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Membrane protein oxidation determines neuronal degeneration

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Abstract

Oxidative stress is an early hallmark in neurodegenerative disorders such as Alzheimer's and Parkinson's diseases. However, the critical biochemical effector mechanisms of oxidative neurotoxicity have remained surprisingly elusive. In screening various peroxides and potential substrates of oxidation for their effect on neuronal survival, we observed that intramembrane compounds were significantly more active than aqueous or amphiphilic compounds. To better understand this result, we synthesized a series of competitive and site-specific membrane protein oxidation inhibitors termed aminoacyllipids, whose structures were designed on the basis of amino acids frequently found at the protein-lipid interface of synaptic membrane proteins. Investigating the aminoacyllipids in primary neuronal culture, we found that the targeted protection of transmembrane tyrosine and tryptophan residues was sufficient to prevent neurotoxicity evoked by hydroperoxides, kainic acid, glutathione-depleting drugs, and certain amyloidogenic peptides, but ineffective against non-oxidative inducers of apoptosis such as sphingosine or Akt kinase inhibitors. Thus, the oxidative component of different neurotoxins appears to converge on neuronal membrane proteins, irrespective of the primary mechanism of cellular oxidant generation. Our results indicate the existence of a one-electron redox cycle and its functional relevance to membrane protein aromatic surface amino acids, whose disturbance or overload leads to excessive membrane protein oxidation and neuronal death.

Introduction

Oxidative stress-related biochemical alterations are characteristic of the biological aging process and many age-associated degenerative disorders (Halliwell, 2006; Lin and Beal, 2006; Sayre et al., 2008). In particular, enhanced prooxidative activity, or aspects of antioxidative failure have been observed in the majority of neurodegenerative diseases

investigated. Still, there has been little progress in the development of functional redox regulatory medicines for these disorders (Moosmann and Behl, 2002a; Jones, 2008; Sayre et al., 2008).

Central to the oxidative hypothesis of neurodegeneration have been analytical investigations in post mortem material. Regarding Alzheimer's disease as a case-in-point, there is evidence of significant and early oxidation of DNA, RNA, proteins and lipids (Nunomura et al., 1999; Pamplona et al., 2005; Markesbery and Lovell, 2007; Lovell et al., 2011). Specifically, protein oxidation appears to affect soluble, filamentous, and aggregated proteins (Martinez et al., 2010), potentially resulting in protein carbonylation (Hensley et al., 1995), nitration (Smith et al., 1997), adduct formation (Pamplona et al., 2005; Zhu et al., 2012), or protein cross-linking via dityrosine bridges (Hensley et al., 1998).

However, comparative investigations of post mortem biomarker oxidation also have inherent limitations. In general, a chemical rationale for the observed structural specificity, if detected, can rarely be assigned. Moreover, it is usually impossible to assess through mere comparison the causal role of an oxidative event in disease progression. A causal contribution of an individual oxidized protein, for instance, cannot be proven by its mere detection even if the same protein was less oxidized in age-matched, healthy controls, as the detected oxidation in the disease might just represent an epiphenomenon related to general redox dysregulation. In fact, it might be argued that the mere existence of an oxidized marker protein in a very slowly progressing disorder like Alzheimer's disease indicates its relative irrelevance: after all, its presence appears not to promote any fast synaptic or neuronal degeneration, and cells appear not to consider their repair or replacement to be a high-priority task. Advances in the field of redox proteomics have yielded some novel ideas regarding the causality problem in recent years (Martinez et al., 2010; Sultana and Butterfield, 2012); however, the structural specificity problem has remained largely unsettled.

Most oxidants do not specifically target a certain individual protein, lipid, or nucleic acid, but rather a certain chemical group or moiety occurring in many individual molecules, such as the bis-allylic positions in all polyunsaturated fatty acids, the thioether moiety in all surface-exposed methionine side chains, or the sulphur atoms in the many proteins that possess iron-sulphur clusters (Levine et al., 1996; Dean et al., 1997; Imlay, 2003; Halliwell, 2006). Moreover, the thiol groups of exposed protein cysteine residues establish a large redox network whose oxidative modifications may be regulatory (Jones, 2008; Groitl and Jakob, 2014), detrimental (Moosmann and Behl, 2008), or both. For this reason, the relevance of each redox target is difficult to assess without a means of specific prevention of each of the observed oxidative modifications, as it is then equally impossible to demonstrate any potential amelioration of a neurodegenerative phenotype in a disease model.

In an attempt to overcome some of these difficulties, we have employed an unbiased chemical biology strategy to identify potential points of convergence of the different redox cascades that are induced in neurons by neurotoxins. Analyzing the prevention of neuronal cell death as primary endpoint, we have identified the one-electron oxidation of integral membrane proteins from within the lipid bilayer as such a point of convergence. In addition, we confirm that it is particularly tyrosine and tryptophan residues in transmembrane domains that are the mediators of membrane protein redox activity.

Materials and methods

Chemical syntheses

Amino acid-lipid hybrid molecules (aminoacyllipids) were prepared essentially as described (Fincher et al., 1996; Moosmann and Behl, 2000) using a Schotten-Baumann procedure to couple the corresponding amine precursors and fatty acid acyl halides by base catalysis in tetrahydrofuran or dimethylformamide. Identity and purity were confirmed by analytical

TLC, ^{1}H-NMR spectroscopy, and, for some compounds, by HPLC and mass spectrometry (MALDI-MS or ESI-MS).

Cell culture

Cerebellar granule neurons were prepared from postnatal (P2) Sprague-Dawley rats (Bayatti et al., 2003) and cultivated in MEM supplemented with 10% horse serum for 12 h, before changing to Neurobasal medium supplemented with B27 (without antioxidants). The cultures were used for experiments beginning 36 h post preparation. Dissociated hippocampal neurons were prepared from embryonal (E17) Sprague-Dawley rats and were directly cultivated in Neurobasal medium supplemented with B27 nutrients (without antioxidants) for 10-14 days. Further details are given in the Supplemental Methods. All animal procedures were conducted in accordance with federal law and following the institutional guidelines of the Central Animal Facility of the University of Mainz, which also provided the animals for this study.

Cell survival and viability assays

Neuronal survival and viability was quantified by metabolic assays (MTT tetrazole reduction), by immunostaining for microtubule-associated protein 2 (MAP-2) and subsequent counting of positively stained neurons, or by staining and counting of fragmented nuclei with the DNA-binding dye Hoechst 33342, as indicated for each experiment. These procedures were carried out as described (Hajieva et al., 2009) and further specified in the Supplemental Methods. All viability and survival assays were performed on at least n = 3 independent cultures, with varying numbers of technical replicates. Regarding the MAP-2 stainings, visual fields amounting to ~750 live neurons (in untreated cultures) were counted per treatment group, and were related to that number to calculate percentages. With respect to the Hoechst

stainings, n = 8 independent cultures were scored, counting ~2500 cells (total and apoptotic) per treatment group. Here, percentages were calculated for each independent n by dividing the number of apoptotic cells by the total number of cells. If not otherwise stated, cells were preincubated for 1 h with any potentially protective compound before the addition of the respective toxin. The duration of toxin exposure is given in the Figure Legends.

Membrane oxidation

Membrane oxidation experiments were carried out as described (Moosmann et al., 2001). In brief, a rat cortical membrane preparation pre-treated to remove surface-adherent proteins (0.5 M NaCl for 30 min, followed by ultracentrifugation) was incubated with the aminoacyllipids at 37°C for 6 h, after initiating the peroxidation reaction by 50 µM ascorbate. Low-level chemiluminescence, indicative of peroxidation reactions in progress, was monitored by single photon counting in a Beckman LS 6500 scintillation counter.

Aqueous oxidation

Bleaching of the aqueous, fluorescent protein B-phycoerythrin from *Porphyridium cruentum* (10 nM in PBS) was used to determine pro- and antioxidative capacity in the aqueous phase (Moosmann and Behl, 2002b). The loss of fluorescence of the protein 6 h after the addition of 2,2'-azo-bis-(2-methylpropionamidine) (AIBN; 500 µM) at 37°C was monitored by flash fluorometry (excitation 340 ± 50 nm; emission 572 ± 6 nm) with a multilabel counter (Wallac Victor; Perkin-Elmer).

