Nurr1 expression is regulated by voltage-dependent calcium channels and calcineurin in cultured hippocampal neurons

Hirofumi Tokuoka¹, Takayuki Hatanaka¹, Daniel Metzger²⁻⁵, and Hiroshi Ichinose^{1*}

¹Graduate School of Bioscience and Biotechnology, Tokyo Institute of Technology, Yokohama, Japan; ²Institut de Génétique et de Biologie Moléculaire et Cellulaire, Illkirch F-67400, France; ³CNRS UMR7104, Illkirch, France; ⁴INSERM U964, Illkirch France; ⁵Université de Strasbourg,, Strasbourg, France.

*To whom correspondence should be addressed: Hiroshi Ichinose, Graduate School of Bioscience and Biotechnology, Tokyo Institute of Technology, 4259 B-7, Nagatsuta, Midori-ku, Yokohama, 226–8501 Japan.

Tel.: 81-45-924-5822, Fax: 81-45-924-5807;

E-mails: hichinos@bio.titech.ac.jp

The abbreviations used are: CaMK, calcium/calmodulin-dependent protein kinase; DL-APV, DL-2-Amino-5-phosphonopentanoic acid; CNQX, 6-Cyano-7-nitroquinoxaline-2,3-dione; TTX, tetrodotoxin; VDCC, voltage-dependent calcium channel.

Abstract

Nurr1 is an orphan nuclear transcription factor expressed in the brain. While Nurr1 is assumed to be an immediate early gene, it is not fully understood how Nurr1 expression is regulated in an activity-dependent manner in the central nervous system. Here, we investigated the molecular mechanisms underlying the regulation of Nurr1 expression in cultured hippocampal and cortical neurons. We found that upregulation of neural activity by high KCl and bicuculline enhances Nurr1 levels, while blockade of its activity by tetrodotoxin reduces Nurr1 levels. The induction of Nurr1 expression was mediated by voltage-dependent calcium channels (VDCCs), as shown by cadmium and VDCC-specific inhibitors. Furthermore, calcineurin, but not calcium/calmodulin-dependent protein kinase (CaMK) was critical for the induction. Thus, Nurr1 expression is regulated by VDCC and calcineurin in a cell-autonomous, neural activity-dependent manner.

Keywords

calcineurin, hippocampal neurons, Nurr1 (Nr4a2), voltage-dependent calcium channel, transcription

Introduction

Nurr1 is an orphan nuclear receptor that belongs to the Nr4a subfamily composed of NGFI-B (Nr4a1), Nurr1 (Nr4a2) and Nor1 (Nr4a3). Nurr1 is essential for the differentiation of midbrain dopaminergic neurons [6, 34], and sustained Nurr1 expression is essential to maintain the expression of genes in dopaminergic neurons in the adult mouse midbrain [17].

Nurr1^{+/-} mice have decreased dopamine levels in the brain [16], and exhibit hyperactive behaviors potentially relevant to schizophrenia [24, 31, 33]. Importantly, gene mutations were reported in the *Nurr1* allele in schizophrenia patients [1, 5, 9].

Nr4a genes are not only expressed in dopaminergic neurons, but also in cortical and hippocampal neurons [2, 32]. Studies with glial cells [25], immune cells [27], and other cell types of peripheral tissues have reported diverse roles of Nurr1 [23]. Nurr1 may play a role in learning and memory as suggested by *Nurr1* gene knock-down experiments [10, 13, 14].

NR4a family members are supposed to be immediate early genes. NGFI-B (NR4a1) was originally identified as an immediate early gene, which was rapidly activated by serum stimulation of quiescent fibroblasts [15]. Nurr1 is inducible by membrane depolarization with KCl in PC12 cells [20], and in young culture of cerebellar granule cells [3]. Nurr1 is also induced by kainic acid in rat brains [12]. However, it is not understood well how Nurr1 gene expression is regulated in an activity-dependent manner in CNS neurons. Thus, we investigated the mechanisms underlying the regulation of Nurr1 gene expression in central neurons.

Materials and methods

Cell culture

Dissociated cultures of hippocampal neurons were prepared from embryonic day 16 mice (C57Bl/6) as described previously with minor modifications [28, 29]. Mice bearing *Nurr1*-null alleles were established from ES cell cells harboring floxed *Nurr1* alleles [17] that were electroporated with a Cre-expressing vector. *Nurr1*^{+/-} mice were intercrossed to generate *Nurr1*-null fetuses. All animal experiments were performed in accordance with the general guide lines of the Tokyo Institute of Technology.

