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European Journal of Pharmacology 406 (2000) 319–324

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Blockade of voltage-sensitive Na^+ channels by the 5-HT_{1A} receptor agonist 8-OH-DPAT: possible significance for neuroprotection

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Received 8 June 2000; received in revised form 31 July 2000; accepted 4 September 2000

Abstract

The present study was undertaken to determine whether 5-hydroxytryptamine$_{1A}$ (5-HT_{1A}) receptor agonists interact with voltage-sensitive Na^+ or N- and P/Q-type Ca^{2+} channels to reduce the influx of Na^+ and/or Ca^{2+}. The 5-HT_{1A} receptor agonist 8-hydroxy-2-(di-*n*-propylamino)tetralin (8-OH-DPAT) inhibited both [^{3}H]batrachotoxinin binding to neurotoxin site 2 of the Na^+ channel in rat cortical membranes ($IC_{50} = 5.1$ μM) and veratridine-stimulated Na^+ influx into rat synaptosomes ($EC_{50} = 20.8$ μM). The 5-HT_{1A} receptor agonist flesinoxan and the 5-HT_{1A} receptor antagonist *N*-(2-(4-(2-methoxyphenyl)-1-piperazinyl)ethyl)-*N*-(2-pyridinyl) cyclohexanecarboxamide (WAY-100635) also displaced [^{3}H]batrachotoxinin binding with similar affinities to 8-OH-DPAT, but were much less effective in reducing veratridine-stimulated Na^+ influx. All three serotonergic agents also increased [^{3}H]saxitoxin binding to neurotoxin site 1 of the Na^+ channel. In contrast, none of these agents interacted with radioligand binding to N- or P/Q-type Ca^{2+} channels. These data show that 8-OH-DPAT directly interacts with voltage-sensitive Na^+ channels to reduce Na^+ influx so providing an additional mechanism to explain how it functions as a neuroprotectant.

Keywords: 8-OH-DPAT; 5-HT_{1A} receptor; Na^+ channel; Ca^{2+} channel; Neuroprotection; Ischemia

1. Introduction

5-Hydroxytryptamine$_{1A}$ (5-HT_{1A}) receptor agonists have been found to reduce neuronal cell death in several in vivo and in vitro paradigms of ischemia/excitotoxicity (Shibata et al., 1992; Oosterink et al., 1998; Ahlemeyer et al., 1999; Torup et al., 2000; Osborne et al., 2000). The neuroprotective effects of 5-HT_{1A} receptor agonists have been hypothesized to result from 5-HT_{1A} receptor-mediated opening of potassium channels leading to membrane hyperpolarization (Ahlemeyer et al., 1999; Krüger et al., 1999). Membrane hyperpolarization, as elicited by 5-HT_{1A} receptor agonists, would theoretically reduce voltage-gated Ca^{2+} influx and hence alleviate ischemic damage. Such a mode of action is consistent with the finding that 5-HT_{1A} receptor agonists can decrease the Ca^{2+}-dependent release of both arachidonic acid and NO/cGMP evoked by NMDA in rat synaptosomes (Strosznajder et al., 1996) and the NMDA-induced influx of Ca^{2+} in rat cortical cultures (Osborne et al., 2000). Nevertheless, the neuroprotective effects of 5-HT_{1A} receptor agonists appear to be observed at doses higher than those merely needed to stimulate 5-HT_{1A} receptors, suggesting that mechanisms other than 5-HT_{1A} receptor stimulation may also be involved. For example, administration of the full 5-HT_{1A} receptor agonist flesinoxan fails to protect hippocampal neurons against transient global cerebral ischemia in gerbils even at concentrations which are known to stimulate central 5-HT_{1A} receptors (Piera et al., 1995). Moreover, the concentrations of a number of 5-HT_{1A} receptor agonists needed to produce neuroprotection in vitro (> 10 μM) are considerably higher than the concentrations required to activate the 5-HT_{1A} receptor (Shibata et al., 1992; Peruche et al., 1994; Krüger et al., 1999).

Given these data, the aim of this work was to search for additional mechanisms that may contribute to the neuroprotective actions of 5-HT_{1A} receptor agonists. Accordingly, we have examined whether 5-HT_{1A} receptor agonists such as 8-hydroxy-2-(di-*n*-propylamino)tetralin

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0014-2999/00/$ - see front matter
PII: S0014-2999(00)00688-9

(8-OH-DPAT) and flesinoxan interact with either voltage-sensitive N- and P/Q-type Ca^{2+} channels or voltage-sensitive Na^+ channels.

