400 µg/ml protease inhibitors; Complete™ Mini, Boehringer-Mannheim, Germany) and centrifuged at 11,000 rpm for 10 minutes at 4°C. Samples were stored at -20°C until use. Protein concentration was determined by the DC Protein assay kit I (Bio-Rad Laboratories, Marnes-la-Coquette, France) and 15 µg of protein/lane were separated by 8% SDS-PAGE gels. Proteins were transferred to nitrocellulose membranes (Bio-Rad Laboratories, Marnes-la-Coquette, France) at 120 mA for 4 hours. Blocking was performed in TBS buffer (10 mM Tris-HCl pH 7.5, 150 mM NaCl) with 5% (w/v) dry milk powder. Membranes were incubated with primary antibodies overnight at dilutions of 1:100 for NCAM and actin antibodies and 1:70 for SP antibody. The immunoblots were subsequently washed 3 x 10 min in TBS containing 0.05% Tween-20 and incubated for 1 hour with HRP-linked anti-rabbit IgG (Bio-Rad Laboratories, Marnes-la-Coquette, France, 1:2,000) or anti-mouse IgG (Bio-Rad Laboratories, Marnes-la-Coquette, France, 1:2,000). Secondary antibody was detected by Immun-Star HRP chemiluminescent kit detection system (Bio-Rad Laboratories, Marnes-la-Coquette, France) and exposure to Kodak BioMax light film (Sigma-Aldrich, St. Louis, MO, USA). Immunoblots for detection of NCAM and SP expression were reblotted with anti-actin antibody for a loading-control.

Statistical analysis

The data are presented as mean ± standard error of the mean (S.E.M.). The statistical analysis of cell death assays results was performed by ANOVA followed by Fisher's post-hoc test, using SPSS 12.0 statistical software. A value of $p < 0.05$ was considered statistically significant.

Results

Gabapentin increases cell viability in CGN cultures maintained in physiological KCl concentration

Taking advantage of the fact that CGN express both ionotropic glutamate and $GABA_A$ receptors [31] and that a proportion of these neurons die by apoptosis when cultures are maintained in physiological KCl concentration [32], we tested the effects on the cell viability of the anticonvulsants GP and TPM and of GABA, NMDA-receptor antagonist MK801 and AMPA-receptor antagonist NBQX after 6 hours of exposure to these compounds in growth medium (Figure 1). GP (30 µM) significantly increased cell viability as compared to control cells (to 123.1 ± 7.5%, n = 33, p<0.01, ANOVA, Fisher's post-hoc test). In contrast to GP, TPM (30 µM), GABA (2 mM), MK801 (10 µM) and NBQX (100 µM) did not significantly change the cell viability as compared to control cells over 6 hours of treatment (Figure 1).

Staurosporine triggers apoptosis in CGN cultures

CGN cultures were exposed to STS, a well-characterized inducer of apoptosis in neurons [23,33]. We explored cell viability in CGN cultures related to different STS concentrations over 24 hours in a previous study [27]. In this study we selected the concentration of 1 µM STS, which over a 6 hour exposure caused a significant decrease in cell viability measured by MTT assay (to 70.8 ± 3.4%, n = 78, p < 0.001, ANOVA, Fisher's post-hoc test) as compared to control cells (Figure 1). Considering that MTT assay estimates mainly the mitochondrial viability and does not detect the form of cell death, we stained the CGN cultures with an annexin V- PI kit, in order to confirm the apoptotic nature of cell death. As shown in Figure 2, STS exposure over 6 hours generated cells positive for both annexin V and PI, characteristic for late apoptosis. We therefore used the MTT assay to quantify and compare cell viability in the next experiments of the neuroprotection study.

Gabapentin and topiramate protect cerebellar granule neurons against staurosporine-triggered apoptosis through different mechanisms

To explore whether GP and TPM are protective against STS-triggered apoptosis, we further determined cell viability in CGN cultures treated with STS and the hypothesized neuroprotective agent in combination. As

