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## Chromosome conformation capture of transcriptional interactions between cytochrome c oxidase genes and genes of glutamatergic synaptic transmission in neurons

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### Abstract

Neuronal activity and energy metabolism are tightly coupled processes. Recently, we found that nuclear respiratory factor 1 (NRF-1) co-regulates all subunits of cytochrome c oxidase (*COX*, representing oxidative energy metabolism) and glutamatergic neurochemicals, including NR1 (*Grin1*) and NR2B (*Grin2b*) of NMDA receptors, GluR2 (*Gria2*) of AMPA receptors, and neuronal nitric oxide synthase (*Nos1*). Moreover, all 10 nuclear-encoded *COX* subunit genes and 3 transcription factor genes for the 3 mitochondrial-encoded *COX* subunits are transcribed in the same transcription factory. The goal of the present study was to test our hypothesis that genomic loci for *Grin1*, *Grin2b*, *Gria2*, and *Nos1* interact with those for *COX* at the transcriptional level. By means of chromosome conformation capture, interactions were found among all of these genes in neurons, but not in C2C12 muscle cells. *COX* subunit genes also did not interact with neurochemical genes not regulated by NRF-1, nor with genes for calreticulin, a non-mitochondrial protein. Depolarizing stimulation up-regulated interaction frequencies between *COX* and neurochemical genes, whereas impulse blockade with TTX or inhibition of *COX* with KCN down-regulated them in neurons. Thus, an efficient mechanism is in place for coordinating the transcriptional coupling of energy metabolism and glutamatergic neurotransmission at the molecular level in neurons.

### Keywords

energy metabolism; excitatory neurochemicals; interactome; KCl depolarization; transcription factory; TTX

### Introduction

Transcription factories are discrete sites within the nucleus where gene transcription is known to occur. These sites of active transcription are thought to contain RNA polymerase II molecules surrounded by transcription factors and loops of chromatin encoding actively expressing genes that are transcribed together (Jackson et al. 1998; Osborne et al. 2004; Zhou et al. 2006). Increasing evidence suggests that long-range interactions among the genes can be studied using chromosome conformation capture (3C), which generates novel ligation products between DNA sequences that are closely juxtaposed *in vivo* (Dekker et al. 2002; Miele and Dekker 2008). 3C employs formaldehyde crosslinking to trap physical interactions between disparate loci within the genome. Cells are then solubilized and

chromosomes are digested with a chosen restriction enzyme and religated under conditions that favor intra- and inter-molecular ligation. After the reversal of crosslinking, the DNA is purified and interaction frequencies between specific chromosomal loci are determined by quantifying the amount of corresponding ligation product with polymerase chain reactions (PCR). Previously, co-regulated genes from one or two different chromosomes were found to preferentially cluster at specialized transcription factories (Spilianakis and Flavell 2004; Ling et al. 2006; Lomvardas et al. 2006; Schoenfelder et al. 2010). Recently, however, we reported that all 13 genomic loci of a multisubunit, multichromosomal, bigenomic enzyme, cytochrome c oxidase (*COX*), and its mitochondrial transcription factors interacted in the same transcription factory in neurons (Dhar et al. 2009a).

In neurons, energy metabolism as reflected by *COX* activity is tightly coupled to neuronal activity, which consumes the bulk of the energy for repolarizing membrane potentials after excitatory depolarizing activity (Wong-Riley 1989). A major excitatory neurotransmitter in neurons is glutamate (Fonnum 1984). Recently, we found that the same transcription factor, nuclear respiratory factor 1 (NRF-1), co-regulates all subunits of *COX* from the two genomes (Dhar et al. 2008) as well as critical neurochemicals of glutamatergic synaptic transmission, including NR1 (*Grin1*) and NR2B (*Grin2b*) subunits of *N*-methyl-D-aspartate (NMDA) receptors (Dhar and Wong-Riley 2009), GluR2 (*Gria2*) subunit of alpha-amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid (AMPA) receptors (Dhar et al. 2009b), and neuronal nitric oxide synthase *NOS1* (*Nos1*) (Dhar et al. 2009c). Such co-regulation ensures that energy supply keeps pace with energy demand of neuronal activity. Such coupling also begs the question, are the *COX* genes and glutamatergic neurochemical genes co-transcribed in the same transcription factory?

