Published in final edited form as:

Anal Chem. 2016 February 16; 88(4): 2399–2405. doi:10.1021/acs.analchem.5b04420.

Detection of Free and Protein-Bound *ortho*-Quinones by Near-Infrared Fluorescence

Joseph R. Mazzulli^{†,‡}, Lena F. Burbulla[†], Dimitri Krainc[†], and Harry Ischiropoulos^{‡,§}

[†]The Ken and Ruth Davee Department of Neurology, Northwestern University Feinberg School of Medicine, 303 East Chicago Avenue, Ward 12-144, Chicago Illinois 60611 United States

[‡]Department of Pediatrics, Children's Hospital of Philadelphia, Philadelphia, Pennsylvania 19104, United States

[§]Department of Systems Pharmacology and Translational Therapeutics, Perelman School of Medicine, University of Pennsylvania, Philadelphia, Pennsylvania 19104, United States

Abstract

Aging and oxidative stress are two prominent pathological mechanisms for Parkinson's disease (PD) that are strongly associated with the degeneration of dopamine (DA) neurons in the midbrain. DA and other catechols readily oxidize into highly reactive *o*-quinone species that are precursors of neuromelanin (NM) pigment and under pathological conditions can modify and damage macromolecules. The role of DA oxidation in PD pathogenesis remains unclear in part due to the lack of appropriate disease models and the absence of a simple method for the quantification of DA-derived oxidants. Here, we describe a rapid, simple, and reproducible method for the quantification of *o*-quinones in cells and tissues that relies on the near-infrared fluorescent properties of these species. Importantly, we demonstrate that catechol-derived oxidants can be quantified in human neuroblastoma cells and midbrain dopamine neurons derived from induced pluripotent stem cells, providing a novel model to study the downstream actions of *o*-quinones. This method should facilitate further study of oxidative stress and DA oxidation in PD and related diseases that affect the dopaminergic system.

Graphical abstract

*Corresponding Author Phone: 312-503-3933. Fax: 312-503-3951. jmazzulli@northwestern.edu.

ASSOCIATED CONTENT

Supporting Information

The Supporting Information is available free of charge on the ACS Publications website at DOI: 10.1021/acs.analchem.5b04420.

Supplemental figures, methods and references (PDF)

The authors declare no competing financial interest.

Dopamine (DA) and other catecholamines regulate the response of a wide range of synaptic circuits involved in voluntary and coordinated movements, feeding, and motivated behaviors relating to reward.^{1–3} Degeneration of neuromelanin (NM)-containing midbrain dopaminergic neurons in the substantia nigra pars compacta (SNpc) leads to the manifestation of motor symptoms that characterize Parkinson's disease (PD), Dementia with Lewy Bodies (DLB), and related synucleinopathies.^{4,5} In addition to neurodegenerative disorders, oxidants produced from DA metabolism have been implicated in synaptic terminal damage that occurs from psychoactive drugs of abuse including methamphetamine, 3,4-methylenedioxymethamphetamine (MDMA), and cocaine.^{6–8} Attenuation of DA synthesis has been shown to abrogate the neurotoxic effects of methamphetamine, as well as α -synuclein (α -syn)-induced toxicity in PD models.^{9,10} Furthermore, exposure to environmental toxins such as 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine (MPTP), rotenone, and paraquat can cause specific damage to dopaminergic neurons through oxidative processes that may be amplified by excessive and inappropriate DA metabolism.^{11–13}

The DA-dependent damage to neurons is thought to proceed through an aberrant increase in cytosolic levels of DA, which may lead to oxidative stress through rapid oxidation at physiological pH. Indeed, recent *in vivo* studies of rodent models have shown that disruption of synaptic vesicle storage of DA through reduced vesicular monoamine transporter 2 (VMAT2) can induce neurodegeneration.^{14,15} In agreement with this, increasing VMAT2 levels in mice can protect DA neurons from toxicity induced by methamphetamine or MPTP.^{16,17} Although valuable insight into DA-induced neurotoxicity has been achieved through rodent models, they are limited by differences in the handling and/or metabolism of DA compared to human neurons. This is most apparent by the lack of NM in the SNpc of rodent neurons.¹⁸ The limitations of rodent model systems indicate a need for more accurate methods and human models to study the toxic mechanisms of DA oxidation.

