

Oxyhomologation of the Amide Bond Potentiates Neuroprotective Effects of the Endolipid *N*-Palmitoylethanolamine

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d) Nonstandard abbreviations: NAE, *N*-acylethanolamines; *N*-arachidonoylethanolamine, AEA; PEA, *N*-palmitoylethanolamine; TRPV1, transient receptor potential type vanilloid 1 receptor; FAAH, fatty-acid amide hydrolase; NAAA, *N*-acylethanolamine-hydrolyzing acid amidase; AMT, anandamide membrane transporter;

NMDA, *N*-methyl-D-aspartate; (+)-MK 801, (5*R*,10*S*)-(+)-5-Methyl-10,11-dihydro-5*H*-dibenzo[*a,d*]cyclohepten-5,10-imine; AM251, *N*-(piperidin-1-yl)-5-(4-iodophenyl)-1-(2,4-dichlorophenyl)-4-methyl-1*H*-pyrazole-3-carboxamide; AM630, (6-iodo-2-methyl-1-[2-(4-morpholinyl)ethyl]-1*H*indol-3-yl)(4-methoxyphenyl)methanone DMEM, Dulbecco's modified Eagle's medium; FCS, fetal calf serum; *t*-BOOH, *tert*-buthylhydroperoxide; MTT, 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide; PBS, phosphate buffered saline; LDH, lactic acid dehydrogenase; TBARS, thiobarbituric acid-reacting substances; F_r , fluorescence ratio; RT-PCR, reverse transcriptase-polymerase chain reaction; GAPDH, glyceraldehyde-3-phosphate dehydrogenase;

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ABSTRACT

The endolipid *N*-palmitoylethanolamine (PEA) shows a pleiotropic pattern of bioactivities, whose mechanistic characterization is still unclear, and whose pharmacological potential is substantially limited by rapid metabolism by the amide hydrolyzing enzymes fatty acid amide hydrolases (FAAH) and *N*-acyl ethanolamine hydrolyzing acid amidase (NAAA). To overcome this problem, we have synthesized a new series of PEA homologues and characterized their activity on two *in vitro* models of neurodegeneration (oxidative stress, excitotoxicity). PEA partially prevented *tert*-butylhydroperoxide (*t*-BOOH; 100 μ M; 3 h)-induced cell death (maximum effect $26.3 \pm 7.5\%$ in comparison to *t*-BOOH untreated cells at 30 μ M), while it was ineffective against the L-glutamate (1 mM; 24 h)-induced excitotoxicity at all concentrations tested (0.01 – 30 μ M). Oxyhomologation of the amide bond, while leading to an increased enzymatic stability, also potentiated neuroprotective activity, especially for *N*-palmitoyl-*N*-(2-hydroxyethyl)hydroxylamine ($EC_{50} = 2.1 \mu$ M). These effects were not mediated by cannabinoid/vanilloid dependent mechanisms, but rather linked to a decreased *t*-BOOH-induced lipoperoxidation and reactive oxygen species formation, and L-glutamate-induced intracellular Ca^{2+} overload. The presence of the hydroxamic group and the absence of either redox active or radical scavenger moieties suggests that the improved neuroprotection is the result of increased metal chelating properties, that boosts the antioxidant activity of these compounds.

Introduction

N-Acylethanolamines (NAE) are a class of endogenous lipids whose biomedical relevance was highlighted by the discovery that *N*-arachidonylethanolamine (anandamide; AEA) binds to and activates the cannabinoid receptors (Devane et al., 1992). NAE are produced from the corresponding *N*-acylphosphatidylethanolamines through the action of Ca²⁺-activated D-type phosphodiesterases. Under physiological conditions, NAE occur at low levels in virtually all mammalian cells, tissues and certain body fluids (Bisogno et al., 1999), and their production is increased by stress, especially under conditions of cell and tissue degeneration (Hansen et al., 2001). Under these conditions, the greatest increase regards saturated and monounsaturated members of this class of endolipids, all cannabinoid-receptor inactive compounds (Schmid and Berdyshev, 2002).

N-Palmitoylethanolamine (PEA) is a fully saturated, bioactive and endogenous NAE, first identified half a century ago in lipid extracts of various tissues (Bachur et al., 1965). PEA is endowed with anti-allergic, anti-inflammatory, and anti-nociceptive properties (Calignano et al., 1998), and may elicit a wide range of effects *in vivo* that includes analgesia, inhibition of food intake, reduction of gastrointestinal motility, inhibition of cancer cell proliferation, and cytoprotection (Lambert and Di Marzo, 1999). Under physiological conditions, PEA is present at high concentrations in the CNS (Cadas et al., 1997), where its concentrations significantly increases under pathological conditions, like brain ischemia (Franklin et al., 2003; Berger et al., 2004), excitotoxicity (Di Marzo et al., 1994; Hansen et al., 1995), and neuroinflammation (Darmani et al.,

2005). *In vitro* and *in vivo* experiments suggest that PEA has neuroprotective activity: it protects cerebellar granule neurons against excitotoxicity (Skaper et al., 1996), inhibits electroshock- and chemically-induced seizures in mice (Sheerin et al., 2004), and enhances microglial cell motility (Franklin et al., 2003). PEA exhibits poor affinity for cannabinoid CB₁ or CB₂ receptors (Sheskin et al., 1997), but, surprisingly, its anti-nociceptive effects are inhibited by SR144528, a selective CB₂ receptor antagonist (Calignano et al., 1998). PEA produces a two-fold decrease in the K_i value for the AEA binding at the transient receptor potential vanilloid-type 1 receptor (TRPV1) (De Petrocellis et al., 2001), and acts as an endogenous ligand for the peroxisome-proliferator activated receptor- α (Lo Verme et al., 2005). PEA is hydrolyzed by at least two enzymes: the fatty-acid amide hydrolase (FAAH) (Schmid and Berdyshev, 2002) and the *N*-acylethanolamine-hydrolyzing acid amidase (NAAA) (Ueda et al., 2001). FAAH and NAAA are widely expressed in mammalian tissues (Ueda et al., 2001; Cravatt and Lichtman, 2002), and mice lacking the *faah* gene show reduced PEA hydrolysis and corresponding increased PEA levels in brain and liver tissues (Patel et al., 2005).

Taken together, these observations suggest that PEA is endowed with pharmacological properties potentially useful for inducing neuroprotection; however, the *in vivo* efficacy of PEA is low due to its rapid hydrolytic inactivation, and PEA does not qualify as suitable drug candidate. To overcome this problem, and as part of a series of investigations on new neuroprotective agents, we have synthesized a series of homologues of PEA, characterizing their activities on two *in vitro* models of neurodegeneration (oxidative stress, excitotoxicity). The affinities and/or activities of these compounds for a series of molecular end-points highly sensitive to variation in the polar head of endolipids (CB₁, CB₂, TRPV1, FAAH and anandamide membrane

transporter; AMT) have been recently published (Appendino et al., 2006). We present evidence that oxyhomologation of the amide bond of PEA, while leading to an increased stability toward amido hydrolyzing enzymes, also potentiates the neuroprotective properties of the parent compound, and propose a mechanistic rationale for this effect.

Methods

Drugs and Chemicals

Synthesis of Compounds. PEA (**1a**), PEA oxyhomologues: *N*-palmitoyl-*N*-(2-hydroxyethyl)hydroxylamine (**1b**), *N*-palmitoyl-*O*-(2-hydroxyethyl)hydroxylamine (**1c**), *O*-palmitoyl-*N*-(2-hydroxyethyl)hydroxylamine (**1d**); and the fatty acid hydroxamates: *N*-hydroxypalmitamide (**2a**), *N*-hydroxynonamide (**2b**), *N*-hydroxyicosamide (**2c**) (Fig. 1) were available from previous studies (Appendino et al., 2006, Ech-Chahad et al., 2005). (5*R*,10*S*)-(+)-5-Methyl-10,11-dihydro-5*H*-dibenzo[*a,d*]cyclohepten-5,10-imine [(+)-MK 801] was obtained from Tocris Bioscience (Bristol, UK). *N*-(Piperidin-1-yl)-5-(4-iodophenyl)-1-(2,4-dichlorophenyl)-4-methyl-1*H*-pyrazole-3-carboxamide (AM251) and (6-iodo-2-methyl-1-[2-(4-morpholinyl)ethyl]-1*H*indol-3-yl)(4-methoxyphenyl)methanone (AM630) were from Alexis Bioscience (Vinci, Italy). Dulbecco's modified Eagle's medium (DMEM), DMEM–Nutrient Mixture Ham's F12, Neurobasal medium, B27 supplement and fetal calf serum (FCS) were from Gibco (Invitrogen, Milan, Italy). L-Glutamine, penicillin, streptomycin, all-*trans*-retinoic acid, *tert*-butylhydroperoxide (*t*-BOOH), vitamin E (α -tocopherol), 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT), were purchased from Sigma-Aldrich (Milan, Italy). All other chemicals were of analytical grade and purchased from Merk (Darmstadt, Germany).