The goal of the present study was to test our hypothesis that genes critical for energy metabolism (*COX*) and glutamatergic transmission (*Grin1*, *Grin2b*, *Gria2*, and *Nos1*) that are co-regulated by NRF-1 interact in the same transcription factory in neurons. Our 3C results indicate that such interactions of multiple genes from multiple chromosomes did exist in primary neurons, thereby ensuring precise co-regulation of agents of energy metabolism and those of neuronal activity.

## Materials and methods

### *In silico* analysis of mouse *COX* subunit and neurochemical gene sequences

Genomic sequences, surrounding non-coding sequences, and chromosomal positions of murine genes for nuclear-encoded *COX4i1* and *COX8a* subunits, NMDA receptor subunits *Grin1*, *Grin2b*, and *Grin3a*, AMPA receptor subunits *Gria2* and *Gria4*, and nitric oxide synthase isoforms *Nos1* and *Nos3* were obtained from mouse genome database (Eppig et al. 2007). *COX*, *Grin1*, *Grin2b*, *Grin3a*, *Gria2*, *Gria4*, *Nos1*, and *Nos3* subunit genes were independently verified by comparing the coding sequences with known cDNA sequences and confirming the presence of intron-exon structures. DNA sequences corresponding to each of these verified genes and its surrounding 10,000 bp region were analyzed for the presence of *Bgl II* restriction sites.

### Primary cortical neuronal cultures and treatment with KCl or tetrodotoxin (TTX)

All experiments were carried out in accordance with the US National Institutes of Health Guide for the Care and Use of Laboratory Animals and the Medical College of Wisconsin regulations. All efforts were made to minimize the number of animals and their suffering.

Primary visual cortical neurons were prepared as described previously (Dhar et al. 2009a). Briefly, 1–2 day old Sprague–Dawley rat pups were anesthetized with CO<sub>2</sub> and killed by decapitation. The brains were removed, cleansed of meninges and surface blood vessels, and

the cortices were dissected, trypsinized, and triturated to dissociate individual neurons. Cells were plated onto poly-L-lysine- (Sigma, St. Louis, MO, USA) coated 100 mm dish at a density of  $\sim 5 \times 10^6$  cells/ml in Dulbecco's minimal essential medium (DMEM) (Invitrogen, Carlsbad, CA, USA) with 10% fetal bovine serum (Hyclone, Logan, UT, USA) for 3 h. The medium was changed to neurobasal/B27 supplement (Invitrogen) and cultures were maintained at 37°C with 5% CO<sub>2</sub> in a humidified incubator. Cytosine arabinoside (Ara-C) (Sigma) was added on the second day of plating neurons to inhibit the replication of non-neuronal cells. Neuronal cultures were maintained by replacing half of the medium every 5 days. Cells were cultured for 7–12 days before exposure to 20 mM KCl for 5h. TTX (Sigma) at a final concentration of 0.4  $\mu$ M was added to the culture media from the 5<sup>th</sup> day after plating. Cultures were exposed to TTX for 6 days. All cells were harvested on the same day for each experiment and 3C samples were prepared for each group.

Control cultures were neither depolarized with KCl nor inactivated by TTX. Real time quantitative PCRs (RTqPCR) were carried out in a Cepheid Smart Cycler Detection system (Cepheid, Sunnyvale, CA, USA). iQ SYBR Green Supermix (Bio-Rad, Hercules, CA) was used following the manufacturer's protocols and as described previously (Dhar et al. 2009a).

### Cell culture and KCN treatment

Mouse N2a neuroblastoma cells (ATCC, Manassaa, VA) and C2C12 mouse myoblast cells (gift of Dr. John Lough, Medical College of Wisconsin, Milwaukee, WI) were grown in DMEM (Gibco, Carlsbad CA) supplemented with 10% Fetal Bovine Serum (FBS, Hyclone, Logan, UT). Cells were passaged every 2–3 days and harvested at 80–90% confluency for 3C experiments.

N2a cells were cultured for 3–4 days before exposure to 300  $\mu$ M KCN for 5 hr. To test for the optimal dosage of KCN treatment, N2a cells were also exposed to 100 and 200  $\mu$ M KCN for 5 hr, 24 hr, 3 days and 5 days. All cell studies were done in covered Petri dishes to avoid the evaporation of hydrogen cyanide. Control cultures were not treated with KCN. All cells were harvested on the same day for each experiment and 3C samples were prepared for each group.

