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# Regional Brain Effects of Sodium Azide Treatment on Cytochrome Oxidase Activity: A Quantitative Histochemical Study

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The objective of the present study was to determine if regional variation in brain cytochrome oxidase activity was observed following systemic administration of sodium azide. An image analysis system calibrated with internal standards of known cytochrome oxidase activity was used to quantify cytochrome oxidase in histochemically stained brain sections. Rats receiving chronic infusion of sodium azide (400 µg/hr), which were sacrificed after two weeks, showed a substantial decrease in brain cytochrome oxidase activity over those infused with saline. All of the 22 regions sampled from telencephalic, diencephalic, and mesencephalic levels, showed a significant activity reduction which ranged between 26% and 37%. The regions that appeared significantly more vulnerable to the sodium azide effects were the mesencephalic reticular formation and the central amygdala, which displayed the largest decrease in activity. In addition, interregional correlations of activity showed a deeply modified pattern of correlative metabolic activity between hippocampal, amygdaloid and cortical areas after azide treatment. The regional effects found were consistent with azide-induced learning and memory dysfunctions.

**Key words:** cytochrome oxidase, quantitative histochemistry, sodium azide, correlations, learning, memory

## INTRODUCTION

Cytochrome oxidase (CO: E.C. 1.9.3.1), the terminal enzyme of the mitochondrial respiratory chain, is essential for aerobic respiration and oxidative phosphorylation

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(Wikström *et al.*, 1981). Seligman *et al.* (1968) developed a histochemical technique based on the oxidative polymerization of diaminobenzidine to a reaction product that chromatically labeled CO in heart, liver, and kidney. Wong-Riley (1979) modified this technique, applying it to the nervous system and later demonstrating that the optical density of histochemically stained sections is closely correlated with the amount of CO in the tissue (Hevner and Wong-Riley, 1989). Histochemistry has become the tool of choice for displaying visually the regional oxidative metabolic capacity of the nervous system. In 1991, Gonzalez-Lima and Garrosa introduced a method to quantify changes in regional cerebral CO activity within histochemically stained brain sections, utilizing internal standards of known CO activity coupled with computerized image analysis. A modification of this method has been applied to quantify changes in regional cerebral CO activity in rats, mice and gerbils (Gonzalez-Lima, 1992; Gonzalez-Lima and Cada, 1994; Gonzalez-Lima and Jones, 1994).

Histochemical analyses of CO activity measures across many species have shown that there is heterogeneity in the distribution of regional metabolic capacity (Wong-Riley, 1989 for review). In addition to its interregional heterogeneity, CO activity can be altered by manipulating neuronal activity. For example, CO activity in the auditory nuclei can be inhibited by blocking auditory input for a period of days (Wong-Riley *et al.*, 1978). A similar inhibition of CO activity has been demonstrated in the visual system of cats by a variety of techniques that block neural input from one eye (Wong-Riley and Riley, 1983). Nobrega *et al.* (1993) induced an increase in CO activity restricted to limbic areas of rat brains measured 28 days after a series of electroconvulsive treatments. Taken together, these results imply that CO activity is positively correlated with neuronal activity.

Recently, Bennett *et al.* (1992a,b) developed a rat model of persistent systemic CO inhibition. Continuous subcutaneous infusion of sodium azide in rats produced a chronic partial inhibition (35-39%) of CO activity in mitochondrial fractions of whole brain homogenates, without significantly affecting other enzymes of the respiratory chain (complexes I, II, and III). Azide treatment also impaired spatial learning and memory, and the expression of hippocampal long-term potentiation (LTP), a physiological model of long-lasting synaptic plasticity (Bennett *et al.*, 1992a,b; Bennett and Rose, 1992). Therefore, there is *a priori* evidence to assume that particular regions related to spatial memory functions will be more sensitive than others; for example the hippocampal formation, the amygdala and the frontal cortex have been implicated in numerous studies (Aggleton, 1992; Olton *et al.*, 1989).

Cytochrome oxidase has been shown to be more vulnerable to insult in Alzheimer's disease than are other enzymes of the respiratory chain (Parker *et al.*, 1990; 1994a,b; Kish *et al.*, 1992, Beal *et al.*, 1993, Mecocci *et al.*, 1994, Mutisya *et al.*, 1994). The clinical finding that a CO defect in Alzheimer's disease occurs in the periphery as well as in the brain suggests that a defect in metabolism in Alzheimer's disease might be widespread (Blass *et al.*, 1988; Blass, 1993). Yet the genetic studies on differences between CO subunits in different brain regions suggest that systemic inhibition of CO activity may still be associated with selective regional vulnerability within the brain (Chandrasekaran *et al.*, 1992).

The objectives of the present experiment were to quantify histochemically the effects of sodium azide treatment on CO activity in rat brain and to determine whether there is differential vulnerability among brain regions in response to this treatment. The goal was not to measure CO activity in all regions of the brain. Rather, it was to select a sample of 22 regions from telencephalic, diencephalic, and mesencephalic levels to test the hypothesis that regional effects of systemic azide are heterogeneous in the brain. The findings are discussed in the context of how preferential sodium azide inhibition of CO in some brain regions could contribute to the learning and memory deficits that are induced by this treatment. These results were reported previously in abstract form (Cada *et al.*, 1994).

## MATERIALS and METHODS

### Subjects

Twenty one adult male Sprague-Dawley rats were used for this experiment: Twelve rats for the preparation of the CO standards and nine rats for the CO histochemistry. Rats were housed for at least one week prior to surgery in AAALAC-approved facilities under standard laboratory conditions (12:12, light:dark cycle) and with food and water freely available.

### Surgery and treatment

The nine rats subsequently used for CO histochemistry were 4 months of age and weighed approximately 415 g at the time of surgery. Using methods described previously (Bennett *et al.*, 1992a; Bennett and Rose, 1992), each rat was anesthetized with secobarbital (40 mg/kg) and implanted subcutaneously with an Alzet 2ML4 osmotic minipump (Alza) containing either sodium azide solution (160 g/L) or the 0.9% saline vehicle. The Alzet 2ML4 minipump has a 2 ml reservoir and provides a constant infusion rate of 2.5 microliters/hr for 28 days; therefore, the dose of sodium azide delivered was 400 micrograms/hr. This treatment regimen has been shown to decrease CO activity by 35-39% in mitochondria extracted from rat forebrain homogenates (Bennett *et al.*, 1992b).

