest that the neuroprotective effect of dexmedetomidine is mediated by activation of the α_{2A} adrenergic receptor subtype.

Keywords: Neonatal asphyxia; Cell culture; α_2-Adrenoceptor antagonist; Neuronal injury; Neurological function

1. Introduction

Perinatal cerebral hypoxia-ischemia remains a frequent cause of chronic neurological morbidity. Estimates suggest that between 2 and 4/1000 full-term newborn infants suffer asphyxia at or shortly before birth (Vannucci and Perlman, 1997). Various biochemical pathways contribute to the development of hypoxic-ischemic brain injury, including oxygen-free radical formation, release of excitatory neurotransmitters (including glutamate and catecholamines) and consequent elevation of intracellular calcium, culminating in excitotoxic neuronal death (Vannucci and Palmer, 1997). High levels of norepinephrine exhibit detrimental effects on neuronal tissue (Stein and Cracco, 1982); furthermore, the norepinephrine surge which occurs during birth, especially in the setting of asphyxia, may compromise the defence of the fetus resulting in neonatal asphyxia (Lagercrantz and Slotkin, 1986). Because similar pathogenic mechanisms have been invoked in the development of acute or chronic brain injuries in adults (e.g., stroke, head injury or neurodegenerative diseases), models of neonatal asphyxia have proven useful in seeking effective therapies for these devastating conditions.

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We, and others, have previously reported on the ameliorative effect that α_2-adrenoceptor agonists, especially dexmedetomidine, exhibit in acute neuronal injury (Hoffman et al., 1991a; Hoffman et al., 1991b; Maier et al., 1993; Halonen et al., 1995; Kuhmonen et al., 1997; Jolkkonen et al., 1999; Laudenbach et al., 2002). Conversely, pretreatment with the nonselective α-adrenoceptor antagonist phentolamine is associated with a reduced ability to survive anoxia (Yuan et al., 1997), stressing the functional significance of α_2-adrenoceptors in resistance to hypoxic-ischemic brain damage. The reason for the neuroprotective effect of dexmedetomidine is thought to be due to its action in attenuating the massive release of catecholamines that occurs with cerebral hypoxic-ischemia in multiple parts of the brain (Globus et al., 1988; Globus et al., 1989; Matsumoto et al., 1993); this action may be mediated by pre-synaptic α_2-adrenoceptos (Harsing and Vizi, 1991).

Molecular cloning has led to the identification of three independent genes encoding α_2-adrenoceptor subtypes, termed α_{2A}, α_{2B}, and α_{2C}, which are ubiquitously distributed (Calzada and De Artinano, 2001). We (Lakhlani et al., 1997) and others (Callado and Stamford, 1999; Hein et al., 1999; Hein, 2001) have suggested that the α_{2A} adrenoceptor subtype modulates the release of catecholamines; in that case, this same receptor subtype may be responsible for the neuroprotective properties of α_2 agonists.

Using both in vitro and in vivo models of perinatal neuronal injury, we investigated which of the α_{2A}-, α_{2B}-, or α_{2C} adrenoceptor subtypes contributes to the neuroprotective effects of dexmedetomidine. In vitro mixed glial-neuronal co-cultures exposed to combined oxygen and glucose deprivation were used to simulate the environment associated with ischemic neuronal death in vivo (Koh and Choi, 1987). This approach affords precise control of temperature, pH, and O_2/CO_2 tension, each of which may independently affect the degree of injury (Choi, 1990). Lactate dehydrogenase (LDH) release was used as an indicator of neuronal injury. Furthermore, pure neuronal cultures were exposed to pathological levels of glutamate and cell viability was assessed by flow cytometry using Annexin V and propidium iodide staining to distinguish between apoptotic and necrotic cell death, respectively. In vivo we then employed the unilateral common carotid artery ligation method which is a validated as a model of cerebral hypoxia-ischemia (Vannucci et al., 1999; Vannucci et al., 2001). In rat pups, this procedure exposes the neurons to an environment not dissimilar from that seen with neonatal asphyxia (Andine et al., 1990). Brain weight deficit of the ipsilateral hemisphere (either as an absolute change or as a ratio of the unaffected contralateral hemisphere) was used as a measure of brain injury and correlates with the loss of evoked responsiveness, enzymatic neuronal markers and tissue destruction as evaluated by tissue histopathology (Roohey et al., 1997). Herein we demonstrate the neuroprotective effects of dexmedetomidine in these various models and characterise the adrenoceptor subtype dependence of this effect via pharmacological and transgenic methods.

2. Materials and methods

This study conforms to the UK Animals (Scientific Procedures) Act of 1986 and the Home Office (UK) approved the study protocol.

2.1. In vitro experiments

2.1.1. Cell culture

The methodology for preparing mixed cortical cell cultures containing both neuronal and glial cell elements in mice was reported elsewhere (Wilhelm et al., 2002). Briefly, whole cerebral neocortices (devoid of the hippocampal formation and basal ganglia) were prepared from early postnatal (days 1–2) pups of three different strains of mice: BALB/c, C57B6 or D79N transgenic mice (kindly donated by Professor Lee Limbird, Department of Pharmacology, Vanderbilt University, Nashville, TN). The D79N mice have a dysfunctional α_{2A}-adrenoceptor gene on a C57B6 genetic background. After trypsination and resuspension, cells were plated at a density of 6.25×10^4 cells/cm^2 on 24-multiwell plates (Costar, Cambridge, MA) and cultured in a medium consisting of Eagle's minimum essential medium augmented with 20 mM glucose, 26 mM $NaHCO_3$, 10% fetal bovine serum, 10% heat-inactivated horse serum, antibiotic-antimycotic solution (Gibco, Paisley, UK), 2 mM glutamine (Sigma, Poole, UK) and 10 ng/ml murine epidermal growth factor (EGF) (GibcoBRL). Glial cells reached confluence about 1 week after plating (Fig. 1A). Cortical neuronal cells were obtained from fetal BALB/c, C57B6 or D79N mice at 14–16 days of gestation and plated at a density of 1.25×10^5 cells per cm^2 on the confluent monolayer of glial cells derived from the corresponding genetic strain. Neuronal cells reached confluence 1 week after plating (Fig. 1B). BALB/c pure cortical neuronal cultures were also prepared for use in flow cytometry using a modified technique employed previously (Choi et al., 1987). The neurons were seeded into 24-multiwell plates pre-coated with poly-L-lysine and fed with neurobasal medium (Gibco) with the addition of B27 supplement (×1) and glutamine (25 μM). The mixed glial-neuronal cells and the pure neurons were used at 14 ± 1 and 8 ± 1 days, respectively. All cultures were maintained at 37 °C in a humidified 95% air–5% CO_2 atmosphere.

