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Functional regulation of neuronal nitric oxide synthase expression and activity in the rat retina

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Abstract

In the nervous system within physiological conditions, nitric oxide (NO) production depends on the activity of nitric oxide synthases (NOSs), and particularly on the expression of the neuronal isoform (nNOS). In the sensory systems, the role of NO is poorly understood. In this study, we identified nNOS-positive cells in the inner nuclear layer (INL) of the rat retina, with distinct characteristics such as somata size, immunolabeling level and location. Employing mathematical cluster analysis, we determined that nNOS amacrine cells are formed by two distinct populations. We next investigated the molecular identity of these cells, which did not show colocalization with calbindin (CB), choline acetyltransferase (ChAT), parvalbumin (PV) or protein kinase C (PKC), and only partial colocalization with calretinin (CR), revealing the accumulation of nNOS in specific amacrine cell populations. To access the functional, circuitry-related roles of these cells, we performed experiments after adaptation to different ambient light conditions. After 24 h of dark-adaptation, we detected a subtle, yet statistically significant decrease in nNOS transcript levels, which returned to steady-state levels after 24 h of normal light–dark cycle, revealing that nNOS expression is governed by ambient light conditions. Employing electron paramagnetic resonance (EPR), we demonstrated that dark-adaptation decreases NO production in the retina. Furthermore, nNOS accumulation changed in the dark-adapted retinas, with a general reduction in the inner plexiform layer. Finally, computational analysis based on clustering techniques revealed that dark-adaptation differently affected both types of nNOS-positive amacrine cells. Taken together, our data disclosed functional regulation of nNOS expression and activity, disclosing new circuitry-related roles of nNOS-positive cells. More importantly, this study indicated unsuspected roles for NO in the sensory systems, particularly related to adaptation to ambient demands.

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Introduction

As a gaseous, diffusible chemical transmitter, nitric oxide (NO) may interact with intracellular targets to trigger several signal transduction pathways (Benarroch, 2011). Besides classically related to the modulation of neurotransmission and synaptic plasticity (Calabrese et al., 2007), NO seems to play additional roles in the central nervous system (CNS), such as in developmental processes (Contestabile and Ciani, 2004) and in the progression of neurodegenerative diseases (Bonnin et al., 2012; Hall et al., 2012; Virarkar et al., 2013). Indeed, NO is essential for CNS homeostasis, as a product from the conversion of L-arginine to L-citrulline by the members of the NO synthase (NOS) family of proteins (Xu et al., 2004).

This regard, previous studies determined the expression of neuronal NOS (nNOS) in the retina of several species. In rats, it seems consensus that nNOS accumulates in amacrine cells, although the molecular identity of these cells remains subject to debate (Cellerino et al., 1999; Haverkamp et al., 2000; Kim et al., 1999). Indeed, NO plays several roles in the retina, including regulation of phototransduction and cone glutamate release (Savchenko et al., 1997). In addition, NO inhibits dopamine release (Bugnon et al., 1994) and decreases the intercellular homologous coupling between horizontal cells (Xin and Bloomfield, 2000) and the heterologous coupling between cone bipolar and amacrine cells (Mills and Massey, 1995). Furthermore, regulation of nNOS...
expression and activity in physiological conditions has been investigated. Notably, increasing evidences indicated that NO modulates light responses in the retina (Pang et al., 2010). For example, it was shown that NO donors increased calcium currents in rods, with NOS inhibition favoring rod hyperpolarization (Noll et al., 1994), supporting a modulation of signal transmission to second-order neurons (Kourenyi et al., 2004).

In this study, we combined morphological analysis with computational clustering techniques to determine the number and molecular identity of nNOS amacrine cell populations. Moreover, we directly measured NO production in the retina after dark-adaptation using electron paramagnetic resonance. Finally, combining experimental methods and mathematical analysis, we were able to quantify the effects of dark-adaptation on nNOS gene expression and protein distribution in distinct retinal layers and specific amacrine cell populations. Taken together, our results contribute for disclosing new roles for NO in the sensory systems.