Here, we describe a new method for the detection of free or protein-bound α -quinone-containing compounds extracted from cultured cells or tissue. This method utilizes the specific inherent excitation–emission properties of α -quinones in the near-infrared spectrum and allows for rapid and absolute quantification into the picomole range. We utilized this method to establish novel human models for the study of catechol oxidation, including human neuroblastoma cells and iPSC-derived midbrain DA neurons induced to produce excess cytosolic catechols. This method and human model system may facilitate future

mechanistic studies of dopaminergic cell death that occurs upon exposure to psychoactive drugs or in neurodegenerative disorders such as PD and DLB.

EXPERIMENTAL PROCEDURES

Detection of Free Oxidized Catechols by NIRF

Catechol-containing compounds, closely related derivatives, and sodium periodate were all obtained from Sigma (St. Louis, MO). Most compounds were dissolved in Dulbecco's Phosphate buffered saline (D-PBS) (Life Technologies) at stock concentrations of 10 mM. To dissolve tyrosine, D-PBS was adjusted to pH 5.0 with HCl. Ellagic acid, baicalein, and taxifolin were dissolved in dimethyl sulfoxide (DMSO) at stock concentrations of 10 mM. Compounds were diluted to 0.5 mM in D-PBS and 10 μ L was slowly spotted onto dry nylon membranes (Pall Life Sciences, Biodyne B 0.45 μ m, no. 60209) and allowed to dry to completion in a fume hood. To oxidize compounds, equimolar sodium periodate was added followed by 5 min incubation at 25 °C. Alternative methods to induce oxidation were done by either incubating samples at 37 °C for 24 h in aerobic conditions (auto-oxidation) or by incubation with equimolar iron(II) sulfate in D-PBS for 24 h, 37 °C. To control sample diffusion, samples were spotted onto membranes at 2–3 μ L at a time in a fume hood while under a gentle air stream from a compressed air source. Dry membranes were scanned on an Odyssey infrared imager (Li-Cor Biosciences, ex = 685 nm) and quantified with Image Studio V3.1.4.

Quantification of 3,4-Dihydroxyphenylacetic acid (DOPAC)- α -Quinone Modified α -syn

Purification of α -syn has been previously described.¹⁹ α -syn (346 μ M) was incubated with 1.5 equiv of DOPAC in D-PBS for 48 h at 37 °C. The sample was boiled in 2% SDS, and free DOPAC was removed by filtering through 10 000 kDa cutoff membranes (EMD Millipore). The sample was spotted onto a nylon membrane as described above. To generate a standard curve, DOPAC was oxidized to completion by equimolar sodium periodate, spotted onto a nylon membrane, and intensity values were fitted to a polynomial function.

In-Gel Detection of Catechol-Bound- α -syn

DA, DOPAC, or baicalein were incubated with α -syn for 0, 24, or 48 h as described above. In some samples, 10 equiv of *n*-acetyl cysteine (NAC) were added to prevent catechol oxidation, or 100 μ M diethylenetriaminepentaacetic acid (DTPA) was added to chelate trace metals. Protein (50 μ g) was boiled in sample buffer (20 mM Tris, 1% glycerol, 180 mM β -mercaptoethanol, 0.003% bromophenol blue, and 2% SDS, pH 6.8) and analyzed by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE). The gels were analyzed using a near-infrared imager as described above and subsequently stained with colloidal blue (Life Technologies) to visualize total protein.

Detection of α -Quinone Proteins from Mouse Brain Homogenate

Triton X-100 soluble lysate (1 mg) from striatal sections of C57Bl6 mouse brain were incubated with 500 μ M of preoxidized DOPAC (DOPAC oxidized by equimolar sodium periodate) for 30 min at 37 °C. Lysates were clarified by centrifugation and injected on a

Superdex 200 10/300 GL column (GE Healthcare). Fractions were concentrated and analyzed by SDS-PAGE/nIRF as described above.