PEA, PEA oxyhomologues and fatty acid hydroxamates were dissolved in dimethylsulfoxide, all other drugs used were dissolved in the experimental media. Final drug concentrations were obtained by dilution of stock solutions into the experimental media. Final concentration of organic solvents was always less than 0.1%, and they

have no effects on cell viability. Drugs were added to the experimental buffer 1 h before the insults, and were maintained for the entire experiment. Cells exposed to solvent alone were considered as controls.

Cell Cultures

Human neuroblastoma cell lines, SH-SY5Y and SK-N-BE, were cultured in DMEM–Nutrient Mixture Ham's F12 and DMEM, respectively, supplemented with 10% (v/v) FCS, penicillin (100 IU/ml), streptomycin (100 µg/ml), and L-glutamine (2 mM). The cell culture medium was replaced every 2 days, and the cultures were maintained at 37°C, 95% air–5% CO₂ in a humidified incubator. The cells were differentiated into neuron-like type by treatment with all-*trans*-retinoic acid (10 µM), that was added to the cell culture medium every day for either 7 days (SH-SY5Y) or 14 days (SK-N-BE). The day before the experiments, differentiated cells were plated in a six-well culture plate (0.5 – 1 × 10⁶ cells/well).

In Vitro Models of Neurodegeneration

Oxidative Stress. The *in vitro* model of oxidative stress was performed as previously described (Lombardi et al., 2002). Briefly, differentiated SH-SY5Y cells were washed twice with phosphate buffered saline (PBS) and exposed to *t*-BOOH (100 µM) in the experimental buffer (in mM: NaCl 138, KCl 2.7, CaCl₂ 1.2, MgCl₂ 1.2, PBS 10, glucose 10; pH 7.4) for 3 h at 37°C.

Excitotoxicity. For the *in vitro* model of excitotoxicity differentiated SK-N-BE cells were washed twice with PBS and incubated in Neurobasal medium supplemented with

B27 (16 h at 37°C) to increase their sensitivity to the excitotoxic insult. Afterwards, the medium was changed and cells were exposed to 1 mM L-glutamate for 24 h at 37°C.

Evaluation of Cell Viability and Cell Death

Cell Viability. Cell viability was evaluated with the MTT assay. Absorbance was measured at 570 – 630 nm by using an Ultramark microplate reader (Bio-Rad Laboratories, Milan, Italy). The percentage of neuroprotection was calculated as follows: percentage of neuroprotection = $100 - [(x-z) \times 100 / (x-y)]$, where x is the absorbance read in control samples, y is the absorbance read in t -BOOH- or L-glutamate-treated samples, and z is absorbance read in drug- and either t -BOOH- or L-glutamate-treated samples.

Cell Death. Cell death was evaluated by measuring the lactic acid dehydrogenase (LDH) activity in the experimental medium at the end of the experiment by using a commercial kit (Roche Diagnostic, Penzberg, Germany) according to the manufacturer's instructions.

Studies on the Mechanisms of Action

Determination of Free Radical Production. Free radical production was measured by incubating the cells with the fluorescent probe 2',7'-dichlorodihydrofluorescein diacetate as previously described (Lombardi et al., 2002). Accumulation of 2',7'-dichlorofluorescein in the cells was measured as an increase in fluorescence at 525 nm, when the sample was excited at 488 nm using a Jasco (FP-777) spectrofluorometer (Jasco International, Tokyo, Japan).

Determination Lipid Peroxidation. Lipid peroxidation was evaluated by measuring the levels of thiobarbituric acid-reacting substances (TBARS) in the cells at the end of the experiments as previously described (Miglio et al., 2004). Briefly, the cells were washed and harvested with ice-cold 50 mM phosphate buffer, pH 7.4, then 500 μ l of 1% thiobarbituric acid and 500 μ l of 8 N HCl were added to each sample. The samples were boiled for 20 min and subsequently cooled with tap water. 1-Butanol, 1.5 ml, was then added to the samples and the mixture was shaken for 2 min. After centrifugation at $2000\times g$ for 10 min, the fluorescence intensity at 550 nm (excitation) and 532 nm (emission) in the butanol phase was measured by a Jasco (FP-777) spectrofluorometer.

Measurement of Intracellular Calcium Concentration

Intracellular calcium concentration was measured at single cell level by using a digital calcium image-system. Fura-2-loaded cells (5×10^5 cells/ml) were firmly attached (15 min at 37°C) to poly-L-lysine (0.1 mg/ml)-coated thin (0.2 mm) round glass coverslips (4 cm), washed, transferred to a perfusion chamber (Biopetechs, Butler, PA, USA) and mounted on an inverted microscope (Eclipse TE 300; Nikon, Tokyo, Japan). Experiments were performed at a chamber temperature of 37°C. All measurements were taken at 40-fold magnification. Excitation wavelengths were alternately selected at 340 and 380 nm by a monochromator system (Polychrome IV; TILL Photonics, Gräfelfing, Germany) and fluorescence, filtered at 505 nm, was taken with a grey-scale CCD camera (SensiCam; PCO, Kelheim, Germany). Images were acquired and analyzed with Axon Imaging Workbench 4.0 software (Axon Instruments, Union City,

CA). Data were expressed as the fluorescence ratio (F_r) mean of n monitored cells when they were excited at 340 and 380 nm respectively.

Reverse Transcriptase-Polymerase Chain Reaction (RT-PCR) Analysis

Total RNA was extracted from cells with the GeneElute Mammalian Total RNA Kit (Sigma–Aldrich) and treated (1 h, 25°C) with 5 U DNase I, RNase-free (Roche Diagnostics). DNase was then inactivated by heating for 5 min at 95°C. Human thalamic RNA was purchased from Clontech Laboratories (Milan, Italy) and used as the positive internal control. Resulting RNA was reverse-transcribed with oligo(dT) primers using 10 µg of total RNA and the ThermoScript RT-PCR System (Invitrogen, Milan, Italy). PCR was performed in a 25 µl reaction mixture containing 2 µg of cDNA, 2.5 µl of $\times 10$ buffer, 1.5 ml of 50 mM MgCl₂, 0.5 µl of a 10 mM dNTPs mix (Invitrogen), 2.5 U of Taq DNA polymerase (Invitrogen) and 2.5 ml of each primer (Table 1). RT-PCR amplicons were resolved in a 2% agarose gel by electrophoresis and visualized with ethidium bromide.

Data Analysis

Results are expressed as means \pm S.E.M. of at least six experiments. Statistical significance was evaluated by Student's t -test for paired varieties. Differences were considered statistically significant when $p < 0.05$. Origin version 6.0 (Microcal Software, Northampton, MA, USA) was used as a non-linear regression model for analysis of the concentration–response data to obtain a 50% effective concentration (EC₅₀).

Results

Neuroprotective Effects Against Oxidative Stress. Since oxidative stress is a common feature of the neurodegenerative processes (Lipton and Rosenberg, 1994), we studied possible neuroprotective effects of PEA (**1a**), PEA oxyhomologues (**1b–d**) and fatty acid hydroxamates (**2a–c**) against *t*-BOOH-induced (100 μ M, 3h) oxidative stress. *t*-BOOH significantly reduced the percentage of cell survival ($49.1 \pm 4.0\%$; $p < 0.01$; $n = 6$) in comparison to controls (*t*-BOOH un-treated cells). During *t*-BOOH exposure, cell morphology was continuously observed by phase-contrast microscopy, and progressive necrosis of cell body and neurites was evident (data not shown). When the cells were treated with increasing concentrations (0.1 – 30 μ M) of PEA the percentage of cell death was significantly reduced ($p < 0.05$; $n = 6$). The maximum neuroprotective effect was $26.3 \pm 7.5\%$ at 30 μ M of PEA (Fig. 2a). Experimental problems, due to both low water solubility and toxic effects of PEA, did not allow us to use higher concentrations of this compound.