Coverslips were coated with 0.25% polyethyleneimine in 0.15 M borate buffer (pH 8.5) overnight. Hippocampal neurons were dissociated by papain and plated with Neurobasal medium (Life Technologies, Carlsbad, CA) containing B-27 (Life Technologies), 0.5 mM GlutaMAX I (Life Technologies) and 5% fetal bovine serum. 1 μ M Cytosine arabinoside (Sigma, St Louis, MO) was added to culture media to prevent proliferation of astrocytes at 3 or 4 DIV. Cultured neurons were used for experiments at 13 – 17 DIV.

Cells were stimulated by addition of 20 μ M bicuculline (WAKO, Osaka, Japan) and 20 mM KCl to culture media (K⁺/bic stimulation). In some experiments, the following channel blockers were added to the culture media 5 to 10 min before K⁺/bic stimulation: 100 μ M CdCl₂, 10 μ M 6-Cyano-7-nitroquinoxaline-2,3-dione (CNQX) (WAKO), 100 μ M DL-2-Amino-5-phosphonopentanoic acid (DL-APV) (Sigma), 1 μ M tetrodotoxin (TTX) (Sigma), 10 μ M nimodipine (Sigma), 1 μ M Ω -conotoxin MVIIC (Peptide Institute, Osaka, Japan), 0.5 μ M Ω -conotoxin GVIA (Peptide Institute), or 0.1 μ M SNK-482 (Peptide Institute). To examine intracellular signaling, cells were pretreated with 1 μ M cyclosporin A, 0.1 μ M FK506 or 1 μ M KN-93 for 1 hr before stimulation with K⁺/bic.

Immunocytochemistry

Immunocytochemistry was performed as described previously [28, 29]. Cells were stimulated as indicated, and fixed by 4% paraformaldehyde in PBS for 10 min at room temperature. The

following primary antibodies were used: anti-Nurr1 (1/200, Peruseus proteomics, Tokyo), and anti-NeuN (1/500, Millipore, Billerica, MA). The following secondary antibodies were used: Alexa546-conjugated anti-mouse IgG (1/2,000, Life Technologies) and Alexa488-conjugated anti-rabbit IgG (1/2,000, Life Technologies). Fluorescence images were captured with a E800 microscope (Nikon, Tokyo, Japan) with 20× or 40× objective lens [numerical aperture (NA) = 0.50, 0.90, respectively; Nikon], equipped with a cooled CCD camera (VB-6010, Keyence, Osaka, Japan).

Fluorescence signals were quantified with Image J. For each batch of experiment, threshold value of the Nurr1 signal was determined from the images of neurons stimulated with K^+ /bic. Each histogram of Nurr1 images was fitted with Gaussian curve, and the averaged $10 \times SD$ above the peak value was set as a threshold. This thresholding provided almost no positive nuclear signal in *Nurr1*^{-/-} neurons, while highlighting distinct nuclear signals in *Nurr1*^{+/+} neurons. The same method was used for the threshold of NeuN signals.

Western blot

For Western blot analysis, P0 mouse brains were dissected out, and homogenized using a PelletMixer (TreffLab) in 4×w/v buffer containing 50 mM Tris-HCl (pH6.8), 1% SDS, 5% sucrose, followed by sonication and boiling. Protein concentrations were determined with DC assay, and 20 µg of protein of each sample was subjected for Western blot.

For Western blot of cultured neurons, mouse cerebral cortex neurons from E16 embryos of C57Bl/6J mice were cultured in 6-well plates at a density of 1×10^6 per well. Cells were stimulated as described above, collected in cold PBS, and lysed with 300 µl RIPA buffer composed of 50 mM Tris-HCl (pH 7.5), 150 mM NaCl, 1% SDS, 1% Triton X-100, 1% deoxycholate, 1 mM phenylmethylsulfonyl fluoride, 1 µg/ml leupeptin, and 1 µg/ml pepstatin. After centrifugation at $20,000 \times g$ for 10 min, protein concentrations were determined by

Bradford protein assay, and 40 mg protein of each sample was used for Western blot with anti-Nurr1 antibody (1/500, Peruseus proteomics). Because Nurr1 signal from brain and cultured neuron samples showed multiple or smear bands near 57 kD and 70 kD markers, we took all these bands signals for quantification.

Results

Neural activity-dependent regulation of Nurr1 expression

First, we examined the expression level of Nurr1 by immunostainings and Western blot using a monoclonal antibody, which specifically detects Nurr1 expression (**Fig. 1A and B**). When the neural network activity of cultured hippocampal neurons was enhanced by application of 20 mM KCl and 20 μ M bicuculline (K^+ /bic), Nurr1 expression was greatly increased within 3 hr (**Fig. 1C and D**). This effect was also confirmed by Western blot in cultured cortical neurons (**Fig. 1E**).