### Tissue processing

CO standards were made from the brains of 12 adult male rats. Each animal was killed by decapitation, the brain was rapidly removed, and then stored at 4°C in sodium phosphate buffer (pH 7.4) until all were collected. The brains were homogenized at 4°C and half of the total weight was inactivated by microwaving and subsequently brought to its fresh tissue weight by the addition of sodium phosphate buffer (pH 7.6). Standards consisting of increasing proportions of active and inactive tissue were mixed at 4°C, packed into 2 ml cryovials, briefly centrifuged to remove air bubbles, and frozen in isopentane at -40°C. In addition, active brain paste sectioned at varying thicknesses was included with the incubation medium of other experiments for subsequent comparison and generation of a single regression equation for all tissue in the present experiment.

After 14 days of azide or saline infusion, the nine operated subjects were sacrificed by decapitation. The brains were immediately removed and divided longitudinally before being

frozen in isopentane at -40°C. One hemi-brain from each subject was used for the CO histochemistry. Tissue was sectioned in the transverse plane into 40 μm sections and picked up on slides in a Frigocut 2800 cryostat at -20°C. Two sets of brain paste standards sectioned at 40 um were included with each incubation. Slides were processed for CO quantitative histochemistry using previously described procedures (Gonzalez-Lima and Garrosa, 1991; Gonzalez-Lima, 1992; Gonzalez-Lima and Cada, 1994, Gonzalez-Lima and Jones, 1994). To summarize, slides were treated in 10% sucrose phosphate buffer (0.1 M, pH 7.6) containing 0.5% glutaraldehyde for 5 min to facilitate adherence of the sections and standards to the slides. To remove blood cells, four changes of 10% phosphate buffer at 5 min each were used. Then slides were preincubated for 10 min in Adam's (1977) Tris buffer (0.05 M, pH 7.6) containing 275 mg/l cobalt chloride, 10% sucrose, and 0.5% dimethylsulfoxide (Silverman and Tootell, 1987). The slides were then rinsed in the phosphate buffer and incubated at 37°C for 60 min in 700 ml of an oxygen saturated reaction solution containing 350 mg diaminobenzidine tetrahydrochloride (Sigma), 52.5 mg cytochrome *c* (Sigma, Type III, horse heart), 35 g sucrose, 14 mg catalase (Sigma), and 1.75 ml dimethylsulfoxide (Fisher) in phosphate buffer. To stop the reaction and fix the tissue, a 30 min immersion in 10% sucrose phosphate buffer with 4% formalin (v/v) was used before dehydrating, clearing, and coverslipping with Permount.

### Spectrophotometric assessment of cytochrome oxidase activity

A method adapted from Wharton and Tzagoloff (1967) and Hevner *et al.* (1993) was used to spectrophotometrically measure the CO enzyme activity. A solution of 1.0% cytochrome *c* in 0.05 M potassium phosphate buffer (pH 7.0) was reduced with sodium ascorbate. The excess reducing agent was removed by dialysis (Spectrapor 3 tubing) against 0.05 M potassium phosphate buffer for 24 hours (3 changes) and was diluted to 0.07% cytochrome *c* in 0.05 M potassium phosphate buffer and chilled on ice. A 20% homogenate consisting of 1.00 g of tissue paste was suspended in 4 ml isolation buffer (0.32 M sucrose, 1 mM dipotassium ethylenediaminotetraacetic acid (EDTA), 10 mM Tris, pH 7.4) and chilled on ice. Hand-held glass/glass homogenizers were used to prepare the tissue/isolation buffer homogenate and subsequent assay mixture. The assay mixture was prepared by diluting an aliquot of the 20% homogenate with isolation buffer and 10% sodium deoxycholate to obtain final concentrations of 0.25% tissue and 0.5% sodium deoxycholate. The assay mixture was then equilibrated at room temperature (22°C) for 5 min with frequent gentle mixing and then placed on ice. For the assay, 10 μl of the assay mixture and 1 ml 0.07% cytochrome *c* solution were reacted together at 37°C. The change in absorbance at 550 nm was taken for one min beginning immediately after placement of the cuvette in the spectrophotometer. Each homogenate was reacted three times. Additionally, three control trials were run by adding one drop (0.015 ml) of saturated potassium ferricyanide to the reaction mixture (1.0 ml cytochrome *c*/10 μl assay mixture) and measuring the resulting absorbance. All control trials resulted in the complete inhibition of the reaction by the potassium ferricyanide. Activity units were defined at pH 7 and 37°C as in our original quantitative method (Gonzalez-Lima and Garrosa, 1991) where 1 unit oxidizes 1 μmol of reduced cytochrome *c* per min (μmol/min/g tissue wet weight).

Homogenate samples were frozen and stored at -20°C until their protein content was measured by the method of Lowry *et al.* (1951). Activity units can also be expressed in terms of protein content by multiplying the reported values by 10 since our brain standards contained an average of 10% protein (Gonzalez-Lima and Cada, 1994).

## Image analysis and statistics

An image-processing system and JAVA software (Jandel Scientific) were used as previously described (Gonzalez-Lima *et al.*, 1993). All standards and structures were measured in optical density (O.D.) units. The more general image analysis encompassed all the integrated activity of the gray matter located at telencephalic, diencephalic and mesencephalic levels, by sampling three consecutive sections at 40 µm intervals per level per brain. The levels sampled were centered at approximately 3.7, -2.3 and -6.3 mm from Bregma respectively (Paxinos and Watson, 1986) as illustrated in Figure 1. The regional image analysis encompassed each region listed in Table II, including its cell bodies and neuropil, with four adjacent readings taken of each structure per section (or standard) with three sections per animal (a total of 12 readings per structure per animal or standard). The part of each region sampled was its central part, to avoid any inaccuracies that may result from sampling near the edges next to the interregion boundaries. The central part sampled corresponded to about 50% of the area of each region quantitated, and it was held constant in size for the same region in different sections and brains.

Four types of statistical analyses were done. First, a comparison of mean O.D. was done between the tissue paste standards included with each incubation medium (Table I). Second, between-group (absolute) comparisons of mean activities in the experimental and control groups were done initially at three rostrocaudal levels and then for each one of the 22 sampled regions (Table II). Mean ± standard error (S.E.) values were obtained by averaging CO activity units from five control and four treated rats. ANOVA followed by subsequent comparisons with corrected t-tests were used to evaluate univariate differences between groups for each of the brain regions.

