Učinki gabapentina in etanola na regulirano celično smrt astrocitov v primarni kulturi

The effects of gabapentin and ethanol on the regulated cell death of astrocytes in primary culture

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Izvleček

Namen: *V raziskavi smo proučevali vpliv gabapentina na regulirano celično smrt astrocitov v primarni kulturi. Astrociti so relativno odporni na propadanje po apoptotični poti, zato nas je zanimalo, kakšen je vpliv različnih koncentracij gabapentina na apoptozo in nekroptozo kot drugo obliko regulirane celične smrti. Dodatno nas je zanimalo, kakšen je vpliv gabapentina na smrt astrocitov, ki so bili izpostavljeni etanolu.*

Metode: *Kot eksperimentalni model smo uporabili primarne kulture astrocitov, ki smo jih pridobili iz možganske skorje novorojenih podgan. Celice smo izpostavili različnim koncentracijam gabapentina oz. kombinacijam gabapentina in etanola ter s pomočjo pretočne citometrije določili deleže živih, zgodnje*

Abstract

Purpose: *In this study, the effect of gabapentin on the regulated cell death of astrocytes in primary culture was examined. Because astrocytes are relatively resistant to decay by apoptotic pathways, the effect of different concentrations of gabapentin on apoptosis in necroptosis was tested as another form of regulated cell death. In addition, the impact of gabapentin on the death of astrocytes that were exposed to ethanol was also examined.*

Methods: *Primary cultures of astrocytes that were obtained from the brain cortex of newborn rats were used as the experimental model. Cells were exposed to different concentrations of gabapentin only, ethanol only or to a combination of ethanol and gabapentin. Using flow cytometry, the proportions of vi-*

apoptotičnih, nekroptotičnih in sekundarno nekrotičnih celic.

Rezultati: *Učinek gabapentina na zgodnjo apoptozo in nekroptozo astrocitov je odvisen od koncentracije; medtem ko v koncentracijah do 10 µg/ml gabapentin nima vpliva na smrt astrocitov, se pri višjih koncentracijah poveča delež nekroptotičnih celic. Sočasna izpostavljenost celic gabapentinu (10 µg/ml) in etanolu (100 mM) za 24 ur ne vpliva značilno na celično smrt, sproženo z etanolom. Pri celicah, ki so kronično izpostavljene etanolu (50mM) 7 dni, gabapentin rahlo zmanjša delež nekroptotičnih celic.*

Zaključek: *Gabapentin v koncentracijah do 10 µg/ml ne vpliva na viabilnost astrocitov. Sočasna izpostavljenost astrocitov etanolu in gabapentinu za 24 ur ne zmanjša toksičnosti etanola. Pri astrocitih, ki so kronično izpostavljeni etanolu, gabapentin rahlo zmanjša vpliv etanola na nekroptozo.*

able, early apoptotic, necroptotic, and secondary dead cells were determined.

Results: *The effect of gabapentin on early astrocytic apoptosis and necroptosis was dependent on concentration. In concentrations of up to 10 µg/mL, gabapentin did not affect astrocyte deaths; whereas at higher concentrations, the proportion of necroptotic cells increased. The concomitant exposure of the cells to gabapentin (10 µg/mL) and ethanol (100 mM) for 24 hours did not significantly affect cell death caused by ethanol. For cells that are exposed to 50 mM ethanol for 7 days, gabapentin slightly reduced the proportion of necrotic cells.*

Conclusion: *Gabapentin did not affect the viability of astrocytes in concentrations up to 10 µg/mL. The concomitant exposure of astrocytes to ethanol and gabapentin for 24 hours did not reduce the toxicity of ethanol. In astrocytes that are chronically exposed to ethanol, gabapentin slightly reduced the effect of ethanol on necroptosis.*

INTRODUCTION

Gabapentin is an amino acid, developed as a structural analogue of gamma butyric acid (GABA) for the management of epileptic seizures (1). It is also recommended for the treatment of neuropathic pain caused by diabetic neuropathy (2), post herpetic neuralgia (3), central neuropathic pain (4), hot flashes, and restless legs syndrome (5, 6). The pharmacological action of gabapentin is not fully understood (7 - 9). Although it has a similar structure to the endogenous neurotransmitter GABA, gabapentin does not bind to GABA receptors at concentrations up to 1 mM (10) while it increases GABA biosynthesis through modulatory effect on glutamate decarboxylase and aminotransferase. One of the important mechanisms of the action of gabapentin is its ability to block the $\alpha 2\delta$-1 subunit of voltage dependent calcium ion channel at selective presynaptic sites and indirectly modulating GABA neurotransmission (11). Modulation of $\alpha 2\delta$-1 transmitted signalling also influences apoptotic cell death, anatomic reorganization, excitatory synaptogenesis, astrocytosis, and network hyperexcitability in a model of insult-induced cortical malformation (12).