SH-SY5Y Cell Culture, Infection with Tyrosine Hydroxylase-Containing Lentiviral Constructs, and nIRF Detection of o-Quinone Modified Cytosolic Proteins

SH-SY5Y cells expressing A53T α -syn were utilized.²⁰ Stable cell line and lentiviral infection has been described.²⁰ Tyrosine hydroxylase (TH) was detected by Western blot (mouse anti-TH 1:4000, EMD Biosciences, La Jolla, CA), and loading was determined by antineural specific enolase (NSE, Polysciences, Warrington, PA). Catechols were quantified by HPLC with electrochemical detection as described.²⁰ One day after infection, cells were treated with 20 μ M retinoic acid for 8 days, extracted in 1% Triton buffer, and analyzed as in “Detection of o-quinone proteins from mouse brain homogenate”.

Measurement of o-Quinones in Human Midbrain Dopamine Neurons

Generation of neurons is described in the Supporting Information. Neurons were cultured in Neurobasal Media (Life Technologies) containing 1% penicillin/streptomycin and Neurocult SM1 supplement (Stem Cell Technologies). To induce catechol oxidation, either 10 nM H₂O₂, 50 μ M L-3,4-dihydroxyphenylalanine L-DOPA, or H₂O₂/L-DOPA combination was added at day 40 in the media and replaced every 3 days, for 20 days total. Neurons were harvested at day 60, and detergent insoluble extracts were dissolved in 1 N NaOH, washed with water, and spotted onto membranes as described above. Please see the Supporting Information for details.

RESULTS

Enhanced Near-Infrared Fluorescence of α -syn by Catechol Oxidation

During the course of our studies on catechol-modified α -syn,^{19,20} we realized that a rapid and reproducible methodology for the detection of oxidized catechols would facilitate our understanding of this pathway in disease. We inadvertently discovered that treatment of purified recombinant α -syn with catechols enhanced the near-infrared fluorescence (nIRF) of the protein (Figure 1A,B). Catechol-treated α -syn protein was boiled in 2% SDS, subjected to SDS-PAGE analysis, and without further treatment the gel was directly analyzed on a near-infrared imaging scanner by excitation at 685 nm and detection at ~700 nm. Incubation of α -syn with catechol-containing compounds DA, DOPAC, or baicalein for 0–48 h increased integrated band intensity in a time-dependent manner (Figure 1A,B). DOPAC-treated α -syn resulted in the most dramatic modification and increase in nIRF signal compared to DA or baicalein (Figure 1A), an effect that is likely due to more efficient modification of α -syn by DOPAC as previously noted.¹⁹ DOPAC lacks the reactive amine group required to form cyclic aminochrome and subsequent self-polymerization to form melanin and since it cannot be consumed into a melanin-like polymer, it is more likely to react with nucleophilic amino acids of α -syn. Treatment of DOPAC/ α -syn reactions with the antioxidant *n*-acetyl-cysteine (NAC) or removal of oxygen from the reaction buffer completely eliminated the nIRF signal, indicating that oxidation was required to enhance the fluorescence of the protein (Figure 1A; lanes 3,5,8; Figure S1). Including the metal chelator DTPA into the DOPAC/ α -syn reaction had no effect, suggesting that the nIRF signal was not

a result of oxidation by trace metals in the buffer (Figure 1A, lane 6). Finally, treatment of α -syn with hydrogen peroxide, a byproduct of catechol oxidation that is known to oxidize α -syn,²¹ had no effect on the nIRF signal of α -syn (Figure 1A, lane 9). This indicates that enhanced fluorescence was specifically due to the reaction with catechol-containing structures rather than general oxidation of the protein.

