Spermine induces cell death in cultured human embryonic cerebral cortical neurons through NMDA receptor activation.

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Abstract

The polyamines putrescine, spermidine and spermine play important roles in cell proliferation, differentiation and the modulation of ion channel receptors. However, the function of increased concentrations of these compounds in brain injury and disease is unclear since they have been proposed as being both neuroprotective and neurotoxic. The effects of spermine and putrescine were studied in human primary cerebral cortical cultures containing both neurons and glia. No toxic effects were induced at 8 days *in vitro* (DIV) by either of the two polyamines at concentrations ranging from 0.3 μ M to 2 mM. However, when the oxidative metabolism of spermine that generates toxic by-products was induced by the presence of foetal calf serum, spermine caused cellular death with a LC₅₀ of approximately 50 μ M. At 14 DIV, the coapplication of spermine 2 mM and glutamate 5 mM induced neuron cell death, but the effect of applying both components separately was null. Both spermine and glutamate were toxic to older neurons (26 to 42 DIV cultures), and here the coapplication of glutamate was found always to intensify the effect of spermine. Spermine showed greater toxicity than glutamate in neurons. Another effect observed is that glutamate, but not spermine, induced astrocyte *swelling*. Spermine toxicity was inhibited by both MK801 and ifenprodil, indicating a mechanism involving NMDA-receptor activation. Moreover, a strong spermine modulation of the NMDA receptor was demonstrated by the inhibition of glutamate toxicity by ifenprodil. Putrescine induced minor effects also as a neurotoxic agent. In conclusion, neuronal death by spermine can be induced by its toxic by-products as well as through NMDA-receptor action. The present results confirm the potentially harmful role of the polyamines in excitotoxicity-related human disorders.

Keywords: polyamines, spermine, putrescine, glutamate, NMDA receptor, neuronal death, human neurons.

1. Introduction

The polyamines putrescine spermidine and spermine are natural cell components. They play important roles in cell proliferation, differentiation and the modulation of ion channel receptors. The synthesis of polyamines is well regulated and putrescine is sequentially transformed into spermidine and spermine (Seiler, 2005; Takano et al., 2005a). Acute brain insults inevitably activate the biosynthesis of polyamines. The induction of ornithine decarboxylase (ODC, the enzyme responsible for the first step in polyamine synthesis) and early alterations in polyamine levels have been described in various pathological states, including: epilepsy, ataxia, Alzheimer's disease and ischemia, (de Vera et al., 2002; Hynd et al., 2004; Paschen, 1992).

The effects of anomalous increases in the concentrations of polyamines in the nervous system are not clear. Both neuroprotective and neurotoxic roles have been proposed. In *in vivo* models of ischemia, systemically administered polyamines have been shown to protect brain tissue in rats (Gilad and Gilad, 1991). Other authors have found that, although spermine exerts a protective effect, spermidine and putrescine are ineffective (Clarkson et al., 2004; Shirhan et al., 2004). The intracerebroventricular application of spermine in rats induces cerebral damage (Conway, 1998). The observation that ischemic cerebral damage is ameliorated by the ODC inhibitor difluoro-methyl-ornithine indicates that putrescine has a toxic effect (Temiz et al., 2005). However, it has also been suggested that putrescine protects against the damage caused by the convulsant agent pentylenetetrazol in a transgenic mouse model with a high tissue putrescine content (Kaasinen et al., 2003). In *in vitro* models, polyamines have been described as protecting cerebellar granule neurons from apoptotic death (Harada and Sugimoto, 1997), but other authors report that spermine and putrescine kill these neurons (Segal and Skolnick, 2000; Sparapani et al., 1997). In addition, spermine potentiates the toxicity of amyloid β in rat hippocampal neuronal cultures (Yatin et al., 2001).

A number of studies have shown that the *in vivo* and *in vitro* toxicity caused by polyamines is indirectly mediated by the by-products of their amino oxidase-catalyzed oxidation (Schipper et al., 2000; Takano et al., 2005b). In this regard, high amino oxidase activity has been demonstrated in the plasma of some animal species such as bovines, which distinctly increases polyamine toxicity (Facchiano et al., 2001; Gilad and Gilad, 1986; Sharmin et al., 2001). However, other authors have suggested that the polyamines themselves can exert toxic effects (Schipper et al., 2000; Segal and Skolnick, 2000; Sparapani et al., 1997).