2. Materials and methods

2.1. Radioligand binding to N- and P / Q-type Ca^{2+} channels

[^{125}I]ω-Conotoxin MVIIA and [^{125}I]ω-conotoxin MVIIC binding to N-type and P/Q-type Ca^{2+} channels, respectively, was determined as described by Nielsen et al. (1999) with some modifications. Aliquots of adult Wistar rat cortical membranes (1 μg protein) were incubated for 60 min at 25°C with 15 pM [^{125}I]ω-conotoxin MVIIA or 15 pM [^{125}I]ω-conotoxin MVIIC and various concentrations of the drugs tested in 300 μl of 20 mM HEPES buffer containing 75 mM NaCl, 0.1 mM EDTA, 0.1 mM EGTA and 0.1% bovine serum albumin (pH 7.2). Non-specific binding was determined in the presence of 20 nM ω-conotoxin MVIIA or 100 nM ω-conotoxin MVIIC.

2.2. Radioligand binding to Na^+ channels

[^{3}H]Saxitoxin and [^{3}H]batrachotoxinin-A 20-α-benzoate binding to sites 1 and 2 on the Na^+ channel was determined essentially as described previously (Chidlow et al., 2000). For [^{3}H]batrachotoxinin competition experiments, aliquots of cortical membranes (200–400 μg) were incubated for 60 min at 37°C with 10 nM [^{3}H]batrachotoxinin and various concentrations of the drugs tested in 200 μl of Na^+-free buffer containing 1 μM tetrodotoxin, 30 μg scorpion venom (*Leiurus quinquestriatus*), and 0.1% bovine serum albumin. Non-specific binding was determined in the presence of 300 μM veratridine. For [^{3}H]saxitoxin inhibition binding experiments, aliquots of cortical membranes (200–400 μg) were incubated for 30 min at 37°C with 2.5 nM [^{3}H]saxitoxin and various concentrations of the drugs tested in 200 μl of Na^+-free buffer. Non-specific binding was determined in the presence of 1 μM tetrodotoxin.

2.3. Determination of Na^+ influx into rat synaptoneurosomes

Na^+ influx into rat cortical synaptosomes was determined as described previously (Chidlow et al., 2000). Aliquots of freshly prepared synaptosomes (containing 350–450 μg protein) were preincubated for 10 min at 37°C with or without the test compounds. After preincubation, 0.5 μCi of ^{22}NaCl diluted in low Na^+ buffer was added and the samples incubated for further 10 min at 37°C. Na^+ influx was initiated by the addition of 100 μM veratridine and terminated after 30 s by dilution of the samples and rapid vacuum filtration through Whatman GF/B filters presoaked in 0.1% polyethylenimine. Radioactivity trapped on the filters was measured by liquid scintillation spectrometry. Tetrodotoxin (1 μM) was used to determine the non-specific influx of $^{22}Na^+$.

2.4. Analysis of data

Slope factors and IC_{50} or EC_{50} values were obtained using a nonlinear method (GraphPad Prism 1.0). All experiments were performed in duplicate and data are expressed as means ± S.E.M. Statistical analyses were performed by Student's *t*-test.

2.5. Materials

[^{125}I]ω-Conotoxin MVIIA (2200 Ci/mmol), [^{125}I]ω-conotoxin MVIIC (2200 Ci/mmol), [^{3}H]batrachotoxinin (34 Ci/mmol) and ^{22}NaCl (1 mCi/ml) were obtained from NEN Research Products (Stevenage, UK), and [^{3}H]saxitoxin (14.9 Ci/mmol) from Amersham (Amersham, UK). ω-Conotoxins MVIIA and MVIIC were purchased from TCS Biologicals (Botolph Claydon, UK), scorpion venom and veratridine from Sigma (Poole, UK) and tetrodotoxin from Semat Technical (St. Albans, UK). 8-OH-DPAT and *N*-(2-(4-(2-methoxyphenyl)-1-piperazinyl)ethyl)-*N*-(2-pyridinyl) cyclohexanecarboxamide (WAY-100635) were from RBI (Poole, UK) and flesinoxan and buspirone were kindly provided by Duphar (Weesp, Netherlands) and Bristol-Meyers Squib (Wallingford, USA), respectively. Protein concentration was determined using a bicinchoninic acid protein assay kit (Sigma) with bovine serum albumin as standard.