Methods

Ethics statement

Experiments with animals were conducted in accordance with the guidelines of the NIH and the Brazilian Scientific Society for Laboratory Animals. Experimental protocol was approved by the Ethics Committee in Animal Experimentation of the Institute of Biomedical Sciences/University of São Paulo (ICB/USP). All animals were housed in cages with free access to food and water throughout the study.

Animal procedures

Experiments were carried out with adult Long Evans rats (Rattus norvegicus) kept on a 12 h light/dark cycle (light phase 80–100 lx) with lights on at 06:00 a.m. Rats were euthanized with a lethal dose of ketamine (30 mg/100 g of body weight, i.m., Parke-Davis, Ann Arbor, MI, USA) and xylazine (2 mg/100 g, i.m., West Haven, CT, USA) between 11:00 and 12:00 a.m. In dark-adaptation experiments, there were four groups of animals: Control group, which are adult animals from 12 h light/dark cycle, and those that were kept in the dark for 3 h, 24 h or 24 h followed by a 12:12 light/dark cycle, all euthanized between 11:00 and 12:00 a.m. Next, retinas were removed for different methodological procedures.

RNA isolation, cDNA synthesis and Real-Time PCR

Retinas (n = 6) were directly homogenized in 1 ml TRizol reagent (Invitrogen, Carlsbad, CA, USA) and total RNA was extracted following the manufacturer’s suggested protocol and as previously described (Belmonte et al., 2006; Kihara et al., 2005). In brief, following two chloroform extraction steps, RNA was precipitated with isopropanol and the pellet washed twice in 70% ethanol. After air-drying, RNA was resuspended in DEPC-treated water and the concentration of each sample obtained from A_{260} measurements. Residual DNA was removed using DNase I (Amersham, Piscataway, NJ, USA) following the manufacturer’s protocol. Quantitative analysis of gene expression was carried out with a Rotor-Gene 6000 Real-Time Rotary Analyzer (Corbett Robotics Inc., San Francisco, CA, USA) with specific primers for rat nNOS (forward, 5'-CCAATGGTTCACAAAAACGAGCTT-3'; reverse, 5'-TCGGCTGGACTAGGGCTTT-3'), GAPDH (forward, 5'-TTCAAAAGGCGCCAGCTTC-3'; reverse, 5'-GCCACCTGTTTATTTATCCA-3') expression was determined by the labeling of one channel, and analysis was performed by pairwise comparisons (Tukey’s HSD test), with the significance level set at 5%. Deoxyribonuclease I (Invitrogen) was used to decontaminate samples before cDNA synthesis (Invitrogen). For each 20 μl reaction, 4 μg total RNA was mixed with 1 μl oligo-T primer (0.5 μg; Invitrogen) and incubated for 10 min at 65 °C. After cooling on ice the solution was mixed with 4 μl 5× first strand buffer, 2 μl of 0.1 M DTT, 1 μl of dATP, dCTP and dGTP (each 10 mM), and 1 μl SuperScript III reverse transcriptase (200 U; Invitrogen) and incubated for 60 min at 50 °C. Reaction was inactivated by heating at 70 °C for 15 min. All PCR assays were performed as follows: after initial activation at 50 °C for 2 min and 95 °C for 10 min, cycling conditions were 95 °C, 10 and 60 °C, 1 min. Dissociation curves of PCR products were obtained by heating samples from 60 °C to 95 °C, in order to evaluate primer specificity.

PCR statistical analysis

Relative quantification of target gene expression was evaluated using the comparative CT method as previously described in detail (Kihara et al., 2008; Medhurst et al., 2000). In the present study, control refers to animals from the 12:12 light/dark cycle. Values were entered into a one-way analysis of variance (ANOVA), followed by pairwise comparisons (Tukey’s HSD test), with the significance level set at 5%.