Membrane protein separation

Fractionated protein samples were prepared by phase separation of native lysates with the detergent Triton X-114 (Bordier, 1981) as detailed in the Supplemental Methods.

Protein immunoblotting

Protein dityrosine was determined by SDS-free, high urea immunoblotting using a monoclonal anti-dityrosine antibody kindly provided by Dr. Y. Kato (Kato et al., 2000). Protein carbonyls were quantified by Western blotting after derivatization with 2,4-dinitrophenyl hydrazine (DNPH). Both procedures are described in the Supplemental Methods.

Measurement of reactive oxygen species

A fluorimetric method based on the specific reactivities of *cis*-parinaric acid (PA) and 6-carboxy-2',7'-dichlorodihydrofluorescein diacetate bisacetoxymethyl ester (6-DCFA) was employed to measure oxidant generation in the lipid bilayer and the aqueous compartment of naïve cerebrocortical cells. Freshly dissociated cortical cells from newborn rats were prepared as described above for cerebellar cells. After centrifugation and resuspension in phenol red-free MEM, the cells were preincubated with aminoacyllipids (10 μM) for 1 h at 37˚C. The cells were then loaded with 2.5 μM PA or 10 μM 6-DCFA for 15 min. After centrifugation (200 g for 4 min), the cells were washed twice with pre-warmed PBS supplemented with 1 g/l glucose to remove the surplus dye. The cells were then resuspended in the same medium at a density of 10^6 cells/ml and treated with aminoacyllipids (10 μM) and tBuOOH (100 μM) as amphiphilic oxidant. Changes in fluorescence of the cell suspensions were monitored with a multilabel counter (Wallac Victor; Perkin-Elmer). PA fluorescence was measured at 355 ± 40 nm excitation and 460 ± 25 nm emission; 6-DCFA fluorescence was recorded at 485 ± 15 nm excitation and 535 ± 25 nm emission.

Proteomic sequence analyses

Annotated human protein sequences were obtained from Uniprot (www.uniprot.org). Helical protein transmembrane domains were detected and sampled with the program TMHMM 2.0, which is based on a hidden Markov model algorithm (Krogh et al., 2001). The cumulated sequence fragments were numerically analyzed using customized Perl scripts as described (Schindeldecker et al., 2011).

ESR spectrometry, mass spectrometry, and HPLC

These procedures are described in the Supplemental Methods.

Statistical analysis

Data are presented as mean ± standard deviation (SD). If not otherwise stated, statistically significant differences are labelled by asterisks (*, $p < 0.001$), which were detected by one-way analysis of variance (ANOVA), followed by Bonferroni's post hoc test. Pertaining to all experiments involving amyloidogenic peptides, statistical analysis was done by two-way analysis of variance (ANOVA), followed by Holm-Sidak's post hoc test as multiple comparison procedure. Statistical analyses were conducted with the commercially available software package SigmaStat (Systat).

Results

Site of oxidation in neurotoxicity

In order to delineate the critical sites of oxidative modification for neuronal survival under conditions of oxidative stress, a stepwise biochemical strategy was employed, based on (i) the different potential of exogenous redox agents to induce neuronal cell death and (ii) the different potential of oxidizable target molecules (mimicking endogenous cellular structures)

to prevent cell death by competitive action, i.e. by protecting their endogenous counterparts from oxidative destruction (Fig. 1).

Hydroperoxides exhibited very different neurotoxic potentials depending on their lipophilicity. The polar compound hydrogen peroxide was characterized by an EC_{50} value of 150 μM, whereas the EC_{50} value of the lipophilic substance octadecadienoic acid hydroperoxide was approximately 15 μM (Fig. 1A). Thus, peroxide activity in the lipid phase of the cell seems to be more destructive than primarily aqueous reactivity. We therefore performed competitive reversibility experiments employing chemical constituents of the lipid bilayer to block the neurotoxicity of *t*-butyl hydroperoxide, which was chosen for its favorable octanol-water partition coefficient. After screening of the major chemical moieties of lipid bilayers that are known to be subject to oxidation, we were unable to identify any that would have prevented neuronal death (Fig. 1B). Neither polyunsaturated fatty acids such as linolenic acid, α,β-unsaturated alcohols such as sphingosylphosphorylcholine, or cell-permeable cholesterol derivatives such as 25-hydroxycholesterol exhibited a protective effect, suggesting that it might rather be the oxidation of integral membrane proteins that would have to be prevented to counteract oxidative neurotoxicity.

An experimental strategy for membrane protein antioxidation

In an effort to make integral membrane protein oxidation amenable to specific experimental perturbation, we have chemically synthesized a variety of low molecular mass compounds, which were assembled from a long-chain fatty acid attached to an amino acid ester, resulting in a charge-free, lipophilic amino acid derivative (Fig. 1C). Corresponding compounds have been explored in the past as dermal penetration enhancers (Fincher et al., 1996) and, by us, as cytoprotective antioxidants in cell culture (Moosmann and Behl, 2000). Model compounds were synthesized for six of the most enriched amino acids in integral membrane proteins,

which collectively make up 57% of all amino acids in the transmembrane domains found in the human proteome (data not shown). Experiments in primary neuronal cells (Fig. 1D) indicated that lipophilic derivatives of two aromatic amino acids, tyrosine and tryptophan, were effective antagonists of neuronal cell death induced by *t*-butyl hydroperoxide. Similarly lipophilic derivatives of phenylalanine, leucine, valine, or alanine had no effect on cell survival. Short-chain fatty acid derivatives of tryptophan and tyrosine with significantly lower lipophilicity, or charged variants of these amino acids were likewise ineffective (Fig. 1D). These findings indicate that a direct chemical effect of the aminoacyllipids was responsible for their neuroprotective effect, and they suggest that integral membrane protein amino acids may be critical for neuronal viability.

Molecular mechanism of aminoacyllipid neuroprotection

To gain further insight into the mechanism behind the neuroprotective effect of the lipophilic tyrosine and tryptophan derivatives, a series of biochemical and analytical investigations were performed (Tab. 1 and Fig. 2). Electron spin resonance experiments with three representative aminoacyllipids (NDo-W-OEt, NDo-Y-OEt, and NDo-F-OEt) indicated that the tyrosine and tryptophan derivatives, but not the phenylalanine derivative were direct spin quenchers, providing evidence for an antioxidant activity against peroxide-derived free radicals in a defined chemical setting (Fig. 2A).

The full series of amino acid derivatives already examined in neuronal cell culture (Fig. 1D) were further analyzed in two biochemical paradigms of oxidative stress in the lipid bilayer and in the aqueous phase; native rat brain membrane chemiluminescence and aqueous fluorescent protein bleaching (Fig. 2B). In the cell-free brain membrane preparation, lipophilic derivatives of tyrosine and tryptophan, but not phenylalanine, were highly protective against peroxidation. In the aqueous protein oxidation assay, however, all

sufficiently hydrophilic tyrosine- and tryptophan-, but not phenylalanine-based structures acted as antioxidants. For instance, palmitoylated tryptophan ester was ineffective, the dodecanoylated version exhibited a slight protective effect, the acetylated form was even more effective, and the most effective compound was tryptophan ester itself. Hence, the pattern of cytoprotective activities of the investigated compounds (Fig. 1D) was rather precisely paralleled by the pattern of direct membrane protection exerted, but opposed to the pattern of aqueous protein protection (Fig. 2B).

Consequently, both tyrosine and tryptophan seem to possess universal antioxidant properties due to their specific reactivity; however, their contribution to redox reactions in a living cell must be strongly dependent on the precise topological location of these amino acids and the local concentrations that are reached. For that reason, we have measured the relative distribution of NDo-W-OEt, NDo-Y-OEt, and NDo-F-OEt after application to neuronal cell culture under the same conditions as used in the cytoprotection experiments in Figure 1. The results (Tab. 1) show that after 1 h of incubation, between 4% (NDo-Y-OEt) and 11% (NDo-F-OEt) of the recovered compound was associated with the cell layer. Considering the approximate volume ratio in these cultures of 1:500 (cell volume:medium volume), these numbers translate into an at least 20-fold accumulation of the dodecanoyl aminoacyllipids in the cells. Notably, NDo-F-OEt showed the strongest cellular accumulation, ruling out distributional effects as origin of this compound's lack of cytoprotective activity. Given the rapid and avid accumulation of the aminoacyllipids in cells, it is likely that they will not be restricted to the plasma membrane, but will be distributed to basically all intracellular membranes either directly or via vesicular transport. This idea is supported by measurements on closely related compounds carrying methionine and isoleucine headgroups (NDo-M-OMe and NDo-I-OMe) that we have recovered from mitochondrial as well as nuclear and Golgi membranes (Bender et al., 2008).