The mechanism of excitotoxicity is proposed in order to explain the brain damage found in a variety of neurological disorders such as ischemia, epilepsy, traumatic brain injury and neurodegenerative disorders, in particular through NMDA-receptor overactivation (Hynd et al., 2004; Obrenovitch and Urenjak, 1997). Polyamines interact with a variety of receptors, including ionotropic glutamate receptors. A role for these compounds as modulators of the NMDA receptor subtype has been proposed. It has also been proposed that polyamines have multiple recognition sites in NMDA receptors. This would account for their complex modulatory activity *in vitro* (Johnson, 1996). Spermine and, to a lesser extent, spermidine stimulate the NMDA receptor. Prolonged activation of this receptor could be responsible for neuronal damage. Unlike spermidine and spermine, putrescine is believed to act as a weak antagonist of the NMDA receptor. The results of experiments with transgenic mice and rats that overexpress ODC strongly suggest that enhanced accumulation of putrescine in the brain is neuroprotective (Jänne et al., 2004; Seiler, 2005).

The aim of this study was to assess the effect of an increase in polyamine concentration on human neuronal viability. For this we used primary cell cultures from the embryonic human cerebral cortex. For comparative purposes, some experiments were also performed in rat cerebral cortical cultures.

2. Materials and methods

2.1. Materials

Cell culture media, serum and reagents were purchased from Gibco (Invitrogen, Paisley, Scotland). Plastic culture plates were from Nunc (Roskilde, Denmark) and glass coverslips from Menzel (Braunschweig, Germany). Mouse anti-NeuN antibody was from Chemicon (Temecula, CA), rabbit anti-GFAP and mouse anti-CD68 antibodies were from Dako (Glostrup, Germany), rabbit anti-galactocerebroside was from Sigma (St Louis, Mo, USA) and secondary Alexa Fluor antibodies and propidium iodide were from Molecular Probes (Leiden, The Netherlands). The Apoptosis Detection System, Fluorescein kit was purchased from Promega. Mowiol 488 mounting medium for immunofluorescence was from Calbiochem (Nottingham, UK). All other reagents were from Sigma.

2.2. Cell cultures

a) Human cultures: Cultures were established using human cortical brain tissue obtained from normal, legally aborted fetuses at 14-16 weeks' gestation. Permission to use human foetal

tissue was obtained from the ethics committee of the Spanish National Research Council (CSIC). Mixed neuron-glia cultures were prepared as described elsewhere (Sebastia et al., 2004). Cells were suspended in minimum Eagle's medium, which was supplemented with 5% heat-decomplemented horse serum, 28 mM of D-glucose (final concentration 33 mM) and 100 µg/ml gentamicin (MEM-HS). Cells were seeded at a density of $3 \times 10^5/\text{cm}^2$ in multiwell plates or on glass coverslips. No antimitotic was added and glial cells were allowed to grow during the culture time. Fresh medium was added weekly until the end of the study.

b) Rat cultures: Pregnant OFA rats were obtained from Charles River, Iffa Credo (St. Germain-sur-l'Arbreste, France). Mixed neuron-glia cultures were prepared from the cerebral cortices of 15-day-old rat foetuses. Pregnant animals were killed by cervical dislocation and foetuses extracted. Neocortices were dissected free of meninges and the brain tissue was processed as previously described for human cerebral cortical cultures. Cytosine arabinoside 10 µM was added after 3 days in vitro (DIV) to reduce glial proliferation and maintain the percentage of neurons to the same obtained in human cultures at 8 DIV. Animals were handled in compliance with protocols of the University of Barcelona, approved by the Generalitat de Catalunya, Spain, in accordance with EU guidelines.

2.3. Percentage of neuronal and non-neuronal cells in the cultures

The time course of changes in neuronal and glial cell content in cultures grown on coverslips was analysed from 8 to 48 DIV for human cultures and at 8 DIV for rat cultures. Neurons were identified immunocytochemically with anti-NeuN antibody (1:100 dilution). Immunostaining for astrocytes, microglia and oligodendrocytes was performed with anti-GFAP (1:500 dilution), anti-CD68 (1:100 dilution) and anti-galactocerebroside (1:100 dilution) respectively. Cultures were fixed with 4% paraformaldehyde, permeabilized with 0.2 % Triton X-100, and incubated with 3% normal goat serum to block non-specific binding. Cultures were incubated overnight with the primary antibody in the presence of 1.5% blocking serum. The cells were washed and incubated with Alexa Fluor 488-conjugated secondary antibody (1:1500 dilution) for 1 h. Nuclei were then stained with bisbenzimidazole (5 µM) for 30 min to visualize the total cell content. Coverslips were mounted in Mowiol. Alternatively, the total cell content was assessed by phase-contrast microscopy.

For the neuron and glial cell count, at least two fields (1.3 mm^2 per field) were microphotographed per coverslip or well and all cells were counted using the Analysis

program (Soft Imaging System, Münster, Germany). The area and the circularity of their nuclei, stained with bisbenzimidazole, were analyzed using the ImageJ 1.34s program (Wayne Rasband, National Institute of Health, USA). Circularity: $\text{area}/\text{perimeter}^2$, as the circularity becomes more proximal to 1 the shape is more circular.