### Chromosome conformation capture (3C)

3C experiments were performed as described in published methods (Dhar et al. 2009a; Miele and Dekker 2009) with minor modifications. Briefly, N2a cells, primary neurons and C2C12 cells were harvested by trypsinization and resuspended in fresh culture media with 10% FBS. To cross-link chromatin,  $1 \times 10^7$  N2a cells,  $5 \times 10^6$  primary neurons, or  $1 \times 10^7$  C2C12 cells in 10 ml of cell culture media were treated with 2% formaldehyde for 10 min at room temperature (RT). Cross-linking was stopped by quenching with 0.125 M glycine for 5 min at RT. Nuclei were isolated by incubating cells in a lysis buffer (10 mM Tris, pH 8.0, 10 mM NaCl, 0.2% NP-40) on ice with agitation for 30 min. Chromatin was subsequently released by treating isolated nuclei with 0.3% SDS and digested with 400 units of *Bgl II* (Fermentas, Hanover, MD) overnight. Afterwards, restriction enzyme was heat-inactivated and chromatin was diluted in T4 ligation buffer to achieve a low genomic DNA concentration of 2.5 ng/ $\mu$ l to facilitate intermolecular re-ligation. Chromatin re-ligation was performed by incubating diluted chromatin with 100 Weiss units of T4 ligase (Fermentas) for 4 h at 16°C. Following RNase A (Roche, Nutley, NJ) digestion to remove RNA contaminant, the 3C chromatin was purified by phenol:chloroform extraction.

### Generation of control templates

To generate individual PCR template controls for comparison with 3C product, *COX4i1* or *COX8a* subunit genes were paired with *Grin1*, *Grin2b*, *Grin3a*, *Gria2*, *Gria4*, *Nos1*, and

*Nos3*, respectively, as were the pairing of other genes or gene loci as shown in the controls' lanes (left columns) of Figs. 1–2. For example, to generate the control template for *COX4i1/Grin1*, we used PCR to amplify the regions spanning a single *Bgl II* site of interest with 100–150 bps on either side for each subunit gene. Approximately 30 µg of each PCR product were digested with 300 units of *Bgl II* overnight at 37°C. DNA was extracted using phenol chloroform and ethanol precipitation. Equimolar amounts of digested product (*COX4i1* and *Grin1*) were ligated at a high concentration (~10 ng/µl for each product) to ensure formation of intermolecular cross-linked product. One primer of a given restriction fragment (*COX4i1*) was combined with a primer of a second (*Grin1*) in all pairwise combinations of ligated orientations (e.g., forward primer of *COX4i1* with forward primer of *Grin1*; reverse primer of *COX4i1* with forward primer of *Grin1*, etc.). For all other pairing of *COX* genes, the same steps were used to generate a large number of pairing among subunits and were used as control templates for comparison with 3C samples. The final chosen control template for each pairing had the same orientation as the 3C-generated product. By comparing the signals obtained by quantitative PCR for the 3C cross-linked templates versus the control templates, we corrected for the differences in amplification efficiency between primer sets and also for differences in signal intensities due to the size of the PCR products (as all of the primers were designed to give PCR products of 150–300 bp) (Table 2). All primers were designed to have an annealing temperature of 58–60°C, and they all yielded a product when used with the positive control templates. These control templates and 3C samples for each primer pair were performed during the same PCR run.

A positive 3C control targeted an “internal” cross-linking product between two *Bgl II* sites located 1.6 kb apart within the coding region of calreticulin (*CalR*) gene (+2.8 kb and +4.4 kb relative to *CalR* transcriptional start point) (Tolhuis et al. 2002) (Fig. 1A).

Three 3C negative controls were employed: a) possible cross-linking between the *CalR*'s 4.4 kb *Bgl II* site and another *Bgl II* site on *COX 4i1*; b) no cross-linking agent (formaldehyde); and c) no ligase (Fig. 1A and B).