Antioxidant activities in biochemical systems without recycling mechanisms are inevitably linked to the gradual destruction and loss of the antioxidant itself. Hence, the fate of the three model aminoacyllipids in response to *t*-butyl hydroperoxide-induced oxidative stress in vitro was traced by mass spectrometry. The obtained spectra (Fig. 2C) indicate that NDo-F-OEt does not have a single, preferred oxidation pathway, but rather disintegrates into numerous products, several of which contain the characteristic +16 amu oxygen adduct (Xu and Chance, 2007). In contrast, NDo-W-OEt and particularly NDo-Y-OEt were characterized by simple dimerization as the predominant reaction pathway.

Prevention of integral membrane protein oxidation

If competitive dimerization of the aminoacyllipids was part of their antioxidative mechanism in living cells, cytotoxic oxidants should induce corresponding, membrane protein-restricted amino acid dimers in neuronal cells, whose formation should be suppressed by the aminoacyllipids. To test this hypothesis, we have investigated the formation of dityrosine in primary neurons treated with a toxic concentration of *t*-butyl hydroperoxide (100 µM) at a time at which microscopic signs of toxicity were still very limited (5 h post treatment). We found that this oxidant induced a strong increase in dityrosine exclusively in membrane proteins, but not in the aqueous protein fraction (Fig. 3A), and the observed increase was completely prevented by NDo-W-OEt and NDo-Y-OEt. In addition, baseline levels of dityrosine in untreated cells appeared to be much lower in the aqueous fraction than in the membrane fraction. To probe for additional recycling pathways of radicalized membrane proteins, we conducted a Western blot analysis for carbonyl-modified proteins (Fig. 3B). Here, the baseline carbonyl levels in membrane and aqueous proteins were similar; however, carbonyls were exclusively inducible in membrane proteins, particularly those of high molecular weight. Again, membrane-bound tyrosine and tryptophan, but not phenylalanine

prevented the increase in protein carbonyls. In these experiments, *t*-butyl hydroperoxide was chosen as an oxidative toxin because of its preferred theoretical distribution (logP = 1.05) and its unique behaviour in organic chemistry oxidation reactions, in which it is widely employed for its amphiphilic reactivity in water as well as organic solvents (Li and Chan, 2007; Mannam and Sekar, 2007). Therefore, *t*-butyl hydroperoxide should act as a cellular oxidant of comparable potency in aqueous and membrane phases, which has already been established for lymphoma cells (Takahashi et al., 2001). To verify that this criterion was also met in acutely dissociated primary neurons, the reactivity of *t*-butyl hydroperoxide in these cells was analyzed by fluorescence spectroscopy. Two site-specific oxidant probes were employed, the fatty acid *cis*-parinaric acid (PA), and a strictly aqueous, charged derivative of dichlorodihydrofluorescein termed 6-DCFA (Hockenbery et al., 1993). In fact, *t*-butyl hydroperoxide led to a pronounced increase in the aqueous 6-DCFA fluorescence (Fig. 3C), and it concomitantly quenched the membrane-bound PA fluorescence (Fig. 3D), thus fulfilling the predicted criterion. In turn, the aminoacyllipids were ineffective in preventing the increase in 6-DCFA fluorescence, but they effectively protected PA from oxidative decomposition. In summary, the data in Figure 3 suggest a structural rationale for the functional relevance of intramembrane aromatic amino acids in neuronal cell death.

In order to scrutinize the arising conclusion that membrane protein surfaces were a sink even for free radicals of exclusively aqueous origin, we have employed buthionine sulfoximine (BSO), an inhibitor of γ-glutamyl cysteine synthetase, to induce aqueous oxidative stress as it results from cytoplasmic glutathione depletion. We found that even with BSO as neurotoxic agent, the cellular sites that had to be protected to achieve neuroprotection were associated with the membrane, as only the most lipophilic aminoacyllipids were able to prevent aqueous BSO neurotoxicity (Fig. 4A and 4B). In addition, comparison of BSO with the lipophilic, direct oxidant cumene hydroperoxide did not reveal any differences regarding the structural

requirements for antioxidant neuroprotection by amino acid derivatives (Fig. 4C and 4D). These results agree with recent findings that dopaminergic cells are able to survive for days even in the complete absence of intracellular glutathione if a sufficiently lipophilic, chain-breaking antioxidant is added (Hajieva et al., 2009), indicating that the burden of aqueous glutathione loss weighs most heavily on lipid bilayers.

Protection from disease-related neurotoxicity

The pathophysiological relevance of these results was investigated in several neurotoxicity paradigms related to neurodegenerative disease. Kainic acid is a cyclic glutamate receptor agonist inducing excitotoxic cell death in different neuronal populations in vitro and in vivo (Wang et al., 2005). We have found that NDo-W-OEt and NDo-Y-OEt were almost fully protective against kainate neurotoxicity in hippocampal neurons (Fig. 5A and 5B), implicating membrane protein oxidation in at least some forms of excitotoxicity. Apoptotic chromatin condensation and fragmentation are typical features of neuronal death evoked by neurotoxic amyloidogenic peptides in vitro. In particular, the Alzheimer's disease-associated amyloid β protein can induce chromatin fragmentation by triggering oxidative processes (Behl et al., 1994). Using chromatin fragmentation as a measure of neurotoxicity, we found that aminoacyllipids were significantly protective against amyloid β_{25-35} toxicity and prion $protein_{106-126}$ toxicity (Fig. 5A and 5B). However, chromatin condensation was not induced under these conditions by amyloid β_{1-42}, which also did not trigger any other form of microscopically detectable toxicity (data not shown). Still, it had a clear and specific inhibitory impact on MTT reduction, described to be due to an effect on MTT formazan exocytosis (Liu and Schubert, 1997) that may involve several mechanisms (Abe and Saito, 1999). This effect was fully resistant to aminoacyllipid modulation (Fig. 5B).

Significant cytoprotection by the antioxidant aminoacyllipids was also noted for the highly toxic oxidant peroxynitrite (Fig. 5C). However, integral membrane protein damage is apparently not involved in pathways to neurodegeneration that are dominated by directly activated proapoptotic cascades. Neuronal death induced by sphingosine, a ceramide-derived lysosphingolipid and fast trigger of apoptosis (Suzuki et al., 2004), could not be forestalled by any aminoacyllipid (Fig. 5C). Similarly, neuronal cell death evoked by blockade of the PI3-kinase pathway (Miller et al., 1997) could not be prevented, confirming that the aminoacyllipids seem to be specific membrane protein antioxidants rather than antiapoptotic agents or general enhancers of cell survival. Thus, the protective action of the aminoacyllipids against different neurotoxins appears to involve their interference with at least one common, oxidative, membrane-protein damaging chemical reaction step.

Discussion

What is the nature of the common step towards neuronal cell death?

Selective vulnerability of a topological site or biochemical residue within the cell does not per se indicate that the corresponding residue's modification is relevant to cell death or any other physiological result. To circumvent this general shortcoming of correlative stress marker analysis in neurodegenerative disease, we have employed a systematic screening strategy to identify oxidative modifications preceding neuronal cell death, whose selective prevention would be sufficient to prevent neuronal demise as a whole. Based on a series of site-specific inhibitors of protein oxidation, we have found that the intra-bilayer attack on integral membrane proteins represents such a common step towards oxidative cell death, which is linked to the reversible or irreversible oxidation of tyrosine and tryptophan residues at the protein-lipid interface. Notably, this reaction is shared by diverse toxicity cascades as

initiated by peroxides, glutathione depletion, certain amyloidogenic peptides, or the excitotoxin kainic acid. How might this unexpected coalescence be explained?