3. Results

3.1. Radioligand binding to N- and P / Q-type Ca^{2+} channels

8-OH-DPAT and flesinoxan at concentrations ranging from 100 nM to 1 mM did not significantly inhibit either [^{125}I]ω-conotoxin MVIIA or [^{125}I]ω-conotoxin MVIIC binding to N- or P/Q-type Ca^{2+} channels, respectively, in rat cortical membranes. ω-Conotoxin MVIIA displaced [^{125}I]ω-conotoxin MVIIA specific binding with an IC_{50} of 8.2 pM ($-\log IC_{50} = 11.08 \pm 0.07$, $n = 6$), while ω-conotoxin MVIIC showed an IC_{50} of 88.7 pM ($-\log IC_{50} = 10.05 \pm 0.05$, $n = 6$) in inhibiting [^{125}I]ω-conotoxin MVIIC specific binding.

3.2. Radioligand binding to Na^+ channels

Both 8-OH-DPAT and flesinoxan inhibited [^{3}H]batrachotoxinin specific binding in a concentration-dependent fashion (Fig. 1). 8-OH-DPAT displaced [^{3}H]batrachotoxi-

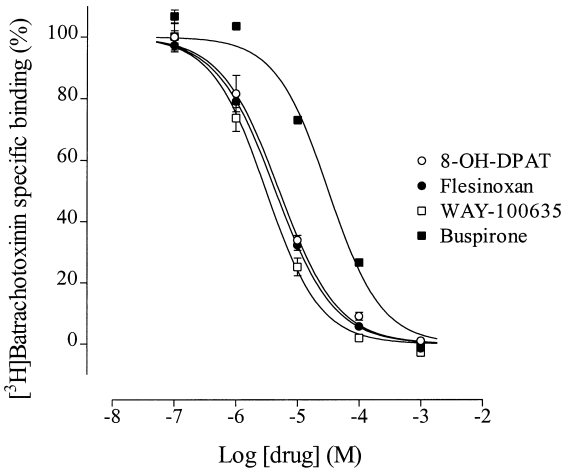

Fig. 1. Effects of 8-OH-DPAT, flesinoxan, WAY-100635 and buspirone on [^{3}H]batrachotoxinin specific binding to rat cortical membranes. Each point represents the mean ± S.E.M. of three independent experiments performed in duplicate.

nin binding with an IC_{50} of 5.1 μM ($-\log IC_{50} = 5.29 \pm 0.04$) and a slope factor of 0.91 ± 0.07 ($n = 3$), whereas the calculated IC_{50} and slope factor values for flesinoxan were 4.4 μM ($-\log IC_{50} = 5.36 \pm 0.02$) and 0.91 ± 0.03 ($n = 3$), respectively. The 5-HT_{1A} receptor antagonist WAY-100635 showed an affinity similar to that of 5-HT_{1A} receptor agonists 8-OH-DPAT and flesinoxan for [^{3}H]batrachotoxinin binding sites (Fig. 1), with an IC_{50} of 3.1 μM ($-\log IC_{50} = 5.50 \pm 0.05$) and a slope factor of 0.98 ± 0.09 ($n = 3$). In contrast, the 5-HT_{1A} receptor partial agonist buspirone displayed a significantly lower affinity ($IC_{50} = 32.8$ μM; $-\log IC_{50} = 4.50 \pm 0.11$; slope factor = 1.00 ± 0.19; $n = 3$). Veratridine (data not shown) completely inhibited [^{3}H]batrachotoxinin specific binding with an IC_{50} of 4.4 μM ($-\log IC_{50} = 5.36 \pm 0.03$) and a slope factor of 0.97 ± 0.03 ($n = 3$).