Third, within-group (relative) comparisons of interregional differences in the degree of sodium azide inhibition in the experimental group were made between the telencephalic, diencephalic and mesencephalic levels, and among the sampled regions to determine if some region was inhibited significantly more than another. For this relative comparison, percent differences in the degree of azide inhibition in each experimental subject were calculated as 100 x (Experimental - Control) / Control. The mean percent differences were compared using nonparametric tests (Mann-Whitney U - Wilcoxon Rank Sum W Test).

Fourth, interregional correlations of activity were calculated for each group to determine the patterns of functional coupling among the regions in control and experimental groups (Tables III and IV). For interregional correlations, CO activity values were normalized by dividing the measured activity of each structure by the average CO activity value for all structures measured for each animal (whole-brain ratio). This was done to reduce variation in the intensity of the CO staining not resulting from the experimental manipulation. We compensated for the small sample size by taking advantage of the fact that four measures of CO activity per section in three brain sections were obtained for each structure (McIntosh

and Gonzalez-Lima, 1991, 1992, 1993, 1994). The normalized values were then averaged over each brain section so that three normalized values for each subject for each area were used in calculating partial correlation coefficients (increasing the "effective" n by a multiple of three). The extra source of variance resulting from multiple measures for each subject used in the calculations was removed using a partial correlation procedure (Pedhazur, 1982). This removed between-section variance and the resultant observations were mathematically independent for the purpose of calculating interregional correlations (McIntosh and Gonzalez-Lima, 1994; Gonzalez-Lima and McIntosh, 1994).

Correlation matrices were obtained by generating partial correlation coefficients for normalized CO activity values for the sodium-azide group and separately for the control group. A partial correlation coefficient r was considered "reliable" using similar criteria to that commonly used in the analysis of cerebral glucose metabolism correlations (Horwitz, 1989; Horwitz *et al.*, 1987, 1992). The first condition was that any coefficient had to correspond to a statistical significance level of $p < 0.05$. The coefficient r had to be greater than 0.5. Coefficients that met the first two criteria were then submitted to the "jacknife" procedure where subjects are sequentially eliminated and the matrices calculated using the remaining subjects. If a coefficient remained significant for all jacknifed correlations it was then deemed reliable.

Correlation coefficients of metabolic activity among brain regions represent the degree to which the variance of CO activity values in one region are related to the variance in another. When two regions increase in CO activity in a proportional way, they are positively correlated. On the contrary, when one region increases while the other decreases, they are negatively correlated. Thus positive and negative correlation coefficients measure the direction of the covariance relationship between pairs of regional activity values. CO activity is time integrated and is the cumulative effect of tissue oxidative metabolic demand (Wong-Riley, 1989). Therefore, it is inappropriate to interpret the sign (positive or negative) of a correlation coefficient as representing excitatory or inhibitory postsynaptic influences in an electrophysiological sense; or to expect a morphological feature such as the number of direct synaptic connections between regions to represent the value of a correlation coefficient between regions in brains under different metabolic conditions. If functional interactions between brain regions underlie brain operations such as learning and memory (Gonzalez-Lima and McIntosh, 1994), then correlation coefficients may be interpreted as an indication of functional influences or how much the variance in the metabolic capacity of a brain region is related to the variance in another. Thus the emphasis is on whether the interregional correlations change between groups.

Reliable between-group differences in interregional correlations represent changes in the functional interaction between regions and can be of two kinds. One is represented as a difference in the sign of the correlation coefficient without a significant difference in absolute magnitude. A difference in sign of the correlative relationship between two regions may be assumed to reflect a reversal in the interactions (from positive to negative or vice versa) between two regions. This may be interpreted as a qualitative difference in the nature of the interaction between two regions (McIntosh and Gonzalez-Lima, 1994). If the difference between coefficients is in absolute magnitude, but not sign, this is interpreted as

a change in the strength of their correlative activities. This may indicate a quantitative shift (increase or decrease) in the interaction between two regions. Although an individual coefficient provides an indication of how a particular regional CO activity is correlated with another, the overall pattern of interregional correlations can give a clearer picture of how brain regions are interacting in control and azide-treated rats.

## RESULTS

### Methodological findings

Internal calibration curves were generated using histochemically measured O.D. of the brain paste standards and the spectrophotometrically determined CO activity units of the brain paste thickness standards. The three sets of standards, though reacted in separate incubation media, showed no significant differences (t-tests, $p>0.21$) between their optical densities (Table I). The interassay coefficient of variation (standard deviation X 100/ mean) was 0.82% for the three staining batches. For each one of the paste standards, its average value showed a standard error (S.E.) below 0.5% of the mean measured across the batches (Table I). A regression equation generated using the activity of the thickness standards from separate incubations and the O.D. of the brain paste standards resulted in a linear function: $r=0.978$, $y=6377.24x - 29.89$. Using this regression equation, the O.D. obtained from each structure was converted to activity units (μmol/min/g tissue wet weight). The total CO activity was 170.43±0.1 units (mean ± S.E.) for the control brains, and 121.06±0.1 units for the azide-treated brains. This corresponded to an overall -29% difference between the groups. The maximal activity among the regions sampled was 228 units in the occipital cortex (Area 18) of the control brains. The maximal activity in the azide-treated brains was below 180 units, found in the basolateral amygdaloid nucleus (BlA).

For the image analysis of regions, the central part of each region was located and measured by two experimenters. The intra- and inter-rater reliability for this sampling technique was assessed by comparing 15 measures made twice from the same 15 loci, separated by a three-month interval. For the intra-rater reliability, the same experimenter made the measures, and this resulted in a mean coefficient of variation of 5.41% and a correlation of $r=0.93$. For the inter-rater reliability, a second experimenter made the second measures, and this resulted in a coefficient of variation of 5.81% and a correlation of $r=0.87$ between measures of the same regions.

| Standards (% Active Paste) | Staining Batches (Optical Densities) Batch 1 | Batch 2 | Batch 3 | Average |
|---|---|---|---|---|
| 0 | 0.0057±0.00003 | 0.0059±0.00002 | 0.0060±0.00004 | 0.0059±0.00001 |
| 15 | 0.0123±0.00007 | 0.0113±0.00006 | 0.0123±0.00021 | 0.0119±0.00005 |
| 50 | 0.0228±0.00014 | 0.0232±0.00004 | 0.0230±0.00007 | 0.0230±0.00003 |
| 100 | 0.0339±0.00011 | 0.0334±0.00014 | 0.0334±0.00014 | 0.0336±0.00004 |
| Average | 0.0187±0.00027 | 0.0184±0.00027 | 0.0187±0.00027 | 0.0186±0.00009 |

**Table I. Interassay variability of brain paste standards stained in separate batches.** Values are mean ± standard error of O.D. from tissue stained with the described CO histochemical procedure. In each bach, a given % standard was measured 12 times in consecutive sections.