## PCR assays

The PCR conditions used were very strict in order to detect only specific signals. PCR cycles used were: initial denaturing step for 2 min at 94°C followed by 36 repeats of a cycle with 30 sec at 94°C, 15 sec at 59°C, and 15 sec at 72°C, followed by a final step of 1 min at 72°C. Thirty six PCR cycles were sufficient to detect the specific PCR fragments generated after digestion and ligation that captured rare molecular phenomenon occurring in the nucleus. Cross-linking among *COX* and NMDAR, AMPAR and nNOS subunit gene loci in 3C samples were detected using semi-quantitative PCR. Specifically, a pair of primers targeting two different genes was used to amplify cross-linked DNA generated from 3C reactions. Each primer targeted a DNA region 100–150 bps from the *Bgl II* site and generated 200–300 bp amplicons. Primers were designed for all genes as shown in Table 2. The linear range of amplification was determined for 3C samples by serial dilution as described previously (Tolhuis et al. 2002; Lomvardas et al. 2006; Dhar et al. 2009a; Miele and Dekker 2009). An appropriate amount (150 µg) of 3C DNA within the linear range was added to each PCR reaction. PCR product was run on 2% agarose gel stained with ethidium bromide. All PCR reactions were performed in triplicates.

## Results

### Mouse chromosomal distribution of *COX* subunit and neurochemical genes

Chromosomal distributions of *COX4i1*, *COX8a*, *Grin1*, *Grin2b*, *Grin3a*, *Gria2*, *Gria4*, *Nos1*, and *Nos3* genes in the mouse genome are summarized in Table 1. The ubiquitously

expressed *COX* subunit genes *4i1* and *8a* are on chromosomes 8 and 19, respectively. *Grin1*, *Grin2b*, and *Grin3a* are located on chromosomes 2, 6, and 4, respectively, whereas *Gria2* and *Gria4* are on chromosomes 3 and 9, respectively. Both *Nos1* and *Nos3* isoforms are located on the same chromosome 5 but are 94.42 Mb apart and do not form a gene cluster. *Nos1* is located on the 5 F locus, whereas *Nos3* is on the 5A3 locus. As all 10 nuclear-encoded *COX* subunit genes have recently been shown to interact with each other (Dhar et al. 2009a), the present study chose only *COX4i1* and *COX8a* (representing the largest and smallest nuclear-encoded subunits of *COX*) and their interactions with each of the glutamatergic neurochemical genes to be tested in neurons.

### Interactions between *COX* subunit genes and glutamatergic neurochemical genes

To identify interactions between two noncontiguous DNA regions, a positive control PCR was attained using primers targeting an “internal” cross-linkable product between two *Bgl II* sites located 1.6 kb apart within the coding region of calreticulin (*CalR*) gene (Fig. 1A). *CalR* and *COX 4i1* are unrelated genes and would co-localize only at a background frequency. As Figure 1A illustrates, the internal cross-linking within the *CalR* gene was detectable by PCR of our 3C samples. In contrast, any chance cross-linking event between *CalR* and *COX 4i1* was not detectable by our PCRs. In the absence of a cross-linking agent or T4 ligase, PCR products were not detected among *CalR*, *COX4i1*, *Grin1*, *Gria2* and *Nos1* genes (Fig. 1B).

Possible interactions between *COX4i1* or *COX8a* and neurochemical genes in primary neurons were tested by PCR using primers for *COX4i1*, *COX8a*, *Grin1*, *Grin2b*, *Grin3a*, *Gria2*, *Gria4*, *Nos1* and *Nos3* genes (Table 2). *COX4i1* and *COX8a* were individually documented to form a cross-linking pair with *Grin1*, *Grin2b*, *Gria2*, and *Nos1* genes, respectively (Fig. 2A–C). All possible interactions among these genes were consistently detected by 3C in our PCR product. However, no cross-linking product between *COX4i1* or *COX8a* and *Grin3a*, *Gria4*, or *Nos3* was detected in our PCR product (Fig. 2A–C).

To determine whether these interactions are neuron-specific, C2C12 muscle cells were tested for possible interactions between *COX4i1* and neurochemical genes. *COX4i1* did not form a crosslinking pair with *Grin1*, *Grin2b*, *Gria2*, or *Nos1* genes (Fig. 2D). However, muscle-specific transcription factors *Myod1* and *Mef2a* did show positive interactions and were consistently detected by 3C in our PCR product (Fig. 2D).

### The effect of KCl stimulation and TTX inactivation on gene interactions in primary neurons

Primary neurons were subjected to depolarizing stimulation with 20 mM KCl for five hours or impulse blockade with TTX for 6 days to verify the effect of functional perturbations on *COX4i1* interactions with *Grin1*, *Grin2b*, *Gria2*, and *Nos1* genes, respectively. As shown in Fig. 3A–D, KCl treatment induced a 224% to 540% increase in crosslinking frequency ( $P < 0.05$ ), whereas TTX blockade resulted in a 68 to 83% decrease in crosslinking frequency ( $P < 0.05$ ) among the genes, as monitored by real-time quantitative PCR.