2.1.2. Pathological stimulation

Mixed neuronal-glial cultures were exposed to oxygen and glucose deprivation (OGD) and pure cortical neuronal cultures were exposed to 300 μM glutamate to provoke injury. The method for OGD-induced neuronal injury was as previously reported (Ma et al., 2003a); briefly, prior to OGD exposure the cultures were carefully washed twice with

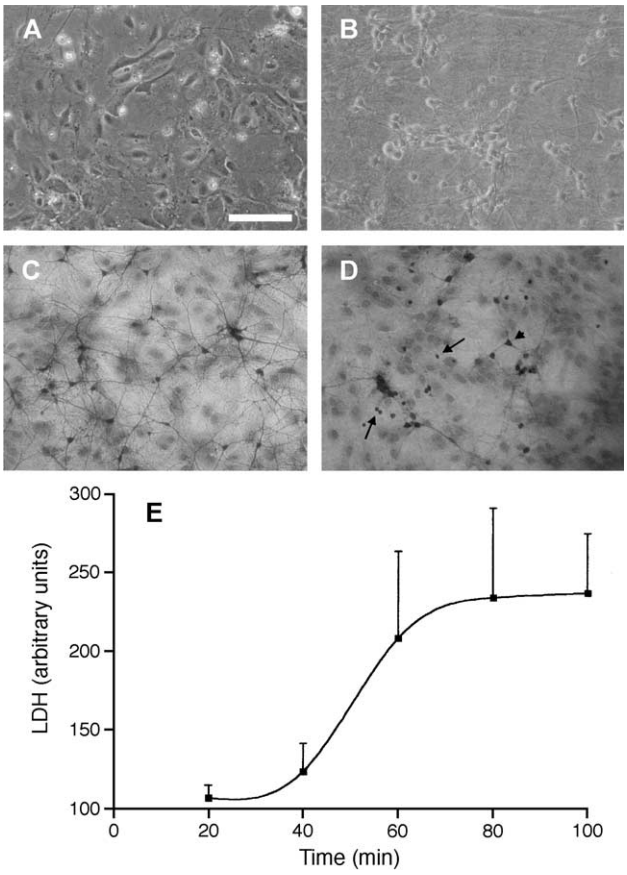

Fig. 1. Morphology of live cells (A, B) or cells fixed with 4% paraformaldehyde followed by cresyl violet staining (C, D) viewed under phase-contrast microscopy. (A) Glial cells—1 week after plating; (B) neurons—1 week after plating on top of a glial bed. (C) Control cells; (D) cells exposed to oxygen and glucose deprivation (OGD) for 75 min followed by a 6-h recovery period. The arrow and arrow head in D indicate pyknotic, and normal neurons, respectively. Bar=100 μm. (E) Time course of lactate dehydrogenase (LDH) release following oxygen and glucose deprivation. Co-cultures of neuronal and glial cells were deprived of oxygen and glucose for varying periods of time (0–100 min). Release of LDH into the culture medium was measured 6 h later.

HEPES buffer. Thereafter, the pre-warmed (37 °C), deoxygenated balanced salt solution was added to each well. Immediately after this replacement, cell cultures were transferred into a temperature-controlled anaerobic chamber (95% N_2, 5% CO_2) for periods varying between 20 and 100 min at 37 °C to establish that the optimal exposure time for maximal injury is 75 min (Fig. 1E) which has been confirmed morphologically (Fig. 1D) when compared with the normal control (Fig. 1C). Therefore, 75-min period of OGD was used in all subsequent experiments. Exposure to these experimental conditions was terminated by exchanging with oxygenated Eagle's minimal essential medium (MEM). The cultures were returned to a normoxic incubator (95% air, 5% CO_2) for the subsequent 6 h at 37 °C (this time period is required for LDH release to reach its maximal level after injury) (Wilhelm et al., 2002).

To examine the subtype dependence of the possible neuroprotective effect of dexmedetomidine, cells were exposed to various α_2-adrenoceptor ligands (Docherty, 1998) in media (either BSS during OGD or experimental MEM post-OGD) at appropriate doses. The ligands used in experiments were: dexmedetomidine, 0.001–10 μM, subtype-nonpreferring α_2 agonist (Orion Pharm, Finland); 2-[(4,5-Dihydro-1*H*-imidazol-2-yl)methyl]-2,3-dihydro-1-methyl-1*H*-isoindole maleate (BRL44408), 100 μM, an α_{2A} subtype-preferring antagonist (α_{2A} anti); 2-[2-(4-(2-Methoxyphenyl)piperazin-1-yl)ethyl]-4,4-dimethyl-1,3-(2*H*,4*H*)-isoquinolindione dihydrochloride (ARC239), 100 μM, an α_{2B} subtype preferring antagonist (α_{2B} anti); rauwolscine, 100 μM, an α_{2C} subtype-preferring antagonist (α_{2C} anti; Tocris, Avonmouth, UK); yohimbine, 100 μM or atipamezole, 100 μM subtype-nonpreferring α_2 antagonists (Sigma). The doses of dexmedetomidine were chosen from pilot studies to effect the desired pharmacologic action and the doses chosen for its antagonist was based on an antagonist/agonist binding affinity ratio for the individual receptor subtype (Renouard et al., 1994; Svensson et al., 1996). For example, dexmedetomidine displays 5- and 11-fold affinity for the α_{2A} adrenoceptor over the α_{2B} and α_{2C} adrenoceptor subtype, respectively (MacDonald et al., 1997); while rauwolscine displays a 6-fold selectivity for the human α_{2C}-adrenoceptor subtype over the α_{2A}-adrenoceptor subtype (Uhlen et al., 1994; Uhlen et al., 1998), though in the rat this difference is 30-fold (Uhlen et al., 1992).