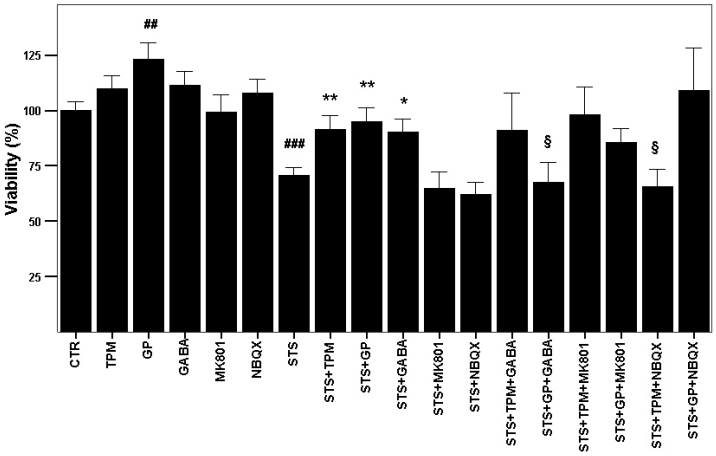

Figure 1. Study of effects of antiepileptic drugs and receptor operators on cell viability in staurosporine-triggered cell death. Rat cerebellar granule neuron cultures were exposed in growth medium for 6 h to the various treatments indicated by abbreviations. Cell viability estimated by MTT assay was calculated in percentages of cell viability in control cells, which were exposed to vehicle (DMSO) in growth medium for 6 h. An average of optical density of all control wells from all experiments was taken as 100% (n = 78). TPM = topiramate (30 µM), GP = gabapentin (30 µM), GABA = gamma-aminobutyric acid (2 mM), MK801 = 5-methyl-10,11-dihydro-5H-dibenzo[a,d]cyclohepten-5,10-imine (dizocilpine) maleate, NMDA receptor antagonist (10 µM), NBQX = 6-nitro-7-sulfamoylbenzo(f)quinoxaline-2,3-dione, AMPA receptor antagonist (100 µM), STS = staurosporine (1 µM). Error bars indicate standard error mean (SEM). Statistical analysis of the results was carried out using ANOVA followed by Fisher's post-hoc test. ##, $p < 0.01$; ###, $p < 0.001$ ($24 \le n \le 78$) mark the statistical significance in cell viability as compared to control values. *, $p < 0.05$; **, $p < 0.01$; ***, $p<0.001$ ($9 \le n \le 78$) mark the statistical significance in cell viability for cells treated with STS + cotreatment as compared to cells treated with STS alone. §, $p < 0.05$ ($9 \le n \le 78$) mark the statistical significance in cell viability for cells treated with STS + GP + GABA as compared to cells treated with STS + GP and for cells treated with STS + TPM + NBQX as compared to cells treated with STS + TPM.

shown in Figure 1, after a 6 hour exposure in growth medium, cell viability was significantly higher in cultures treated with STS+GP (94.8 ± 6.3%, n = 33, p < 0.01, ANOVA, Fisher's post-hoc test) and STS+ TPM (91.3 ± 6.2%, n = 45, p < 0.01, ANOVA, Fisher's post-hoc test) as compared to cultures treated with STS alone (70.8 ± 3.4%, n = 78). Moreover, both GP and TPM were able to reverse the STS-induced typical apoptotic PI-positive and annexin V positive cells (Figure 2).

We also tested the effects of GABA, MK801 and NBQX on STS-induced cell death. We found that addition of GABA significantly increased the cell viability in STS-treated cultures (to 90.1 ± 5.9%, n = 24, p < 0.01, ANOVA, Fisher's post-hoc test). In contrast, the addition of MK801 or NBQX did not significantly change the cell viability of STS-treated cultures.

Even though it is well-documented that STS triggers apoptosis independent of glutamate and GABA receptors [34], considering that endogenous glutamate is released in CGN cultures and that these cultures are sensitive to changes in receptor-operated intracellular ionic concentrations [31] and that the main mechanism of TPM neuroprotection is the blockade of AMPA receptors [24], we tested whether we can reverse the protective effect of GP and TPM by GABA or by glutamate ionotropic receptor antagonists.

In these conditions we found that GABA (2 mM) was able to completely reverse cell viability increase determined by GP in STS-treated cells. Cell viability in STS+GP+GABA treated cells was 67.6 ± 9.6% (n = 12, p < 0.05 for comparison to STS+GP treated cells and p = 0.79 for comparison with STS treated cells, ANOVA, Fisher's post-hoc test). In contrast, blockade of NMDA or AMPA receptors by MK801 or NBQX respectively did not affect the neuroprotection conferred by GP to STS-treated cells. Unlike GP, cell viability increase determined by TPM in STS-treated cells was completely reversed by the AMPA-receptor antagonist NBQX (100 μM). Cell viability in STS+TPM+NBQX treated cells was 65.7 ± 7.7% (n = 15, p < 0.05 for comparison to STS+TPM treated cells and p = 0.65 for comparison with STS treated cells, ANOVA, Fisher's post-hoc test). GABA receptors stimulation or blockade of NMDA receptors by MK801 did not affect neuroprotection conferred by TPM to STS-treated cells.