Enhanced nIRF Occurs from the Oxidation of Catechols to Form o-Quinone

We next sought to determine the structural requirements for nIRF signal enhancement by measuring the fluorescence of a series of structurally related compounds upon oxidation. Catechol-containing compounds were chemically oxidized by sodium periodate (NaIO₄), spotted onto a nylon membrane, and analyzed by nIRF. While the reduced forms of most compounds did not generate a detectable signal, oxidation of catechol-containing compounds resulted in enhanced nIRF (Figure 2A). Signal variation was observed between oxidized species although synthetic melanin produced the greatest signal enhancement upon oxidation (Figure 2A,B, compounds 1–13). Using oxidized DOPAC, we found the limitation of sample detected by nIRF was 39 pmol (Figure S2). Other methods of oxidation, including 24 h auto-oxidation at 37 °C, pH 7.4, and Fe(II)-oxidation, also resulted in enhancement of nIRF signal (Figure S3). Oxidation of closely related compounds including tyrosine, tyramine, and para-quinone yielded an essentially undetectable signal (integrated intensity < 1) (Figure 2A,B, compounds 14–20), indicating a requirement of oxidized hydroxyl moieties at the 1,2 position of a benzene ring. Therefore, the data indicated that oxidation of 1,2 dihydroxybenzene-containing compounds to form 1,2 benzoquinones (*o*-quinone) are detected by nIRF.

Absolute Quantification of Protein-Bound o-Quinones by nIRF

To determine if the nIRF assay can be used for absolute quantification of *o*-quinone structures on proteins, we incubated α -syn with 1.5 mol equiv of DOPAC under physiological conditions for 48 h to modify the protein. The sample was then boiled in 2% SDS and filtered with 10 000 kDa cutoff membranes to remove free, oxidized DOPAC. DOPAC-modified α -syn was immobilized onto a nylon membrane, and the nIRF signal intensity was compared to a known amount of fully oxidized, free DOPAC-*o*-quinone (Figure 3A). Extrapolation from the standard curve revealed that 670 pmol of DOPAC-*o*-quinone were bound to 346 pmol of α -syn protein under these conditions, which is consistent with a DOPAC/ α -syn molar ratio of 2 (Figure 3B). These results show that the nIRF assay provides a simple and rapid method for the quantification of *o*-quinone-modified proteins.

nIRF-Based Detection of o-Quinone-Modified Proteins from Mouse Brain Homogenate and Cultured Neuroblastoma Cells

To determine if nIRF can be utilized to detect *o*-quinone-containing proteins from complex homogenate, mouse brain lysates were incubated with preoxidized DOPAC (DOPAC-*o*-quinone) gel filtered through a superdex 200 column and analyzed by SDS-PAGE/nIRF. This revealed an increase in protein band intensity in DOPAC-*o*-quinone treated samples compared to PBS-treated controls (Figure 4A) indicating the utility of nIRF for detecting modified proteins from complex mixtures.

To determine if nIRF can detect α -quinone modified proteins generated within catechol-producing cell lines, we utilized a SH-SY5Y cell culture model. Previous analysis of this cell line revealed that cellular catechol levels were below the detection limits.²⁰ Therefore, catechol levels were elevated by overexpression of the rate-limiting enzyme, tyrosine hydroxylase (TH) using a lentiviral expression system.²⁰ As expected, transduction of cells with TH-containing lentivirus increased the protein levels of TH as well as L-DOPA, DA, and DOPAC (Figure 4B). We tested if increasing cytosolic catechol levels resulted in the formation of catechol-bound proteins by analysis of triton-soluble cell lysates that were gel filtered and analyzed by SDS-PAGE/nIRF. This revealed an increase in fluorescent intensity of proteins in lysates from TH-transduced cells compared to empty vector-transduced cells (Figure 4C). This indicates that the nIRF assay is suitable for the detection of α -quinone-modified proteins that occur through the elevation of endogenous catechol levels.

nIRF-Based Detection of α -Quinones in Human Midbrain Dopamine Neurons

We next sought to determine if nIRF assay would be suitable for detection of oxidized catechols in human midbrain DA neurons. We use a previously established protocol²² to develop midbrain DA neurons from human induced pluripotent stem cells of a healthy control individual (line 2135, described in ref 23). Expression of midbrain markers was confirmed by immunofluorescence, demonstrating that the majority of cells expressed neural specific β -III-tubulin, and a subset of these neurons expressed TH and FOXA2 (β -III-tubulin = $94\% \pm 2.53$; β -III-tubulin with TH = $89\% \pm 4.66$, values are the mean $\pm$ SEM) (Figure 5A). We cultured midbrain neurons with excess L-DOPA for 20 days to induce α -quinone production,²⁴ as well as 10 nM hydrogen peroxide (H_2O_2) to promote oxidation of endogenous catechols, and combined H_2O_2 /L-DOPA. Neurons were extracted in detergent-containing buffer, centrifuged at 100 000g, and the resulting insoluble pellets contained brown pigment consistent with the presence of oxidized catechols²⁵ (Figure 5B). Insoluble pellets were dissolved in sodium hydroxide (NaOH), washed with water, and nIRF intensity was determined. We observed a ~5 fold enhancement of signal in neuronal extracts treated with H_2O_2 , ~10–15-fold increase in extracts of the L-DOPA and combined H_2O_2 /L-DOPA treated cultures (Figure 5B). The data suggests that the nIRF assay is suitable for absolute quantification of α -quinones in human midbrain neurons.