Both 8-OH-DPAT and flesinoxan produced an increase in the specific binding of [^{3}H]saxitoxin to rat cortical membranes that reached statistical significance ($P < 0.05$ by paired Student's *t*-test) at a concentration of 10 μM (Fig. 2). The 5-HT_{1A} receptor antagonist WAY-100635 significantly ($P < 0.05$ by paired Student's *t*-test) increased [^{3}H]saxitoxin binding only at 250 μM concentration (Fig. 2). In contrast, [^{3}H]saxitoxin binding was potently displaced by tetrodotoxin ($IC_{50} = 37.4$ nM, $-\log IC_{50} = 7.43 \pm 0.02$, slope factor = 1.16 ± 0.05, $n = 3$).

3.3. Na^+ influx into rat synaptoneurosomes

Veratridine (100 μM) induced approximately a three-fold increase in the influx of $^{22}Na^+$ into rat cortical synaptosomes from $35\,890 \pm 2700$ cpm/mg protein (in the presence of 1 μM tetrodotoxin) to $103\,460 \pm 10\,170$ cpm/protein ($n = 9$). Both 8-OH-DPAT and flesinoxan displayed no significant effect on Na^+ influx alone (data not shown) but dose-dependently inhibited veratridine-stimulated Na^+ influx into synaptosomes (Fig. 3). 8-OH-DPAT completely blunted the veratridine-stimulated Na^+ influx with an EC_{50} of 20.8 μM ($-\log EC_{50} = 4.68 \pm 0.07$, slope factor = 0.60 ± 0.06, $n = 4$), while flesinoxan exhibited a significantly lower potency ($P < 0.01$ by Student's *t*-test) with an EC_{50} of 176.0 μM ($-\log IC_{50} = 3.75 \pm 0.09$, slope factor = 0.61 ± 0.08, $n = 4$). The 5-HT_{1A} receptor antagonist WAY-100635 was also found to reduce the veratridine-stimulated Na^+ influx alone, although with a significantly lower efficacy than 8-OH-DPAT. At 100-μM concentration, WAY-100635 elicited a 20.7 ± 4.37%

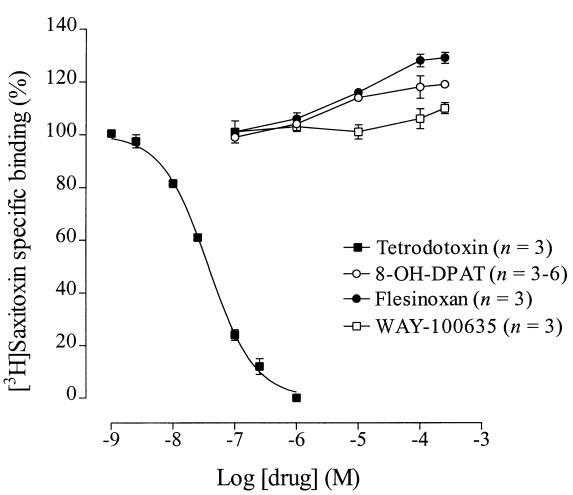

Fig. 2. Effects of 8-OH-DPAT, flesinoxan and WAY-100635 on [^{3}H]saxitoxin specific binding to rat cortical membranes. The effect of tetrodotoxin is also shown for comparison. Each point represents the mean ± S.E.M. of the number of experiments shown in parentheses, with each determination performed in duplicate.

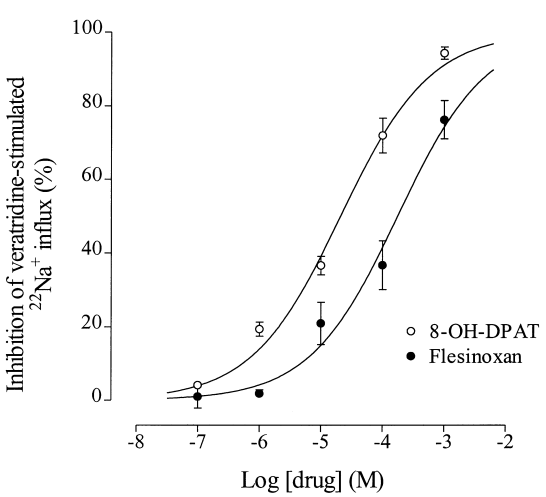

Fig. 3. Inhibition of veratridine-stimulated $^{22}Na^+$ influx into rat cortical synaptosomes by 8-OH-DPAT and flesinoxan. Each point represents the mean ± S.E.M. of four independent experiments performed in duplicate.