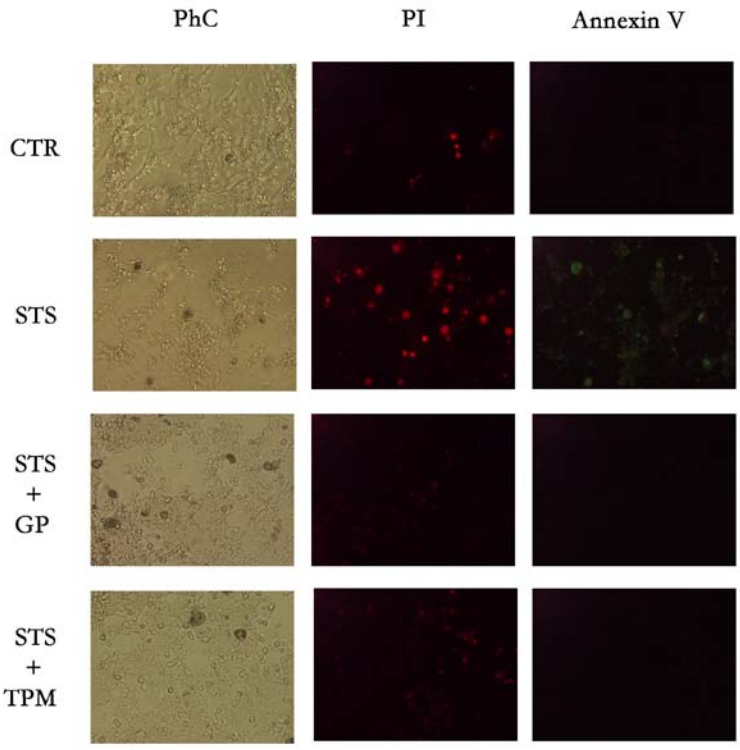

Figure 2. Phase contrast (PhC) and fluorescence microscopy of rat cerebellar granule neuron (CGN) cultures stained with an apoptosis detection kit containing propidium iodide (PI) and annexin V, in control (CTR) condition (cells exposed to vehicle, DMSO) or after 6 h treatment with staurosporine (STS, 1 μM), STS (1 μM) + GP (gabapentin, 30 μM) or STS (1 μM) + TPM (topiramate, 30 μM). For space reasons, microscopy images of cultures treated with GP or TPM were not included, these being similar to the control condition. Many typical PI positive (late apoptosis) and annexin V positive (early apoptosis) cells can be noticed in CGN cultures treated with STS, in contrast to control cultures and cultures treated with STS + GP or STS + TPM.

Neuroprotective concentrations of gabapentin and topiramate do not affect expression of neuronal cell adhesion molecule and of synaptophysin in CGN cultures

Furthermore, taking into account that the blockade of glutamate excitotoxicity by neuroprotective agents (which act directly on glutamate receptors) could result in decreased neuronal plasticity, we set out to determine the effects of GP and TPM on the expression of NCAM and SP, two proteins involved in synaptic plastic changes [25]. We therefore treated CGN cultures with vehicle (DMSO 1%, control cells), GP (30 μM), TPM (30 μM), STS (1 μM), STS (1 μM) +GP (30 μM) or STS (1 μM) + TPM (30 μM), using the concentrations of these drugs that previously proved to be protective against cell death in our experimental paradigm. Observation of neuronal endings in phase-contrast microscopy did not show any notable changes either with GP or TPM treatments (data not shown).

NCAM antiserum identified a single and specific band of ~180 kDa, corresponding to the high molecular weight isoforms, and SP antiserum identified a single and specific band of ~ 38 kDa, typical for the whole-length synaptic vesicle protein.

As Figure 3 shows, neither GP nor TPM significantly influenced NCAM (panel A) or SP (panel B) expression over 6 hours of treatment. STS exposure resulted in a decreased NCAM expression, which was not restored by

treatments with GP or TPM that efficiently rescued cells from apoptosis (Figure 3A). On the contrary, STS treatment did not alter expression of SP and STS+GP or STS+TPM treatments had no effects on SP expression either (Figure 3B).