Immunohistochemistry

Eyes from six rats were dissected out and the retinas were fixed for 30 min in 4% paraformaldehyde (PFA) in phosphate buffer 0.1 M pH 7.3 (PB), and cryoprotected in 30% sucrose solution for at least 24 h at 4 °C. After embedding in O.C.T. compound (Sakura Finetek, Torrance, CA, USA) they were cut transversally (12 μm) on a cryostat. Retinal sections were incubated overnight with primary antibodies in a solution containing 5% normal donkey serum and 0.5% Triton-X 100 in PBS at room temperature. All antibodies and specific concentrations used in this study are listed in Table 1. After several washes, retinal sections were incubated with donkey antiserum against rabbit, mouse or goat IgG tagged to Alexa 488 (1:250–1:500, Invitrogen) diluted in 3% normal donkey serum containing 0.5% Triton-X 100 in PBS for 2 h at room temperature. For double-labeling experiments, we used secondary antibodies conjugated to Alexa 546 and Alexa 647 (1:500, Invitrogen). Controls for the experiments consisted of the omission of primary antibodies; no staining was observed in these cases. Counter-staining of retinas was achieved using 4',6-diamidino-2-phenylindole (DAPI), by incubating sections at room temperature for 10 min. After washing, the tissue was mounted using Vecta Shield (Vector Labs, Burlingame, CA, USA), and analyzed in a Nikon TS100F inverted microscope (Nikon Instruments Inc., Melville, NY, USA). Figures were mounted with Adobe Photoshop CS. Manipulation of the images was restricted to brightness and contrast adjustments of the whole image.

Image quantification

Image analysis (n = 6) was performed with Image-Pro Plus (Media Cybernetics, Bethesda, MD, USA) and NIS Elements (Nikon Instruments Inc.), as previously described (Paschon et al., 2012). After channel separation (RGB) of the color images, we performed bitmap analysis. X–Y axis analysis generated numerical appended data files corresponding to pixel values. The bitmap analysis was used to view the pixel values of the active window (or area of interest, AOI) in numeric format, where values correspond to the brightness of the pixels. In some cases, AOI was defined by the labeling of one channel, and analysis was performed in another channel, as for instance, labeling of nNOS in the green channel, defined by DAPI labeling in the blue channel. Values were exported to

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Table 1

Primary antibodies used in this study.
Excel (Microsoft, Redmond, WA, USA) for the appropriate mathematical analyses.

Cluster analysis

From retinal images (n = 6) we measured three distinct parameters of individual cells: brightness, width, and distance to the inner border of the inner nuclear layer. These attributes span a three-dimensional vector space in which each cell corresponds to a data point. The data was clustered around two different centers that can be identified by a k-means clustering analysis. In short, the algorithm works as follows: first, the number of clusters k was assumed a priori. Then, the algorithm finds the clusters and their corresponding centers by minimizing the d distortion. The d distortion is the sum of squared distances between the cluster centers and their assigned data points. In order to estimate the number of clusters in our dataset, we employed a previously developed method (Sugar and James, 2003), which applies rate distortion theory. In this method, a distortion curve d(k) for different values of k is generated by the k-means algorithm. The distortion curve is then rescaled by the negative power −p/2, based on the dimensionality p of the dataset. In our three-dimensional dataset, d(k) → d−3/2(k).

Electron paramagnetic resonance (EPR)

EPR spectra were recorded in a Bruker EMX Plus Electron-Spin Resonance Spectrometer System instrument, operating at X-band frequency, in the Multiuser Facility Center at UFABC. We utilized standard Willmad quartz tubes in a high sensitive cavity. A digital temperature control system using liquid and gaseous nitrogen was used to control the temperature at 100 K. DPPH (2,2′-diphenyl-1-picrylhydrazyl) was used for calibrating frequency (g = 2.0036) with samples in a frozen water solution at 100 K. The quantification of radical production was measured with double signal integration of standard aqueous solutions of 4-hydroxy-2,2,6,6-tetramethyl-1-piperidinylxyloxy (TEMPOL), in the same conditions as in the experiments. Typical conditions used in these measurements were: frequency 9.41 GHz, power 20.00 mW, 4.48 × 10^3 gain, 5 G modulation amplitude, time constant 20.48 ms, sweep time 80.92 ms, 12 scans, resolution 1024 points, temperature (100 ± 1) K.