### The effect of KCN treatment on gene interactions in neurons

To determine if specific inhibition of COX would disrupt the co-regulation of *COX* and neurochemical genes, N2a cells were subjected to 300  $\mu$ M KCN, a specific inhibitor of COX, for 5 hr. As shown in Fig. 4A–D, KCN inhibition resulted in a 60 to 80% decrease in crosslinking frequency ( $P < 0.05$ ) among the genes, as monitored by real-time quantitative PCR. Other concentrations of KCN and time points were tested. 100  $\mu$ M and 200  $\mu$ M KCN for 5 hr yielded no change in interactions, but at 24 hr the frequencies of interactions were reduced but did not reach statistical significance (data not shown). Cells started to die after



24 hr of 300  $\mu\text{M}$  KCN, after 3 days of  $\geq 200 \mu\text{M}$  KCN, or after 5 days of  $\geq 100 \mu\text{M}$  KCN (data not shown). At 300  $\mu\text{M}$  KCN for 5 hours, COX was inhibited but cells all survived.

## Discussion

The present study documents for the first time that genes responsible for energy metabolism (COX) and those involved in glutamatergic neurotransmission (*Grin1*, *Grin2b*, *GluR2*, and *Nos1*) are transcribed in presumably the same transcription factory in neurons. Such intermolecular interactions are further enhanced by depolarizing stimulation and suppressed by impulse blockade. The results substantiate our previous findings that these genes are co-regulated by the same transcription factor, nuclear respiratory factor 1 (Dhar and Wong-Riley, 2009; Dhar et al., 2009b,c). The interactions are also neuron-specific, as they were not present in C2C12 muscle cells, where *Myod1* and *Mef2a* genes did interact (present study). Furthermore, specific inhibition of COX by KCN significantly reduced the frequency of interactions among these genes, indicating that, indeed, COX and neurochemical genes are co-regulated.

The 3C technique was developed based on the theory that actively co-expressed or co-regulated genes are physically localized in a “transcription factory” that contains copies of active RNA polymerases, transcription factors, and associated genomic loci undergoing transcription (Xu and Cook 2008; Schoenfelder et al. 2010). Initially, the 3C method was used in studying chromosomal conformation in yeasts (Dekker et al. 2002), but the approach was found to expose folding of complex gene loci and active chromatin hub in mammalian cells as well (Jackson et al. 1998; Osborne et al. 2004; Zhou et al. 2006). As shown in the present study, such interactions are not random but are highly specific. For example, genes that do not share a common transcription factor, in the present case, did not interact, such as the absence of ligation pairs between *COX4i1* or *COX8a* with *Grin3a*, *Gria4*, or *Nos3*. Until recently, 3C has uncovered interactions only between genomic loci of the same chromosome or at most of two different chromosomes (Spilianakis and Flavell 2004; Ling et al. 2006; Lomvardas et al. 2006). However, we found interactions among 13 genomic loci from 11 chromosomes related to the bigenomic enzyme, COX, in neurons (Dhar et al. 2009a). Ten of the loci represent 10 nuclear-encoded COX subunit genes from 9 different chromosomes (Kadenbach et al. 1983; Eppig et al. 2007), whereas the other 3 denote mitochondrial transcription factors *Tfam*, *Tfb1m*, and *Tfb2m* genes important in transcribing the 3 mitochondrial-encoded COX subunit genes (Virbasius and Scarpulla 1994; Gleyzer et al. 2005; Scarpulla 2008a). The present study extended these findings to include interactions of COX subunit genes with genes of glutamatergic neurotransmission that are functionally coupled to energy metabolism.