($n = 3$) inhibition of veratridine-stimulated Na^+ influx, which was significantly lower than that observed with an equimolar dose of 8-OH-DPAT ($71.93 \pm 4.72\%$, $n = 3$, $P < 0.01$ by Student's *t*-test). The inhibition of veratridine-stimulated Na^+ influx caused by 8-OH-DPAT alone (100 μM) was not significantly different from that produced by 8-OH-DPAT plus WAY-100635 (both 100 μM; $83.97 \pm 6.96\%$, $n = 3$).

4. Discussion

In the present study, the 5-HT_{1A} receptor agonist 8-OH-DPAT was found to interact directly with voltage-sensitive Na^+ channels and to reduce the veratridine-stimulated influx of Na^+ into rat cortical synaptosomes. On the contrary, the drug did not show any significant affinity for N- or P/Q-type voltage-gated Ca^{2+} channels.

The 5-HT_{1A} receptor agonists 8-OH-DPAT and flesinoxan completely inhibited [^{3}H]batrachotoxinin binding to neurotoxin site 2 of the Na^+ channel, which is associated with the gating mechanism of the channel, yet the compounds increased [^{3}H]saxitoxin binding to neurotoxin site 1, which is located at the channel pore. [^{3}H]Batrachotoxinin binding to rat cortical membranes was displaced in a concentration-dependent fashion by both 8-OH-DPAT and flesinoxan with similar affinities (IC_{50} values of 5.1 and 4.4 μM, respectively) and showing in both cases a slope factor close to unity. These data are suggestive of a competitive mode of inhibition with a single class of sites, although it should be noted that if the magnitude of negative heterotropic interaction is large, an allosteric modulator can inhibit the specific binding of a radioligand in an apparently competitive fashion (Ehlert, 1988). It is likely, therefore, that these ligands indirectly modulate radioligand binding to neurotoxin site 2, as described for local anaesthetics, anticonvulsants and antiarrhythmics (Catterall, 1987), β-adrenoceptor antagonists like propranolol and betaxolol (Chidlow et al., 2000), the general anaesthetic propofol (Ratnakumari and Hemmings, 1996), Ca^{2+} channel blockers (Grima et al., 1988), and other neuroprotective compounds (Shimidzu et al., 1997). The mechanism of the interaction of 8-OH-DPAT and flesinoxan with the Na^+ channel, however, appears to be more complex than for the above-mentioned compounds, since these 5-HT_{1A} receptor agonists also caused a small but significant increase in [^{3}H]saxitoxin binding to neurotoxin site 1. The data resemble, in some respects, the binding of dihydropyridines to the L-type voltage-gated Ca^{2+} channel (Mir and Spedding, 1987) and suggest perhaps that 8-OH-DPAT and flesinoxan interact with a site, which is coupled to the [^{3}H]saxitoxin binding site in a positive heterotropic allosteric manner. The complex nature of the interaction between 5-HT_{1A} receptor agonists and the Na^+ channel is further illustrated not only by the shallow slopes (0.6) of the concentration–response curves for the inhibition of veratridine-stimulated Na^+ influx into synaptosomes by 8-OH-DPAT and flesinoxan, but also by the clear disparity between their EC_{50} values in this assay (20.8 and 176 μM, respectively), which would not be expected given their very similar affinities for [^{3}H]batrachotoxinin binding sites.

Since the 5-HT_{1A} receptor antagonist WAY-100635 failed to blunt the effect of 8-OH-DPAT on veratridine-induced Na^+ influx, a potential role for 5-HT_{1A} receptor activation in this effect seems unlikely. WAY-100635 itself displaced [^{3}H]batrachotoxinin binding as effectively as 8-OH-DPAT or flesinoxan, and reduced, although less potently, the veratridine-induced influx of Na^+ into synaptosomes. Taken together, these observations suggest that the ability to interact with Na^+ channels could be a feature generally shared by drugs having an affinity for 5-HT_{1A} receptors. The fact that buspirone, a 5-HT_{1A} receptor partial agonist, also showed an affinity for the [^{3}H]batrachotoxinin binding site supports this view. Interestingly, 8-OH-DPAT has recently been shown to cause an inhibition of approximately 50% of voltage-dependent Na^+ currents in rat taste receptor cells (Herness and Chen, 2000). The data presented here are consistent with this finding and indicate that this inhibition of voltage-dependent Na^+ currents is produced by a direct interaction with the Na^+ channel rather than by stimulation of 5-HT_{1A} receptors.