To determine whether the oxyhomologation of the polar head of PEA causes changes in its neuroprotective activity, similar experiments were performed in the presence of increasing concentrations (0.1 – 30 μ M) of PEA oxyhomologues (**1b–d**). Cell treatments with either **1b**, **1c** or **1d** significantly reduced the *t*-BOOH-induced cell death in a concentration-dependent manner. The compound **1b** (10 μ M) abolished the effect of *t*-BOOH (the EC₅₀ calculated value was 2.1 μ M), while **1c** and **1d** only partially reduced the effect of *t*-BOOH. The maximal neuroprotective effects were $33.2 \pm 5.5\%$ for **1c** and

39.2 ± 7.8% for **1d** at 30 μM; the EC₅₀ calculated values were 2.6 μM and 2.8 μM, respectively (Fig. 2b).

Finally, to clarify the role of the hydroxamic group the neuroprotective effects of three fatty acid hydroxamates (**2a–c**) were tested with the same experimental protocol. The *N*-hydroxypalmitamide (**2a**; 10 μM) abolished the effects of *t*-BOOH (the EC₅₀ calculated value was 1.0 μM), while the *N*-hydroxynonamide (**2b**) was both less effective (the maximum neuroprotection observed was 36.4 ± 4.7% at 30 μM) and less potent (the EC₅₀ calculated value was 6.2 μM) than **2a**. No neuroprotective effects were observed for the *N*-hydroxyicosamide (**2c**) at all concentrations tested (Fig. 2c).

Effects of Cannabinoid Receptor Antagonists. Previous results from our laboratory show that PEA oxyhomologues have poor affinity for CB₁/CB₂ receptors and do not inhibit either the FAAH-mediated AEA hydrolysis or the AMT-mediated AEA cellular uptake (Appendino et al., 2006). To determine if the neuroprotective effects evoked by these compounds are mediated by cannabinoid receptors, we repeated the above experiments in the presence of AM251, a selective cannabinoid CB₁ receptor antagonist (Gatley et al., 1996), and AM630, a selective cannabinoid CB₂ receptor antagonist (Ross et al., 1999). Neither AM251 (10 μM) nor AM630 (10 μM) antagonized the neuroprotective effects induced by increasing concentration (0.1 – 30 μM) of either the compound **1b** (Fig. 3a) or **2a** (Fig. 3b). The EC₅₀ calculated values, in the absence or presence of AM251 or AM630 respectively, were: 1.7 μM, 1.8 μM, 2.1 μM for **1b**; 0.9 μM, 1.2 μM and 1.2 μM for **2a**.

Antioxidant Effects of PEA Hydroxamic Derivatives. *t*-BOOH treatment induces a rapid and progressive increase of free radical production, [Ca²⁺]_i rise, activation of

caspases and calpains, leading to cell death (Lombardi et al., 2002; Annunziato et al., 2003). To study the mechanisms underlying the neuroprotective effects of the **1b** and **2a** compounds, we measured TBARS and free radical levels in *t*-BOOH (100 μ M; 3 h)-treated cells in the absence or presence of increasing concentrations of PEA, **1b**, or **2a**. *t*-BOOH treatment (100 μ M; 3 h) induced a significant increase in both the TBARS ($47.8 \pm 8.6\%$; $p < 0.01$; $n = 6$) and the cellular free radical levels (11-fold increase of the measured fluorescence; $p < 0.01$; $n = 6$), if compared to *t*-BOOH-untreated cells. The compounds **1b** and **2a** significantly prevented these increases in a concentration-dependent manner. Both **1b** and **2a** abolished TBARS formation at 10 μ M, and the EC_{50} calculated values were 0.12 and 0.34 μ M, respectively. On the contrary, the fold of free radical increase was only partially reduced by either **1b** or **2a** ($81.3 \pm 2.8\%$ and $57.1 \pm 5.2\%$ respectively; $p < 0.01$; $n = 6$) at 1 μ M. PEA did not prevent either TBARS formation or free radical increase at all concentrations tested (Fig. 4, a-b).

Moreover, to determine if these compounds are capable to prevent the $[Ca^{2+}]_i$ increase elicited by free radicals (Annunziato et al., 2003), we measured the $[Ca^{2+}]_i$ increase evoked by *t*-BOOH treatment (100 μ M; 3 h) in fura-2-loaded cells treated or not with compounds **1b** or **2a**. Both compounds, added to the experimental medium 60 min before *t*-BOOH, prevented the $[Ca^{2+}]_i$ increase elicited by oxidative stress (Fig. 4c).

Excitotoxicity. Since controversial results have been reported on the neuroprotective effects of PEA against excitotoxicity (Skaper et al., 1996; Andersson et al., 2000), we then studied the effects of PEA and the new PEA homologues (**1b** and **2a**) in an experimental model of excitotoxicity.

Differentiated SK-N-BE neuroblastoma cells express NR1, NR2A and NR2B subunits of the NMDA receptor, as assessed by RT-PCR analysis (Fig. 5; line 2). When these cells were exposed to L-glutamate (1 mM; 24 h) a significant increase ($60.5 \pm 2.8\%$; $p < 0.01$; $n = 6$ in comparison to L-glutamate-untreated cells) in the level of LDH activity was measured in the experimental medium at the end of experiments. (+)-MK 801 (1 μ M), a non-competitive NMDA receptor antagonist, was able to antagonize L-glutamate-induced LDH increase (Fig. 6a). When cells were exposed to L-glutamate (1 mM; 24 h) in the presence of increasing concentration (0.01 – 30 μ M) of either **1b** or **2a**, the levels of LDH activity were significantly decreased. The maximum protection ($68.4 \pm 0.1\%$ and $67.2 \pm 3.3\%$ respectively; $p < 0.01$; $n = 6$) was measured at 10 μ M of **1b** or **2a**; the EC_{50} calculated values were 0.35 and 0.69 μ M respectively. On the contrary, PEA resulted ineffective at all concentrations tested (0.01 – 30 μ M) (Fig. 6b).

Effects of PEA Homologues on the L-Glutamate-Induced $[Ca^{2+}]_i$ Increase.

Protracted entry of Ca^{2+} into neurons is one of the mechanisms leading to excitotoxicity (Choi, 1988). Therefore, we studied if cell exposure (60 min before L-glutamate) to either **1b** or **2a** may prevent the delayed L-glutamate-induced $[Ca^{2+}]_i$ increase in fura-2-loaded cells. The F_r mean measured from control cells was 0.71 ± 0.04 ($n = 50$). Cell exposure to L-glutamate (1 mM, 24 h) produced a significant increase in the $[Ca^{2+}]_i$ (F_r mean = 1.22 ± 0.06 ; $n = 54$; $p < 0.01$), that was prevented by (+)-MK 801 (1 μ M; 60 min before L-glutamate), a non-competitive NMDA receptor antagonist (F_r mean = 0.81 ± 0.05 ; $n = 55$). Both compounds **1b** (10 μ M) and **2a** (10 μ M) significantly reduced ($-50 \pm 10\%$ in

comparison with L-glutamate-treated cells) the L-glutamate-induced $[Ca^{2+}]_i$ increase (F_T mean were 0.91 ± 0.07 and 0.92 ± 0.08 respectively; $n = 52$ for each compound; $p < 0.05$), whereas PEA (up to $10 \mu M$) had no effect (Fig. 7). The significant ($p < 0.05$) $[Ca^{2+}]_i$ increase still present, compared to control (L-glutamate-untreated cells), in cells exposed to **1b** and **2a** supports the residual excitotoxicity ($\sim 30\%$) measured (Fig. 6b).