On the basis of the current findings, we conclude that integral membrane proteins are permanently subjected to a cyclic one-electron redox reaction (Fig. 6) in which tyrosine and tryptophan are the decisive players. Integral membrane proteins are characterized by a striking accumulation of exposed tyrosine and tryptophan residues in a particular layer of their transmembrane domains (Fig. 6A), which is the transition zone between the hydrophobic core of the membrane and the region of the polar lipid head groups (zone 2 in a four-zone model of the biomembrane) (Tieleman et al., 1997). This characteristic of membrane protein architecture has been noted since the first crystallization of membrane proteins (Schiffer et al., 1992). Phenylalanine is similarly enriched in transmembrane domains, but primarily on the level of the inner hydrophobic core of the membrane (Landolt-Marticorena et al., 1993; Wallace and Janes, 1999; Lizardi-Ortiz et al., 2008). We reviewed the validity of these observations for the annotated human proteome, and for a set of 220 synaptic membrane proteins, and we found that the accumulation of tyrosine and tryptophan was in fact a global feature of their transmembrane domains (Tab. 2). The crystal structure of bovine cytochrome c oxidase illustrates the described principle (Fig. 6B).

Consequently, there is a high concentration of phenol (tyrosine) and indole (tryptophan) groups present in lipid bilayers, especially in membranes with high protein content, as found in brain gray matter (O'Brien and Sampson, 1965). Notably, these residues are usually solvent-exposed, i.e. in contact with the surrounding lipid bilayer (Palsdottir and Hunte, 2004). Therefore, they are inevitably confronted with all diffusible lipid peroxyl radicals and various other types of reactive oxygen and nitrogen species that are present in lipid bilayers (Cordeiro, 2014), such as nitric oxide (Liu et al., 1998) or peroxynitrite (Marla et al., 1997). Upon contact with intramembrane radicals like lipid peroxyl radicals, tyrosine and tryptophan

become readily radicalized, by donating a hydrogen atom to the attacking species (Fig. 6C, i). Rapid hydrogen atom donation to ambient free radicals represents an essentially unique behaviour of these two amino acids (Supplemental Discussion S1). As a consequence, the attacking lipid peroxyl radical is quenched, and any potential free radical chain reaction within the lipid bilayer comes to a standstill. The resulting lipid hydroperoxide is much more inert than its free radical precursor and may await reduction by glutathione peroxidase 4 (GPx4) or other membrane-associated peroxidases.

Fate of the generated tyrosine and tryptophan radicals

Both radicals may initiate a variety of reactions towards re-reduction, which can be staged according to the degree of irreversible macromolecular damage that is caused in the course of each pathway. We argue that the default recycling pathway does not cause any irreversible damage and may in fact be viewed as a protein-based antioxidant cycle. Only in the case of an overload of this primarily initiated, favourable recycling pathway may other pathways become relevant, as detailed in the following.

The "default pathway" and its relevance to neuronal health

Upon radicalization, tyrosine and tryptophan become substantially more polar. This promotes their flipping into the more dipolar region of the membrane lipid head groups (zone 1 according to Tieleman et al. (1997)) and, concomitantly, their interaction with water-borne one-electron reducing agents such as ascorbate. Ascorbate spontaneously reduces the amino acid radicals, which will then re-immerge into the less polar layers of the membrane (zones 2 and 3), whereas the generated ascorbyl radicals will be re-reduced by one of various enzymes that have evolved for that purpose.

The default pathway is supported by the following evidence: chemical calculations (Supplemental Methods) indicate that tyrosine and tryptophan side chain radicals, like most organic radicals, are more dipolar than their diamagnetic parent compounds (tryptophan: 1.77 D, tryptophan radical: 2.84 D, tyrosine: 1.36 D, tyrosine radical: 4.34 D; water, for comparison: 1.84 D). In cooperation with this polarity gain as directional driving force, there appears to be sufficient side chain flexibility to allow the relocation of the oxygen atom of a radicalized tyrosine residue by ~ 9 Å (the thickness of a lipid bilayer leaflet is ~ 17-25 Å). In addition, extensive motility of all lipid bilayer components perpendicular to the plane of the membrane has been demonstrated (phospholipids: ~ 4-10 Å; single-span α-helical peptides: ~ 6 Å) (Shen et al., 1997), enabling even hidden constituents of the lipid bilayer to reversibly come into contact with aqueous molecules (Shen et al., 1997; Tieleman et al., 1997; Marrink et al., 2009). The subsequent, fast repair of tyrosine and tryptophan radicals by ascorbate has been demonstrated in vitro (Domazou et al., 2009). Hence, the combined effects of the polarity gain and the particular diffusional flexibility of the lipid bilayer at the protein-lipid interface (Shen et al., 1997; Marsh, 2008) appear to well allow transmembrane tyrosine and tryptophan residues to sequentially encounter intramembrane radicals as well as, after radicalization, aqueous reducing agents.

Further supportive of the default pathway is the strong similarity between intramembrane tyrosine and tryptophan residues and low-molecular weight lipophilic phenols. Lipophilic phenols such as tocopherol are well-established to convert into membrane-bound radicals upon free radical attack, before being repaired by aqueous one-electron reduction. In particular, tocopherol has been extensively characterized to be re-reduced by ascorbate at the lipid bilayer boundary (zone 1) in a non-enzymatic fashion (Sharma and Buettner, 1993; Bisby and Parker, 1995; Kagan and Tyurina, 1998; Atkinson et al., 2008). The functional complementarity of tocopherol and ascorbate has even been evidenced in vivo (Bruno et al.,

2006). Thus, tocopherol and transmembrane tyrosine and tryptophan residues share a common membrane topology (Landolt-Marticorena et al., 1993; Wallace and Janes, 1999; Atkinson et al., 2008) and a highly similar free-radical chemistry (Jonanovic and Simic, 1985; Jonanovic et al., 1986; Kagan and Tyurina, 1998; Lien et al., 1999; Atkinson et al., 2008), suggesting that the established recycling mechanisms for radicalized tocopherol also operate on radicalized membrane protein tyrosine and tryptophan residues.

Beyond the direct, aqueous recycling of protein tyrosine and tryptophan radicals, indirect recycling mechanisms may become significant under special circumstances, for example, in the presence of ubiquinone, tocopherol, or nitric oxide. These pathways, which might explain some of the seemingly paradoxical behaviours of compounds like nitric oxide, are outlined in Supplemental Discussion S2. Finally, the in vivo importance of the default pathway for neuronal health is underscored by the central role of aqueous one-electron reducing agents, especially ascorbate, in neuronal injury (Supplemental Discussion S3).

The "overflow pathway" and its signatures in neurodegenerative disease

The tyrosine and tryptophan radical reduction system will become overloaded if the rate of generation of these radicals is too high, as would be the case in widespread lipid peroxidation, or if the rate of repair is too low, as would be the case in ascorbate deficiency. The accumulating tyrosine and tryptophan radicals will then pursue other recycling pathways in their immediate chemical neighborhood, which may vary considerably. The most relevant seem to be dimerization, carbonyl formation, and the (re-)initiation of lipid peroxidation.

Dityrosine formation is a well-characterized result of tyrosine radicalization (Malencik and Anderson, 2003) that might be particularly detrimental if two adjacent membrane proteins are cross-linked and thereby rigidified (Fig. 6C, iv). Correspondingly, a profound increase in membrane-associated dityrosine was detected in neurons under oxidative stress (Fig. 3A),

which was prevented by the competitive addition of NDo-Y-OEt. In addition, dimers of the latter compound were detected by ESI-MS as key products in an ascorbate-free, cell-free trapping assay (Fig. 3C).

Strongly elevated dityrosine levels have been found in affected brain regions in Alzheimer's disease (Hensley et al., 1998), in which it may also be involved in extracellular amyloid β protein crosslinking (Atwood et al., 2004). Dityrosine formation appears to trigger the fibrillogenesis of α-synuclein in Parkinson's disease (Krishnan et al., 2003). Moreover, it was found to be selectively increased in the MPTP mouse model of this disease (Pennathur et al., 1999), even if separate analyses for membrane proteins versus aqueous or fibrillary proteins have not been reported. Similarly, there is only limited knowledge about the role of tryptophan oxidation in neurodegenerative disease. While ditryptophan formation can be easily achieved in vitro (Matthews et al., 2001), in vivo reports on its potential physiological role are lacking. Still, tryptophan oxidation involving *N*-formylkynurenine formation has been detected in various instances, especially affecting membrane proteins (Taylor et al., 2003).