Recent studies revealed that gabapentin protects cultured neurons from staurosporine-induced apoptosis, and reduces the intensity of reactive gliosis, both in microglial and astroglial cells (13). Gabapentin has also been found beneficial in the treatment of alcohol withdrawal in alcoholics (14, 15) and has a selective action in decreasing the convulsive and anxiety related signs of ethanol withdrawal in mice (16).

In the CNS, astrocytes represent the major cellular location of ethanol metabolism and have been postulated to protect neurons from ethanol induced oxidative stress (17 - 19). Ethanol can induce apoptosis as well as necroptosis of astrocytes (20). In the present study the aim was to explore the effect of gabapentin on different forms of regulated cell death using cultured astrocytes as well as the influence of gabapentin on ethanol induced cell death.

MATERIAL AND METHODS

Materials

Foetal bovine serum (FBS), L-15 Leibowitz medium, Dulbecco's Modified Eagle Medium and Ham's nutrient mixture F-12 (DMEM / F12), penicillin-streptomycin (P/S) (10,000 IU/mL – 10,000 UG/mL) and Dulbecco's phosphate-buffered saline (PBS) were supplied from Gibco BRL (Life Technologies, Paisley, Scotland). Gabapentin and ethanol were obtained from Merck (Darmstadt, Germany). Bovine serum albumin was obtained from Sigma-Aldrich Chemical Co. (St. Louis, MO, USA). Petri plates were supplied from Nunc (Wiesbaden, Germany), and tissue culture flasks were supplied from TPP AG (Trasadingen, Switzerland). Annexin V-fluorescein isothiocyanate (annexin V-FITC) and 7-Aminoactinomycin D (7-AAD) staining kit for flow cytometry were obtained from Beckman-Coulter, Inc. (Brea, CA, USA). Flow cytometry experiments were carried out on the Quanta SC MPL flow cytometer (Beckman Coulter, USA).

Animals

Two-day-old Wistar rats were obtained from our own breeding colony. The animals were maintained under constant environmental conditions, with an ambient temperature of 22°C, relative humidity of 55% and a natural light-dark cycle. The breeding colony was kept in Ehret type-4 cages (Emmerdingen, Germany). The bedding material was Lignocel 3/4 (Altromin, Germany). The colony received a standard rodent diet (Altromin, Germany) and had free access to food and water. All animal studies were approved by the Veterinary Authority of the Republic of Slovenia (licence number 34401-7/2012/3) and performed in accordance with the EU Directive 2010/63/EU and the European Convention for the protection of vertebrate animals used for experimental and other scientific purposes (ETS 123).

Astrocyte Culture Preparation

Cultures of rat cortical astrocytes were prepared from the brains of new born rats in DMEM/F12 (1:1), 10% FBS, 1% P/S culture medium as described previously (21). Cells were grown at 37°C in a humidified environment containing 10% CO2, until they became confluent, then they were used for the treatment.

Treatment of the Cells

In the first set of experiments, the astrocytes were treated with different concentrations of gabapentin (1, 10, 50 or 100 µg/mL) for 24 hours. After the treatment, the cells were allowed to regenerate for 22 hours in gabapentin free medium. The control cells were not exposed to gabapentin.

In the second set of experiments, the astrocytes were treated simultaneously with gabapentin (10 µg/mL) and ethanol (100 mM) for 24 hours. After the treatment, the cells were allowed to regenerate for 22 hours in gabapentin and ethanol free medium.

In the last set of experiments, the cells were grown in a culture medium containing ethanol (50 mM) for 7 days. The cells were then treated with gabapentin (10 µg/mL) for 24 hours, then regenerated in gabapentin and ethanol free medium for 22 hours. The control cells were not exposed to either gabapentin or ethanol. After the regeneration, the cells were trypsinized and stained for analysis with a flow cytometer. The concentrations of ethanol used in the present study were selected from our previous studies where a dose-response relationship for ethanol on cell viability and cell proliferation was studied (17, 19).