Discussion

Oxidative stress and excitotoxicity are critical components of series of integrated cellular and biochemical events (“vicious circle”) that may culminate in the neuronal death associated to brain ageing and many neurodegenerative conditions, like Alzheimer’s disease (AD), Parkinson’s disease (PD), stroke, epilepsy, amyotrophic lateral sclerosis and prion diseases (Lipton and Rosenberg, 1994; Barnham, 2004). Exogenous or endogenous compounds capable to prevent or block the progression of these harmful events may be relevant for neuroprotection (Levi and Brimble, 2004). NAE are endogenous compounds that are part of a protective physiological system providing on-demand defence in case of abnormal neuronal activity (Marsicano et al., 2003). Neurodegeneration is associated with a massive accumulation of PEA (Hansen et al., 2001), whose neuroprotective effects are, however, still a matter of debate. The beneficial effects, reported in some studies (Skaper et al., 1996), have been questioned and could not be reproduced by others (Andersson et al., 2000; Franklin et al., 2003). Recently, Zolese et al. (2005) demonstrated a combination of both anti-oxidative and slightly pro-oxidative effects of PEA on Cu²⁺-induced low-density lipoprotein oxidation. Anti-oxidative effects were observed at low PEA concentrations (0.01 - 0.1 μM), while pro-oxidative effects dominated the profile of this compound at higher concentrations (1 μM). From our findings, 30 μM PEA partially (26.3 ± 7.5% in comparison to *t*-BOOH-untreated cells) protected neurons against oxidative stress, but toxicity was observed at higher (> 30 μM) concentrations. In addition, PEA was incapable to prevent L-glutamate-induced excitotoxicity in our experimental model. Discrepancies in the results from

different laboratories might be related to differences in experimental models (i.e., cell culture type, potency of the insult, time exposure to the insult, experimental buffers, chemical stability of solutions), but it seems also clear that the problems associated to the metabolic stability of PEA are compounded by a substantially bi-phasic (anti- or pro-neurotoxic) profile of activity.

PEA metabolizing enzymes are widely expressed in mammalian tissues, and the cells used for these experiments can, in principle, metabolize PEA and PEA derivatives, since they express FAAH (RT-PCR analysis; data not shown). Therefore, the stabilization of PEA molecule appears to be essential to enhance its bioactivity.

To increase the metabolic stability of PEA and shift its bi-phasic biological profile toward neuroprotection, we modified the polar head group of the molecule. We reasoned that oxyhomologation of the amide bond, while having a stabilizing effect toward enzymatic hydrolysis, because of topologic and electronic changes in the amide bond, should also induce metal chelating properties potentially capable to interfere with oxidative stress (Gaeta and Hider, 2005). Indeed, when compared to the parent compound, the oxyhomologues of PEA (**1b–d**) showed greater efficacy and potency in protecting neurons against oxidative stress and excitotoxicity, with *N*-homologation being more efficient in this respect compared to carbonyl or alkyl oxyhomologation (cf **1b** with **1c,d**). Spurred by the activity of the *N*-hydroxyamide **1b**, we also investigated the activity of its parent compound, palmitoyl hydroxamic acid (**2a**). This compound showed potent neuroprotective activity, even larger than that of the oxyhomologues of PEA. Shortening or lengthening of the acyl chain, as in capryl (C-9; **2b**) and arachyl (C-20; **2c**) hydroxamates, led to a decrease of activity, showing that the activity associated to the hydroxamate group is modulated in a specific fashion, by the lipid moiety. While

hydroxamic acids have been well investigated for their bioactivity (Yoo and Jones, 2006), remarkably little is known on their *N*-alkyl derivatives (*N*-hydroxyamides). The observation that *N*-palmitoyl-*N*-(2-hydroxyethyl)hydroxylamine retains most of the neuroprotective activity of palmitoyl hydroxamate despite its decreased chelating properties, is a significant observation, worth pursuing by in a systematic way with a larger sets of substrates.

Remarkably, oxyhomologation had little, if any, effect on the interaction of PEA with cannabinoid receptors and the enzymes involved in the degradation of endocannabinoid (Appendino et al., 2006). For these reasons, amide oxyhomologues could achieve substantially increased concentrations and a longer half-life at their sites of activity.

Rather than depending on the interaction with cannabinoid receptors and their associated proteins, the neuroprotective effects of the oxyhomologues (**1b–d**) or the hydroxamates (**2a,b**) are seemingly related to their metal chelating and antioxidant activity (Gaeta and Hider, 2005). Moreover: *a*) neither AM251, a selective CB₁ receptor antagonist, nor AM630, a selective CB₂ receptor antagonist, interfered with the neuroprotective effects of **1b** or **2a**; *b*) compounds **1b–d** showed negligible affinity for cannabinoid CB₁ and CB₂ receptors, and did not prevent AEA hydrolysis catalyzed by FAAH or its cellular uptake mediated by the putative neuronal AMT (Appendino et al., 2006); *c*) compounds **1b** and **2a** prevented *t*-BOOH-induced lipid peroxidation/free radical accumulation; *d*) compounds **1b** and **2a** prevented the [Ca²⁺]_i increase elicited by both free radical actions (Annunziato et al., 2003) and L-glutamate activation of NMDA receptors (Choi 1988). It is therefore possible that these compounds protect neurons against oxidative insults by inhibiting chain reactions leading to free radical accumulation, seemingly by a metal chelating mechanism, a well know property of

hydroxamates (Gaeta and Hider, 2005). Direct inhibition of NMDA receptor channels is unlike, since most of the NMDA ligands are, indeed, amino acids, phosphono amino acids, or amines that can be protonated under physiological conditions (SAR studies) (Brauner-Osborne et al., 2000). Two classes of amphiphilic compounds, lysophospholipids and arachidonic acid, have been demonstrated to modulate NMDA receptors through insertion into plasma membranes (Casado and Ascher, 1998). Our compounds might elicit similar effects, but the molecular basis for the neuroprotective activity of PEA is still substantially unclear. However, it does not seem unreasonable to assume that the hydroxamate moiety potentiates the intrinsic neuroprotective properties of this compound.

Taken together, our results suggest that implanting a metal chelating motif on endolipid structures leads not only to an increase of metabolic stability, but also to a potentiation of the neuroprotective properties, without substantially changing the profile of interaction with the endocannabinoid/endovanilloid system and their associated proteins.

Iron metals, particularly iron and copper, play a critical role in both oxidative stress and abnormal metal–protein interaction associated with several neurodegenerative disorders (Zecca et al., 2004; Barnham et al., 2004). Non-toxic lipophilic brain-permeable iron chelators might offer potential therapeutic benefits for these progressive diseases. An ideal chelating agent should have the capacity to first scavenging the free redox active metal, present in excess in the brain, into nontoxic and excretable metal complexes, and, secondly, to cap the metal at its labile binding site (i.e. β -amyloid, α -synuclein, prion protein and neuromelanine), preventing any mediated toxic action (Fenton activity and/or aggregation) (Gaeta and Hider, 2005). Lipids endowed with the

hydroxamate group represents an interesting addition to the growing inventory of compounds potentially capable to achieve this goal, and deserves further investigation in *in vivo* models of neurodegeneration.

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References

- Andersson M, Jacobsson SO, Jonsson KO, Tiger G and Fowler CJ (2000) Neurotoxicity of glutamate in chick telencephalon neurons: reduction of toxicity by preincubation with carbachol, but not by the endogenous fatty acid amides anandamide and palmitoylethanolamide. *Arch Toxicol* **74**:161-164.
- Annunziato L, Amoroso S, Pannaccione A, Cataldi M, Pignataro G, D'Alessio A, Sirabella R, Secondo A, Sibaud L and Di Renzo GF (2003) Apoptosis induced in neuronal cells by oxidative stress: role played by caspases and intracellular calcium ions. *Toxicol Lett* **139**:125-133.
- Appendino G, Minassi A, Berton L, Moriello AS, Cascio MG, De Petrocellis L and Di Marzo V (2006) Oxyhomologues of anandamide and related endolipids: chemoselective synthesis and biological activity. *J Med Chem* **49**:2333-2338.
- Bachur NR, Masek K, Melmon KL and Udenfriend (1965) Fatty acid amides of ethanolamine in mammalian tissues. *J Biol Chem* **240**:1019-1024.
- Barnham KJ, Masters CL and Bush AI (2004) Neurodegenerative diseases and oxidative stress. *Nat Rev Drug Discov* **3**:205-214.
- Berger C, Schmid PC, Schabitz WR, Wolf M, Schwab S and Schmid HH (2004) Massive accumulation of *N*-acylethanolamines after stroke. Cell signalling in acute cerebral ischemia? *J Neurochem* **88**:1159-1167.
- Bisogno T, Berrendero F, Ambrosino G, Cebeira M, Ramos JA, Fernandez-Ruiz JJ and Di Marzo V (1999) Brain regional distribution of endocannabinoids: implications for their biosynthesis and biological function. *Biochem Biophys Res Commun* **256**:377-380.