Discussion

Antiepileptic drugs have been used extensively in clinical practice and it has been suggested that they might be neuroprotective by a large array of experimental studies [6]. Unlike TPM, for which neuroprotection in conditions linked to both epilepsy and ischemia was demonstrated [18-21], studies regarding GP neuroprotection are scarce and contradictory [15,17]. Together with pregabalin and vigabatrin, GP belongs to the GABA analog group of anticonvulsants and it is a well-tolerated drug in most patients, with a mild side-effect profile. For GP, the main antiepileptic mechanism of action is binding to the voltage-gated Ca^{2+} channel α2-δ1 auxiliary subunit with selective inhibition of the channel [10]. Considering that neuroprotective drugs like TPM, which block ionotropic glutamate receptors [24], could interfere with neuronal plasticity, which is also regulated through glutamate neurotransmission [35], we considered it important to explore whether an anticonvulsant with a different mechanism of action, like GP, has neuroprotective properties.

We chose to explore the effects of GP in an experimental paradigm suitable to the detection of apoptosis and neuroprotection. Rat CGN cultures represent a well-described and valuable model to the study of the mechanisms of neurodegeneration and test molecules for neuroprotection [22]. These cultures contain glutamatergic cells which express ionotropic glutamate and $GABA_A$ receptors [31] and are characterized by spontaneous synapse formation and electrical activity when CGN are cultured in physiological medium [36]. In CGN cultures a proportion of naturally occurring apoptosis is documented 3-5 days after plating, which can be rescued by elevated KCl concentration through the activation of voltage-gated Ca^{2+} channels and depolarization [32].

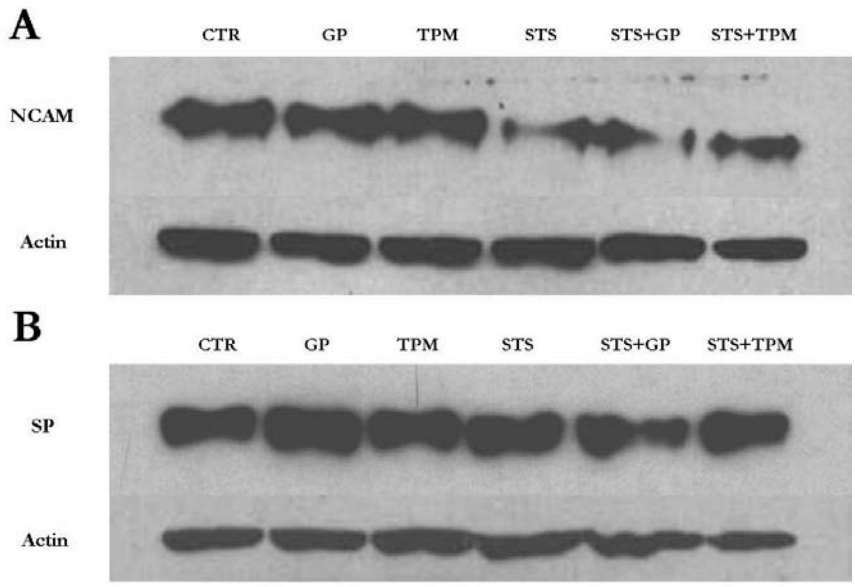

Figure 3. Expression of neural plasticity markers in rat cerebellar granule neuron (CGN) cultures in control (CTR) condition (cells exposed to vehicle, DMSO) or after 6 h treatment with staurosporine (STS, 1 μM), gabapentin (GP, 30 μM), topiramate (TPM, 30 μM), STS + GP or STS +TPM. Equivalent amounts of total lysate protein (15 μg / lane) were separated by 8% SDS-PAGE gels and immunoblotting with neural cell adhesion molecule (NCAM) antiserum (panel A, bands at ~ 180 kDa) and with synaptophysin (SP) antiserum (panel B, bands at ~ 38 kDa) was performed. Reblotting of the same membranes with anti-actin antibody was used as a loading control (bands at ~ 42 kDa) which is shown below the immunoblots with anti-NCAM and anti-SP antibodies in each panel.