Pools of 8 retinas (n = 3) were isolated from adult rats after 24 h of dark-adaptation or controls maintained in a 12:12 light/dark cycle. These attributes span a three-dimensional vector space in which each cell corresponds to a data point. The data was clustered around two different centers and this result was applied to the rate distortion theory (Fig. 1F), which corroborated for the evidence that there are two populations of positive nNOS cells in the inner border of INL. Due to the morphological characteristics, cells corresponding to the red point dataset (width = 8.77 ± 0.09; brightness = 26.05 ± 0.84; distance = 5.06 ± 0.07) were collectively named “Round” cells, whereas the blue dataset (width = 11.45 ± 0.16; brightness = 70.15 ± 2.57; distance = 9.15 ± 0.15) corresponded to the “Balloon” cells.

Horizontal cells, bipolar cells and specific amacrine cells do not accumulate nNOS

In order to determine the presence of nNOS in specific retinal cell types, we performed double-labeling experiments. We did not detect colocalization of calbindin (CB), a marker for horizontal cells in the rodent retina (Oguni et al., 1998; Wassle, 2004), in nNOS-positive cells located in the outer margin of the inner nuclear layer (Figs. 2A–E). Similarly, we failed to detect colocalization of nNOS and PKC, a marker for rod bipolar cells (Greff et al., 1999). Although in the pixel profile and in high magnification images it was possible to observe some red and green channel superposition, which clearly originated from distinct cells (Figs. 2F–K). Finally, we were not able to detect accumulation of nNOS in ChAT- and parvalbumin (PV)-positive cells, markers for starburst and All glycnergic amacrine cells (Kunzevitzky et al., 2013; Wassle, 2004), respectively (Figs. 2L–U).

Calretinin-positive amacrine cells accumulate nNOS

In order to further analyze the molecular identity of nNOS amacrine cells, we employed another antibody raised against calretinin (CR), which is in the inner border of the inner nuclear layer is a marker for All amacrine cells, diffuse amacrine cells and A19 (Kolb et al., 2002; Wassle, 2004). In these experiments, we observed that some CR-positive cells also accumulated nNOS (Figs. 3A–B). Interestingly, distinct nNOS-positive cells were also CR-positive, including those that we previously classified as round (Figs. 3C–E) and balloon (Figs. 3F–H) cells. However, colocalization degree of both round and balloon nNOS-positive cells was only partial, 30.78 ± 2.95% and 38.96 ± 4.49%, respectively (Fig. 3I).

Ambient light conditions govern nNOS gene expression in a reversible manner

To access the functional regulation of nNOS in the retina, we carried out dark-adaptation experiments. Experiments were performed in 4 groups: control, 3 and 24 h of dark-adaptation, and 24 h of dark-adaptation followed by 24 h in the 12:12 light/dark cycle. Significant downregulation (2^-1.02 ± 0.26, -50.85 ± 6.80%, P < 0.05) of nNOS transcripts was observed after 24 h of dark-adaptation (Figs. 4A–B). Interestingly, 24 h after the return to the 12:12 cycle the gene expression levels returned approximately to the steady-state, control levels. Taking together, these results revealed that nNOS gene expression levels depend on the ambient light conditions. GAPDH gene expression levels were used as internal control (Fig. 4C).

Dark-adaptation downregulates NO production in the retina

Downregulation of nNOS transcripts triggered by dark rearing might indicate that NO plays a role in retinal adaptation mechanisms. Thus, we next aimed to directly measure NO production in the retina after dark-adaptation. With this purpose, we employed the spin trap technique to capture the NO free radical generated in the rat retinas. The spin trap Fe(2+), is able to enter in the plasmatic membrane and form the stable radical adduct complex NO–Fe(2+), easily identified by EPR spectra (Mikoyan et al., 1997; Piel et al., 2007; Shen et al., 1998; Tsuchiya et al., 1996). The g-factor to identify this complex has already been established, g = 2.038, and we used this value to determine the NO concentration in the samples. In tissues, other dinitrosyl iron species
Neuronal nitric oxide synthase (nNOS) immunolabeling in transverse sections of the rat retina. (A) In transverse sections of the rat retina counterstained with 4′,6-diamidino-2-phenylindole (DAPI, blue), nNOS labeling (green) was mainly observed in the inner plexiform layer (IPL) and inner nuclear layer (INL). (B) In the inner border of the INL, we observed nNOS-positive cells with distinct characteristics, including width and brightness, as shown in the pixel profile corresponding to the horizontal line shown in A. We observed the presence of both weak (white arrows) and strong signals (white arrowhead). (C, D) From high magnification of selected areas 1 and 2, we observed that the center of cells’ soma were located at different distances from the inner border of INL. (E) Three-dimensional representation of the cells according to width, brightness and distance (from the inner border of the INL). The dataset was segregated using k-means algorithm into k = 2 clusters (blue/balloon cells and red/round cells). The position of centroids is represented by gray dots. (F) Rescaled distortion curve (gray squares) and jumps in the rescaled distortion curve (black dots). The highest jump occurs at k = 2, which strongly indicates the presence of two different clusters in the dataset. (G) According to the projection of the centroid in the three axes, width = 8.77 ± 0.09; brightness = 26.05 ± 0.84; and distance = 5.06 ± 0.07 for the red dataset and width = 11.45 ± 0.16; brightness = 70.15 ± 2.57; and distance = 9.15 ± 0.15 for the blue dataset, values were used for a computational design of the typical round (dark green) and balloon (light green) cells. Red and black arrows indicated typical width and position of round and balloon cells, respectively. Scale bar = 20 μm. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