- Brauner-Osborne H, Egebjerg J, Nielsen EO, Madsen U and Krogsgaard-Larsen P (2000) Ligands for glutamate receptors: design and therapeutic prospects. *J Med Chem* **43**:2609-2645.
- Cadas H, di Tomaso E and Piomelli D (1997) Occurrence and biosynthesis of endogenous cannabinoid precursor, *N*-arachidonoyl phosphatidylethanolamine, in rat brain. *J Neurosci* **17**:1226-1242.
- Calignano A, La Rana G, Giuffrida A and Piomelli D (1998) Control of pain initiation by endogenous cannabinoids. *Nature* **394**:277-2781.
- Casado M and Ascher P (1998) Opposite modulation of NMDA receptors by lysophospholipids and arachidonic acid: common features with mechanosensitivity. *J Physiol* **513**:317-330.
- Choi DW (1988) Glutamate neurotoxicity and diseases of the nervous system. *Neuron* **1**:623-634.
- Cravatt BF and Lichtman AH (2002) The enzymatic inactivation of the fatty acid amide class of signaling lipids. *Chem Phys Lipids* **121**:135-148.
- Darmani NA, Izzo AA, Degenhardt B, Valenti M, Scaglione G, Capasso R, Sorrentini I and Di Marzo V (2005) Involvement of the cannabimimetic compound, *N*-palmitoylethanolamine, in inflammatory and neuropathic conditions: Review of the available pre-clinical data, and first human studies. *Neuropharmacology* **48**:1154-1163.
- De Petrocellis L, Davis JB and Di Marzo V (2001) Palmitoylethanolamide enhances anandamide stimulation of human vanilloid VR1 receptors. *FEBS Lett* **506**:253-256.
- Devane WA, Hanus L, Breuer A, Pertwee RG, Stevenson LA, Griffin G, Gibson D, Mandelbaum A, Etinger A and Mechoulam R (1992) Isolation and structure of a brain constituent that binds to the cannabinoid receptor. *Science* **258**:1946-1949.

- Di Marzo V, Fontana A, Cadas H, Schinelli S, Cimino G, Schwartz JC and Piomelli D (1994) Formation and inactivation of endogenous cannabinoid anandamide in central neurons. *Nature* **372**:686-691.
- Ech-Chahad A, Minassi A, Berton L and Appendino G. (2005) An expeditious hydroxyamidation of carboxylic acids. *Tetrahedron Lett* **46**: 5113-5115.
- Franklin A, Parmentier-Batteur S, Walter L, Greenberg DA and Stella N (2003) Palmitoylethanolamide increases after focal cerebral ischemia and potentiates microglial cell motility. *J Neurosci* **23**:7767-7775.
- Gaeta A and Hider RC (2005) The crucial role of metal ions in neurodegeneration: the basis for a promising therapeutic strategy. *Br J Pharmacol* **146**:1041-1059.
- Gatley SJ, Gifford AN, Volkow ND, Lan R and Makriyannis A (1996) ¹²³I-labeled AM251: a radioiodinated ligand which binds in vivo to mouse brain cannabinoid CB1 receptors. *Eur J Pharmacol* **307**:331-338.
- Hansen HH, Ikonomidou C, Bittigau P, Hansen SH and Hansen HS (2001) Accumulation of the anandamide precursor and other *N*-acylethanolamine phospholipids in infant rat models of *in vivo* necrotic and apoptotic neuronal death. *J Neurochem* **76**:39-46.
- Hansen HS, Lauritzen L, Strand AM, Moesgaard B and Frandsen A (1995) Glutamate stimulates the formation of *N*-acylphosphatidylethanolamine and *N*-acylethanolamine in cortical neurons in culture. *Biochim Biophys Acta* **1258**:303-308.
- Lambert DM and Di Marzo V (1999) The palmitoylethanolamide and oleamide enigmas: are these two fatty acid amides cannabimimetic? *Curr Med Chem* **6**:757-773.
- Levi MS and Brimble MA (2004) A review of neuroprotective agents. *Curr Med Chem* **11**:2383-2397.

- Lipton SA and Rosenberg PA (1994) Excitatory amino acids as a final common pathway for neurologic disorders. *N Engl J Med* **330**:613-622.
- Lo Verme J, Fu J, Astarita G, La Rana G, Russo R, Calignano A and Piomelli D (2005) The nuclear receptor peroxisome proliferator-activated receptor- α mediates the anti-inflammatory actions of palmitoylethanolamide. *Mol Pharmacol* **67**:15-19.
- Lombardi G, Varsaldi F, Miglio G, Papini MG, Battaglia A and Canonico PL (2002) Cabergoline prevents necrotic neuronal death in an in vitro model of oxidative stress. *Eur J Pharmacol* **457**:95-98.
- Marsicano G, Goodenough S, Monory K, Hermann H, Eder M, Cannich A, Azad SC, Cascio MG, Gutierrez SO, van der Stelt M, Lopez-Rodriguez ML, Casanova E, Schutz G, Zieglgansberger W, Di Marzo V, Behl C and Lutz B (2003) CB1 cannabinoid receptors and on-demand defense against excitotoxicity. *Science* **302**:84-88.
- Miglio G, Varsaldi F, Francioli E, Battaglia A, Canonico PL and Lombardi G (2004) Cabergoline protects SH-SY5Y neuronal cells in an in vitro model of ischemia. *Eur J Pharmacol* **489**:157-165.
- Patel S, Carrier EJ, Ho WS, Rademacher DJ, Cunningham S, Reddy DS, Falck JR, Cravatt BF and Hillard CJ (2005) The postmortal accumulation of brain *N*-arachidonylethanolamine (anandamide) is dependent upon fatty acid amide hydrolase activity. *J Lipid Res* **46**:342-349.
- Ross RA, Brockie HC, Stevenson LA, Murphy VL, Templeton F, Makriyannis A and Pertwee RG (1999) Agonist-inverse agonist characterization at CB1 and CB2 cannabinoid receptors of L759633, L759656, and AM630. *Br J Pharmacol* **126**:665-672.

- Schmid HH and Berdyshev EV (2002) Cannabinoid receptor-inactive *N*-acylethanolamines and other fatty acid amides: metabolism and function. *Prostaglandins Leukot Essent Fatty Acids* **66**:363-376.
- Sheerin AH, Zhang X, Saucier DM and Corcoran ME (2004) Selective antiepileptic effects of *N*-palmitoylethanolamide, a putative endocannabinoid. *Epilepsia* **45**:1184-1188.
- Sheskin T, Hanus L, Slager J, Vogel Z and Mechoulam R (1997) Structural requirements for binding of anandamide-type compounds to the brain cannabinoid receptor. *J Med Chem* **40**:659-667.
- Skaper SD, Buriani A, Dal Toso R, Petrelli L, Romanello S, Facci L and Leon A (1996) The ALIAmide palmitoylethanolamide and cannabinoids, but not anandamide, are protective in a delayed postglutamate paradigm of excitotoxic death in cerebellar granule neurons. *Proc Natl Acad Sci* **93**:3984-3989.
- Ueda N, Yamanaka K and Yamamoto S (2001) Purification and characterization of an acid amidase selective for *N*-palmitoylethanolamine, a putative endogenous anti-inflammatory substance. *J Biol Chem* **276**:35552-35557.
- Yoo C and Jones PA (2006) Epigenetic therapy of cancer: past, present, future. *Nature Rev Drug Discov* **5**: 37-50.
- Zecca L, Youdim MB, Riederer P, Connor JR and Crichton RR (2004) Iron, brain ageing and neurodegenerative disorders. *Nat Rev Neurosci* **5**:863-873.
- Zolese G, Bacchetti T, Ambrosini A, Wozniak M, Bertoli E and Ferretti G (2005) Increased plasma concentrations of palmitoylethanolamide, an endogenous fatty acid amide, affect oxidative damage of human low-density lipoproteins: an *in vitro* study. *Atherosclerosis* **182**:47-55.

Footnotes

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- b)** Reprint requests to:
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Legends for figures

Figure 1. Chemical structure of the compounds used in this study

Figure 2. Concentration-response curves of the neuroprotective effects of PEA, PEA oxyhomologues and fatty acid hydroxamates. Panel a shows the effects of increasing concentrations of PEA (0.1 – 30 μ M) on *t*-BOOH-induced cell death (black bar), expressed as percentage of cell survival over *t*-BOOH-untreated cells (white bar). Panel b and c show the neuroprotective effects of increasing concentrations (0.1 – 30 μ M) of either PEA oxyhomologues (**1b–d**) or fatty acid hydroxamates (**2a–c**), respectively, expressed as percentage of neuroprotection. The EC₅₀ calculated values were: 2.1 μ M, 2.6 μ M, 2.8 μ M, 1.0 μ M and 6.2 μ M for **1b**, **1c**, **1d**, **2a** and **2b**, respectively. The data represent mean \pm S.E.M. of at least six experiments runs in triplicate. # $p \leq 0.01$ vs. cells treated with vehicle alone; * $p \leq 0.05$ vs. *t*-BOOH-treated cells.