STS is a broad-spectrum protein kinase inhibitor widely used to trigger apoptosis in all cell types including neurons [23,33,37]. We used STS to trigger apoptosis because this model has well-characterized mechanisms of cell death (release of cytochrome c, increase in reactive oxygen species, calcium dysregulation and caspase activation) and is not operated primarily by glutamate receptors [34]. We also considered the fact that in CGN cultures, glutamate is endogenously released by granule cells in a physiological concentration at synapse level [31], which can interfere with cell survival, and therefore we used glutamate ionotropic receptor antagonists to explore effects of glutamate release. It is nonetheless possible for dying neurons with altered membranes to release excess non-synaptic glutamate in these cultures. In our experiments we also used GABA, which was shown to protect CGN from death triggered by excessive endogenous glutamatergic stimulation [38].

We first examined the effects of GP and TPM on CGN cultured in physiological medium and we showed that GP was able to increase cell viability, while TPM lacked this effect. Once NBQX and MK801 were also unable to significantly increase cell viability in this paradigm, we concluded that the GP effect was glutamate receptor independent. Moreover, from other studies it could have been predicted that blockade of glutamate receptors in CGN cultured in physiological medium would not result in neuroprotection [36,38].

Next, we investigated the neuroprotective capabilities of GP and TPM in STS-induced cell death and we found that both anticonvulsants were able to significantly rescue neurons from apoptosis. As expected, and due to the fact that STS does not induce cell death through glutamate receptors, blockade of NMDA or AMPA receptors by MK801 or respectively, NBQX did not result in any significant alteration of cell viability. In contrast, we found that GABA significantly protected neurons against STS-triggered apoptosis. One possible explanation for this result is that GABA was shown to increase the intracellular Ca^{2+} concentration

$[Ca^{2+}]_i$ by activation of $GABA_A$ receptors and voltage-gated calcium channels of L-subtype [39]. Meanwhile, an abnormally low $[Ca^{2+}]_i$ was associated to apoptosis triggered by STS and raising $[Ca^{2+}]_i$ rescued cells from death [23]. Therefore, the protective effect of GABA against STS-induced apoptosis found in our study could be due to compensation of $[Ca^{2+}]_i$ by activation of calcium channels. Moreover, reversal of the protection conferred by either GP or GABA against STS-triggered cell death when CGN cultures where treated with both compounds could be due to the antagonizing effects on voltage gated calcium channels, once GP is an inhibitor and GABA an activator of these channels [10].

Neuroprotection of GP was insensitive to the blockade of glutamate ionotropic receptors, in contrast to neuroprotection of TPM, which was reversed by AMPA-receptor antagonist NBQX. NBQX itself was not able to rescue cells from apoptosis, suggesting that neuroprotection of TPM is mediated through AMPA-receptors but not by a similar mechanism such as NBQX. This result is in conflict with studies which demonstrated neuroprotection with TPM in ischemia models [21,24]. However, a different apoptosis inducer was used in these studies [21,24], which does not primarily operate on glutamate receptors.

Finally, we explored the effect of treatment with GP or TPM on the expression of NCAM and synaptophysin, proteins involved in neuronal plasticity and synapse functionality. No alteration in the expression of these proteins or of the morphology of neuronal endings was caused by treatment with GP or TPM, either when they were used alone or added to STS-exposed cells. We noted instead an obvious decrease of NCAM expression caused by STS treatment, which can be due either to diminution in protein synthesis or to acceleration of turnover, and one of these might be regulated by an STS-sensitive protein-kinase. A recent study showed that NCAM expression is regulated through p75 receptor [40], which operates by tyrosine kinases, possibly susceptible as well to STS treatment. Moreover, NCAM effects on neuritogenesis are mediated through PKC isoforms [41-43] and it is possible that the expression of the protein is regulated at least partially by the same biological system. However, to this end, our results suggest that treatments with anticonvulsants do not alter expression of neuronal proteins important for synaptic contacts and functionality.

To our knowledge, there are no reports looking at progression of neurodegenerative diseases or at stroke evolution in patients under treatment with antiepileptic drugs, even though a neuroprotective rationale for the treatment of Alzheimer's disease patients with sodium valproate was previously suggested [44]. Such studies could confirm a clinical efficacy for anticonvulsants by delaying the disease progression or limiting the neurological deficit.

In summary, we report that GP is neuroprotective through glutamate-receptor independent mechanisms and without alteration of neuronal plasticity markers in our model of cultured rat cerebellar neurons. The neuroprotective potential of anticonvulsants with similar properties could be an important issue for developing combined treatment strategies in stroke or neurodegenerative diseases, such as Alzheimer's disease or Parkinson's disease.

Acknowledgments

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