2.4. Cell viability and apoptosis

Cultures in 96 wells were stained with propidium iodide (7 μM) for 1 h to quantify cell membrane damage. The fluorescence of the propidium iodide bound to nucleic acids was measured using a fluorescence plate reader set at 485-nm excitation/530-nm emission, as described elsewhere (Sebastia et al., 2004). Cultures were then immediately loaded with 0.5 mg/ml of MTT, to detect any decrease in cell metabolic activity using the MTT reduction assay. The assay was performed following standard procedures (Hansen et al., 1989).

Terminal deoxynucleotidyl transferase-mediated dUTP nick-end labelling (TUNEL) was performed with the Apoptosis Detection System, Fluorescein kit. Cultures in coverslips were stained following manufacturer's instructions.

Cell death and apoptosis were also analysed by double staining cells in coverslips or in 24-well plates with propidium iodide and bisbenzimidazole. At termination, cell cultures were incubated with propidium iodide (7 μM) for 1 h, washed with PBS and fixed with 4% paraformaldehyde. Cultures were then incubated with bisbenzimidazole (5 μM) for 30 min to identify both apoptotic nuclei and the total number of cells. Dead cells, apoptotic cells and the total cell content were counted as described above.

2.5. Treatment of cultures.

Human and rat cultures at 8 DIV were incubated with spermine or putrescine (ranging from 0.3 μM to 2 mM, dissolved in PBS) for 24 h. Experiments were performed in three different media: 1) with 5% horse serum (MEM-HS), 2) serum-free by replacing the medium with MEM without serum before the addition of polyamines (MEM serum-free), and 3) replacing the 5% horse serum with 10% foetal calf serum (heat-decomplemented) (MEM-FCS) 24 h before the addition of polyamines. In some experiments, cultures in MEM-HS were incubated to 48 h with polyamines. Cell viability was measured by MTT assay and propidium iodide fluorescence. The subsequent experiments were all carried out with MEM-HS.

For the following toxicity studies, human cultures at 14 to 42 DIV in MEM-HS were incubated with spermine (2 and 4 mM), putrescine (8 mM) and/or glutamate (1, 5 and 10 mM, dissolved in PBS) for 24 h.

To study pharmacological interactions, putrescine (2, 4 and 8 mM), MK-801 (50 μ M) or ifenprodil (100 μ M) were added for 1 h prior to the addition of other agents and throughout the subsequent 24 h incubation. The presence of apoptosis was assessed (10 mM) for 30 min or for 15 h, followed by a change to fresh medium up to 24h. The cells were double stained with propidium iodide and bisbenzimidazole or by using the TUNEL method, as described in section 2.4. Dead or apoptotic neurons and total cell number were counted as described in section 2.3.

2.6. Data analysis

One-way or two-way analyses of variance (ANOVA) were performed with a post-hoc Newman-Keuls test for one-way, using the GraphPad Prism version 4.00 for Windows, San Diego, California, USA. Differences were considered significant at $p < 0.05$. All experiments were carried out on at least three independent primary cultures. Results are shown as the mean $\pm$ SEM.

3. Results

3.1. Characterization of cultures

The neuronal content in human cultures at 8, 14, 32, 42 and 48 DIV is shown in Fig 1 A. The number of neurons fell from 8 DIV (667 ± 132 , $n=3$) to 48 DIV (189 ± 75 , $n=3$) (one-way ANOVA, $F_{4,11}=6.92$, $p=0.0049$). The decrease in the percentage of neurons in the cultures became steeper as the number of days *in vitro* increased (Fig 1 A, insert) ($F_{4,11}=42.49$, $p < 0.0001$), due to the concomitant growth of astrocytes. There were about 80% of neurons at 8 and 14 DIV and this percentage fell to 48% at 32 DIV and then further to 25% at 48 DIV. In a previous study (Sanfeliu et al., 1999), the human cultures established at 8-14 DIV consisted of 75%-85% neurons, 15%-25% astrocytes, 1%-2 % microglia and 0.1%-1% oligodendrocytes, which is in agreement with the present results. Further immunostainings at 32 and 42 DIV showed that the content of microglia and oligodendrocytes remained below 2%. Rat cultures at 8 DIV contain $79 \pm 3\%$ of neurons.

The photomicrographs in Figure 1 B illustrate the field of a human culture at 14 DIV, showing abundant neurons and few astrocytes, and the field of a culture at 48 DIV, filled with

bodies and processes from astrocytic cells and presenting a smaller number of neurons. At 14 DIV, there was a mean of 506 ± 101 neurons and 115 ± 55 ($n=3$) astrocytes; at 48 DIV, there was a mean of 189 ± 75 neurons and 676 ± 156 ($n=3$) astrocytes per field.