Figure 3. Effects of CB₁ and CB₂ receptor antagonists on either the **1b**- or the **2a**-induced neuroprotection. The cells were exposed to the CB₁ (AM251; 10 μ M) or the CB₂ (AM630; 10 μ M) receptor antagonist 5 min before exposure to increasing concentrations of either **1b** (0.1 – 30 μ M) (Panel a) or **2a** (0.1 – 30 μ M) (Panel b). The values are expressed as percentage of neuroprotection. The EC₅₀ calculated values, in the

absence or presence of AM251 or AM630 respectively, were: 1.7 μM , 1.8 μM , 2.1 μM for **1b**; 0.9 μM , 1.2 μM and 1.2 μM for **2a**. The data represent mean \pm S.E.M. of at least six experiments runs in triplicate.

Figure 4. Anti-oxidant effects of PEA hydroxamic homologues. Panel a shows the ability of increasing concentrations (0.001 – 10 μM) of either **1b** or **2a** in reducing membrane lipoperoxidation (TBARS) in *t*-BOOH-treated cells (black bar), expressed as percentage of TBARS over *t*-BOOH-untreated cells. The EC_{50} calculated values were 0.34 μM and 0.12 μM for **1b** and **2a**, respectively. Panel b shows the ability of increasing concentrations (0.001 – 10 μM) of either **1b** or **2a** in reducing reactive oxygen species (ROS) formation in *t*-BOOH-treated cells (black bar), expressed as fold increase of 2',7'-dichlorofluorescein's fluorescence over *t*-BOOH-untreated cells. The EC_{50} calculated values were 8.2 nM and 49 nM for **1b** and **2a**, respectively. Panel c shows the effect of 10 μM **1b** and **2a** compounds on the $[\text{Ca}^{2+}]_i$ increase evoked by 100 μM *t*-BOOH. The values are expressed as F_r mean of 55 cells monitored. The data represent mean \pm S.E.M. of at least six experiments runs in triplicate. # $p \leq 0.01$ vs. cells treated with vehicle alone; * $p \leq 0.05$, ** $p \leq 0.01$ vs. *t*-BOOH-treated cells.

Figure 5. Expression of NMDA receptor subunit genes in differentiated SK-N-BE neuroblastoma cells. RT-PCR analysis was performed on total RNA isolated from

human thalamic nucleus (lane 1) or SK-N-BE cells (lane 2) using specific primer pairs directed against either NR1, NR2A–D subunits or GAPDH.

Figure 6. Effects of PEA hydroxamic homologues on L-glutamate-induced excitotoxicity. The cells were exposed to L-glutamate (1 mM; 24 h) in the absence (black bar) or presence of (+)-MK 801 (1 μ M) (dashed bar). Cell death was evaluated as percent of LDH activity increase over sample treated with vehicle alone (panel a). Panel b shows the protective effects of increasing concentration (0.01 – 30 μ M) of either PEA, **1b** or **2a** on L-glutamate-induced cell death. The values are expressed as percentage of neuroprotection in comparison to sample treated with L-glutamate. The EC₅₀ calculated values were 0.34 μ M and 0.12 μ M for **1b** and **2a**, respectively. The data represent mean \pm S.E.M. of at least six experiments runs in triplicate. # $P \leq 0.01$ vs. cells treated with vehicle alone; * $p \leq 0.05$, ** $p \leq 0.01$ vs. *t*-BOOH-treated cells.

Figure 7. Effects of PEA hydroxamic homologues on the L-glutamate-induced $[Ca^{2+}]_i$ increase. $[Ca^{2+}]_i$ was measured in fura-2/AM loaded cells exposed to L-glutamate (1 mM; 24 h) in the absence or presence of either (+)-MK 801 (1 μ M), PEA (10 μ M), **1b** (10 μ M) or **2a** (10 μ M). The values are expressed as F_r mean of 50 – 55 cells monitored. # $p \leq 0.05$, # # $p \leq 0.01$ vs. cells treated with vehicle alone; * $p \leq 0.05$, ** $p \leq 0.01$ vs. L-glutamate-treated cells.

TABLE 1

PCR primers and protocols used in this study

<i>Template</i>	<i>Primers</i>	<i>Size (bp)</i>	<i>Denaturation</i>	<i>Annealing</i>	<i>Extension</i>	<i>Cycles</i>
NR1	F: 5'-GATGTCTTCCAAGTATGCGGA-3'	667	94°C, 30 s	58°C for 30 s	72°C for 60 s	35
	R: 5'-GGGAATCTCCTTCTTGACCAG-3'					
NR2A	F: 5'-CCGGCCTGGGTTGCTCTTC-3'	458	94°C, 10 s	66°C for 10 s	72°C for 60 s	35
	R: 5'-AGTTCGCTTTGGATTCTGTGCTCA-3'					
NR2B	F: 5'-CTGCCGGACATCACCACCAACA-3'	442	94°C, 10 s	70°C for 10 s	72°C for 60 s	35
	R: 5'-CATCACGCGACCCACAGCCTTACC-3'					
NR2C	F: 5'-GAACGGCATGATTGGGGAGGTGTA-3'	460	94°C, 10 s	67°C for 10 s	72°C for 60 s	35
	R: 5'-CGTGTAGCTGGCGAGGAAGATGAC-3					
NR2D	F: 5'-CCGCCGTGTGGGTGATGATGTTCG-3'	457	94°C, 10 s	69°C for 10 s	72°C for 60 s	35
	R: 5'-ACGCGGGGCTGGTTGTAG-3'					
GAPDH	F: 5'-GGTCGGAGTCAACGGATTTGG-3'	1000	96°C, 30 s	60°C for 30 s	72°C for 45 s	25
	R: 5'-ACCACCCTGTTGCTGTAGCCA-3'					