DISCUSSION

We describe a new method for detection and absolute quantification of free and protein-bound α -quinones based on the enhancement of nIRF signal intensities. Our data indicate that nIRF enhancement occurs through changes in catechol moieties induced by oxidation to form α -quinone structures. Since this effect occurred even in the most basic catechol structure (compound 11, pyrocatechol) but not para-quinone (compound 17), we conclude that oxidation of 1,2 hydroxybenzene to 1,2 benzoquinones results in nIRF enhancement. Pyrocatechol, DOPAC, and other structures that cannot polymerize into melanin-like polymers also show increased nIRF, indicating that the polymerization itself is not required to produce the signal. The method was then used to document quantification of catechol-modified proteins generated *in vitro* or in cells that generate cytosolic catechols in excess. We show that prolonged H_2O_2 or L-DOPA treatment in human midbrain DA neurons can

generate insoluble, black/brown species consistent with oxidized catechols formed synthetically.²⁵ Previous studies have utilized rodent SN cultures treated with L-DOPA to produce NM and have generated important insights into the mechanisms of NM formation.²⁴ However, unlike human SN neurons, rodent SN neurons are not known to form NM by natural mechanisms.¹⁸ The model and quantification method described here will provide further insights into the formation, degradation and biological role of insoluble oxidized polymers of *o*-quinones in the context of the human brain as well as the toxic mechanisms of *o*-quinones.

The presence of NM in the SNpc provides *in vivo* evidence that DA neurons in this region are exposed to oxidants and electrophiles. While normally tolerated in aging healthy individuals, the increased exposure to oxidants may render SNpc neurons vulnerable toward additional toxic insults. For example, exposure to pesticides, other environmental toxins, or genetic risk factors for PD may provide additional impairment leading to accelerated degeneration of SNpc DA neurons. Interestingly, none of the mutated familial PD genes are selectively expressed in SNpc or other neurons that selectively degenerate in PD brain. This indicates that additional factors, such as oxidative stress, may be involved in the pathogenesis of circumscribed brain regions in familial PD. Indeed, elevated oxidative stress has been documented in PD brain through the detection of oxidized α -syn within Lewy inclusions.²⁶ Previous studies have also shown that SNpc neurons exhibit unique pacemaking activity that results in mitochondrial-derived oxidative stress.²⁷ Excessive mitochondrial-derived oxidants will likely accelerate catechol oxidation in DA neurons leading to accelerated cell death of SNpc neurons by multiple mechanisms.^{20,28–31}

The nIRF technique provides a simple, quantitative method to measure oxidized catechols from biologically complex samples. Current techniques for the absolute quantification of *o*-quinones or molar ratios of catechol-modified proteins require several extraction steps and specialized analysis equipment, such as acid hydrolysis of proteins followed by high-performance liquid chromatography-electrochemical detection. In contrast, the nIRF assay only requires immobilization of standards and sample on a nylon membrane followed by scanning on a near-infrared imager. Previously established techniques for the detection of *o*-quinone-containing proteins, such as detection of formazan from the reduction of nitro-blue tetrazolium (NBT), are useful for relative quantification but require transfer of proteins from gels to membranes followed by incubation with the detecting agent.³² The nIRF method offers direct in-gel detection and is nondestructive. Therefore, quantification of *o*-quinone proteins can be achieved by SDS-PAGE/nIRF, and the same gel can be utilized for other purposes such as Western blotting. Since the nIRF method relies on fluorescence detection of a continuously produced signal and does not require derivatization, it is inherently more linear and quantitative compared to NBT detection that relies on the nonlinear production of formazan.