3.2. Sensitivity of human and rat cultures to polyamines.

3.2.a. Sensitivity of human and rat cultures at 8 DIV to polyamines in different sera.

No cellular death was induced by spermine or putrescine when incubated for 24 h in human cultures at 8 DIV in MEM-HS or in MEM serum-free (Fig. 2, A and B). Similarly, incubation in MEM-HS to 48 h did not induce cellular death (not shown). However, the lack of sensitivity of human cell cultures to spermine at 8 DIV changed when foetal calf serum was present. At concentrations below 30 μM of spermine there were no effects, but a loss of cells was seen at higher concentrations with a LC_{50} of 46.1 μM (Fig. 2 A). Maximal cell death was reached at 100 μM . In contrast, no such effect was found for putrescine (Fig. 2 B). However, rat cultures were sensitive to spermine in all incubation media used (Fig. 2 C). The LC_{50} was 28 μM and 38 μM for cultures in MEM-HS and MEM serum-free, respectively; in MEM-FCS the toxicity of spermine was higher, with a LC_{50} of 13 μM . The maximal cell death was reached at 1 mM for cultures in MEM-HS and MEM serum-free and at 100 μM for cultures in MEM-FCS. Putrescine was toxic to rat cultures (Fig. 2 D) but at the highest doses assayed (1 and 2 mM). No differences in putrescine toxicity were observed between the different media used. The results in Fig. 2 were obtained by MTT assay and were confirmed by propidium iodide fluorescence measurement (not shown).

3.2.b. Sensitivity of human cultures at 14 to 42 DIV to polyamines. Effect of glutamate.

In order to avoid the toxicity of polyamine by-products from foetal bovine serum, the following experiments (this section and the section 3.2.c) were carried out in MEM-HS.

A dose-response curve for spermine (range 10 μM to 4 mM) was performed to establish the concentrations to be used. Results in cultures of 26 DIV showed no effects at concentrations from 10 μM to 1 mM and toxicity was present at 2 mM and 4 mM (not shown). Therefore the latter concentrations were chosen for spermine studies.

In younger cultures (14 DIV) spermine 2 mM was not toxic (Fig 3, A and B). Glutamate 1 or 5 mM had also no effect (Fig 3, A and B). However, the combination of both compounds spermine plus glutamate (1 or 5 mM) induced a similar percentage (about 18%)

of neuronal death (Figure 3, A and B) (one-way ANOVA $F_{3,7}=361.20$, $p<0.0001$ and $F_{3,8}=18.55$, $p<0.0006$). When the concentration of spermine was doubled to 4 mM, we found 28% of neuronal death (Fig 3 C). However, increasing glutamate to 10 mM was not lethal for these cultures. Nevertheless, the combination of both components, spermine 4 mM and glutamate 10 mM, potentiated the toxic effect of spermine 4 mM to 57% (Fig. 3 C) ($F_{3,11}=105.90$, $p<0.0001$).

After more days *in vitro*, spermine 2 mM killed neurons of the cultures. Thus, there was about 20% of neuronal death at 26, 32 and 42 DIV (Fig 3, D-F). Similar results were found with glutamate 5 mM (Fig 3, D-F). When spermine 2 mM and glutamate 5 mM were combined, the toxic effect was additive (Fig 3, D-F) ($F_{3,6}=237.30$, $p<0.0001$, $F_{3,9}=23.71$, $p<0.0001$, and $F_{3,14}=25.39$, $p<0.0001$). The lower glutamate concentration 1 mM was non toxic by itself but it potentiated the spermine 2 mM effect as observed at 14 DIV (Fig 3, F insert for 42 DIV; 26 DIV and 32 DIV not shown), ($F_{3,18}=73.11$, $p<0.0001$).

The influence of the age of cultures on the toxic effects of spermine 2 mM, glutamate 5 mM and spermine 2 mM plus glutamate 5 mM was also considered. The two-way ANOVA indicated a strong influence of the treatment ($F_{3,37}=81.21$, $p<0.0001$) and DIV ($F_{3,37}=20.82$, $p<0.0001$) and the corresponding interaction ($F_{9,37}=2.67$, $p=0.0169$).

Cultures were noticeably more sensitive to spermine than to glutamate. Thus, 4 mM spermine killed neurons at 14 DIV (Fig. 3 C), whereas 10 mM glutamate did not. In older cultures, 2 mM spermine killed the same percentage of neurons as were killed by 5 mM glutamate (Fig. 3, D-F). The culture time of 32 DIV was selected for the following experiments.