Cell Staining and Flow Cytometric Analysis of Cellular Death

The cells were stained simultaneously with annexin V-FITC and 7-AAD dye according to the modified manufacturer's instructions. Cells were centrifuged at 500 rcf for five min at 4°C and washed once in the ice-cold PBS. Then cells were resuspended in 100 µl of ice cold 1× binding buffer and stained with 10 µL (0,025 µg) of annexin V-FITC and with 20 µL of 7-AAD solution. After staining, the mixture was incubated in the dark at 4°C for 15 minutes. After incubation, an additional 400 µl of 1X binding buffer was added. Data acquisition was carried out in a flow cytometer, with 10.000 cells analysed in each sample. The differentiation of early apoptotic, secondary necrotic, necroptotic and viable cells was made according to their phenotype. Annexin V+/7-AAD- were considered early apoptotic, annexin V-/7-AAD+ necroptotic, annexin V+/7-AAD+ secondary necrotic and annexin V-/7-AAD as viable cells.

Statistical Analysis

Statistical analyses were made with SPSS 19 software (SPSS, Inc, USA). For each treatment and controls, ten samples from two independent groups of animals were analysed. In the cell death experiments, data (means ± SEM) were expressed as the percentage of cell death. For statistical comparisons, only the proportions of early apoptotic and necroptotic cells were considered. The differences between various groups were examined for significance using Mann-Whitney U test. In all cases, a value $P < 0.05$ was considered statistically significant.

RESULTS

The Influence of Gabapentin on Regulated Cell Death of Cultured Astrocytes

To determine the effect of gabapentin on cell death, the cultured astrocytes were treated with different concentrations of gabapentin, from one to 100 µg/mL for 24 hours. Using flow cytometry, four different sub-populations of astrocytes were detected, which included viable cells, early apoptotic and necroptotic cells and secondary necrotic cells. The proportions of early apoptotic and necroptotic cells were detected simultaneously due to the binding of annexin V-FITC and uptake of 7-AAD dye as described in materials and methods. The binding of annexin V-FITC is considered a specific marker of apoptosis and is independent of cell death stimulus. It precedes the loss of ability to exclude viability dyes, membrane ruptures, or occurrence of any morphological changes associated with apoptosis. The uptake of 7-AAD dye is a specific marker of necroptosis and it was reported for Jurkat cells with necrotic morphology, that the binding of annexin V+ precedes the formation of membrane ruptures and necrosis (22). This finding indicated that secondary necrotic cells (annexin V+/7-AAD+) may not necessarily die by apoptosis, so to avoid potential bias in our study, only early apoptotic cells (annexin-V+/7-aad-) were considered apoptotic and secondary necrotic cells were omitted from the analyses. Similarly only cells with annexin V-/7-AAD+ phenotype were considered necroptotic and secondary necrotic cells were omitted from the analyses.

The results showed that gabapentin affected the viability of cultured astrocytes in a dose-dependent manner, because at concentrations up to 10 µg/mL there was no effect on either apoptosis or necroptosis, but higher concentrations of gabapentin enhanced overall cell death. In this study, the proportion of necroptotic cells increased significantly, whereas the early apoptotic sub-population did not differ from the untreated cells (Figure 1).

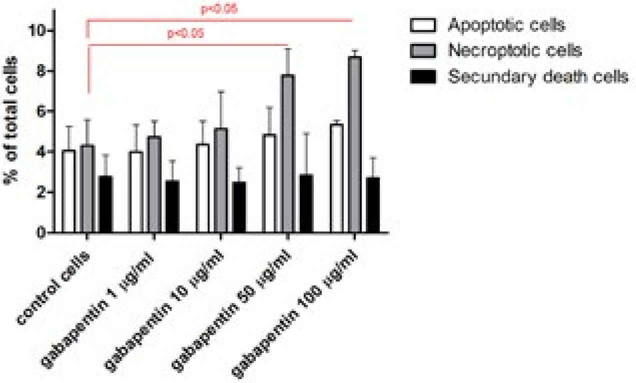

Figure 1. *The effect of gabapentin on different forms of cell death in cultured rat astrocytes. Early apoptosis and necroptosis were determined by the binding of annexin V-FITC and 7-AAD uptake, using a flow cytometry. The cells were exposed to different concentrations of gabapentin for 24 hours, and regenerated for 22 hours in gabapentin free medium. The control cells were not exposed to gabapentin. Each bar is the mean ± SEM of three independent determinations.* $p < 0.05$ *indicate significance.*

The Influence of Gabapentin on Ethanol-induced Cell death in Cultured Astrocytes

The effects of gabapentin on cell death, triggered by either acute or chronic exposure of the cells to ethanol was examined. First, the cultured astrocytes were treated simultaneously with 10 µg/mL of gabapentin and 100 mM ethanol for 24 hours and then were allowed to regenerate for 22 hours. For a positive control, the cells were treated with ethanol only. The results showed that exposure of the cells to 100 mM ethanol for 24 hours induced both apoptosis and necroptosis, the proportion of early apoptotic cells as well as the proportion of necroptotic cells was higher in comparison to untreated cells and the percentage of nonviable cells differed significantly compared to control cells. Co-treatment of the cells with 100 mM ethanol and 10 μg/mL gabapentin did not significantly prevent either apoptotic or necroptotic cell death form, induced by acute exposure of the astrocytes to ethanol (Figure 2).