COX is a critical oxidative enzyme without which oxidative metabolism cannot be carried to completion and mitochondrial ATP cannot be produced (Wikström et al. 1981; Kadenbach et al. 1983; Wong-Riley 1989). Recently, we found that all 13 subunits of cytochrome c oxidase derived from both the nuclear and mitochondrial genomes are regulated by the transcription factor NRF-1 (Dhar et al. 2008). NRF-1 is also known to be a key transcriptional activator of nuclear genes encoding a number of mitochondrial respiratory enzymes, including subunits of the five respiratory chain complexes (Scarpulla 2008a,b). Neuronal activity and energy metabolism are tightly coupled processes, and the bulk of energy in neurons is used to repolarize their membranes subsequent to glutamate-induced depolarization (Wong-Riley 1989). When neuronal activity is altered, such as with TTX-induced impulse blockade or KCl-stimulated depolarization, neuronal COX activity is down- or up-regulated to match the new energy demand (Wong-Riley 1989), and the expressions of glutamatergic NMDA receptors, AMPA receptor GluR2 subunits, and neuronal NOS are adjusted in parallel (Wong-Riley et al. 1998a,b; Wong-Riley and Jacobs 2002; Bai and

Wong-Riley 2003; Dhar et al. 2009b). Remarkably, NRF-1 also directly regulates the expression of critical components of glutamatergic synapses, including NR1 and NR2B of the NMDA receptors, subunit GluR2 of the AMPA receptors, and neuronal nitric oxide synthase (Dhar et al., 2009b,c; Dhar and Wong-Riley 2009). Thus, the tight coupling of neuronal activity and energy metabolism is extended to the molecular level of regulation. The present study further documents that these functionally coupled metabolic and neurochemical genes that are regulated by the same transcription factor interact within the same presumed transcription factory in neurons. These interactions are specific, as genes not regulated by NRF-1 (*Grin3a*, *Gria4*, or *Nos3*) did not interact with *COX* subunit genes. Comparable associations between co-regulated mouse globin genes and their regulatory factors have recently been described in erythroid cells as a transcriptional interactome (Schoenfelder et al. 2010). Such transcription factories or interactomes are likely to be dynamic depending on the functional demands of the cell. As shown in the present study, interactions among functionally associated genes are up-regulated by KCl stimulation and down regulated by TTX. This presumably leads to the concurrent up- or down-regulation of the respective transcripts, including those for *COX*, *Grin1*, *Grin2*, *Gria2*, and *Nos1* (Dhar et al. 2009a).

Besides NRF-1, nuclear respiratory factor 2 (NRF-2) also regulates all 13 *COX* subunit genes from the two genomes (Ongwijitwat and Wong-Riley 2005; Ongwijitwat et al. 2006). Moreover, an important coactivator of NRF-1 and NRF-2, known as peroxisome proliferator activated receptor  $\gamma$  coactivator 1 $\alpha$  (PGC-1 $\alpha$ ) (Wu et al. 1999; Scarpulla 2008a,b), is also involved in the transcriptional regulation of *COX* in neurons (Liang and Wong-Riley 2006; Meng et al. 2007). It is possible that these factors participate in the regulation of the same and/or comparable neurochemicals of glutamatergic neurotransmission. Future studies will be directed at uncovering interactions among these critical factors of transcription in neurons.

## Acknowledgments

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## Abbreviations

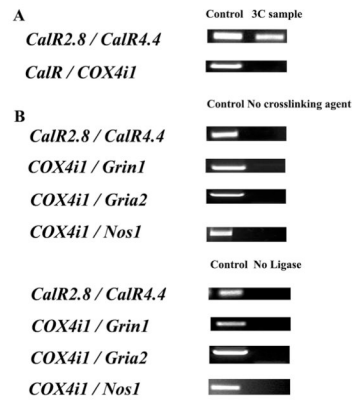
<b>3C</b>	chromosome conformation capture
<b>AMPA</b>	alpha-amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid
<b>COX</b>	cytochrome c oxidase
<b>Nos1</b>	neuronal nitric oxide synthase
<b>Nos3</b>	endothelial nitric oxide synthase
<b>NRF-1</b>	nuclear respiratory factor-1
<b>NRF-2</b>	nuclear respiratory factor 2
<b>PGC-1<math>\alpha</math></b>	peroxisome proliferator-activated receptor- $\gamma$ coactivator 1 $\alpha$
<b>Tfam</b>	transcription factor A of mitochondria
<b>Tfb1m</b>	transcription factor B1 of mitochondria
<b>Tfb2m</b>	transcription factor B2 of mitochondria