Fig. 1

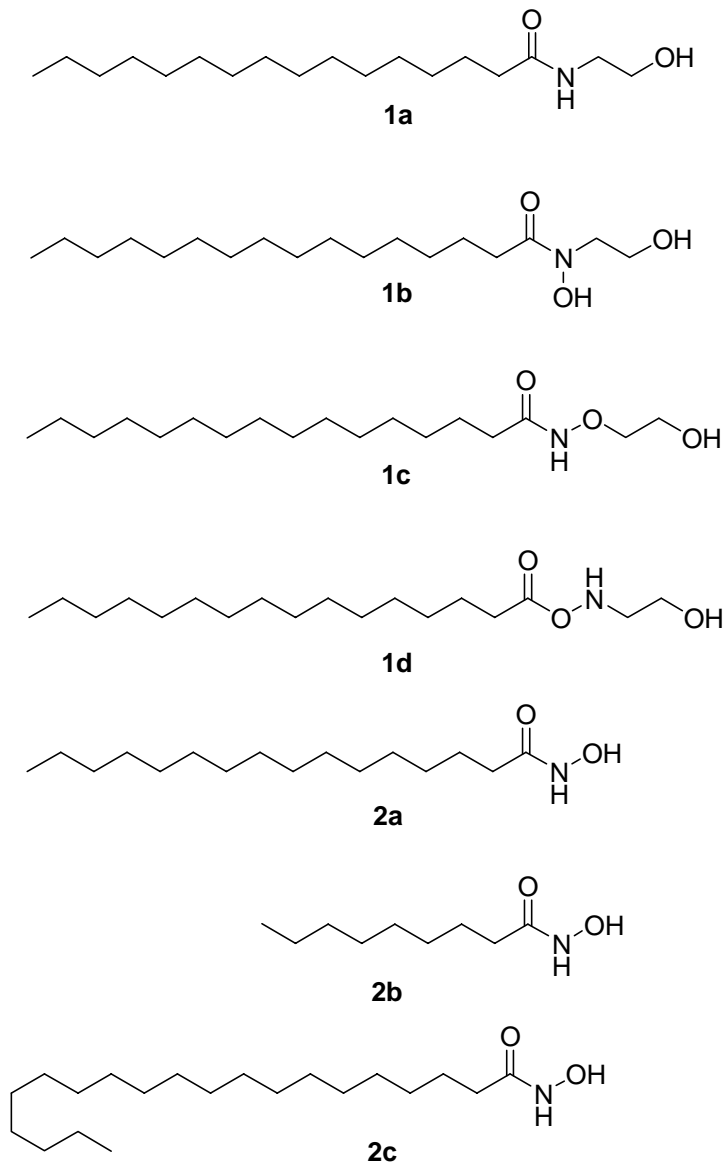


Fig. 2

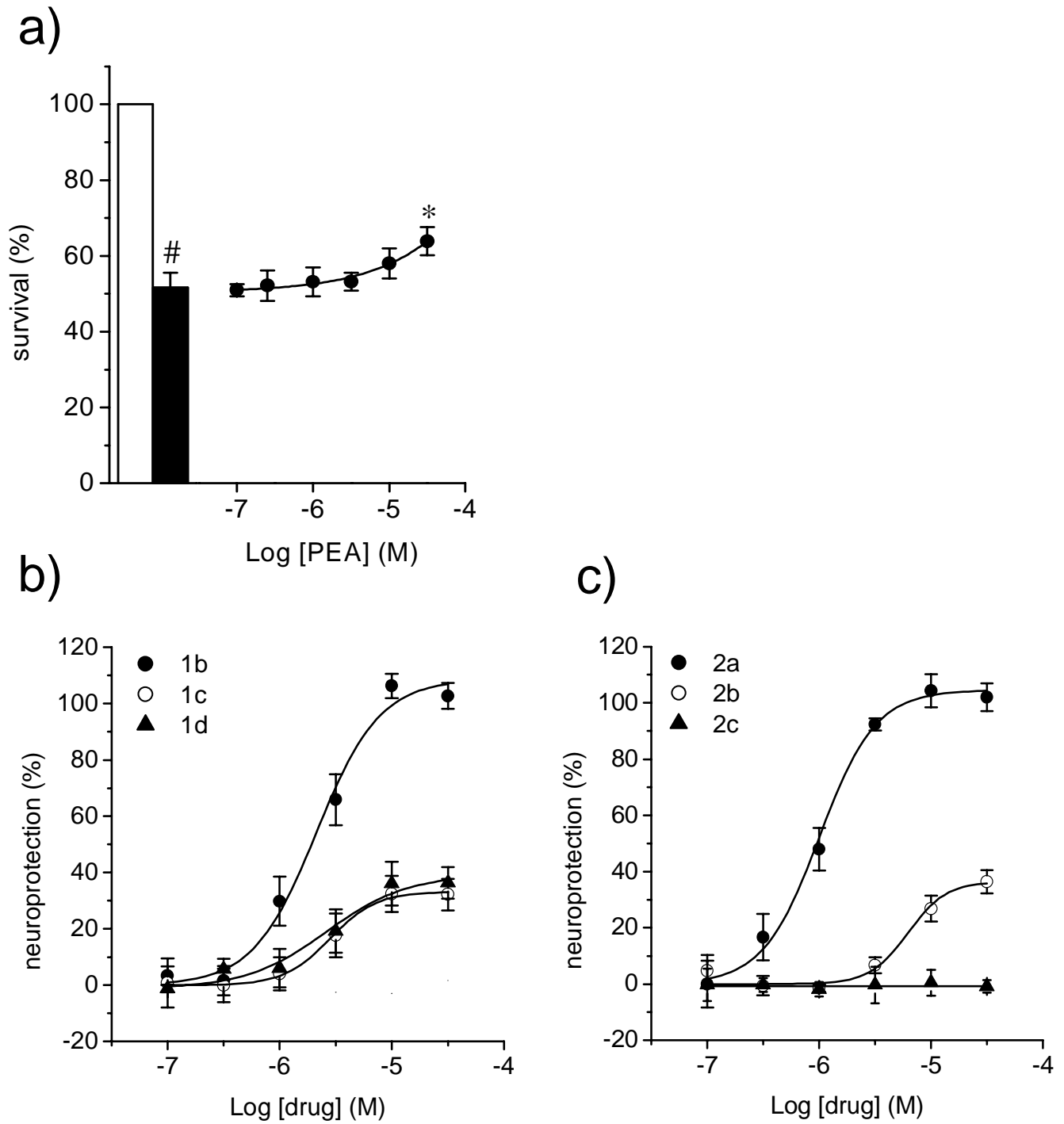


Fig. 3

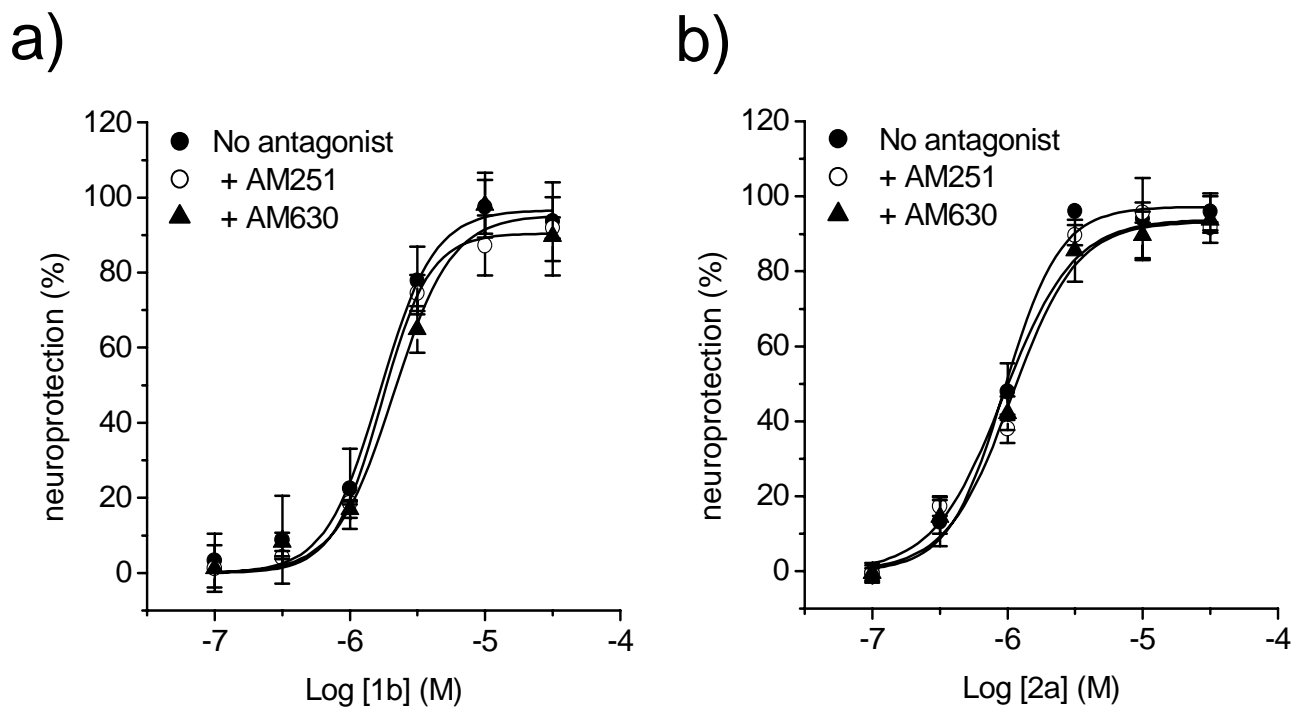