## Regional effects of sodium azide on brain CO activity

The major finding was that all brain regions examined showed a reliable decrement in CO activity after sodium azide treatment (Figure 1). The regional evaluation of CO activity was performed in two steps, from a more general evaluation of integrated activity at three rostrocaudal levels to a more specific evaluation of a sample of 22 separate regions. In both evaluations, the CO activities measured in the sodium azide treated rats were significantly lower than those found in the control rats ($p<0.01$, ANOVA followed by subsequent comparisons with corrected t-tests).

The more general analysis showed that in addition to the between-group reduction in CO activity at each level, there was indication of the mesencephalic levels being more strongly affected than the other levels. The mean percent reductions (± S.E.) for each level were: 27.31±0.77 for the telencephalic, 29.27±1.18 for the diencephalic, and 35.70±0.33 for the mesencephalic levels.

In the sodium azide treated rats, the percentages of CO activity reduction in the sections (n=12/level) at the mesencephalic levels were significantly greater than those at the telencephalic levels when compared using nonparametric tests (Mann-Whitney U - Wilcoxon Rank Sum W tests: $U=10$, $W=88$, $p<0.01$). However, no significant differences in percent reduction were found between telencephalic and diencephalic levels ($p>0.9$).

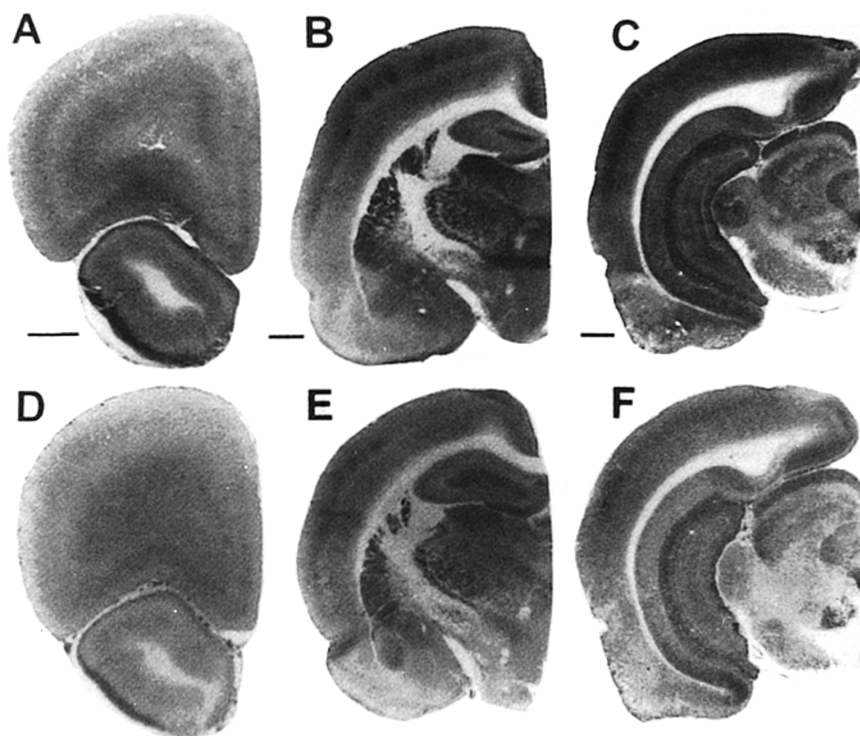


**Figure 1. Inhibitory effect of sodium azide chronic treatment on cytochrome oxidase histochemical staining.** Control brain sections, A, B and C can be contrasted with comparable antero-posterior sections D, E and F from an azide-treated rat. The darker the staining the more cytochrome oxidase activity. The global inhibitory effect of azide treatment was readily seen by visual inspection of stained sections as well as in photographs developed with the same parameters, as shown here. Densitometric image analysis was necessary to determine regional variations in staining reduction produced by the treatment at different levels. Sections at the telencephalic level (A,D) were centered at approximately 3.7 mm from Bregma, sections at the diencephalic levels (B,E) were at about -2.3 mm, and sections at the mesencephalic level (C,F) at -6.3 mm. Sections are of the left side and are oriented such that dorsal is at the top and lateral is to the left, as they correspond to 40 μm transverse sections incubated for 1 hour at 37°C as described in the text.

The more specific analysis involved measuring CO activities in the 22 regions listed in Table II. This evaluation served to confirm the inhibitory effect of azide treatment on a region-by-region basis and allowed the identification of the regions with the highest decrement in activity, corresponding to the deep mesencephalic reticular area (-37.11%) and the central amygdala (-37.02%). These regions showed decrements which were significantly greater than the other decrements found in the telencephalic ($U=28$, $W=106$, $p < 0.05$) and diencephalic ($U=28$, $W=106$, $p<0.05$) levels, but not significantly greater than mesencephalic levels ($U=53$, $W=131$, $p>0.29$).