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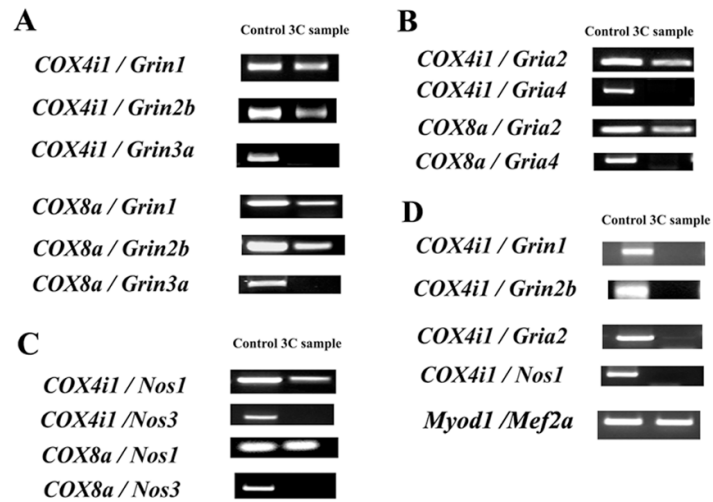
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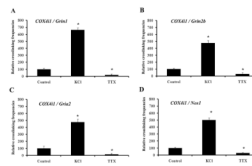
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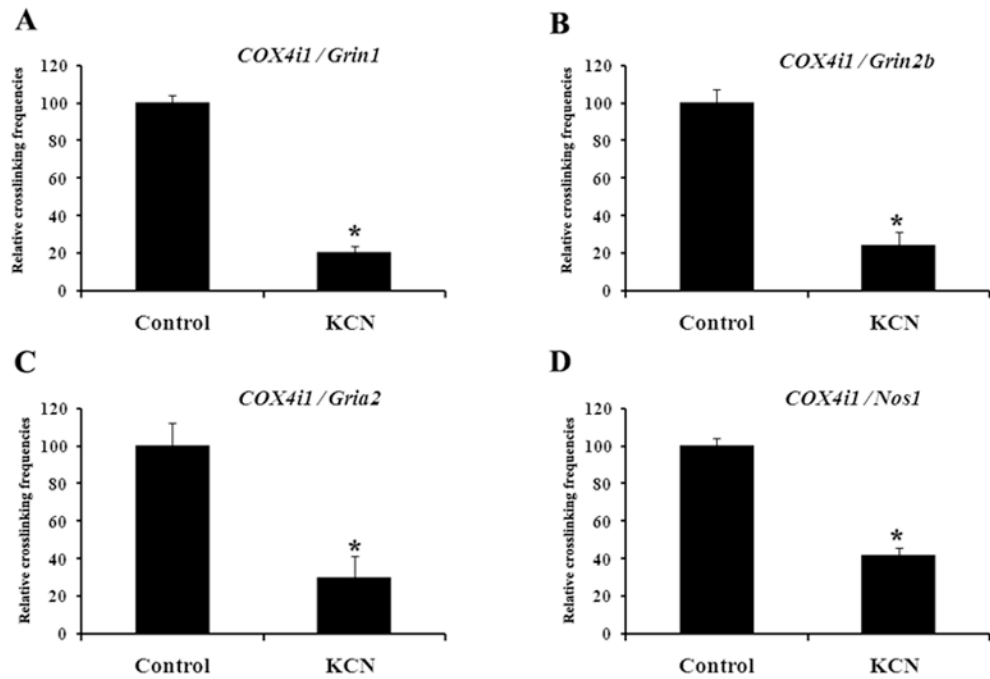
**Fig. 1.** Positive and negative controls for chromosome conformation capture. All possible restriction fragments after *Bgl II* digestions were present in equimolar amounts. A. Two regions of calreticulin gene, *CalR2.8* and *CalR4.4*, were cross-linked and represented a positive control. On the other hand, *COX4i1* and *CalR* were two functionally unrelated genes, and they did not interact in our 3C reactions. B. *CalR2.8* and *CalR4.4*, as well as *COX4i1* or *COX8a* and *Grin1*, *Gria2*, or *Nos1*, respectively, did not interact in the absence of a cross-linking agent (formaldehyde) or T4 ligase. Experiments were performed in triplicates.