Transmembrane proteins are globally characterized by an increased usage of cationic amino acids (lysine, arginine) in the aqueous region adjacent to the aromatic tyrosine and tryptophan rings (Fig. 6B) (Landolt-Marticorena et al., 1993). Concomitantly, both residues are preferred substrates to be converted to carbonyls in response to oxidative attack (Stadtman and Levine, 2003). We argue that the overflow pathway that leads to an accumulation of tyrosine and tryptophan radicals in close proximity to these cationic lysine and arginine residues will result in a significant local generation of protein carbonyls (Fig. 6C, v). This local carbonylation concept is supported by our finding that protein carbonyls were selectively increased in membrane proteins isolated from oxidatively stressed neurons (Fig. 3B).

Application of NDo-W-OEt and NDo-Y-OEt prevented the increase, as they enabled the default pathway to proceed.

A general increase in protein carbonyls has been found in many different neurodegenerative conditions (Halliwell, 2006; Sayre et al., 2008). Moreover, the double-edged character of phenols such as tyrosine has frequently been noted, being antioxidants in a reduced state, yet turning into transmitters of damage if their radicalized forms accumulate. In this respect, the enforced generation of tyrosyl radicals by myeloperoxidase has been shown to mediate the induction of LDL lipid peroxidation (Savenkova et al., 1994), whereas in the absence of this enzyme, tyrosine-containing peptides and numerous other phenols were potent inhibitors of LDL lipid peroxidation (Noguchi and Niki, 2000; Moosmann and Behl, 2002b).

Conclusion: Ianus faces of integral membrane protein redox activity

Under normal conditions, tyrosine and tryptophan accumulation in integral membrane proteins provides a means of antioxidant defence, protecting the lipid bilayer including membrane proteins from oxidation (default pathway). In a state of oxidative overload, however, irreversible covalent protein modification and cross-link formation become significant, eventually leading to protein misfolding and proteotoxicity (overflow pathway).

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Figure legends

Figure 1. Competitive inhibition of oxidative neurotoxicity.

(**A**) Neurotoxicity of four cell-permeable hydroperoxides of different lipophilicity in primary cerebellar neurons: hydrogen peroxide (H_2O_2) (open triangles); *t*-butyl hydroperoxide (tBuOOH) (filled triangles); cumene hydroperoxide (CuOOH) (open circles); octadecadienoic acid hydroperoxide (13S-OOH) (filled circles). With increasing lipophilicity, hydroperoxides became increasingly toxic to neurons. LogP values (octanol-water partition coefficients) of the applied hydroperoxides were: H_2O_2: -1.43; tBuOOH: 1.05; CuOOH: 2.44; 13S-OOH: 5.32.

(**B**) Effect of different lipids (10 µM) on the survival of neurons stressed with tBuOOH (100 µM). The investigated lipids are membrane-bound carriers of oxidizable chemical moieties in the lipid bilayer: OA: oleic acid; LA: linoleic acid; α-LNA: α-linolenic acid; γ-

LNA: γ-linolenic acid; AA: arachidonic acid; DHA: docosahexaenoic acid; LPC: lysophosphatidylcholine; SPC: sphingosylphosphorylcholine; PSY: psychosine; 22-OH-Chol: 22-hydroxycholesterol; 25-OH-Chol: 25-hydroxycholesterol. The enrichment of neuronal cells with oxidizable groups such as those found in unsaturated fatty acids (OA, LA, LNA, AA) or other neuronal lipids did not prevent oxidative cell death. Survival in (A) and (B) was quantified by metabolic MTT reduction (n = 3).

(**C**) Chemical structures of six representative N-alkyl amino acid ethyl esters (aminoacyllipids) that were employed to probe for redox activities of integral membrane proteins.

(**D**) Effect of different aminoacyllipids (10 μM) and corresponding controls on the survival of neurons stressed with tBuOOH (100 μM). Amino acids are represented in single-letter code; NDo indicates N-dodecanoylation of the corresponding amino acid, NPa N-palmitoylation, and NOl N-oleoylation. OEt refers to an ethyl ester modification on the C-terminus. Full chemical names are given in the Supplemental Methods. Lipophilic derivatives of tryptophan and tyrosine were specific and potent inhibitors of oxidative cell death (*, $p < 0.001$; n = 3). Quantification of neuronal survival was carried out 24 h post toxin by MTT reduction.

Figure 2. Biochemical characterization of amino acid derivatives as site-specific antioxidants.

(**A**) Electron spin resonance (ESR) spectra of the spin trap *t*-butyl-α-phenylnitrone (PBN; 50 mM) coincubated with tBuOOH (100 mM) and selected aminoacyllipids (50 mM). NDo-W-OEt and NDo-Y-OEt largely suppressed the radicalization of PBN by tBuOOH, while NDo-F-OEt had no significant spin quenching effect. The shown spectra are overlays of ten scans. The bar indicates 10 G.

(**B**) Differential antioxidant protection of rat brain membranes (upper chart) and an aqueous fluorescent indicator protein (lower chart) by amino acid derivatives (10 µM) with varying lipophilicity. Compound designations are as in Figure 3. Tyrosine and tryptophan derivatives had direct antioxidant potential in both cell-free assay systems. However, only membrane antioxidant properties were predictive of neuroprotective potential as established in Figure 1. Antioxidant protection is displayed relative to controls defined as 0% (prooxidant alone) and 100% (no prooxidant). Asterisks indicate significant protection (*, $p < 0.001$; $n = 4$) compared to prooxidant-treated controls.

(**C**) Fate of NDo-W-OEt, NDo-Y-OEt, and NDo-F-OEt. Electrospray-ionization mass spectrometry (ESI-MS) was used to trace the main reaction products of the aminoacyllipids (1 mM) in 1 mM tBuOOH-containing cell culture medium. Dimerization was the dominating peroxide-induced reaction of NDo-W-OEt and NDo-Y-OEt, whereas NDo-F-OEt was completely fragmented, lacking a single main reaction pathway. The primary peaks (labelled by asterisks) were assigned as follows (with M designating the corresponding molecule ion, and +/– indicating the addition/loss of a chemical group):

NDo-W-OEt: 413: M; 449: M+Cl; 863: M_2+Cl; 1278: M_3+Cl.

NDo-Y-OEt: 390: M; 426: M+Cl; 781: M_2; 817: M_2+Cl; 1173: M_3; 1209: M_3+Cl.

NDo-F-OEt: 255: M–undecyl+Cl; 265: M–benzyl–ethyloxycarbonyl+OH+Cl; 283: M–benzyl; 353: M–ethyloxycarbonyl+OH+Cl; 410: M+Cl; 786: M_2+Cl.

Figure 3. Prevention of integral membrane protein oxidation.

(**A**) Immunoblot analysis of membrane and cytoplasmic fractions of cerebellar neurons for dityrosine content, using a monoclonal anti-dityrosine antibody. Cell cultures preincubated with aminoacyllipids (10 µM; 2 h) were incubated with tBuOOH (100 µM; 5 h) before the cells were harvested, homogenized, and fractionated with Triton X-114. Dityrosine-conjugated bovine serum albumin (DT-BSA) was used as positive control and as antibody blunting reagent. The observed increase in dityrosine in the membrane was fully prevented by NDo-W-OEt and NDo-Y-OEt, whereas NDo-F-OEt did not exhibit any effect.

(**B**) Membrane and cytoplasmic fractions generated as in (A) were analyzed for protein carbonyl content by anti-DNP Western blotting.

(**C**) The aqueous reactivity of tBuOOH in dissociated cortical cells was measured using the hydrophilic, cell compartment-specific fluorescent probe 6-carboxy-2',7'-dichlorodihydrofluorescein (6-DCFA). Neuroprotective aminoacyllipids did not suppress the cytoplasmic oxidation of 6-DCFA.

(**D**) The membrane reactivity of tBuOOH was measured using the lipophilic fluorescent probe *cis*-parinaric acid (PA). The oxidation of PA by tBuOOH was significantly attenuated by the cytoprotective compounds NDo-W-OEt and NDo-Y-OEt (*, $p < 0.001$; $n = 3$).

