Supplemental Data

Multiple Signaling Pathways Regulate The Transcriptional Activity Of The Orphan Nuclear Receptor Nurr1

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Supplemental Material and Methods:

Spot excision and in gel digestion - Protein spots were excised semi-automatically from 1D gels using the "Click and pick" mode of the Ettan Spot Picker (Amersham Biosciences). Approximately ten plugs were excised from each band and put in the same well. In-gel digestion was performed using an automated protein digestion system (Ettan Digester; Amersham Biosciences). Briefly, plugs were washed three times with 50 mM ammonium bicarbonate containing 50 % methanol, then 50 % acetonitrile containing 0.1% TFA. After two washing steps in 70 % acetonitrile, plugs were dried and rehydrated with 10 µL of 20 mM ammonium bicarbonate containing 4 µg/ml trypsin (Promega) for 4 h. Finally, a 20 mM ammonium bicarbonate solution was added overnight. The peptide mixture were then dried, and resuspended in 10 µL 0.1 % TFA before MALDI-MS analysis.

Acquisition of mass spectrometric peptide maps, MALDI-TOF MS and database search and analysis - 1 µL of peptide mixture was mixed with 1 µL of DHB matrix solution (10 mg dihydrobenzoic acid in 50% methanol) on the MALDI target. MALDI-TOF MS was performed using a Voyager DE STR mass spectrometer (PerSeptive Biosystems) equipped with a 337.1 nm nitrogen laser and the delayed extraction facility. All spectra were acquired in a positive ion reflector mode. Typically, 200 laser shots were recorded per sample, and spectra were internally calibrated (using the DataExplorerTM software, PerSeptive Biosystems) using three peptides arising from trypsin autoproteolysis ([M+H]+ 842,5100; [M+H]+ 1045.5642; [M+H]+ 2211.1046). Tryptic monoisotopic peptide masses were searched for in the NCBI, using Protein Prospector (http://prospector.ucsf.edu/) with a mass tolerance setting of 50 ppm, with three missed cleavage site as fixed parameters, and with carbamidomethylation and methionine oxidation as variable modifications. The database search identified multiple proteins in each band. However, the proteins to be further tested were chosen among the possible candidates by comparing the relative abundance of the different peptides, the percentage of recovery of each protein and taking into account the full size of the proteins. MS analysis was carried out 4 times on independent samples and selected peptides were detected at least twice out of four analysis, but in most cases they were detected three or four times out of four analysis. Proteins of interest were selected from this type of analysis if peptide coverage was above 20%. Specific samples were re-analyzed and provided enhanced spectra of protein digests which resulted in significantly increased sequence coverage (above 35%) and confidence in protein identification.

Supplemental Figure Legends:

Supplemental Figure 7 - Nurr1 gene expression was detected by reverse transcription-PCR in total RNAs from rat midbrain tissue, mesencephalic CSM14.1 and PC12 cells using nurr-specific primers. CSM14.1 cells were treated for 2 hr with DMSO (-Forsk) or 25 µM Forskolin (+Forsk) before RNA extraction, while PC12 cells were transfected with empty vector (-nurr1) or pCMX-nurr1 plasmid (+nurr1).

Supplemental Figure 8 - Representative examples of the gradient gels used to identify nurr1 novel interacting partners. Panel A) Original image of the silver nitrate stained gel shown in Fig.1B; these gels were used to evaluate the differences in the various conditions. Panel B) Representative Colloidal Blue stained gel used for MS analysis. Clear differential bands could be identified in the different conditions and excised as described in Supplemental Material and Methods.

Supplemental Figure 9 - Nurr1 interacts directly with multiple proteins identified by MS analysis. (A) ^{35}S-labelled hPARP-1 (G. De Murcia), NCoR, PPARγ, GR or FXR were incubated in the presence of bacterially expressed GST alone (-) and GST-nurr1 fusion proteins (LBD or A/BΔ domain) for 3 h. The first lane corresponds to 10% input. Protein interactions were analyzed by SDS-PAGE (8-9%) and assayed by autoradiography. (B) ^{35}S-labelled full length nurr1 was incubated with STAT3 fused to GST or GST alone (-) expressed, separated and quantified as in *A*. (C) Co-immunoprecipitations with the anti-HA antibody of cellular extracts of CSM14.1 cells co-transfected with HA-tagged human nurr1 and empty vector (-) or expression plasmids of pXJ-GST-p85 βPIX (C.G. Koh) and visualized with anti-GST antibody, and pRC-CMV-Mxi1 (E.V. Prochownik) and detected with anti-Mad antibody.

Supplemental Table 1. Potential interacting partners of nurr1 identified by mass spectroscopy and classified according to their known function

Transcription Factors	***Transcription Repression***	***DNA Repair Apoptosis***	***Signalling pathways***	***Others***
nurr1	NCoR-1	PARP-1	MEK5	Hsp90
RXRα	Mxi1		ERK1, ERK2	
PPARγ			LIMK1	
GR			PAK2	
FXR			p85 βPIX	
STAT3			Akt	