Pure cortical neuronal cultures were exposed to glutamate (250 μM) for 10 min, a stimulus previously shown to induce sublethal neurotoxicity (Wilhelm et al., 2002). Prior to exposure, cells were washed with HEPES buffer (120 mM NaCl, 5.4 mM KCl, 0.8 mM $CaCl_2$, 15 mM glucose and 20 mM HEPES titrated to pH 7.4 using 1 M NaOH). Glutamate exposure was conducted in room air at 22±2 °C with cells in neurobasal media with or without dexmedetomidine (10 μM) or antagonists as above. After 10 min, cells were washed and replaced in neurobasal media with supplement as above. The cells were then returned to the incubator in a normoxic environment for 24 h at 37 °C.

2.1.3. Assessment of neuronal injury: mixed neuronal-glial cultures

Neuronal injury was quantified by the amount of lactate dehydrogenase (LDH) released into the medium during a 6-h period following the termination of OGD exposure using a standardised colorimetric enzyme kit (Sigma). Previous experiments have reported that this convenient and quantitative index linearly correlates with the degree of loss assessed by cell counts using morphological criteria (Koh and Choi, 1987; Goldberg et al., 1987). LDH activity in each sample (medium collected from each well) was measured in triplicate by spectrophotometric measurements (absorbance at 450 nm) at room temperature using a microwell plate reader and a software package (MRX II and Revelation, Dynex Technologies, UK). The amount of LDH released by sham-washed controls (reflecting non-injury provoked spontaneous release) was subtracted to

yield OGD-induced LDH release. The effect of the intervention was calculated by the following equation:

$$\frac{\text{OGD} + \text{intervention} - \text{induced release} - \text{spontaneous release}}{\text{OGD} - \text{induced release} - \text{spontaneous release}}$$

2.1.4. Assessment of neuronal injury: pure neuronal cultures

Twenty-four hours after exposure to glutamate for 10 min, the cells were stained using a previously described method (Schutte et al., 1998) with minor modifications. Briefly, the culture medium was removed, washed twice with HEPES buffer and stained with annexin V (0.4 μg/ml; Sigma-Aldrich, Poole, UK) and then with propidium iodide (0.8 μg/ml; Sigma; both in the binding buffer solution: 50 mM Hepes, 750 mM NaCl, 12.5 mM $CaCl_2$, 5 mM $MgCl_2$, 20% BSA; titrated to pH 7.4 using 1 M NaOH). The cells were detached with 0.25% trypsin/EDTA, harvested and then centrifuged at 1200×*g* for 10 min at 4 °C. The supernatant was removed and the cells were resuspended in 1% FBS in PBS for flow cytometric analysis. A FACSCalibur (Becton Dickinson, Sunnyvale, CA) equipped with a single Argon laser was used. Excitation was carried out at 488 nm and the emission filters used were 515–545 BP (green; FITC) and 600 LP (red; PI). A minimum of 10,000 cells per sample was analysed. Electronic compensation was used among the fluorescence channels to remove residual spectral overlap. Data acquisition was performed with Cell Quest 3.3 (Becton Dickinson) and analysis was performed with Cell Quest Pro (Becton Dickinson).

2.2. In vivo experiments

2.2.1. Hypoxia-ischemia (HI) model

Our in vivo model was that developed by Vannucci et al., (2001) with minor modification. In brief, 7-day-old postnatal Sprague–Dawley rat pups underwent right common carotid artery ligation under surgical anesthesia (1–1.5% isoflurane in pure oxygen). After ligation, the animals were returned to their dams and placed in a specially designed area at constant room temperature (23 °C) and humidity (48%) (Sealsafe IVC cage, Tecniplast UK, Kettering, UK). One hour after surgery, hypoxic-ischemic brain injury was induced by placing the rats into a chamber (1–2 animals/chamber) with 8% oxygen in nitrogen for 30–150 min at 37 °C. On the 7th day after hypoxia-ischemia, rats were sacrificed and their brains removed. The right hemisphere was separated from the left and weighed. The use of hemispheric weight as an indicator of brain damage is well-validated in this model and is preferred to morphologic assessment because the immature brain rapidly loses its structural integrity after the injurious insult (Fig. 2A; Gidday et al., 1999; Calvert et al., 2002). The ratio of the right hemispheric weight to that of the left (*R*/*L*