Data in (C) and (D) are presented as percent values relative to controls set at 0% (unloaded cells) and 100% (control fluorescence). At the employed concentration of 100 µM, tBuOOH was highly oxidizing in the aqueous phase and in the membrane; however, protein dityrosine and carbonyl formation were selectively induced in membrane proteins.

Figure 4. Protection from neuronal cell death evoked by site-specific oxidants.

(**A**) Survival of primary cerebellar neurons treated with 200 µM buthionine sulfoximine (BSO) for 48 h, an inhibitor of glutathione biosynthesis that leads to cytoplasmic glutathione depletion. The metabolic MTT reduction assay was used to quantify cellular survival. Compound abbreviations are as in Figure 1.

(**B**) The same experiment as in (A), consulting neuronal cell counts as measure of antioxidant protection. Neurons were identified by immunocytochemical staining for the neuron-specific marker protein MAP-2.

(**C**) Survival of primary cerebellar neurons treated with 30 µM cumene hydroperoxide (CuOOH), a lipophilic prooxidant, quantified by metabolic MTT reduction 24 h after the addition of the toxin.

(**D**) The same experiment as in (C), using cell numbers of MAP-2 positive neurons as readout. Neurons were fixed and immunostained after 6 h incubation with CuOOH.

In all charts, asterisks indicate significant protection (*, $p < 0.001$; $n = 3$) as compared to cell cultures treated with prooxidant alone. Integral membrane protein protection by lipophilic aminoacyllipid competitors is necessary and sufficient to secure neuronal survival, irrespective of the mode and site of induction of oxidative stress.

Figure 5. Effect of aminoacyllipids against different neurotoxic stimuli.

(**A**) The potential of NDo-W-OEt, NDo-Y-OEt, and NDo-F-OEt to prevent neuronal death induced by the excitotoxin kainic acid (50 µM for 16 h) was investigated in primary hippocampal cell culture and analyzed by immunocytochemistry for MAP-2 (left column). Primary hippocampal neurons were also challenged with amyloid $\beta_{25\text{-}35}$ (50 µM) for five days; nuclear fragmentation indicative of neuronal apoptosis was visualized by Hoechst 33342 staining (right column).

(**B**) Quantification of the results depicted in (A), including the effects of incubation with prion protein$_{106-126}$ (80 µM; Hoechst staining) and amyloid β_{1-42} (20 µM; MTT reduction) for five days. Aminoacyllipids were highly protective against kainite toxicity (*, $p < 0.001$; $n = 3$). Moreover, they had a significant effect against the proapoptotic toxicity of amyloid β_{25-35} (toxicity: $p = 0.014$, protection: $p < 0.001$; two-way ANOVA; $n = 8$) and prion protein$_{106-126}$ (toxicity: $p = 0.024$, protection: $p = 0.001$; two-way ANOVA; $n = 8$); individual significance levels of protection (by post hoc analysis) are given in the charts. The aminoacyllipids had no effect on the significantly compromised MTT reduction effected by amyloid β_{1-42} (toxicity: $p < 0.001$, protection: $p = 0.997$; two-way ANOVA; $n = 8$).

(**C**) Peroxynitrite-induced cytotoxicity (100 µM), sphingosine-induced apoptosis (25 µM), and the effect of Akt inhibition (LY 294002; 50 µM) with concomitant depolarization (KCl; 25 mM) were examined in cerebellar cell culture and quantified by metabolic activity analysis (MTT assay). NDo-W-OEt and NDo-Y-OEt were protective against peroxynitrite toxicity (*, $p < 0.001$; $n = 3$), but not against the effects of sphingosine and LY 294002.

Different redox toxins, but not the more direct inducers of apoptosis, share membrane protein oxidation as critical step towards neuronal cell death.

Figure 6. Model of the proposed redox interaction between membrane proteins and their local environment.

(**A**) Tyrosine and tryptophan as well as phenylalanine are enriched in integral membrane proteins (compare Tab. 2). They differ in the precise site of accumulation, however, with tryptophan and tyrosine being preferably localized in the vicinity of the lipid head groups (lipid bilayer zone 2).

(**B**) Structural representation of bovine cytochrome c oxidase (PDB ID 1v54) with tryptophan, tyrosine, phenylalanine, and lysine residues in space-filling representation,

illustrating the principles outlined in (A). Arrows point to some of the numerous solvent-exposed tyrosine and tryptophan residues of this integral membrane protein. Bars denote the approximate membrane boundaries.

(**C**) Membrane protein tyrosine and tryptophan are part of a one-electron redox cycle (default pathway). Oxidation by lipid bilayer-bound radicals (exemplified by $ROO^{\bullet}$, (**i**)) is reversed by aqueous reducing agents (exemplified by ascorbate, (**ii**)). The depicted redox cycle is afforded by the particular chemical properties of these amino acids. If the velocity of intramembrane tyrosine and tryptophan radicalization (e.g. through the addition of lipophilic peroxides, Fig. 1) exceeds the velocity of aqueous reduction (e.g. as a consequence of the inhibition of glutathione synthesis, Fig. 4), alternative reaction pathways are proceeded (overflow pathway), leading to the formation of membrane protein dityrosine bridges (**iv**), or to carbonyl formation (Fig. 3) on nearby amino acid residues (exemplified by lysine aldehyde, (**v**)). Site-specific prevention of tyrosine and tryptophan oxidation in integral membrane proteins by competitively acting tyrosine or tryptophan-headed lipids is sufficient to prevent intramembrane dityrosine and carbonyl formation (Fig. 3). Concurrently, it is sufficient to prevent neuronal death as induced by certain disease-related neurotoxins (Fig. 5).

Tables

Table 1. Physical distribution of aminoacyllipids in cell culture.

The relative distribution of NDo-W-OEt, NDo-Y-OEt, and NDo-F-OEt in cerebellar cell culture after 1 h of incubation was determined by HPLC. Identical concentrations (10 µM) and cell densities were used as in the neuroprotection experiments (n = 3).

	Recovery from the cell pellet	*Approximate cellular accumulation factor**
NDo-W-OEt	6.88% ± 2.13%	37 ± 21
NDo-Y-OEt	4.20% ± 0.81%	22 ± 10
NDo-F-OEt	11.26% ± 2.21%	63 ± 28

**based on an estimated cell volume of 20 µl ± 5 µl in a 10 cm tissue culture dish supplied with 10 ml medium*

Table 2. Frequency of tryptophan and tyrosine in human proteins.

The usage of these amino acids related to all 20 proteinogenic amino acids is given for the investigated sets of 19806 total proteins, the contained 17040 transmembrane helices, and 445 transmembrane helices identified in 220 synaptic proteins.

	Trp	*Tyr*	*Σ*
Proteome, total	1.23%	2.65%	3.87%
Transmembrane domains, total	2.44%	4.00%	6.44%
Transmembrane domains, 220 synaptic proteins	2.89%	5.07%	7.96%