Morphological and immunocytochemical identification revealed that only neurons were killed by spermine, while astrocytes were preserved (Fig. 3 G). A similar effect was found for glutamate (Fig. 3 G). Furthermore, glutamate, but not spermine, induced *swelling* of astrocyte nuclei. This effect was already present at 14 DIV. Corroborating this observation, the area of astrocyte nuclei was greater in the glutamate-treated cultures than it was in control and polyamine-treated cultures ($F_{9,20}=25.05$, $p<0.0001$). Further, the shape of the glutamate-treated astrocyte nuclei was more circular than those of the control and the polyamine-treated cultures (Table 1; Fig. 3G, bottom) ($F_{9,20}=66.68$, $p<0.0001$). Neuronal death was essentially necrotic. No bisbenzimid-stained apoptotic figures nor TUNEL positive neurons were observed in response to spermine 2 or 4 mM, putrescine 2 mM, glutamate 5 and 10 mM and

the combinations of spermine 2 mM with glutamate 5 mM, spermine 4 mM with glutamate 10 mM or putrescine 2 mM with glutamate 5 mM (not shown).

Unlike spermine, putrescine did not kill neurons at 32 DIV (Fig 4), even at doses as high as 8 mM, nor did putrescine 4 mM or 8 mM protect cultures of 32 DIV from spermine 2 mM or glutamate 5 mM mediated toxicity (Fig. 4, A and B respectively). However, putrescine increased the amount of neuronal death induced by spermine 2 mM plus glutamate 5 mM (Fig. 4 C). This result was observed with 4 and 8 mM putrescine but not with 2 mM.

Control and putrescine 8 mM values were statistically different from spermine 2 mM alone or in combination with putrescine ($F_{4,6}=9.15$, $p<0.01$) (Fig. 4 A), from glutamate 5 mM alone or in combination with putrescine ($F_{4,6}=11.82$, $p=0.0052$) (Fig. 4 B) and from the combination of spermine and glutamate alone or with putrescine ($F_{5,8}=191.80$, $p<0.001$) (Fig. 4 C).

3.2.c. Pharmacological studies

The pharmacological studies were also performed at 32 DIV. The NMDA receptor antagonists ifenprodil and MK-801 protected cultures from the toxicity caused by spermine 2 mM (Fig. 5 A). These drugs reduced the toxicity of spermine 2 mM to that of control values ($F_{5,13}=27.38$, $p<0.0001$). Likewise, ifenprodil and MK-801 also reduced the toxicity of glutamate 5 mM to that of control values ($F_{5,13}=6.85$, $p=0.0024$) (Fig. 5 B). However, neither ifenprodil nor MK-801, when applied separately, prevented the neuronal death induced by the combination of spermine 2 mM with glutamate 5 mM (Figure 5 C). Nevertheless, a mixture of ifenprodil and MK801 partially protected cultures against the toxic effect of spermine 2 mM plus glutamate 5 mM. The values of the control and the combination of ifenprodil plus MK801 were statistically different from all the other combinations ($F_{5,13}=16.51$, $p<0.0001$) (Fig. 5 C). No toxic effect was detected for MK-801, ifenprodil or a combination of both drugs (Fig. 5 A-C). In addition, MK801 and ifenprodil applied separately or in combination did not reduce the *swelling* of astrocytes induced by glutamate (Table 1).

4. Discussion

Glutamate receptor mediated neurotoxicity

Spermine and glutamate at mM concentration are toxic to long-term human cerebral cortical neuron cultures (two-six weeks *in vitro*) but not to younger cultures. Rat neurons are much more sensitive than human neurons to both spermine and glutamate. Indeed, rat cortical

neurons were killed by μM concentrations of spermine (this study; Sparapani et al., 1997) and they are strongly affected by glutamate 1 mM (Mattson et al., 1991).

Differences in rat and human NMDA receptor development may explain this dissimilarity. It has been suggested (Mattson et al., 1991) that glutamate receptors are not present in human cortical neurons at 14 weeks of gestation, but begin to be expressed at a later date. However, data from human foetal brains show that ionotropic glutamate receptor binding sites and subunit mRNA are in fact present in cerebral cortex as early as gestational week 8 (Ritter et al., 2001). Nevertheless, no data on the functionality of these receptors have been reported. Studies using recombinant NMDA receptors have shown that the effects of spermine are dependent on the subunit composition of the receptor (Williams, 1994). Thus, it is conceivable that cortical human neurons may have mature functional NMDA subunit receptors later than rat neurons. Further, possible species differences in the *in vitro* receptor subunit maturation should be taken into account, as it has been seen in mice and rat cortical neurons (Frandsen and Schousboe, 1990; Li JH et al., 1998). Unfortunately, no data for NMDA receptor subunit development in human cultures have been reported. However, it has been seen that whereas all NR1, NR2A and NR2B subunit mRNAs were expressed in human hippocampus and adjacent cortex from neonate to adult ages, their abundance varied across ontogeny with a pattern similar, but not identical, to that seen in rodents (Law et al., 2003).