The blockade of Na^+ influx elicited by 8-OH-DPAT may contribute to its neuroprotective effect. A number of compounds capable of blocking Na^+ channels at concentrations similar to that reported here for 8-OH-DPAT have been found to be neuroprotective in experimental models of cerebral ischemia (see Obrenovitch, 1998). Concentrations of 8-OH-DPAT in the 10–100 μM range are required to produce neuroprotection in vitro (Shibata et al., 1992; Peruche et al., 1994; Krüger et al., 1999), and according to our data, such doses would produce almost complete inhibition of Na^+ channels, yet one would expect 8-OH-DPAT to be neuroprotective at much lower concentrations if the mode of action merely involved stimulation of 5-HT_{1A} receptors. Moreover, a significant reduction of neuronal damage in a model of global cerebral ischemia in gerbils has been reported after i.p. administration of 8-OH-DPAT, but not of buspirone or flesinoxan (Piera et al., 1995). Such a difference may well reflect the higher potency of 8-OH-DPAT relative to flesinoxan and buspirone in inhibiting Na^+ influx, since it could not be ascribed to pharmacokinetic factors as all three drugs completely inhibited locomotor hyperactivity. In in vivo models of ischemia/excitotoxicity in rodents, 8-OH-DPAT elicits significant neuroprotection when administered at doses above 0.5–1.0 mg/kg i.p. or s.c. (Piera et al., 1995; Oosterink et al., 1998; Torup et al., 2000). In rats, a dose of 0.25 mg/kg s.c. (*R*)-8-OH-DPAT has been reported to produce plasma concentrations ranging from 120 to 30 nM during the first 60 min after its administration, 10- to

20-fold higher levels being found in the brain indicating the high lipophilicity of the drug (Yu et al., 1996). Brain levels of 8-OH-DPAT able to cause neuroprotection would therefore be one order of magnitude lower than the EC_{50} value reported here for inhibiting veratridine-stimulated Na^+ influx into rat cortical synaptosomes and in the same order of magnitude that the IC_{50} value for displacing [^{3}H]batrachotoxinin binding. Since it has been shown that the concentrations of some lipophilic drugs in the membrane bilayer are substantially higher than in the aqueous surroundings (Mason et al., 1991), it is not unlikely that brain concentrations of 8-OH-DPAT producing significant inhibition of Na^+ channels would have been achieved in such in vivo models of ischemia/excitotoxicity.

Inhibition of Na^+ channels by 8-OH-DPAT would reduce the energy expenditure of neurons and hence favour their survival in periods of anoxia or energy metabolism deficiency (Obrenovitch, 1998). Additionally, the ability of this drug to decrease depolarization- or NMDA-evoked Ca^{2+} influx (Cheng et al., 1998; Osborne et al., 2000) may also attenuate neuronal damage by decreasing excitatory neurotransmitter release and/or Ca^{2+} overload during ischemia (Kobayashi and Mori, 1998). Such a Ca^{2+}-influx reducing effect of 8-OH-DPAT does not result from a direct interaction of this drug with L-type (Osborne et al., 2000) or, as reported here, N- and P/Q-type voltage-dependent Ca^{2+} channels.

In conclusion, the present data indicate that the 5-HT_{1A} receptor agonist 8-OH-DPAT directly interacts with voltage-gated Na^+ channels to reduce the influx of Na^+. The 5-HT_{1A} receptor agonist flesinoxan and antagonist WAY-100635 displayed affinities for [^{3}H]batrachotoxinin binding site on the Na^+ channel similar to that of 8-OH-DPAT, but exhibited significantly lower potencies in inhibiting veratridine-stimulated Na^+ influx into synaptoneurosomes. Such a Na^+-influx reducing action was not dependent on 5-HT_{1A} receptor activation. This ability of 8-OH-DPAT to reduce the influx of Na^+ into neurons is likely to contribute to its observed neuroprotective action.

Acknowledgements

J. Melena was supported by a post-doctoral Marie Curie grant (TMR programme, European Commission). Support from the Glaucoma Foundation (New York) is also gratefully acknowledged.

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