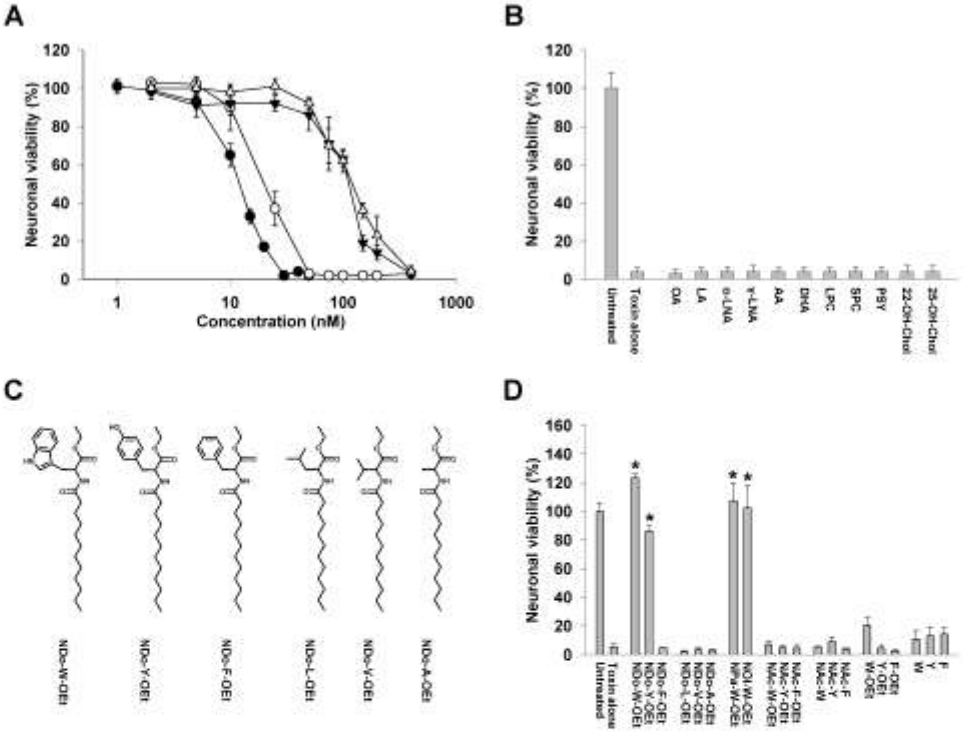

Figure 1

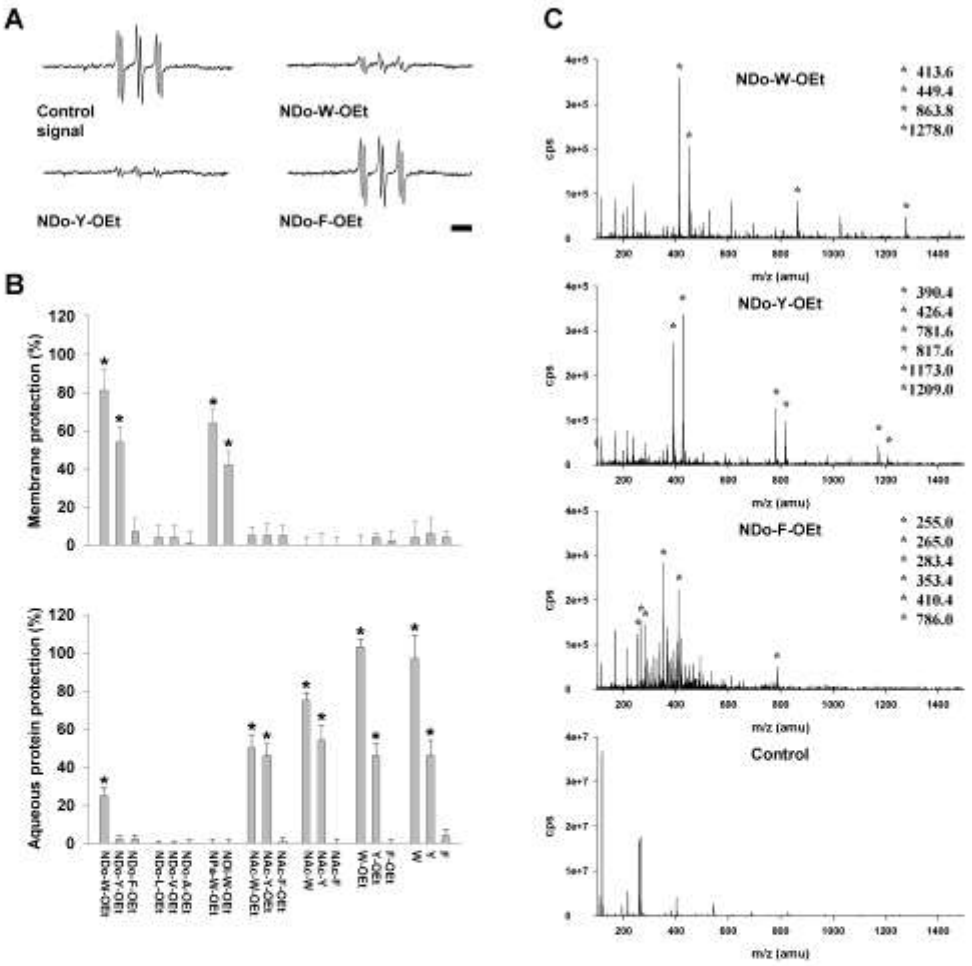

Figure 2

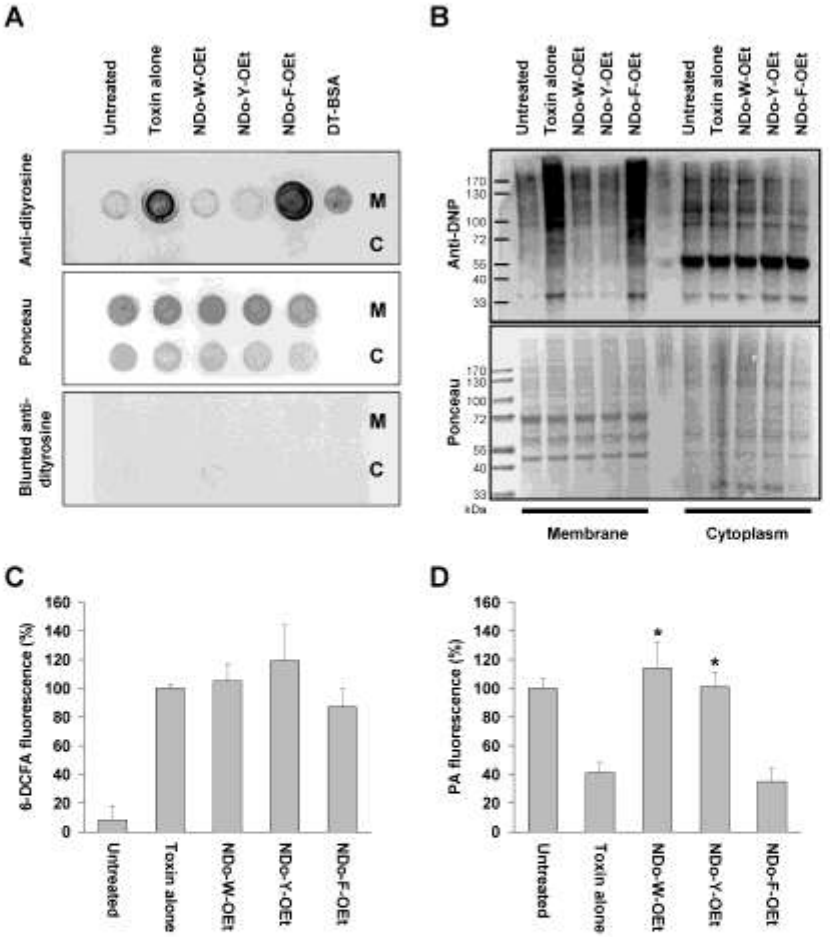

Figure 3