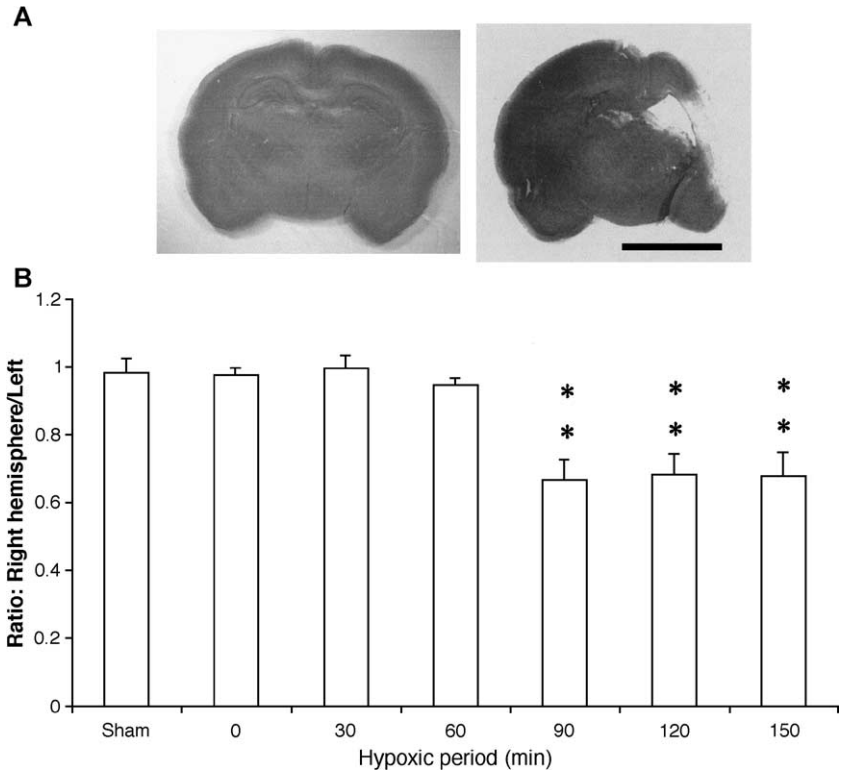

Fig. 2. (A) Representative photomicrographs of brain sections stained with cresyl violet, from an animal that received 90 min of hypoxia-ischemic injury (right panel) and from an uninjured animal (left panel). Scale bar=0.5 cm. (B) The ratio of right to left hemispheric weight in rat pup brains following hypoxic/ischemia. At postnatal day 7, the right carotid artery was ligated and the rat pups were exposed to a hypoxic environment (8% oxygen) for varying times (0–150 min). Seven days later, the brains were removed and the weight of the individual cerebral hemispheres was measured and the ratio of the right to left hemispheric weights was deduced. Results are expressed as the mean±S.E.M. (n=5). **P<0.01 vs. sham control.

ratio) was deduced; maximal brain damage is induced after hypoxia for 90 min (Fig. 2B) which was subsequently used in all experiments.

2.2.2. Treatment with α_2-adrenoceptor ligands

Rat pups were administered dexmedetomidine 6.25, 12.5, 25 or 50 μg/kg, with or without the antagonists mentioned above at a dose of 0.5 mg/kg, s.c. This dose was selected to provide the desired pharmacologic specificity. Fifteen minutes after injection of the α_2 ligands, the pups were placed in a chamber (1–2 animals/chamber) and exposed to the hypoxic mix of oxygen 8% in nitrogen for 90 min at 37 °C.

2.2.3. Assessment of neurological function

Neuromotor function was assessed at the 30th and 40th days post-HI using our validated methodology (Ma et al., 2003b); assessment included measurements of prehensile traction, strength, and balance beam performance graded on a 0–9 scale (maximum score=9). Coordination was tested by placing rats on a rotarod, rotating at 30 rpm (cut-off time being 300 s). For each of the functional assays, the rat pup was tested three times with a 10-min interval between each assessment. The results were presented as the sum of three tests.

2.3. Data analysis

Neurologic outcomes were compared using Kruskal–Wallis nonparametric analysis of variance followed by the Dunn's comparisons test. Statistical analysis for parametric data was performed by analysis of variance followed by the Newman–Keuls test. A $P<0.05$ was considered to be statistically significant.

For assessment of the dose response of dexmedetomidine, data were fitted to a logistic equation of the form

$$E(c) = \frac{\alpha^n}{\alpha^n + c^n}$$

where $E(c)$ is the LDH release or brain matter loss expressed as a fraction of the control at a dexmedetomidine concentration c, α is the IC_{50} and n is a slope factor. The IC_{50} was defined as the concentration of dexmedetomidine that reduced the release to 50% of the maximal control value or the dose of dexmedetomidine that attenuated brain matter loss to 50% of the maximal control value. The results are expressed as means±S.E.M.s.

3. Results

3.1. In vitro-LDH release

3.1.1. Effect of dexmedetomidine on OGD-induced LDH release

Neuronal damage induced by 75-min duration of OGD was dose-dependently diminished by dexmedetomidine with an IC_{50} concentration of 0.083±0.001 μM (Fig. 3).

3.1.2. Effect of α_2-adrenoceptor antagonists on dexmedetomidine-induced inhibition of LDH release provoked by OGD

In cells derived from BALB/c mice, dexmedetomidine at 10 μM inhibited LDH release induced by OGD to 31±2% of maximal release of LDH ($P<0.05$ vs. control; Fig. 4A). Both subtype nonselective α_2 antagonists, i.e., yohimbine and atipamezole, blocked the effect of dexmedetomidine on OGD-induced LDH release (Fig. 4A). BRL44408, 100 μM, the α_{2A} subtype-preferring antagonist, completely reversed the effect of dexmedetomidine on LDH release (103±3%); however, following co-administration with either ARC239 or rauwolscine, the dexmedetomidine effect on OGD-induced LDH release was still significantly different from control values achieving values of 42±8% and 32±5%, respectively.

In cells derived from C57B6 mice (which share the same genetic background as the D79N transgenic mice), a similar pattern of dexmedetomidine-induced neuroprotection and reversal by α_2 antagonists was observed. Thus, dexmedetomidine 10 μM decreased injury to 39±2% of control ($P<0.01$); when combined with either the subtype nonselective α_2 antagonist, atipamezole, or the α_{2A}-preferring antagonist BRL44408, the neuroprotective effect of dexmedetomidine is reversed such that the injury is now no different from cells not treated with dexmedetomidine. Conversely, neither ARC239 nor rauwolscine reversed the neuroprotective effect of dexmedetomidine (Fig. 4B). Dexmedetomidine exerted no neuroprotective effect in cells derived from D79N transgenic mice which lack functional

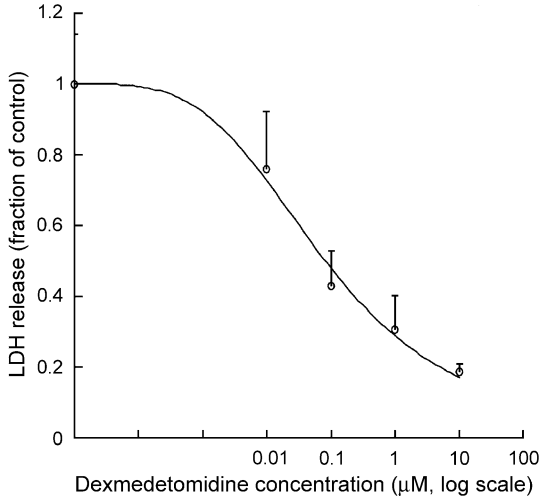

Fig. 3. Effect of dexmedetomidine on release of lactate dehydrogenase (LDH) following oxygen and glucose deprivation (OGD). Co-cultures of neuronal and glial cells were deprived of oxygen and glucose for 75 min in the presence of different concentration of dexmedetomidine (0–10 μM). Release of LDH into the culture medium was measured 6 h later. Data are normalized as a fraction of the release of LDH by OGD without dexmedetomidine.