Fig. 4

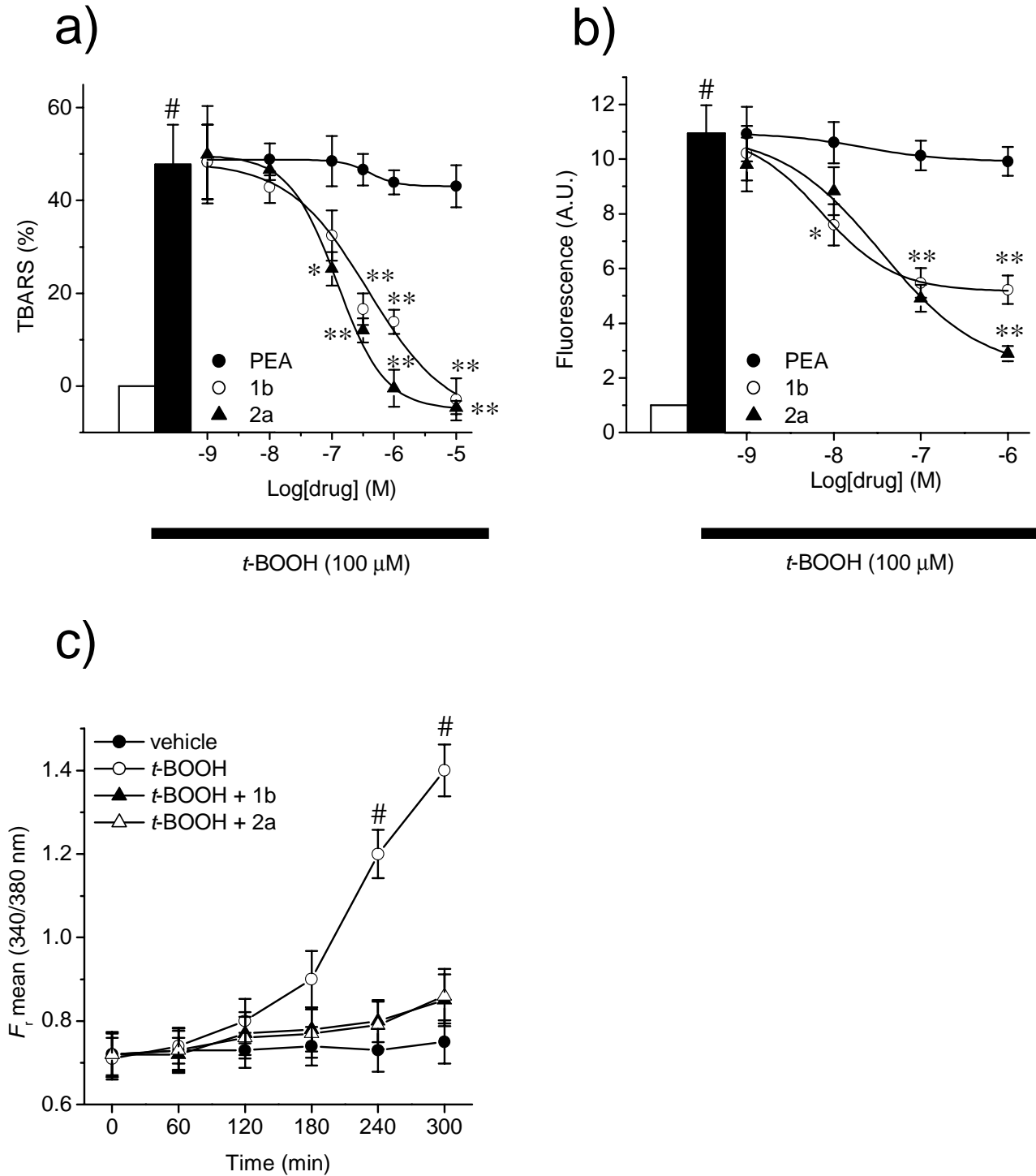


Fig. 5

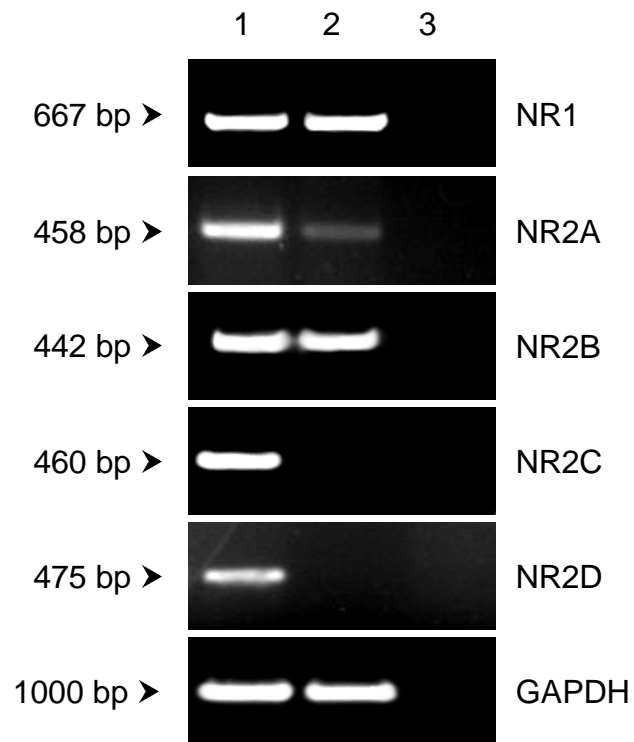


Fig. 6

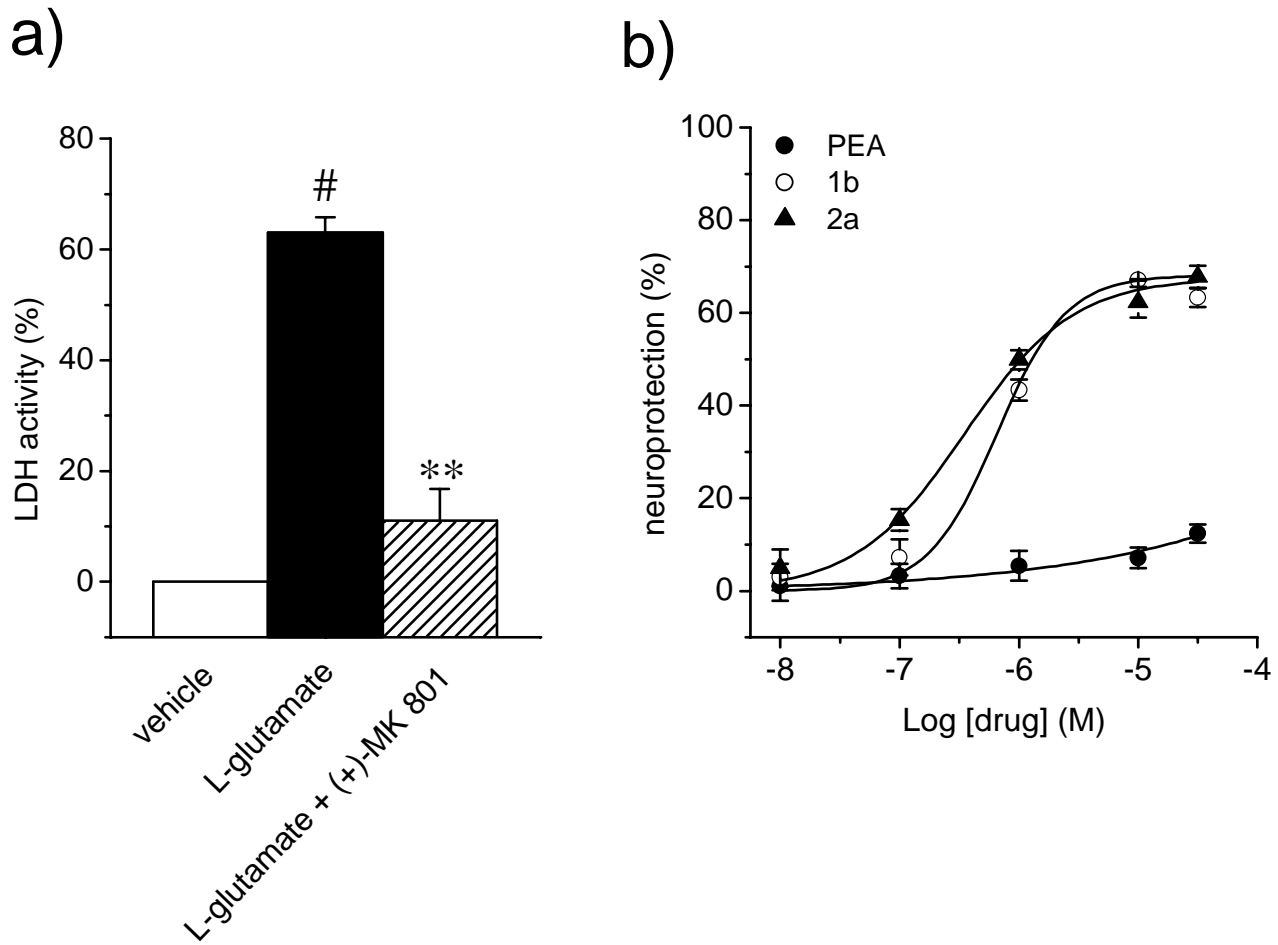


Fig. 7