| Structure | Control | Experimental | Difference |
|---|---|---|---|
| | Mean ± S.E. | Mean ± S.E. | % |
| Deep Mesencephalic Nucleus (DpMe) | 143.75 ± 1.96 | 90.41 ± 1.07 | -37.11 |
| Central Amygdaloid Nucleus (CeA) | 182.12 ± 0.85 | 114.70 ± 2.57 | -37.02 |
| Superior Colliculus -deep (SCDp) | 164.59 ± 1.46 | 106.87 ± 0.87 | -35.07 |
| Central Gray (CG) | 162.79 ± 1.38 | 106.89 ± 1.12 | -34.34 |
| Ventral Tegmental Area (VTA) | 109.28 ± 1.01 | 75.68 ± 1.22 | -30.75 |
| Subiculum (Sub) | 165.77 ± 1.90 | 115.10 ± 1.13 | -30.57 |
| Medial Amygdaloid Nucleus (MeA) | 183.83 ± 0.58 | 129.27 ± 2.36 | -29.68 |
| CA1 of Hippocampus (CA1) | 182.22 ±1.73 | 128.29 ± 1.32 | -29.60 |
| Basolateral Amygdaloid Nucleus (BlA) | 198.69 ± 1.09 | 140.40 ± 2.38 | -29.34 |
| CA2 of Hippocampus (CA2) | 176.28 ± 1.88 | 125.43 ± 1.15 | -28.85 |
| Auditory Cortex (Aud) | 157.41 ± 2.71 | 112.13 ± 1.62 | -28.77 |
| CA3 of Hippocampus (CA3) | 171.58 ± 1.81 | 122.65 ± 1.24 | -28.52 |
| Area 18 (A18) | 180.11 ± 1.93 | 128.92 ± 1.07 | -28.42 |
| Area 18a (A18a) | 178.81 ± 1.84 | 130.30 ± 1.54 | -27.13 |
| Retrosplenial Cortex (Rs) | 179.43 ± 1.96 | 131.09 ± 0.97 | -26.94 |
| Medial Frontal Cortex (MFC) | 178.83 ± 0.90 | 136.64 ± 0.95 | -26.90 |
| Sulcal Frontal Cortex (SFC) | 196.32 ± 1.20 | 143.58 ± 0.79 | -26.86 |
| Area 17 (A17) | 180.86 ± 1.81 | 132.35 ± 1.29 | -26.82 |
| Red Nucleus (Red) | 166.74 ± 0.90 | 122.46 ± 1.18 | -26.56 |
| Lateral Frontal Cortex (LFC) | 187.71 ± 1.26 | 138.36 ± 0.53 | -26.29 |
| Superior Colliculus -superior (SCSu) | 172.39 ± 1.56 | 127.55 ± 1.24 | -26.01 |
| Entorhinal Cortex (Ent) | 147.32 ± 1.25 | 109.54 ± 0.96 | -25.64 |

**Table II. Regional activity of cytochrome oxidase in the brains of azide-treated and control rats.** Mean ± standard error (S.E.) units in μmol/min/g tissue wet weight were obtained by averaging measurements from five control and four treated rats. In each rat, a given structure was measured in three consecutive sections. Percent differences between groups were calculated as 100 x (Experimental-Control)/Control. ANOVA followed by a subsequent comparison with corrected t-tests were used to assess differences statistically. Each structure's mean was significantly lower in the experimental group at $p<0.01$.

The other activity decrements at the regional telencephalic and diencephalic levels showed statistically comparable means. Univariate analysis of mean activity in these regions indicated only that activity within each region differed between the two groups. However, regions that show similar differences in mean activity between groups may have quite different covariance relationships in response to the treatment (McIntosh and Gonzalez-Lima, 1993; 1994). Therefore, analysis of regional means gives an incomplete account of how interactions among brain regions differed between groups. To further evaluate interregional changes, a correlational analysis was performed.

## Interregional correlations between CO activity values

The matrix of partial correlation coefficients for the saline control group is shown in Table III and for the sodium azide, group in Table IV. The major finding was that most of the regional pairs that were significantly correlated in one group were different from the ones in the other group, although the total number of significant correlations between the sodium azide and saline control conditions was not significantly different (20 vs. 19). The pattern of significant correlations in the control group consisted of 9 positive and 10 negative correlations, whereas in the azide group there were 12 positive and 8 negative correlations. In terms of similarities, only 3 significant correlations were the same among the groups, corresponding to positive correlations between the three amygdaloid nuclei (MeA-CeA, BlA-CeA, and BlA-MeA).

In the hippocampal formation, a differential pattern was seen between groups. In control brains, CA1 and CA2 sectors showed positive intercorrelations and CA1 was positively correlated with SCDp. In azide-treated brains these correlations were not observed, and instead the Sub and CA3 showed new patterns of 3 positive and 3 negative correlations with other regions (DpMe, SCDp, SCSu, and LFC correlated with Sub; MeA and BlA correlated with CA3, Table IV). These differences provide evidence for a modified pattern of correlative activity between intrinsic hippocampal regions and their relationship to midbrain regions and amygdaloid nuclei.

Sensory cortical areas (Aud, 18, 18a and 17) showed six significant correlations, always negative, with other regions in control brains. In azide-treated brains, these correlations disappeared or became positive. Again, this indicated a quite different network pattern of correlative activity of sensory cortex after azide treatment. In addition, frontal cortical areas (MFC, SFC and LFC) showed different between-group correlations. The MFC and LFC correlations, always negative, found in control brains disappeared in azide-treated brains, and instead SFC and LFC showed other negative correlations with hippocampal and midbrain regions. Together, these differences in interregional correlations revealed a deeply modified pattern of correlative metabolic activity between hippocampal, amygdaloid, midbrain and cortical areas after azide treatment.