Dark-adaptation changes nNOS distribution in specific retinal layers

Considering that ambient light conditions regulate nNOS gene expression and NO production, we analyzed the distribution of nNOS in the retina after 24 h of dark-adaptation. When compared to controls (Figs. 6A–B), it is possible to see that dark-adapted retinas apparently show faint, weaker labeling in the IPL (Figs. 6C–D), as revealed by the pixel intensity profiles. In addition, we also performed quantification of pixel intensity to confirm changes in nNOS labeling. We quantified the brightness and calculated the IPL/INL ratio for both groups. Values obtained for the control were significant higher when compared to the dark-adaptation group, (1.37 ± 0.07 and 1.11 ± 0.03, respectively; P < 0.01; Fig. 6E). As quantification was taken as the ratio of the two layers, we were interested in knowing where this decrease occurred. We started evaluating the IPL, which apparently showed a decrease of labeling in sublamina off, mainly. Thus, we quantified both on and off sublaminas and calculated the ratio (Fig. 6F). Values obtained from control and 24 h dark-adaptation groups were not statistically different (0.98 ± 0.03 and 0.98 ± 0.01, respectively). When values for off (61.15 ± 2.23 vs. 58.88 ± 1.00) and on (61.71 ± 3.68 vs. 57.42 ± 1.43) sublaminas from control and 24 h dark-adaptation groups were compared, we found a small decrease of the brightness in both sublaminas, although not statistically significant (Fig. 6G).

Dark-adaptation differentially affects nNOS accumulation in balloon and round cells

Since we determined the existence of two distinct nNOS amacrine cell populations, and that nNOS gene expression is regulated by ambient light conditions, we examined whether dark-adaptation differentially regulates nNOS in these amacrine cells. When comparing nNOS labeling in control (Figs. 7A–D) vs. 24 h DA (Figs. 7E–H) groups, it is possible to see that dark-adaptation triggers a general reduction in the labeling of both populations. To specifically assess how the intensity labeling of the two populations changed in the dark, we performed computational cluster analysis. Once cells were classified as previously described, we observed that in the controls (Fig. 7I), data points were displaced upwards when compared to the dataset of the group kept in the dark (Fig. 7J). Thereafter, we evaluated the brightness parameter through the relation of balloon/round cells (Fig. 7K). Medians from several retinal slices were obtained for each
animal (n = 6) and means and EPM were calculated. Values for the control and 24 h DA groups, 1.48 ± 0.07 and 1.24 ± 0.07, respectively, indicated that round and balloon cells were differentially affected by dark-adaptation (P < 0.05).