The method described here should facilitate the study of catechol-derived oxidant stress that occurs within DA neurons. The applications of this method range from the study of neurodegeneration to drug-induced neurotoxicity. Delineation of the neurotoxic processes that occur in these conditions should uncover novel therapeutic pathways focused on the reduction of oxidants and restoration of neuronal function.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

This research was supported by the National Institute of Neurological Disorders and Stroke Grant R01NS092823 (J.R.M.) and Grant R01NS076054 (D.K.), the National Institute on Aging Grant AG13966, and the National Institute of Environmental Health Sciences Center of Excellence in Environmental Toxicology Grant ES013508 (H.I.). L.F.B. was supported by a fellowship within the postdoc program of the German Academic Exchange Service (DAAD).

References

1. Robinson S, Sotak BN, During MJ, Palmiter RD. *Behav. Neurosci.* 2006; 120:196–200. [PubMed: 16492130]
2. Zhou QY, Quaife CJ, Palmiter RD. *Nature.* 1995; 374:640–643. [PubMed: 7715703]
3. Ligmond MJ, Stricker EM. *Science (Washington, DC, U. S.).* 1972; 177:1211–1214.
4. Fearnley JM, Lees AJ. *Brain.* 1990; 113(Pt 6):1823–1842. [PubMed: 2276046]
5. Albin RL, Young AB, Penney JB. *Trends Neurosci.* 1989; 12:366–375. [PubMed: 2479133]
6. LaVoie MJ, Hastings TG. *J. Neurosci.* 1999; 19:1484–1491. [PubMed: 9952424]
7. Granado N, O'Shea E, Bove J, Vila M, Colado MI, Moratalla R. *J. Neurochem.* 2008; 107:1102–1112. [PubMed: 18823368]
8. Dietrich JB, Mangeol A, Revel MO, Burgun C, Aunis D, Zwiller J. *Neuropharmacology.* 2005; 48:965–974. [PubMed: 15857623]
9. Wagner GC, Lucot JB, Schuster CR, Seiden LS. *Brain Res.* 1983; 270:285–288. [PubMed: 6883096]
10. Xu J, Kao SY, Lee FJ, Song W, Jin LW, Yankner BA. *Nat. Med.* 2002; 8:600–606. [PubMed: 12042811]
11. Betarbet R, Sherer TB, MacKenzie G, Garcia-Osuna M, Panov AV, Greenamyre JT. *Nat. Neurosci.* 2000; 3:1301–1306. [PubMed: 11100151]
12. Lotharius J, O'Malley KL. *J. Biol. Chem.* 2000; 275:38581–38588. [PubMed: 10969076]
13. Manning-Bog AB, McCormack AL, Li J, Uversky VN, Fink AL, Di Monte DA. *J. Biol. Chem.* 2002; 277:1641–1644. [PubMed: 11707429]
14. Guillot TS, Shepherd KR, Richardson JR, Wang MZ, Li Y, Emson PC, Miller GW. *J. Neurochem.* 2008; 106:2205–2217. [PubMed: 18643795]
15. Caudle WM, Richardson JR, Wang MZ, Taylor TN, Guillot TS, McCormack AL, Colebrooke RE, Di Monte DA, Emson PC, Miller GW. *J. Neurosci.* 2007; 27:8138–8148. [PubMed: 17652604]
16. Lohr KM, Stout KA, Dunn AR, Wang M, Salahpour A, Guillot TS, Miller GW. *ACS Chem. Neurosci.* 2015; 6:790–799. [PubMed: 25746685]
17. Lohr KM, Bernstein AI, Stout KA, Dunn AR, Lazo CR, Alter SP, Wang M, Li Y, Fan X, Hess EJ, Yi H, Vecchio LM, Goldstein DS, Guillot TS, Salahpour A, Miller GW. *Proc. Natl. Acad. Sci. U. S. A.* 2014; 111:9977–9982. [PubMed: 24979780]
18. Barden H, Levine S. *Brain Res. Bull.* 1983; 10:847–851. [PubMed: 6616275]
19. Mazzulli JR, Armakola M, Dumoulin M, Parastatidis I, Ischiropoulos H. *J. Biol. Chem.* 2007; 282:31621–31630. [PubMed: 17785456]
20. Mazzulli JR, Mishizen AJ, Giasson BI, Lynch DR, Thomas SA, Nakashima A, Nagatsu T, Ota A, Ischiropoulos H. *J. Neurosci.* 2006; 26:10068–10078. [PubMed: 17005870]
21. Uversky VN, Yamin G, Souillac PO, Goers J, Glaser CB, Fink AL. *FEBS Lett.* 2002; 517:239–244. [PubMed: 12062445]
22. Kriks S, Shim JW, Piao J, Ganat YM, Wakeman DR, Xie Z, Carrillo-Reid L, Auyeung G, Antonacci C, Buch A, Yang L, Beal MF, Surmeier DJ, Kordower JH, Tabar V, Studer L. *Nature.* 2011; 480:547–551. [PubMed: 22056989]