**Fig. 2.** 3C interactions in N2a and C2C12 cells between *COX* subunit genes and glutamatergic neurochemical genes. All interactive pairs between *COX4i1* or *COX8a* and *Grin1*, *Grin2b*, *Grin3a*, *Gria2*, *Gria4*, *Nos1*, or *Nos3* were tested by PCR targeting their cross-linking products. A–C. *COX4i1* or *COX8a* consistently interacted with each of the *Grin1*, *Grin2b*, *Gria2*, or *Nos1* genes in N2a cells. However, *COX4i1* or *COX8a* did not yield any positive hybrid product when paired with *Grin3a*, *Gria4*, or *Nos3*. D. In C2C12 cells, *COX4i1* did not interact with each of the *Grin1*, *Grin2b*, *Gria2*, or *Nos1* genes, whereas *Myod1* and *Mef2a* showed consistent interactions. All experiments were performed in triplicates.



**Fig. 3.** 3C interactions in primary neurons exposed to KCl stimulation or TTX impulse blockade. KCl depolarization induced an up-regulation of cross-linking frequencies between *COX4i1* and *Grin1* (A), *COX4i1* and *Grin2b* (B), *COX4i1* and *Gria2* (C), and *COX4i1* and *Nos1* (D), whereas TTX exposure for 6 days down-regulated the interactions of these genes (A–D). \*,  $P < 0.05$ .



**Fig. 4.** 3C interactions in N2a cells exposed to 300  $\mu$ M KCN treatment for 5 hr. KCN inactivation induced a significant down-regulation of cross-linking frequencies between *COX4i1* and *Grin1* (A), *COX4i1* and *Grin2b* (B), *COX4i1* and *Gria2* (C), and *COX4i1* and *Nos1* (D). \*,  $P < 0.05$ .



**Table 1**

Chromosomal location of murine genes arranged by chromosomal number.

Chromosome	Gene
2	<i>Grin1</i>
3	<i>Gria2</i>
4	<i>Grin3a</i>
5	<i>Nos1</i>
5	<i>Nos3</i>
6	<i>Grin2b</i>
8	COX4i1
9	<i>Gria4</i>
19	COX8a

**Table 2**3C primers around *Bgl II* sites.

Gene	Sequence	Amplicon length	T <sub>m</sub>
<b>COX4i1</b>	F 5'TTAGGACCTCTGCTCGCTCT 3' R 5'TCAGCAGTAAGAGCACTGG 3'	204 bps	60°C
<b>COX8a</b>	F 5'CATTATGGTGGGCAACCTTC 3' R 5'TCCATCTGCCATTCTCACTG 3'	261 bps	60°C
<i>Grin1</i>	F 5'CTCTGTGGCGTAGGAGGAAC 3' R 5'TTTGTGTGGTGTGGTGTGTG 3'	220 bps	60°C
<i>Grin2b</i>	F 5'TTCTTCCTTTCCCCTCTTCC 3' R 5'TTCCAGCTTGTGGCTTTCT 3'	271 bps	60°C
<i>Grin3a</i>	F 5'CTTGGCGTACCTGAACTCGT 3' R 5'AAAGAAGGCCATGGAGAAGC 3'	275 bps	60°C
<i>Gria2</i>	F 5'ACAATGCGCCAGAGAGA 3' R 5'CCCAAGAATGGAGTTTAAGTCTTT 3'	250 bps	60°C
<i>Gria4</i>	F 5'TGGGCATCAGTGAAGAAAA 3' R 5'CTGTGTTGCCATGTGAGTCC 3'	315 bps	60°C
<i>Nos1</i>	F 5'CCCAAGGTCCTGAGTTCAA 3' R 5'TCCCTTCCTTCTACCTTCG 3'	190 bps	60°C
<i>Nos3</i>	F 5'CTGCCTGTCATCACTCAGGA 3' R 5'TATCCTTGGGCTCTCCAAAA 3'	166 bps	60°C
<i>Myod1</i>	F 5'TGACCATGTTCTTCCACTGC 3' R 5'CAGGTGGACTGTATGCAGGA 3'	202 bps	60°C
<i>Mef2a</i>	F 5'TCTGGCCCACTGACCTACAT 3' R 5'CTCACAGCCGCCTAGAACTC 3'	206 bps	60°C
<i>CalR</i> (+2.8 kb)	F 5'TTCTTCTACAGGGCGAGTGG 3' R 5'GACTTGAGGTCTGCCAGGAA 3'	197 bps	60°C
<b>CalR</b> (+4.4 kb)	F 5'CTGGGCTCTCCAATAATGT 3' R 5'GGGAGGGACAGAAGGAAAAG 3'	210 bps	60°C