| | DpMe | CeA | SCDp | CG | VTA | Sub | MeA | CA1 | BlA | CA2 | Aud | CA3 | 18 | 18a | Rs | MFC | SFC | 17 | Red | LFC | SCSu |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| DpMe | 1 | | | | | | | | | | | | | | | | | | | | |
| CeA | -0.28 | 1 | | | | | | | | | | | | | | | | | | | |
| SCDp | 0.13 | -0.45 | 1 | | | | | | | | | | | | | | | | | | |
| CG | -0.11 | -0.01 | 0.59 | 1 | | | | | | | | | | | | | | | | | |
| VTA | 0.51 | 0.34 | -0.01 | 0.05 | 1 | | | | | | | | | | | | | | | | |
| Sub | 0.28 | -0.51 | 0.39 | 0.46 | 0.12 | 1 | | | | | | | | | | | | | | | |
| MeA | 0.29 | 0.85* | -0.06 | -0.11 | 0.33 | -0.59 | 1 | | | | | | | | | | | | | | |
| CA1 | 0.14 | -0.57 | 0.71* | 0.45 | 0.08 | 0.71 | -0.39 | 1 | | | | | | | | | | | | | |
| BlA | -0.14 | 0.90* | -0.48 | -0.09 | 0.46 | -0.52 | 0.77* | -0.55 | 1 | | | | | | | | | | | | |
| CA2 | 0.32 | -0.46 | 0.49 | 0.37 | 0.18 | 0.48 | -0.39 | 0.67* | -0.33 | 1 | | | | | | | | | | | |
| Aud | 0.02 | -0.64 | 0.02 | 0.09 | -0.63 | 0.36 | -0.78* | 0.22 | -0.79* | 0.24 | 1 | | | | | | | | | | |
| CA3 | 0.21 | -0.42 | 0.57 | 0.17 | 0.31 | 0.09 | -0.05 | 0.58 | -0.30 | 0.39 | -0.15 | 1 | | | | | | | | | |
| 18 | 0.43 | -0.59 | 0.14 | 0.14 | -0.19 | 0.45 | -0.74* | 0.04 | -0.49 | 0.26 | 0.48 | -0.21 | 1 | | | | | | | | |
| 18a | -0.02 | -0.85* | 0.55 | -0.01 | -0.45 | 0.29 | -0.61 | 0.39 | -0.80* | 0.27 | 0.52 | 0.31 | 0.56 | 1 | | | | | | | |
| Rs | 0.58 | -0.68 | 0.48 | 0.09 | 0.13 | 0.37 | -0.5 | 0.31 | -0.61 | 0.19 | 0.19 | 0.49 | 0.57 | 0.56 | 1 | | | | | | |
| MFC | -0.36 | 0.47 | -0.37 | -0.36 | 0.21 | -0.87* | 0.55 | -0.25 | 0.56 | -0.29 | -0.51 | 0.11 | -0.61 | -0.37 | -0.41 | 1 | | | | | |
| SFC | -0.45 | 0.53 | -0.53 | -0.17 | 0.19 | -0.46 | 0.41 | -0.45 | 0.62 | -0.14 | -0.47 | -0.05 | -0.59 | -0.56 | -0.65 | 0.72 | 1 | | | | |
| 17 | -0.32 | -0.38 | 0.17 | 0.19 | -0.48 | 0.59 | -0.46 | 0.25 | -0.49 | 0.06 | 0.57 | -0.37 | 0.44 | 0.54 | -0.01 | -0.70* | -0.45 | 1 | | | |
| Red | -0.46 | 0.86* | 0.58 | -0.06 | 0.04 | -0.39 | 0.79* | -0.46 | 0.76* | -0.45 | 0.79 | -0.51 | -0.51 | -0.62 | -0.79 | 0.31 | 0.46 | -0.06 | 1 | | |
| LFC | -0.29 | 0.57 | -0.86* | 0.51 | -0.16 | -0.64 | 0.32 | -0.71* | 0.51 | -0.58 | -0.09 | -0.56 | -0.39 | -0.51 | -0.61 | 0.54 | 0.47 | -0.21 | 0.45 | 1 | |
| SCSu | 0.11 | -0.56 | 0.61 | 0.23 | -0.11 | 0.57 | -0.41 | 0.46 | -0.63 | 0.01 | 0.25 | 0.18 | 0.51 | -0.70* | 0.61 | -0.67 | -0.62 | 0.57 | -0.11 | -0.58 | 1 |
| Ent | -0.24 | -0.51 | 0.68* | 0.11 | -0.21 | 0.01 | 0.08 | 0.45 | -0.49 | 0.41 | -0.02 | 0.64 | -0.06 | 0.66 | 0.35 | 0.11 | -0.22 | 0.09 | -0.44 | -0.54 | 0.26 |

**Table III. Partial correlation matrices of cytochrome oxidase activity from the control rats.** Abbreviations are as in Table II. Effective sample size for correlations was increased as multiple samples were taken for each structure for each subject (see text). Effective n=15. *Indicates significant difference ($p<0.05$) from zero.

| | DpMe | CeA | SCDp | CG | VTA | Sub | MeA | CA1 | BlA | CA2 | Aud | CA3 | 18 | 18a | Rs | MFC | SFC | 17 | Red | LFC | SCSu |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| DpMe | 1 | | | | | | | | | | | | | | | | | | | | |
| CeA | 0.03 | 1 | | | | | | | | | | | | | | | | | | | |
| SCDp | 0.56 | -0.19 | 1 | | | | | | | | | | | | | | | | | | |
| CG | 0.53 | -0.69 | 0.51 | 1 | | | | | | | | | | | | | | | | | |
| VTA | 0.21 | -0.28 | 0.16 | 0.13 | 1 | | | | | | | | | | | | | | | | |
| Sub | 0.75* | -0.45 | 0.77* | 0.73 | 0.19 | 1 | | | | | | | | | | | | | | | |
| MeA | -0.06 | 0.94* | -0.32 | -0.68 | -0.08 | -0.59 | 1 | | | | | | | | | | | | | | |
| CA1 | 0.59 | -0.27 | 0.53 | 0.46 | 0.45 | 0.75 | -0.31 | 1 | | | | | | | | | | | | | |
| BlA | -0.04 | 0.96* | -0.31 | -0.78 | -0.12 | -0.47 | 0.92* | -0.39 | 1 | | | | | | | | | | | | |
| CA2 | 0.36 | -0.69 | 0.45 | 0.76 | 0.03 | 0.83 | -0.82 | 0.59 | -0.67 | 1 | | | | | | | | | | | |
| Aud | 0.24 | -0.46 | 0.29 | 0.57 | 0.13 | 0.63 | -0.49 | 0.72 | -0.39 | 0.79 | 1 | | | | | | | | | | |
| CA3 | -0.09 | -0.69 | 0.28 | 0.46 | -0.29 | 0.39 | -0.84* | -0.07 | -0.74* | 0.64 | 0.14 | 1 | | | | | | | | | |
| 18 | 0.35 | -0.57 | 0.14 | 0.46 | 0.76* | 0.48 | -0.45 | 0.56 | -0.44 | 0.47 | 0.48 | -0.04 | 1 | | | | | | | | |
| 18a | 0.06 | -0.74 | 0.38 | 0.74 | 0.12 | 0.53 | -0.71 | 0.59 | -0.74 | 0.76 | 0.80* | 0.42 | 0.53 | 1 | | | | | | | |
| Rs | -0.13 | -0.83 | -0.05 | 0.45 | 0.31 | 0.24 | -0.76 | 0.15 | -0.73 | 0.53 | 0.19 | 0.69 | 0.38 | 0.37 | 1 | | | | | | |
| MFC | -0.01 | 0.16 | 0.28 | -0.28 | -0.18 | 0.02 | 0.01 | -0.56 | 0.11 | -0.18 | -0.51 | 0.35 | -0.54 | -0.56 | 0.09 | 1 | | | | | |
| SFC | -0.54 | 0.54 | -0.55 | -0.71 | 0.19 | -0.81 | 0.52 | -0.91* | 0.49 | -0.75 | -0.47 | -0.13 | -0.83* | -0.76 | -0.27 | 0.47 | 1 | | | | |
| 17 | -0.03 | -0.82 | 0.17 | 0.57 | 0.65 | 0.32 | -0.65 | 0.45 | -0.75 | 0.49 | 0.57 | 0.32 | 0.78* | 0.69 | 0.69 | -0.38 | -0.68 | 1 | | | |
| Red | -0.24 | 0.56 | 0.17 | -0.61 | -0.22 | -0.31 | 0.44 | -0.45 | 0.48 | -0.51 | -0.62 | -0.02 | -0.63 | -0.62 | -0.34 | 0.73* | 0.55 | -0.47 | 1 | | |
| LFC | -0.76 | 0.34 | -0.46 | -0.78* | -0.04 | -0.82* | 0.42 | -0.68 | 0.39 | -0.71 | -0.09 | -0.16 | -0.41 | -0.45 | -0.12 | 0.21 | 0.68 | -0.21 | 0.54 | 1 | |
| SCSu | 0.64 | -0.51 | 0.85* | 0.72 | 0.08 | 0.87* | -0.64 | 0.53 | -0.59 | 0.64 | 0.33 | 0.52 | 0.28 | 0.48 | 0.22 | 0.23 | -0.58 | 0.26 | -0.16 | -0.62 | 1 |
| Ent | 0.08 | -0.59 | -0.14 | 0.51 | 0.23 | 0.39 | -0.54 | 0.47 | -0.46 | 0.71 | 0.84* | 0.17 | 0.66 | 0.65 | 0.47 | -0.61 | -0.65 | 0.59 | -0.78* | -0.56 | 0.05 |