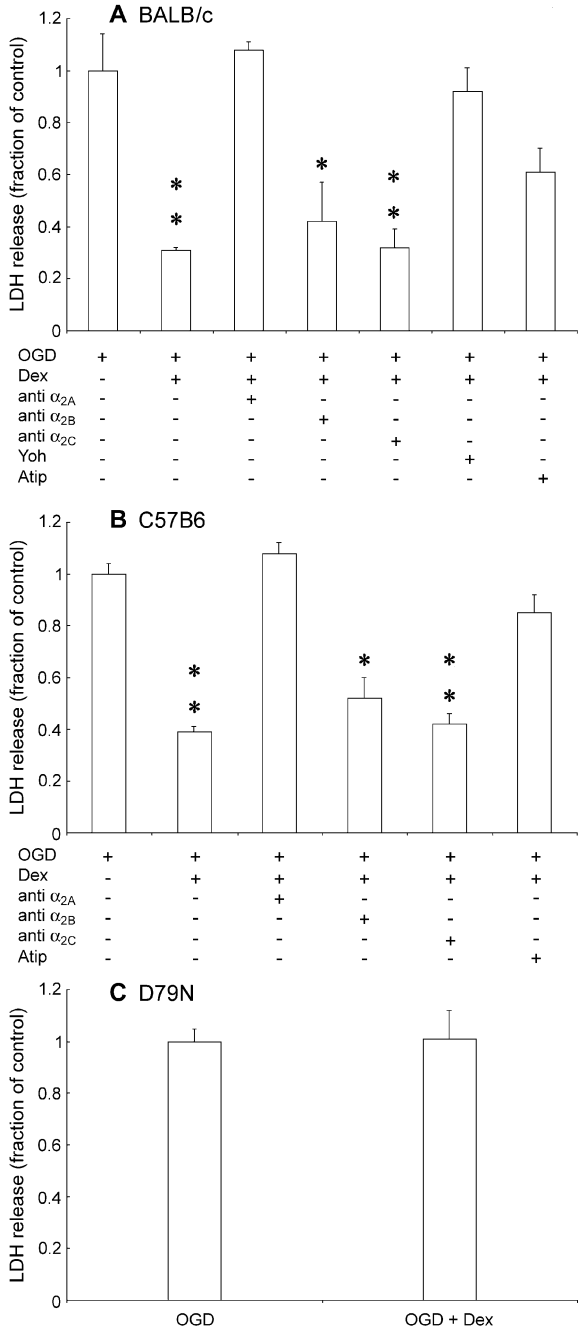

Fig. 4. Effect of dexmedetomidine (Dex)±α_2 antagonists on release of lactate dehydrogenase (LDH) following oxygen and glucose deprivation (OGD) in neuronal-glial co-cultures from different mice strains. Neuronal and glial co-cultures were derived from the cerebral cortex of BALB/c (A), C57B6 (B) and D79N transgenic mice (C) which have a dysfunctional α_{2A}-adrenoceptor gene on a C57B6 genetic background. Co-cultures were deprived of oxygen and glucose for 75 min in the presence of dexmedetomidine, and different α_2 antagonists with subtype preference for the α_{2A} (BRL44408; anti-α_{2A}), α_{2B} (ARC239; anti-α_{2B}), or α_{2C} (rauwolscine; anti-α_{2c}) or the subtype nonselective antagonists, atipamezole (Atip) and yohimbine (Yoh). Release of LDH into the culture medium was measured 6 h later. Data (mean±S.E.M.; n=3–4) are normalized as a fraction of the release of LDH by OGD without dexmedetomidine. *P<0.05; **P<0.01 vs. OGD.

α_{2A} adrenoceptors (Fig. 4C). None of the antagonists affected LDH release when used alone (data not shown).

3.1.3. Effect of dexmedetomidine on neuronal viability

Dexmedetomidine at 10 μM induced neuronal viability, reducing apoptotic and necrotic cell death (demonstrated by reduced annexin V staining and propidium iodide staining, respectively) in pure cortical neuron cultures (Fig. 5). The α_{2A}-preferring antagonist BRL44408 inhibited dexmedetomidine's neuroprotective effect; ARC239 and rauwolscine had no effect (Fig. 5).

3.2. In vivo experiments

3.2.1. Effects of dexmedetomidine on hypoxic-ischemic (HI)-induced brain matter loss

The brain matter loss following HI was 33.7±4%. Dexmedetomidine dose-dependently attenuates HI-induced loss of brain matter in the right hemisphere (Fig. 6) with an IC_{50} dose of 40.3±6.1 μg/kg; at the highest dose of dexmedetomidine (50 μg/kg), brain matter loss was reduced to 14.2±4.5%.

3.2.2. Effects of α_2-adrenoceptor antagonists on attenuation by dexmedetomidine of hypoxic-ischemic (HI)-induced brain matter loss

Dexmedetomidine at 25 μg/kg significantly attenuated brain matter loss in the right hemisphere expressed as R/L ratio (0.90±0.06 vs. 0.71±0.08—the saline control; P<0.05). Both atipamezole, the subtype nonselective antagonist, and BRL44408 reversed the effect of dexmedetomidine on R/L ratio; however, neither ARC239 (0.90±0.07) nor rauwolscine (0.86±0.08) blocked the neuroprotective effect of dexmedetomidine (Fig. 7). The