23. Mazzulli JR, Xu YH, Sun Y, Knight AL, McLean PJ, Caldwell GA, Sidransky E, Grabowski GA, Krainc D. *Cell*. 2011; 146:37–52. [PubMed: 21700325]
24. Sulzer D, Bogulavsky J, Larsen KE, Behr G, Karatekin E, Kleinman MH, Turro N, Krantz D, Edwards RH, Greene LA, Zecca L. *Proc. Natl. Acad. Sci. U. S. A.* 2000; 97:11869–11874. [PubMed: 11050221]
25. Graham DG. *Mol. Pharmacol.* 1978; 14:633–643. [PubMed: 98706]
26. Giasson BI, Duda JE, Murray IV, Chen Q, Souza JM, Hurtig HI, Ischiropoulos H, Trojanowski JQ, Lee VM. *Science (Washington, DC, U. S.)*. 2000; 290:985–989.
27. Guzman JN, Sanchez-Padilla J, Wokosin D, Kondapalli J, Ilijic E, Schumacker PT, Surmeier DJ. *Nature*. 2010; 468:696–700. [PubMed: 21068725]
28. Hastings TG, Lewis DA, Zigmond MJ. *Proc. Natl. Acad. Sci. U. S. A.* 1996; 93:1956–1961. [PubMed: 8700866]
29. Berman SB, Hastings TG. *J. Neurochem.* 1999; 73:1127–1137. [PubMed: 10461904]
30. Kuhn DM, Arthur R Jr. *J. Neurosci.* 1998; 18:7111–7117. [PubMed: 9736634]
31. Conway KA, Rochet JC, Bieganski RM, Lansbury PT Jr. *Science (Washington, DC, U. S.)*. 2001; 294:1346–1349.
32. Paz MA, Fluckiger R, Boak A, Kagan HM, Gallop PM. *J. Biol. Chem.* 1991; 266:689–692. [PubMed: 1702437]

Figure 1.

Modification by catechol oxidation enhances the near-infrared fluorescence (nIRF) of α -syn: (A) α -syn was incubated with DA, DOPAC, baicalein, or H₂O₂ under physiological conditions and samples (50 μ g) were analyzed after 0, 24, or 48 h by SDS-PAGE/nIRF. Gels were scanned using a near-infrared imager, then stained with coomassie brilliant blue (CBB) to visualize total protein (left panel). The contrast of the nIRF scan was enhanced to visualize the low-intensity bands in lanes 2 and 7 (right panel). (B) The fluorescent signal was quantified by obtaining the integrated intensity of the entire gel lane. The molecular

weight marker on the left side of the blot indicates the protein size in kilodaltons. Incubation conditions 1–9 are shown below. $n = 4$, $*p < 0.05$ compared to control (condition 1); $\#p < 0.05$ compared to 24 h time point; $\ddagger p < 0.05$ compared to NAC, Student's t test. DA = dopamine, NAC = *n*-acetyl cysteine, DTPA = diethylenetriaminepentaacetic acid, Bac = Baicalein. See also Figure S-1.

Figure 2.