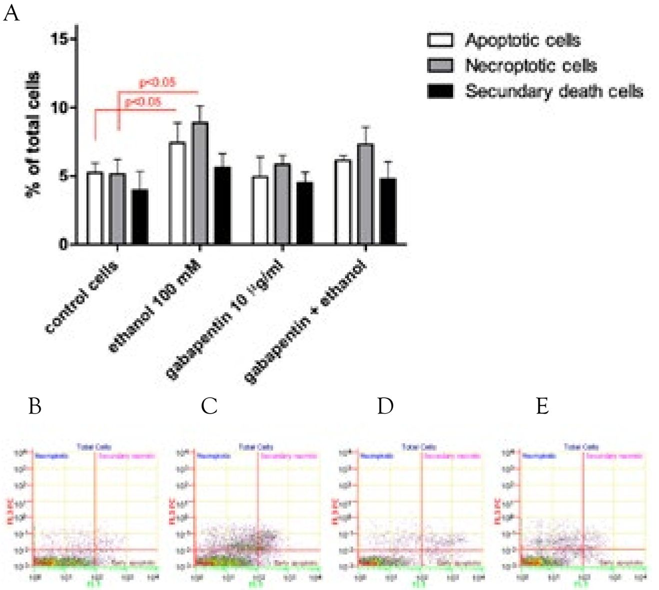

Figure 2. *The effect of gabapentin on cell death in cultured rat astrocytes, exposed to ethanol for 24 hours. A: Sub-populations of early apoptotic, necroptotic and secondary death cells, expressed as percentages of total cells after exposure of the cells to ethanol, gabapentin or combination of ethanol and gabapentin. The control cells were not exposed to either ethanol or gabapentin. Each bar is the mean ± SEM of three to five independent determinations.* $p < 0.05$ *indicate significance. B-E: Examples of two-parameter flow cytometry dot plots showing simultaneous binding of Annexin V (FL1) and 7-AAD (FL3-FC) uptake by cultured rat astrocytes after the induction of cell death. B: Control cells were not exposed to ethanol or gabapentin. C: Cells were exposed to 100 mM ethanol. D: Cells were exposed to 10 μM gabapentin. E: Cells were exposed to 100 mM ethanol and 10 μM gabapentin.*

grown in the culture medium, containing 50 mM ethanol for 7 days. The results showed that chronic exposure to ethanol significantly increased the subpopulations of early apoptotic, necroptotic, and secondary necrotic cells in comparison to untreated cells. The subpopulation of necroptotic cells was bigger in comparison to the early apoptotic one. In the astrocytes that were chronically exposed to ethanol, gabapentin diminished the subpopulation of necroptotic cells whereas the proportion of apoptosis was not significantly changed (Figure 3).