**Table IV. Partial correlation matrices of cytochrome oxidase activity from the azide-treated rats.** Abbreviations are as in Table II. Effective sample size correlation was increased as multiple samples were taken for each structure for each subject (see text). Effective n=12. *Indicates significant difference ($p<0.05$) from zero.

## DISCUSSION

### Methodological considerations

The use of internal standards of known CO activity in combination with quantitative image analysis of histochemical sections formed the basis for the applied quantitative approach (Gonzalez-Lima and Garrosa, 1991). Both of these tools are commonly used in 2-deoxyglucose autoradiography (Gonzalez-Lima, 1992, Gonzalez-Lima *et al.*, 1993) and other metabolic mapping techniques (Biegon and Wolff, 1986, Nobrega, 1992, Nobrega *et al.* 1993). Histochemical techniques such as the one used here contain some variability between tissue processed in different incubation reactions. However, if tissue processing procedures are strictly reproduced and complete sets of standards of known CO activity are included with each incubation medium -- as was done in the present study -- the problem of interassay variability is largely resolved. This indicated that the percent differences found between the various brain regions in response to azide treatment (Table II) could not be accounted for simply by variability between assays or paste standards (Table I). Obviously, variations in the proportion of "gray" and "white" matter between various regions in normal brains contributed to their differences in CO activity. However, the important point is not that the tissue level of CO activity is different in various brain regions (Wong-Riley, 1989, for review); but rather it is that the between-group percent decreases produced by azide treatment are significantly larger in some regions.

The total average for CO activity units in the control brains (170.43±0.1) was very similar to the total content of CO activity in the whole rat brain (158±5) reported by Hevner *et al.* (1993). Our somewhat higher value may be attributed to the fact that it represents a mean from a sample of 22 regions rather than a whole brain homogenate that may contain more white matter (with lower activity). In addition, our CO units were defined at pH 7 and 37°C, as in our original method (Gonzalez-Lima and Garrosa, 1991), as opposed to pH 6 and 30°C as done by Hevner *et al.* (1993). The agreement between these control brain CO values suggests that both assays were optimal in unmasking enzyme activity to maximal or near maximal levels (Hevner *et al.*, 1993). The definition of CO activity units using optimal conditions of measurement may be preferable for studies evaluating absolute CO activity, rather than simpler routine assays (Hess and Pope, 1953) done at room temperature in which CO units can be defined reliably but at below maximal levels (Gonzalez-Lima and Cada, 1994).

### Differences in the vulnerability of brain regions to inhibition of CO activity

There were three major findings in this study. First, there was a general decrement in CO activity following sodium azide treatment. This decrement ranged from 27% to 35% in the integrated activities measured at telencephalic, diencephalic and mesencephalic levels, and between 25% and 37% in the individual regions analyzed. This decrement measured histochemically is consistent with a previous report of 35-39% decrease in biochemically assayed mitochondrial CO activity in brain homogenates from rats given the same azide treatment (Bennett *et al.* 1992b). Second, midbrain reticular formation and central amygdala appeared more vulnerable than other regions to the sodium azide effects. Third, differences

in regional vulnerability were manifested as different patterns of interregional activity correlations found in control and treated brains.

The central nervous system, in particular, is functionally vulnerable to sodium azide treatment because of its disproportionate energy requirement, thus making it strongly dependent on oxidative phosphorylation (Wong-Riley, 1989). Impairments of aerobic respiration have the potential for limiting the activity of the brain (Wikström et al., 1981). Decreases of 25-37% in CO activity may be of potential functional significance for brain functions affected by aging such as learning and memory (Bennett *et al.*, 1992 a,b). For example, in a comparison of CO activity in mitochondria isolated from brains of 4-month-old and 30-month-old rats, a significant age-related decrease of 25% in CO activity was observed in the parietotemporal cortex (Curti et al., 1990). A CO activity decrease of 25% or more may be associated with a reduced capacity for ATP production, as suggested by a parallel regulation of CO and $Na^+$, $K^+$-ATPase activities in brain (Hevner *et al.*, 1992).

Systemic infusion of sodium azide produced heterogeneity both in the degree of CO inhibition and in the correlations of CO activity among brain regions. Whether all brain regions presumably had equal access to this highly diffusible compound is unknown.