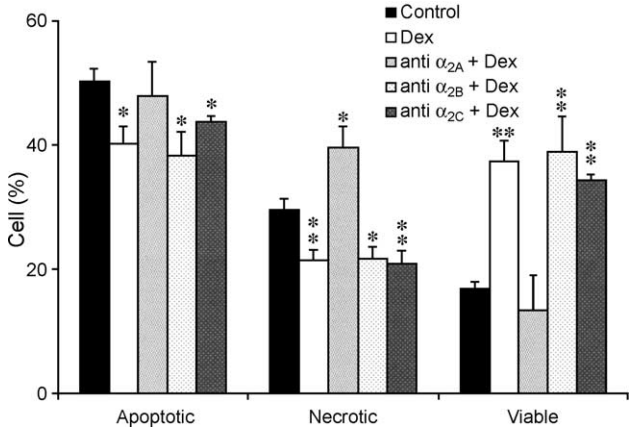

Fig. 5. Effect of dexmedetomidine (Dex)±α_2 antagonists on apoptotic and necrotic cell death induced with glutamate (250 μM). Cells were exposed with glutamate (250 μM) in neurobasal media in the presence or absence of dexmedetomidine (10 μM), the α_{2A} (BRL44408; anti-α_{2A}), α_{2B} (ARC239; anti-α_{2B}), or α_{2C} (rauwolscine; anti-α_{2c}) receptor subtype antagonists. The cultured neurons were stained with annexin V (for apoptosis) and propidium iodide (for necrosis) and then sorted with flow cytometry. *P<0.05; **P<0.01 vs. control (mean±S.E.M.; n=4).

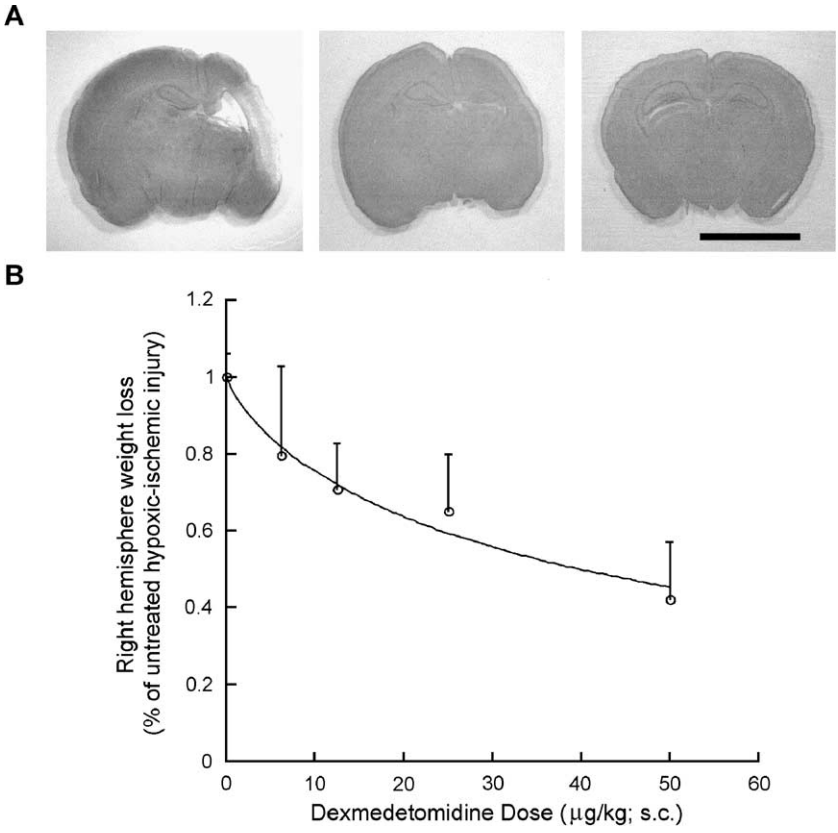

Fig. 6. (A) Representative photomicrographs of brain sections stained with cresyl violet from a rat that has suffered 90 min of hypoxic-ischemic injury with no intervention (left) of a similarly-injured rat pretreated with dexmedetomidine, 50 μg/kg (middle) or an uninjured rat (right). Scale bar=0.5 cm. (B) Effect of dexmedetomidine on loss of weight in the right cerebral hemisphere following an hypoxic/ischemic insult. At postnatal day 7, the right carotid artery was ligated and rat pups were exposed to a hypoxic environment (8% oxygen) for 90 min with varying doses of dexmedetomidine (6.25, 12.5, 25 or 50 μg/kg) subcutaneously administered just prior to hypoxia. Seven days later, the right hemisphere was removed and weighed. Data (mean±S.E.M., n=5) were normalized as percentage of the maximum brain matter loss (33.7±4%) in the absence of dexmedetomidine.

antagonists alone did not significantly affect hemispheric weights in the sham-operated rats (non-ischemic, normoxic; data not shown).

3.2.3. Neurological outcomes

Neurological function was significantly improved by dexmedetomidine at both doses and at both time points tested, although the effect was most apparent in animals treated with 50 μg/kg and tested at 40 days post-injury. At this dose and time, the functional scores were no different from those of sham-operated controls (Fig. 8).

4. Discussion

Using a primary culture of neuronal and glial cells derived from the cerebral cortex of neonatal mice, predictable neuronal injury (as reflected by the amount of LDH released into the culture medium) was induced by exposing the cells to an environment deprived of glucose and oxygen (OGD). Dexmedetomidine, administered at the start of glucose and oxygen deprivation, concentration-dependently attenuated the subsequent neuronal injury provoked by glucose and oxygen deprivation in the cortical neuronal cells. At the maximal dose used (10 μM), dexmedetomidine effected 80% neuronal protection. Furthermore, dexmedetomidine (10 μM) protected neurons exposed to pathological levels of glutamate (300 μM), reducing both apoptotic and necrotic types of cell death. These findings provide confirmation for an earlier finding in which dexmedetomidine dose-dependently decreased excitotoxic neuronal death induced by exogenous NMDA in an in vitro model of perinatal neuronal injury (Laudenbach et al., 2002). Similarly, the α_2-adrenoceptor agonist clonidine was shown to decrease the size of hypoxic-ischemic cortical infarcts produced in newborn rats aged 7–8 days (Yuan et al., 2001). In the current study, dexmedetomidine's neuroprotective effect was completely reversed by the α_{2A}-adrenoceptor subtype-preferring antagonist BRL44408 but not by ligands which had greater efficacy at the α_{2B} and α_{2C} adrenoceptor subtypes. The results of these pharmacologic experiments are corroborated by the finding that dexmedetomidine did not exert a protective effect on OGD-induced neuronal injury in cell cultures derived from D79N transgenic mice which express dysfunctional α_{2A}-adrenoceptors. In a validated in vivo model of brain injury in neonatal rats,