Oxidation by sodium periodate enhances the nIRF signal of catechol-containing compounds. Selected catechol-containing compounds were chemically oxidized with equimolar sodium periodate (NaIO_4). Five nanomoles were spotted on a nylon membrane and scanned with a near-infrared imager ($\text{ex} = 685$, $\text{em} = 700$). The fluorescent signal obtained upon NaIO_4 oxidation was quantified and integrated signal intensities were compared. The compounds are ordered by a fold increase in signal intensity that occurs upon oxidation ($****p < 0.0001$, $***p < 0.001$, $**p < 0.01$, $*p < 0.05$, Student's t test, $n = 3$, values are the mean $\pm$

SEM). Shaded compounds in the table showed an integrated intensity lower than one. See also Figures S-2 and S-3.

Author Manuscript

Author Manuscript

Author Manuscript

Author Manuscript

Figure 3.

Detection and absolute quantification of DOPAC-*o*-quinone moieties in modified α -syn by nIRF. (A) NaIO₄-oxidized DOPAC was spotted onto a nylon membrane (5 μ L), analyzed by nIRF, and integrated intensity of the signal was quantified. These values were utilized to generate a standard curve for quantification of protein-bound DOPAC-*o*-quinone (see methods). The nIRF signal intensity was linear up to 5 nmol; however, a curve suitable for detecting higher amounts DOPAC-*o*-quinone required a polynomial fit. (B) α -Syn (346 μ M) was incubated with DOPAC (0.5 mM) for 48 h under physiological conditions, boiled in 2% SDS, and separated from unbound DOPAC. Dilutions were spotted onto nylon membranes and detected by nIRF. The amount of protein-bound DOPAC-*o*-quinone was determined from the standard curve generated in part A and reveals a 2:1 DOPAC-*o*-quinone/ α -syn ratio.

Figure 4.

In-gel detection of *o*-quinone-modified proteins from mouse brain or human neuroblastoma cells overexpressing tyrosine hydroxylase. (A) Lysate from a mouse striatum was incubated with 0.5 mM DOPAC-*o*-quinone, subjected to size-exclusion chromatography (SEC), and fractions were analyzed by SDS-PAGE/nIRF. CBB indicates total protein loaded on the column. (B) SH-SY5Y cells were transduced with empty vector (V) or TH-containing lentiviral vectors, differentiated for 8 days with retinoic acid, then analyzed for intracellular catechol levels by high-pressure liquid chromatography-electrochemical detection (HPLC-

ECD) ($n = 3$, $*p < 0.05$, values are the mean $\pm$ SEM). Catechol levels were not detectable in empty vector transduced cells (ND). Western blot analysis below shows overexpression of TH compared to empty vector transduced cells. Neural specific enolase (NSE) was used as a loading control. (C) Triton soluble lysates were subjected to SEC and analyzed as in panel A. The horizontal marker indicates the molecular size in angstroms or kilodaltons, while the vertical marker indicates the protein weight in kilodaltons.

Figure 5.

Detection of *o*-quinones in H₂O₂ or L-DOPA treated human midbrain DA neurons. (A)

Generation of human midbrain DA neurons from induced pluripotent stem cells.

Immunostaining analysis of day 60 neurons generated from a healthy control demonstrates the expression of neural-specific β -III-tubulin (green) and tyrosine hydroxylase (TH, red). Nuclei are shown in blue. Scale bar = 50 μ m. Expression of midbrain markers TH (green)

and FOXA2 (red) is demonstrated by immunostaining analysis of day 60 neurons (below). Scale bar = 10 μ m. (B) Neurons at day 40 were treated with peroxide (H₂O₂), L-DOPA, or

L-DOPA + H₂O₂, harvested at day 60, followed by 1 N NaOH extraction and nIRF analysis. Insoluble brown-pigmented pellets were observed after detergent extraction and centrifugation at 100 000g ($n = 3$, * $p < 0.05$, ANOVA with Tukey's posthoc test, values are the mean $\pm$ SEM). NaOH-soluble extracts were loaded according to the protein amount found in the corresponding detergent-soluble fractions. The standard curve shown above was generated with sodium periodate-induced oxidation of DA and used to calculate total picomoles of oxidized catechols found in each sample.