Astrocytes have glutamate transport mechanisms that protect neurons from excitotoxicity by maintaining a low extracellular glutamate concentration (Schousboe and Waagepetersen, 2005). Thus, the presence of astrocytes in our human cultures could explain the resistance of neurons to glutamate cytotoxicity. However, human neurons became more susceptible to spermine and glutamate after two weeks in culture, when the number of astrocytes was progressively increased. In any case, the astrocyte:neuron ratio increased from 1.1:1 at 26 DIV to 2.3:1 at 42 DIV. This is in the range of the ratio 1.4:1 of adult cerebral cortical human brain (calculated from (Pakkenberg et al., 2003; Steiner et al., 2007)). Moreover, the fact that neuronal vulnerability to spermine and glutamate was similar from 26 to 42 DIV discards the progressive vulnerability caused by spontaneous degeneration *in vitro* as has been described for rat cerebellar neurons (Favit et al., 1992). Therefore, neurotoxicity probably depended on specific NMDA receptor changes as discussed above.

In human cultures, the toxicity of spermine was noticeably greater than that of glutamate. Thus, at the early maturation period of 14 DIV, at which glutamate 1-10 mM did not cause neuron mortality, spermine 4 mM induced about 25% neurotoxicity. This effect was probably

mediated through the glutamate receptor because these non-effective concentrations of glutamate potentiated the toxicity of spermine in neurons at this time and at subsequent points in time. The lethal effect of spermine combined with glutamate has been described for rodent neurons (Segal and Skolnick, 2000). Neuronal death induced by spermine 2 mM was fully prevented by MK801 (50 μ M), a non-competitive NMDA- receptor antagonist, and ifenprodil (100 μ M), an antagonist of the polyamine site present in the NMDA receptor complex. Thus, both MK801 blocking the NMDA-receptor channel and ifenprodil acting on the polyamine site inhibited the overactivation of the NMDA receptor induced by spermine. Interestingly, the action of ifenprodil on the polyamine site is enough to prevent the toxicity of 5 mM of glutamate indicating a strong modulation of NMDA-receptor by polyamines. However, the neuronal damage induced by coapplication of spermine and glutamate seems to involve also non-NMDA related mechanisms. The protective effect of ifenprodil and MK801 applied together was partial and the antagonists applied separately failed to rescue neurons. This suggesting that the combination of spermine plus glutamate activates also the non-NMDA receptors. The involvement of both NMDA and non-NMDA receptors in the toxicity induced by spermine has been suggested by *in vivo* and *in vitro* studies (Otsuki et al., 1995; Segal and Skolnick, 2000; Sparapani et al., 1997). In addition, a direct effect of spermine in the production of toxic reactive oxygen species (not related with polyamine catabolism) has been proposed (Segal and Skolnick, 2000).

Putrescine showed very different effects from those of spermine in human cultures: no toxicity was observed at any concentration irrespective of the length of time in culture. By contrast, rat neurons at 8 DIV are killed by putrescine, in agreement with Sparapani et al. (1977). In our experiments, putrescine did not protect human cultures against spermine or glutamate toxicity. Instead, at concentrations of 4 and 8 mM, putrescine increased the number of neurons killed by a combination of spermine and glutamate. This suggests that putrescine might only have an effect on the NMDA-receptor when the receptor is overactivated.

Polyamine by-product mediated neurotoxicity.

While spermine was not toxic in immature human cultures at 8 DIV, in serum-free medium or horse serum, the presence of foetal calf serum in the culture medium induced a dramatic loss of cell viability. This was due to the toxic by-products generated by amine oxidase activity, which is present in high concentrations in ruminant serum. The products of the oxidation of spermine and spermidine that are generated by serum amine oxidase in foetal