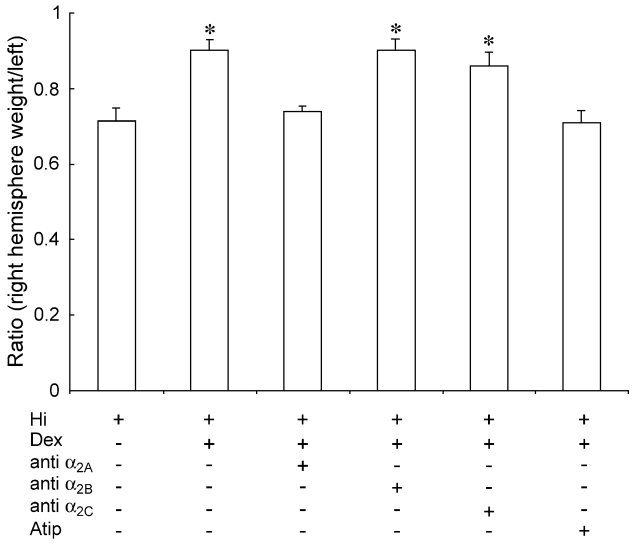

Fig. 7. Effect of dexmedetomidine (Dex)±α_2 antagonists on the ratio of right to left hemispheric weight in rat pup brains following hypoxia-ischemia (HI). At postnatal day 7, the right carotid artery was ligated and rat pups were exposed to a hypoxic environment (8% oxygen) for 90 min with subcutaneously administered dexmedetomidine (25 μg/kg) and different α_2 antagonists with subtype preference for the α_{2A} (BRL44408; anti-α_{2A}), α_{2B} (ARC239; anti-α_{2B}), or α_{2C} (rauwolscine; anti-α_{2c}) subtypes or with the subtype nonselective antagonist, atipamezole (Atip) administered just prior to hypoxia. Seven days later, the brains were removed and the weight of the individual cerebral hemispheres were measured and the ratio of the right to left hemispheric weights was deduced. Data are expressed as the mean±S.E.M. (n=5). *P<0.05 vs. HI alone.

dexmedetomidine, concentration-dependently, attenuated neuronal damage as reflected by a reduction in brain matter loss; this neuroprotective effect of dexmedetomidine is reversed by the α_{2A}-adrenoceptor subtype-preferring antagonist BRL44408, but not by ligands with efficacy at other subtypes. Taken together these data suggest that the neuroprotective effect of dexmedetomidine is mediated via α_{2A}-adrenoceptor subtype.

In the present study, we also examined the effect of dexmedetomidine on late neurological impairment since neuronal injury caused by perinatal asphyxia often results in chronic neurological disability and functional impairment (Vannucci and Palmer, 1997). Dexmedetomidine-treated animals exhibited a significant improvement in functional performance when assessed at remote intervals after the injury (30th and 40th days).

Previously, studies of neuroprotection by dexmedetomidine were conducted largely in adult animal models of hypoxic ischemia (Hoffman et al., 1991a,b; Maier et al., 1993; Halonen et al., 1995; Kuhmonen et al., 1997; Jolkkonen et al., 1999). We used neonatal rats in which neurologic development shares similarities with that of human neonate (Andine et al., 1990). In rodents, α_2-adrenoceptors are detected in the cortex on the first post-natal day (Nomura et al., 1984), whereas α_1-adrenoceptors appear during the second post-natal week (Jones et al., 1985); thus, α_1-adrenoceptors are unlikely to contribute to the effect of dexmedetomidine in our experiments.

All three α_2-adrenoceptor subtypes are widely distributed in the nervous system although α_{2A} and α_{2C} subtypes appear to predominate in the CNS, while the α_{2B} subtype is present in many peripheral tissues and in low abundance in some discrete areas of the CNS (Hunter et al., 1997). Previous studies have shown that α_2 agonist-induced sedation, analgesia, hypotension and hypothermia are mediated by the α_{2A} subtype (Hunter et al., 1997; Kable et al., 2000; Lahdesmaki et al., 2003), the α_{2C} subtype mediated the α_2 agonist effects on the startle reflex, stress response and locomotion (Sallinen et al., 1998; Kable et al., 2000). On the other hand, the hypertensive effect of α_2