Hence a possible basis for the regional effects of azide on CO activity may be differences in access of azide to different regions, but there is presently no experimental support for this mechanism. Another possible explanation for the selective vulnerability of some brain regions may be related to the differential expression of CO genes in different brain regions. Chandrasekaran *et al.* (1992) have provided evidence that this is the case in monkey brain. For example, cDNA clones for the three CO subunits encoded by mitochondrial DNA showed higher levels of mRNA in frontal pole, dorsal lateral frontal cortex, and hippocampus than in the primary visual or somatosensory cortices, in agreement with heterogeneous CO histochemistry in these regions. Chandrasekaran *et al.* (1992) concluded that such differences may be related to differences in the distribution of neuropil versus cell bodies in the brain regions investigated; and they further suggested that these genetically-mediated regional differences may be relevant to selective regional vulnerability in Alzheimer's disease. Indeed, a subsequent study Chandrasekaran *et al.* (1994) investigated the expression of CO-related genes in the temporal cortex and motor cortex of Alzheimer's brains, and found significantly more decreased mitochondrial RNA levels in the temporal region as compared to the motor region or the same regions in healthy age-matched controls.

However, to our knowledge, no studies on CO activity in Alzheimer's brains have been done in the mesencephalic reticular formation or the central amygdala where CO inhibition was greatest in the present study. The fact that histopathological plaques and tangles are only present in humans, and they may or may not be abundant in these regions, is not a sufficient argument to discount the potential role of mitochondrial pathophysiological events in these regions in relation to some behavioral deficits of Alzheimer's patients (Mecocci *et al.*, 1994). Therefore, the studies of Chandrasekaran *et al.* (1992, 1994) provide evidence to suggest that the selective vulnerability of CO inhibition may be linked to genetically-inherent capabilities of some regions. This evidence may be relevant for the observed regional heterogeneity in CO inhibition after azide treatment.

The deep mesencephalic reticular formation and the central amygdaloid nucleus exhibited the largest overall decreases in activity in response to the treatment. The ascending reticular input from the mesencephalic reticular formation represents an important pathway in the reticular activating system linked to behavioral arousal (Gonzalez-Lima and Scheich, 1985) and memory consolidation (Bloch, 1976); and the central amygdala has a well-established role in memory modulation and behavioral dysfunction (Aggleton, 1992).. The significant positive correlations between hippocampal sectors and other regions which existed in the control brains disappeared after sodium azide treatment, a finding that further supports the interpretation that the hippocampal formation as well as the deep mesencephalic reticular formation and the central amygdala are disproportionately affected by sodium azide. This treatment has been shown to produce learning and memory deficits on appetitively- and aversively-motivated tasks that are highly impaired by hippocampal dysfunction (Bennett *et al.*, 1992a,b; Bennett and Rose, 1992). Indeed, this azide treatment impairs the functional organization of long-term potentiation in the hippocampal formation (Bennett *et al.*, 1992a).

The patterns of activity correlations in control brains, but not sodium azide-treated brains, consisted of significant limbic (CA1, CA2, central amygdala) and frontal cortex correlations (lateral and medial frontal cortices). Sodium azide treatment uncoupled these significant correlations in limbic and frontal cortex structures seen in control brains, without significantly altering the number of interregional correlations (azide=20, control=19). Horwitz *et al.* (1987) reported a similar change in the patterns of interregional cerebral glucose metabolic correlations in Alzheimer's disease patients, but no significant difference in the total number of significant correlations, as compared to controls. These investigators reported that the correlation matrix for the Alzheimer's disease group had fewer significant correlations between regions in the frontal lobe and other regions than did the matrix for the control group.

Horwitz (1991) has explained in detail the significance of establishing the presence or absence of a correlation between two regions, that may be "coupled" (by direct anatomical connections) or indirectly "associated"; and how correlations between regional metabolic rates may be interpreted in terms of functional interactions in the brain. Briefly, metabolic data from animals and humans and computer-simulated data demonstrated that the correlation coefficient between normalized metabolic rates is proportional to the strength of the functional coupling constant, and that correlational analysis gives information on regional involvement in neural systems not evident in the pattern of absolute metabolic values (Horwitz, 1991). Indeed, validation for correlational analysis could be obtained with computer models of simulated metabolic data because the underlying patterns of functional couplings in the models were known (Horwitz, 1991). Another consideration refers to the actual value of a correlation coefficient. This value depends on several factors, including a limited sample size. That is, in our correlational analysis using 12 or 15 measures, the sample correlation may be somewhat different from the population value estimated with 4 or 5 subjects. However, it should be emphasized that it is not the actual value, but the change in the correlation coefficient between groups, that reflects the alteration in neural functional relations (Horwitz, 1991).

## Clinical implications

Previous studies have reported that the activities of several mitochondrial enzymes are reduced in Alzheimer's disease (Butterworth and Besnard, 1990; Parker *et al.*, 1994b; Mutisya *et al.*, 1994). The present approach mapping brain CO activity differences in response to sodium azide treatment might prove useful for investigating patterns of brain and behavioral dysfunction secondary to CO inhibition. These differences in CO activity may be a contributing factor to Alzheimer's disease (Chandrasekaran *et al.*, 1992, 1994). However, extreme caution must be exercised in extrapolating our findings in rats to a complex disease such as Alzheimer's in which CO inhibition is only one among many factors. The present findings do indicate that chronic, systemic infusion of sodium azide produced a global inhibition of CO activity in rat brain, with significant variability in degree between some brain regions. Furthermore, this azide treatment uncoupled interregional CO activity correlations found in control brains, induced altered interregional correlations, and impaired learning and memory performance (Bennett *et al.*, 1992a,b). Since Alzheimer's patients do have a systemic metabolic CO defect (Parker *et al.*, 1990, 1994a,b; Beal *et al.*, 1993; Mecocci *et al.*, 1994, Mutisya *et al.*, 1994) and their correlative patterns of brain metabolism (Horwitz *et al.*, 1987) and their spatial memory dysfunctions are similar to those of azide-treated rats (Bennett *et al.*, 1992a,b), our results support the suggestion that sodium azide treatment may be further investigated as a rat model of chronic CO metabolic and degenerative dysfunction which may be relevant to some aspects of metabolic brain diseases.

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