calf serum are H₂O₂, ammonia, 3-aminopropanal and the highly toxic acrolein (Sharmin et al., 2001). Polyamines in a medium containing heat-denatured foetal calf serum (100°C for 15 min) did not induce any damage of cell cultures (Facchiano et al., 2001). However, serum submitted to 56°C for 20 min to inactivate heat-labile complement factors for neuron culture purposes preserves amino oxidase (Gilad and Gilad, 1986) and induces toxic by-products such as acrolein (Sharmin et al., 2001). However, fresh horse serum is deficient in amine oxidase (Facchiano et al., 2001; Sakata et al., 2003) and did not produce toxic polyamine by-products as is shown by the absence of toxicity of spermine in our cultures at 8 DIV. Nevertheless, we did not observe any toxicity caused by putrescine in the presence of foetal calf serum; this is in agreement with the lack of putrescine metabolisation by serum amino oxidase (Gilad and Gilad, 1986; Sharmin et al., 2001). In our human neuron cultures at 8 DIV, polyamines were not toxic in the absence of foetal calf serum. In contrast, cortical rat cultures were sensitive to spermine and putrescine regardless of the presence of foetal calf serum. This finding is in agreement with other authors (Segal and Skolnick, 2000; Sparapani et al., 1997) who found that spermine and putrescine killed rat granule cerebellar neurons in serum-free medium, involving mechanisms different to those of the by-products of polyamines. Human serum also contains polyamine amino oxidase and accumulates toxic by-products in pathological states such as renal failure (Sakata et al., 2003); in addition, it was reported that polyamines are toxic to rat neuronal cerebellar cultures containing human serum (Gilad and Gilad, 1986). Then, human neurons may be killed *in vivo* by spermine metabolic by-products in addition to the excitotoxicity-related mechanism discussed above.

Neuron and astrocyte cellular damage

Necrotic neuronal death was seen in response to the treatments used in human neurons. No apoptotic cell death was observed. In contrast, other authors found that apoptosis was caused by spermine or a combination of spermine and glutamate in rat cerebellar granule cells (Segal and Skolnick, 2000; Sparapani et al., 1997). Therefore, the cell death pathways involved appear to differ depending on the species.

The glutamate-induced *swelling* of astrocytes observed in the cultures in our experiments has also been described in rodents (Chen et al., 2000; Han et al., 2004). We did not observe astrocyte cell death caused by glutamate. This is probably due to the protective presence of cystine (a glutathione precursor) in the incubation medium (Chen et al., 2000). The *swelling* of astrocytes as a response to high glutamate has been attributed to an uptake of

water after membrane depolarization derived from Na⁺-dependent glutamate transport mechanisms. Metabotropic but not ionotropic- NMDA receptor activation would appear to be involved in the glutamate-induced *swelling* of astrocytes (Han et al., 2004). This is in agreement with the absence of any effect of the ionotropic-glutamate receptor antagonists MK801 and ifenprodil in protecting the astrocytes from *swelling* in our cultures.

Conclusion

In this study, spermine, and to a lesser extent putrescine, have been shown to be neurotoxic, rather than neuroprotective, to human cortical cultures. The mechanisms of neuronal death can be induced by toxic polyamine by-products as well as through NMDA-receptor action. Polyamines can be released into the extracellular space in response to brain injuries involving glutamate receptor activation (Fage et al., 1992; Vivó et al., 2002) where they appear to overactivate the NMDA receptor and participate in the mechanisms of neuronal damage. For instance, polyamines are involved in the proposed excitotoxic mechanism of Alzheimer's disease. In support of this, the changes in enzymes involved in polyamine metabolism and the increase in spermidine- the precursor of spermine - have been found in the brains of Alzheimer's disease patients (review Hynd et al., 2004). In addition, increased levels of acrolein were found in the amygdala and hippocampus of Alzheimer's disease patients (Lovell et al., 2001). Although our findings showed human neurons to be less sensitive than rat neurons to the detrimental effects of polyamines, the present results confirm the potentially harmful role of polyamines in excitotoxicity-related human disorders.

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Figure 1.- Neuron cell content evolution in human cerebral cortical cultures after 9 to 48 days *in vitro* (DIV) in MEM-HS. A) Number of neurons per field. Insert: percentage of neurons as related to the whole cell content. Results are shown as the mean $\pm$ SEM of 2 to 5 fields each from a total of 7 cultures. Statistics at the significance level of $p < 0.05$: main graph, * 32, 42 and 48 DIV vs 9 DIV; insert graph, * 32, 42 and 48 DIV vs 9 DIV, B) Photomicrographs show representative fields of cultures at 14 and 48 DIV (phase contrast x20). The field at 14 DIV mainly contains neurons. The field at 48 DIV contains a higher number of astrocytes than neurons. Arrows: neurons; arrowheads: astrocytes. Scale bar = 20 μ m.