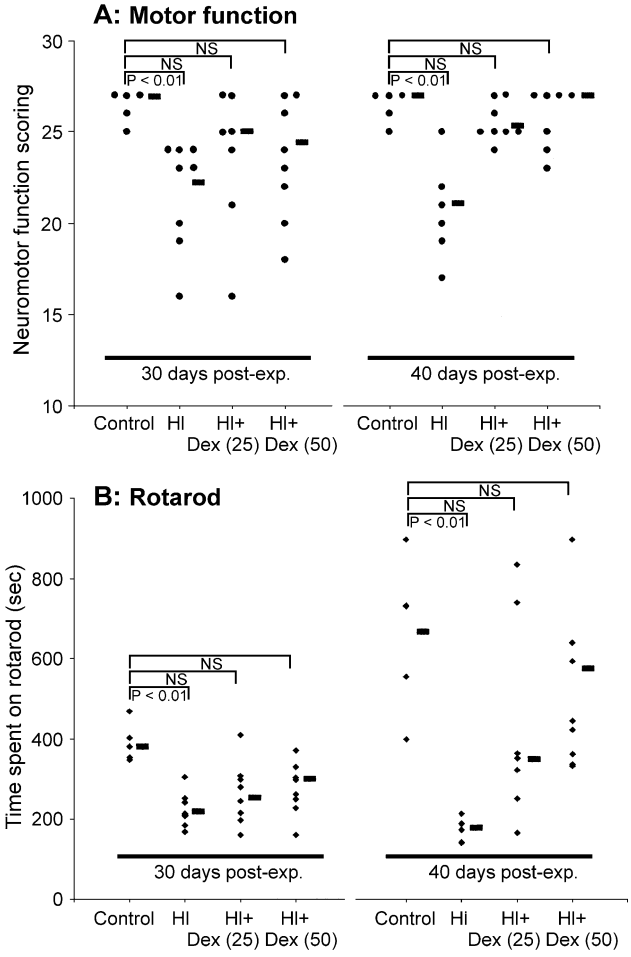

Fig. 8. Effect of dexmedetomidine on neurological functions assessed remotely after hypoxic-ischemic (HI) insult. At postnatal day 7, the right carotid artery was ligated and rat pups were exposed to a hypoxic environment (8% oxygen) for 90 min with subcutaneously administered dexmedetomidine, 25 or 50 μg/kg, administered just prior to hypoxia. Both 30 and 40 days after the insult, rats were evaluated for neuromotor function (A) using a panel that included assays of prehensile traction, strength, and balance beam performance (graded on a 0–9 scale) and (B) balance on the rotarod. Each data point from an individual rat is the sum of three tests. The horizontal bars indicate the median for each group. Control=sham surgery; HI=a permanent ligation of the right carotid artery+hypoxia (8% oxygen) for 90 min; HI+Dex (25)=25 μg/kg Dex (s.c.) prior to HI; HI+Dex (50)=50 μg/kg Dex (s.c.) prior to HI. Nonsignificant (NS) and significant differences are reflected by the p value.

agonists, as well as the peripheral hyperalgesic effect of norepinephrine in experimental pain models, is mediated by the α_{2B} subtype (Khasar et al., 1995; Kable et al., 2000).

In intact animals, cerebral hypoxic-ischemic injury evokes release of norepinephrine as well as glutamate, which compromises the viability of the neurons (Globus et al., 1988; Globus et al., 1989; Vannucci and Palmer, 1997). Norepinephrine increases neuronal metabolism and exaggerates the discrepancy between impaired blood flow to ischemic tissue and an increase in the metabolic demand (Nemoto et al., 1996). Not surprisingly central norepinephrine release, cerebral oxygen consumption, and infarct size during ischemia all positively correlate with each other (Meyer et al., 1974). Dexmedetomidine significantly inhibits norepinephrine release via presynaptic α_2-adrenoceptors in brains of wild-type mice under basal conditions; this effect is absent in mice lacking a functional α_{2A}-adrenoceptor (Hunter et al., 1997). Also we have established that, in a setting in which dexmedetomidine exerts a neuroprotective effect, ischemia-induced release of norepinephrine is significantly attenuated (Lakhlani et al., 1997).

We cannot rule out the possibility that postsynaptically located α_{2A}-adrenoceptor subtypes may also contribute to the neuroprotective effect of dexmedetomidine. At this site, activation of the α_{2A}-adrenoceptor subtype can result in hyperpolarization via K^+ channel activation (Maze and Tranquilli, 1991). Under these conditions, the NMDA receptor may be more vigorously blocked by endogenous Mg^{2+} through a voltage-dependent mechanism.

Our in vitro experiments presented here demonstrate that dexmedetomidine is neuroprotective by attenuating both apoptotic and necrotic cell death. This discovery is of importance because both forms of cell death are thought to contribute to neonatal neurodegeneration following hypoxia-ischemia (Northington et al., 2001; Nakajima et al., 2000), further underpinning the neuroprotective effect observed in vivo. Recent evidence suggests that α_2-adrenoceptor agonists upregulate anti-apoptotic proteins such as bcl-2 and mdm2 during cerebral ischemia (Engelhard et al., 2003); however, it was unknown whether this represented an anti-apoptotic mechanism or whether this was merely a correlate of cell survival. Therefore, this is the first demonstration that dexmedetomidine possesses anti-apoptotic properties likely acting through an α_{2A}-adrenoceptor subtype mediated process.

In immature rats, a reduction in systemic temperature by 6 °C largely protects the perinatal brain from hypoxic-ischemia (Young et al., 1983; Vannucci and Perlman, 1997). Dexmedetomidine administration can produce hypothermia and experiments involving α_{2A}-knockout mice demonstrated that this effect is mediated by that receptor subtype (Hunter et al., 1997; Lahdesmaki et al., 2003). It is unlikely that the neuroprotective effect of dexmedetomidine is due entirely to hypothermia in the current study because rats were kept in a temperature-controlled environment throughout the experimental period. Furthermore, in previous studies, dexmedetomidine exhibited neuroprotection in models in which normothermia was maintained (Kuhmonen et al., 1997; Jolkkonen et al., 1999). In addition, dexmedetomidine-induced hypothermia could not account for neuroprotection observed in vitro, when temperature was controlled.

In our study, we used complementary pharmacologic and genetic approaches to establish the role of the α_{2A}-adrenoceptor subtype in the neuroprotective effect of dexmedetomidine. Both of these strategies have drawbacks, including the relative lack of specificity of the adrenergic ligands, and the possible induction of compensatory changes that may mask the functional outcome of the targeted mutation. However, the fact that these different approaches, independently, ascribed the neuroprotective effect to the α_{2A}-adrenoceptor subtype, reassures us that this subtype is implicated in this important action of dexmedetomidine.

In conclusion, dexmedetomidine is neuroprotective in both in vitro and in vivo models of hypoxic-ischemic injury and this action is mediated via the α_{2A}-adrenoceptor subtype. If our data can be extrapolated to the clinical setting then dexmedetomidine may be a useful adjunct to an anesthetic regimen in a setting where there is a perceived risk of neurological impairment. There is limited experience with the use of dexmedetomidine in children although it has been used for sedation during mechanical ventilation and controlled hypotension during anterior spinal fusion (Tobias and Berkenbosch, 2002) and has been shown to be effective as an analgesic in a neonatal rat pain model (Giombini et al., 2002). Ultimately, data from our study may prove useful in preventing patients with neonatal asphyxia developing chronic neurologic impairment and disability.

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