Discussion

In physiological conditions, NO production in the nervous system depends on the expression and activity of the nNOS enzyme. Previous

Fig. 2. Neuronal nitric oxide synthase (nNOS) and specific retinal markers in transverse sections of the rat retina. In order to evaluate the presence of nNOS in specific cell populations in the inner nuclear layer (INL), we performed double-labeling experiments to simultaneously localize nNOS (green) and specific markers (red) in transverse sections of the retina counterstained with 4',6-diamidino-2-phenylindole (DAPI, blue). (A) Double-labeling of nNOS and calbindin (CB), a marker for horizontal cells. (B) Typically, pixel analysis revealed absence of overlapped signals. (C–E) In high magnification of selected areas, it was possible to observe that nNOS did not accumulate in CB-positive cells. (F–K) Similar analysis was performed for protein kinase C (PKC) labeling, a marker for rod bipolar cells. We did not detect accumulation of nNOS in PKC-positive cells. (L–U) Finally, double-labeling experiments were carried out using antiparvalbumin (PV) and -choline acetyltransferase (ChAT). In both cases, we were not able to detect colocalization of nNOS and these markers. Scale bar=25 μm. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

Fig. 3. Colocalization of neuronal nitric oxide synthase (nNOS) and calretinin (CR) in transverse sections of adult rat retina. (A) In order to confirm the presence of nNOS in specific amacrine cell populations, we performed double-labeling experiments to simultaneously localize nNOS (green) and calretinin (CR, red) in transverse sections of rat retinas counterstained with 4',6-diamidino-2-phenylindole (DAPI, blue). (B) Pixel analysis revealed overlap of green and red signals, in typical profiles of both round (white arrow) and balloon (white arrowhead) cells. We also observed red signals that did not overlap green signals, revealing that some CR-positive cells do not accumulate nNOS (red arrows). (C–E) In high magnification of selected areas, it was possible to observe a typical round cell which is also CR-positive. (F–H) Similarly, we were able to detect balloon cells which also accumulate CR. (I) Graph represents the percentage of round (red, 30.78 ± 2.95%) and balloon (blue, 38.96 ± 4.48%) cells that simultaneously accumulate CR. Bars represent standard errors of mean (n = 6). Scale bar = 25 μm. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)
Intricate molecular mechanisms have been found to participate in retinal adaptation to ambient light levels. Notably, the dopaminergic system has been implicated in this process (Jackson et al., 2014; Witkovsky, 2004). It was proposed that light responses of rod bipolar cells are enhanced by sustained chloride currents via GABA(C) receptor channels. This sensitizing GABAergic input would be controlled by dopamine D1 receptors, with GABA provided by horizontal cells (Herrmann et al., 2011). In addition to chemical synapses, it has been reported that changes in cell coupling provided by connexin (Cx) channels in the electrical synapses provide a mechanism whereby sensory circuits simultaneously adjust and enhance the dynamic range (Kihara et al., 2006a; Kinouchi and Copelli, 2006), a hypothesis not restricted to the visual processing (Christie et al., 2005). Indeed, it has been proposed that fine tuning of the sensory input, including the visual system, should take place in the initial processing layers, as indicated by mathematical/computational modeling (Publio et al., 2009), psychophysical findings (Kihara et al., 2006b) and cellular/molecular evidences (Kihara et al., 2009). This tuning would involve cell–cell coupling provided by electrical synapses, which are regulated by both dopamine and NO (Bloomfield and Volgyi, 2009). In fact, changes in homologous coupling between all amacrine cells triggered by light adaptation seem to be regulated by dopamine, while coupling between all amacrine and cone bipolar cells is mainly affected by NO (Bloomfield and Volgyi, 2004; Xia and Mills, 2004).

Finally, our results revealed that nNOS distribution in the retina is controlled by ambient light conditions. Considering that the presence of nNOS is essential for NO production, it was remarkable that a decrease in the NO levels correlates with downregulation of nNOS expression restores to steady-state levels after returning to the normal light/dark cycle.

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expression, especially in the synaptic contacts located in the inner plexiform layer. Moreover, we determined that accumulation of nNOS is differentially regulated in balloon and round cells. Our findings indicated that these neurons may play specific roles in the adaptation of the retina to the ambient light levels, since nNOS accumulation is differentially affected by dark-adaptation. This particular topic should be investigated in future studies.

Competing interests

The authors have declared that no competing interests exist.

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Author contributions

Conceived and designed experiments: LTW, GVH, ES, ERK, GC, AHK. Performed experiments: LTW, GVH, ES, ERK, CS, GC. Contributed reagents, materials, and analysis tools: SR, DEH, GC, AHK. Wrote the paper: LTW, DEH, GC, AHK.

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