Figure 2.- Toxic effects of polyamines in human and rat cerebral cortical cultures at 8 DIV in different serum. A) Absence of toxicity of spermine to human cultures in medium containing horse serum (MEM-HS, Δ) or in serum-free medium (MEM serum-free, $\square$); loss of viability caused by the presence of foetal calf serum in the culture medium (MEM-FCS, $\blacktriangle$) ($EC_{50} = 4,6 \times 10^{-5} \pm 1,48$ M). B) Absence of toxicity caused by putrescine to human cultures incubated in the same conditions as in A). C) Loss of viability of rat cultures in the three media assayed, $EC_{50} = 2,8 \times 10^{-5} \pm 1,07$ M (MEM-HS), $EC_{50} = 3,8 \times 10^{-5} \pm 1,14$ M (MEM serum-free), $EC_{50} = 1,3 \times 10^{-5} \pm 1,11$ M (MEM-FCS). D) Toxic effect of high concentrations of putrescine to rat human cultures. Incubation time was 24 h. Viability was measured by the MTT reduction assay. Results are shown as the mean $\pm$ SEM from 3-6 determinations in 4 different cultures.

Figure 3.- Toxic effects of spermine and glutamate in cultures at 14, 26, 32 and 42 DIV. A) Spermine 2 mM (SM2) and glutamate 1 mM (G1) did not have a toxic effect on cultures at 14 DIV but the mixture of both compounds induced toxicity. B) The same result as in A) was found with glutamate 5 mM (G5). C) Spermine 4 mM (SM4) was toxic to these cultures. Glutamate 10 mM was not toxic by itself but potentiated the toxicity of SM4. D) At 26 DIV, both SM2 and G5 were toxic to the cultures. When mixed together the toxicity increased. E) At 32 DIV, SM2 and G5 induced toxicity separately or in combination. F) At 42 DIV, SM2 and G5 induced neuronal death. The combination of these substances increased their toxicity. Insert: G1 did not kill neurons, but when mixed with SM2 it potentiated the toxicity of SM2. A-F) Neuronal death was measured by propidium iodide staining. Results are shown as the mean $\pm$ SEM of 2-9 fields each from 3-7 different cultures; statistics ($p < 0.05$): * mixture of

spermine plus glutamate vs. all the other components, # spermine and glutamate vs. control.

G) Photomicrographs of representative fields from the control, SM2, G5 and SM2+G5 conditions at 32 DIV. Top row: dead neurons with bright nuclei stained by propidium iodide, bottom row: cell nuclei stained by bisbenzimidazole. Arrows: neuronal nuclei stained with propidium iodide or bisbenzimidazole. Arrowheads: flat astrocytic nuclei in control and SM2 treated cultures and spherical nuclei in G5 and SM2+G5 treated cultures indicative of cellular *swelling*. Scale bar=20 μ m. Incubation time was 24 h. Cultures were maintained in MEM-HS.

Figure 4.- Effect of different concentrations of putrescine on the toxicity induced by spermine 2 mM (SM2), glutamate 5 mM (G5) and a combination of both compounds (SM2+G5) in human cerebral cortical cultures at 32 DIV. A) Neither a protective nor more toxic effect of putrescine 4 mM (Put4) or 8 mM (Put8) was observed on the lethal effect of SM2. No toxicity was seen for Put8 alone. B) Put4 and Put8 had the same null effect on the toxicity of G5. C) An increase in the neuronal death caused by SM2+G5 was observed when Put4 and Put8 were added but no effects were detected after putrescine 2 mM (Put2). A-C) Neuronal death was measured by propidium iodide staining. Results are shown as the mean $\pm$ SEM of 3 different cultures, using 3-6 fields each from 2-3 wells; statistics ($p < 0.05$): # SM2, G5, SM2+G5 and these agents combined with putrescine vs. control and Put8, * SM2+G5 plus Put 4 or 8 mM vs SM2+G5 and SM2+G5+Put2. Incubation time was 24 h. Cultures were maintained in MEM-HS.

Figure 5.- Protective effect of 100 μ M ifenprodil (IF) and 50 μ M MK801 against the toxicity of spermine 2 mM (SM2), glutamate 5 mM (G5) and a combination of both compounds (SM2+G5) in human cortical cultures at 32 DIV. A) Both IF and MK801 protected neurons from SM2-mediated toxicity. B) Both IF and MK801 also protected neurons from G5-mediated toxicity. C) No protection against SM2+G5-mediated toxicity was observed when IF or MK801 were applied separately. However, the coapplication of IF and MK801 provided partial protection from SM2+G5 induced neuronal death. A-C) Neuronal death was measured by propidium iodide staining. Results are shown as the mean $\pm$ SEM of 4 different cultures, using 2-6 fields each from 1-6 wells; statistics ($p < 0.05$): * SM2 and G5 vs. control and all the other treatments, # SM2+G5 alone or mixed with IF or MK801 vs. control and IF+MK801, & SM2+G5 mixed with IF+MK801 vs. SM2+G5 alone and in presence of IF or MK801. Incubation time was 24 h. Cultures were maintained in MEM-HS.