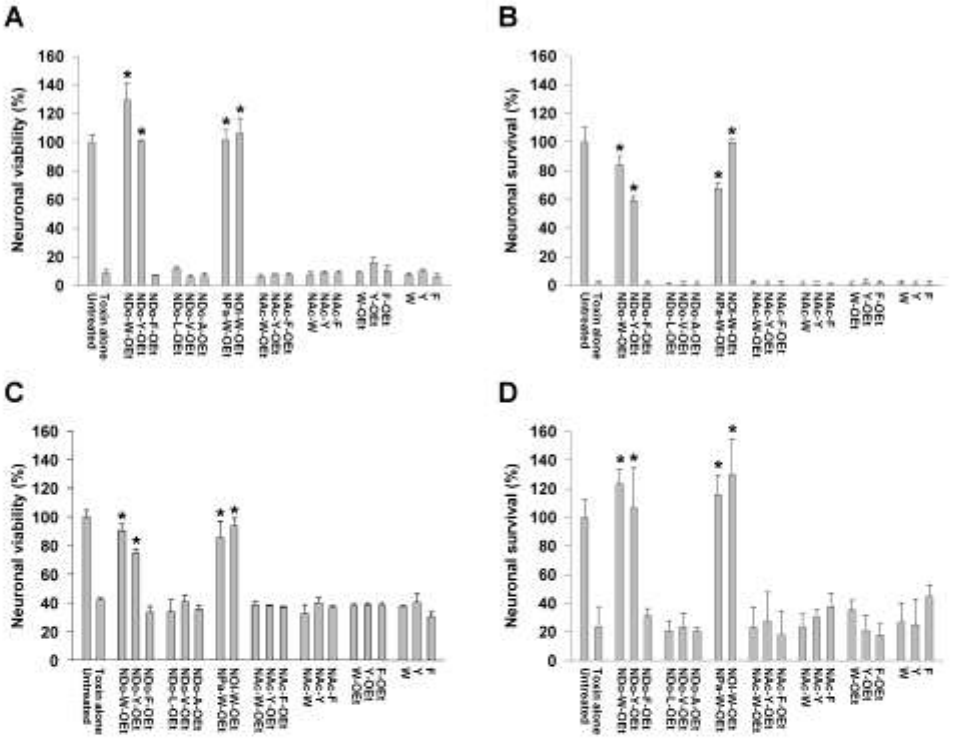

Figure 4

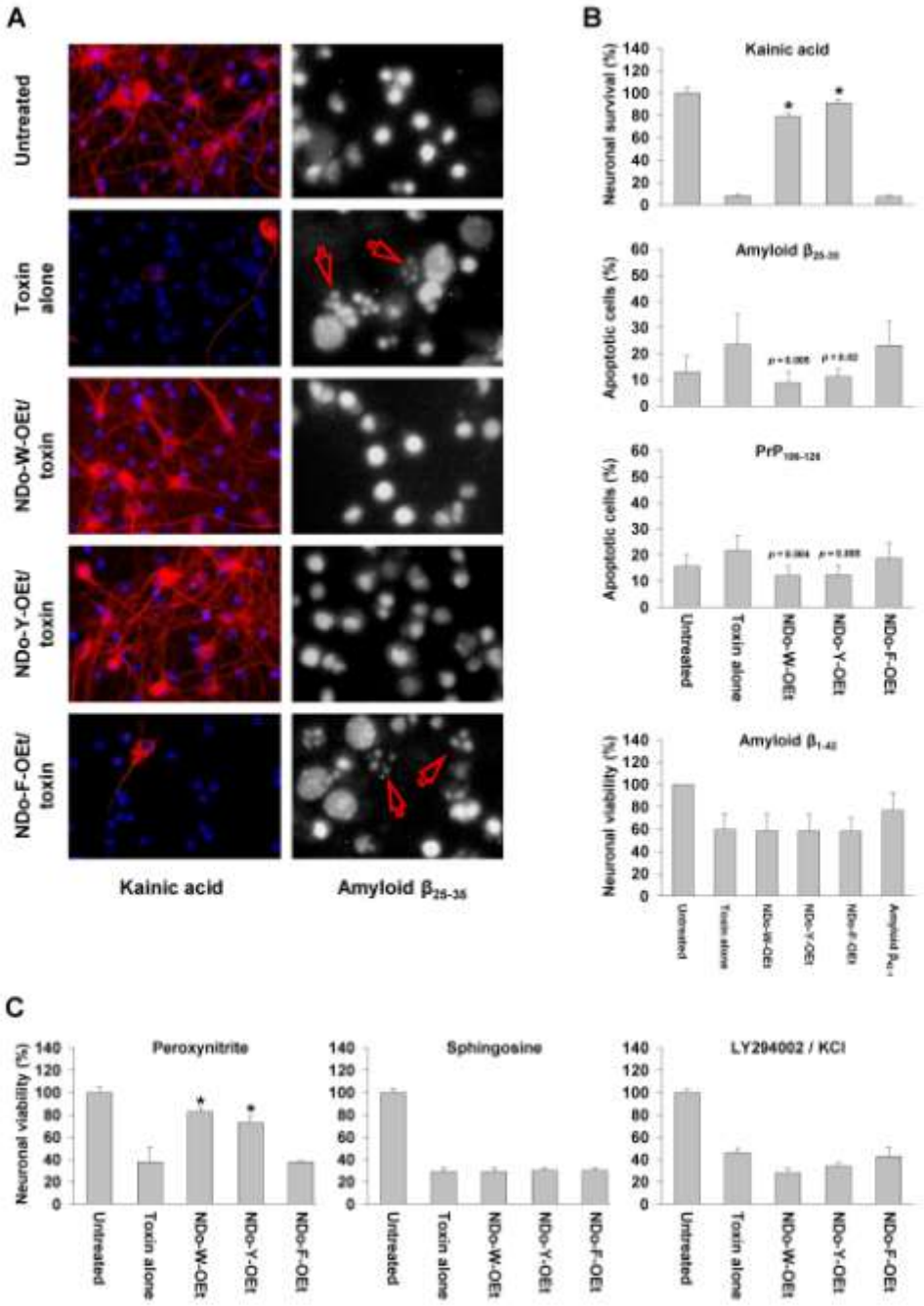

Figure 5

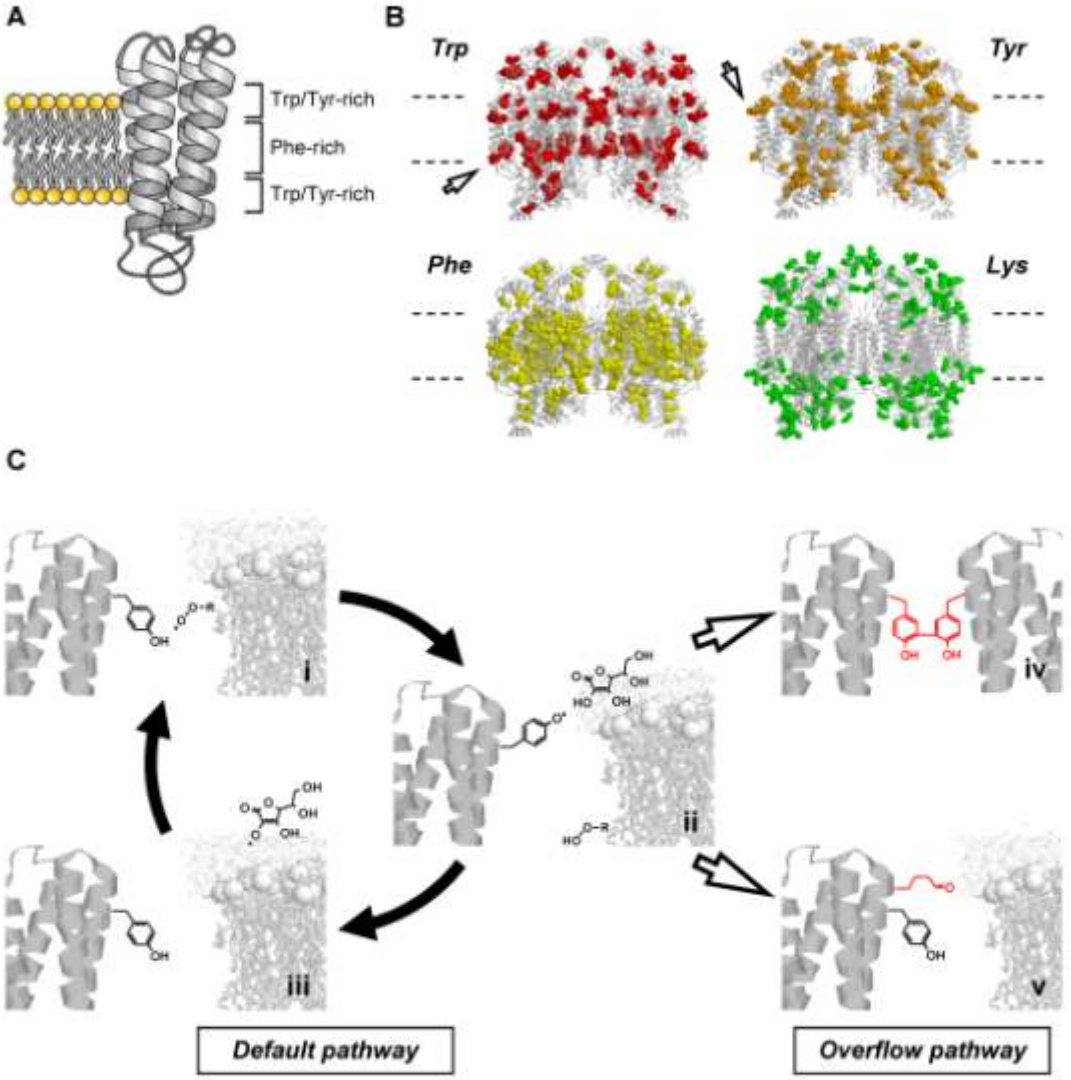

Figure 6