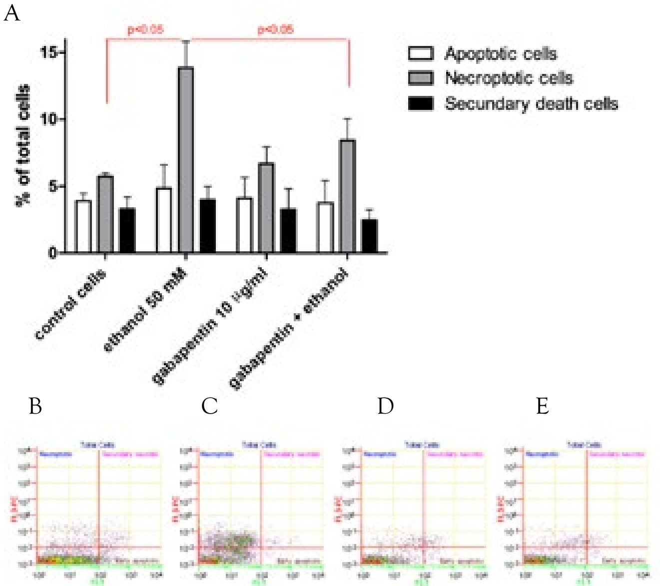

Figure 3. *The effect of gabapentin on cell death in cultured rat astrocytes, exposed to ethanol for 7 days. A: Sub-populations of early apoptotic, necroptotic and secondary death cells, expressed as percentages of total cells after exposure of the cells to ethanol, gabapentin or combination of ethanol and gabapentin. The control cells were not exposed to either ethanol or gabapentin. Each bar is the mean ± SEM of three to five independent determinations.* $p < 0.05$ *indicate significance. B-E: Examples of two-parameter flow cytometry dot plots showing simultaneous binding of Annexin V (FL1) and 7-AAD uptake (FL3-FC) by cultured rat astrocytes after the induction of cell death. B: Control cells were not exposed to ethanol or gabapentin. C: Cells were exposed to 50 mM ethanol. D: Cells were exposed to 10 μM gabapentin. E: Cells were exposed to 50 mM ethanol and 10 μM gabapentin.*

DISCUSSION

Gabapentin exerts several beneficial effects in the brain, including protecting hippocampal neurons, preventing dendritic loss and reducing reactive gliosis and astroglial stellateing, caused by glutamate exposure in rats and some of its effects are probably due to direct interactions with neurons and glia (23). Besides its GABAergic effects, gabapentin interacts with different molecules involved in cell death, like α2δ-1 subunit of voltage dependent calcium ion channels which signalling drives apoptosis, then NMDA receptors and proinflammatory cytokines, modulating cell death processes (24).

In our study we showed that gabapentin at lower concentrations of 1 and 10 µg/mL did not influence regulated cell death in cultured rat astrocytes, whereas at higher concentrations (50 and 100 µg/mL) viability of the cells was diminished (Figure 1). Our result is in line with the observation of Cardile et al. (25, 26), where they showed that gabapentin at concentrations up to 10 µg/mL did not interfere with viability of cultured rat astrocytes whereas at concentration of 50 µg/mL, it reduced the astrocytes ability to metabolize tetrazolium salts. Importantly, we found in our study, that at concentrations of gabapentin greater than 50 µg/mL, only the proportion of necroptotic cells increased and the proportion of early apoptotic cells remained at the same level as untreated cells. It is known that astrocytes are relatively resistant to apoptosis and usually die through the necroptotic pathway (27). Gabapentin also blocks the α2δ-1 subunit of voltage dependent calcium ion channels, present both in neuronal and glial cells, which interferes with apoptosis (23). Because apoptosis and necroptosis are interconnected processes, this may enhance the necroptotic pathway, which should be further explored.

Recent studies showed beneficial effects of gabapentin in alcohol dependent trial participants, where gabapentin reduced craving and disturbances in sleep and mood (28, 29). Electrophysiological findings showed that gabapentin had different effects in nondependent and ethanol dependent rats on GABAergic synaptic transmission in the central amygdala on cellular and pharmacological level. In nondependent rats, gabapentin facilitated GABAergic transmission, but did not affect ethanol intake; but in dependent rats, gabapentin decreased GABAergic transmission in the central amygdala and reduced excessive ethanol intake. It has been shown in the past that ethanol induces cell death in cultured astrocytes, where apoptosis as well as necroptosis can occur (18, 20). In the present study the effect of gabapentin was examined on regulated cell death processes in the astrocytes exposed to ethanol for a short term of 24 hours, as well as for a longer term of 7 days. The results showed that acute and chronic exposure to ethanol was greatly enhanced by necroptotic cells, while early apoptosis was not affected as much. It was also found that 10 µg/mL gabapentin did not affect either early apoptosis or necroptosis of astrocytes significantly, triggered by 24 hour exposure to ethanol. When the cells were exposed to ethanol for seven days, 10 µg/mL gabapentin was able to diminish necroptotic cell death, caused by ethanol (Figure 2, 3).

Although gabapentin was found to diminish apoptotic cell death in some experimental models, this study did not confirm this effect on cultured astrocytes either after acute or chronic exposure to ethanol. This could be partially explained by the resistance of astrocytes to apoptosis and the fact that apoptosis and necroptosis are not separated processes and thus interfere with each other.

CONCLUSIONS

This study showed that gabapentin in concentrations up to 10 µg/mL did not affect regulated cell death in cultured rat astrocytes, whereas at the higher concentrations of 50 and 100 µg/mL, viability of the cells was diminished. Further, 10 µg/mL gabapentin did not protect astrocytes from cell death after acute exposure to ethanol, whereas it was able to diminish necroptotic cell death after chronic exposure to ethanol.

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