On the other hand, the expression of Nurr1 was reduced by suppression of neural network activity with TTX for 3 hr (**Fig. 2A, B**). These data directly demonstrate Nurr1 expression is tightly regulated depending on neural activity in hippocampal and cortical neurons.

VDCC-mediated regulation of Nurr1 expression

We next examined how neurons sense the neural activity for Nurr1 regulation. When neurons were treated with cadmium, a non-specific VDCC inhibitor, Nurr1 induction by K^+ /bic was completely inhibited (**Fig. 3A**). Meanwhile, blockade of AMPA receptors, NMDA receptors, and voltage-dependent sodium channels, by CNQX, APV, and TTX, respectively, did not inhibit Nurr1 induction by K^+ /bic (**Fig. 3A**). Similar results were obtained by Western blot analysis (**Fig. 3C**).

We then used specific inhibitors for VDCC subtypes; nimodipine for L-type VDCC, Ω -conotoxin MVIIC for P/Q-type and N-type VDCC, Ω -conotoxin GVIA for N-type VDCC, and SNX-482 for R-type VDCC. Combined treatment of these inhibitors completely abrogated Nurr1 induction by K^+ /bic (**Fig. 3B, C**), confirming the essential role of VDCC.

We examined which subtype of VDCC was important by omitting one of those inhibitors. We found that withdrawal of nimodipine from the four VDCC inhibitors recovered the Nurr1 induction, while removal of other single inhibitors did not (**Fig. 3B**). The data suggests activation of L-type VDCC is sufficient to induce Nurr1 induction.

However, when only nimodipine or Ω -conotoxin MVIIC was applied, Nurr1 induction by K^+ /bic was not blocked. Rather, we found that the combination of nimodipine and Ω -conotoxin MVIIC effectively inhibited the Nurr1 induction (**Fig. 3C**). Combination nimodipine with Ω -conotoxin GVIA or SNX-482 did not affect Nurr1 expression (data not shown). These data indicate that Nurr1 expression is modulated not only by L-type VDCC, but by multiple VDCCs.

Calcineurin mediates Ca^{2+} signal for the regulation of Nurr1 expression

We further explored what signaling molecule mediates the Ca^{2+} signal downstream of VDCC. Cyclosporin A and FK506, both calcineurin inhibitors, suppressed the induction of Nurr1 by K^+ /bic as demonstrated by immunocytochemistry and Western blot (**Fig. 4A and B**). On the other hand, KN-93, a calcium/calmodulin-dependent protein kinase II/IV (CaMKII/IV) inhibitor, did not affect the Nurr1 induction (**Fig. 4A and B**). These data suggest that calcineurin is a primary mediator of Ca^{2+} signal downstream of VDCC to promote the induction of Nurr1 expression.

Discussion

In this study, we demonstrate that *Nurr1* gene is under regulation of neural activity through VDCC and calcineurin in cultured hippocampal and cerebral cortical neurons. Furthermore, we demonstrated that basal expression of Nurr1 is decreased when neuronal activities are blocked by TTX. These data raise the possibility that Nurr1 expression may not always be transient, but could be constitutive in some cells depending on a basal activity of each neuron.

We found that VDCC is responsible for neural activity-dependent regulation of *Nurr1* gene, while AMPA- and NMDA-type glutamate receptors are not directly involved. These data indicate that Nurr1 expression is cell-autonomously regulated, and could be variable depending on the activity of each neuron. However, the involvement of NMDA receptor was reported previously with young cerebellar granule cells in culture [3]. This difference may be attributable to the difference of cell origin, or maturity of cultured neurons, as we used relatively matured culture of hippocampal and cerebral cortical neurons.

Different VDCC subtypes show different kinetics and localization [8], and different contribution to the total VDCC current when recorded at soma [7]. We found that L-type VDCC was sufficient for enhancement of Nurr1 expression, while activation of other VDCC subtypes could also induce Nurr1 expression. These data suggest that the total amount of Ca^{2+} entry into cells through VDCCs, rather than activation of a specific VDCC subtype, is an important factor for the regulation of Nurr1 expression.

We found that calcineurin was essential for the induction of Nurr1, while CaMK was not. CaMK is known as a primary mediator of calcium signals to transcription of genes [4]. The involvement of calcineurin was reported for NGFI-B induction [19], suggesting that the VDCC-calcineurin pathway might be common for the regulation of NR4a family genes in central neurons. The downstream factor of calcineurin which upregulates Nurr1 gene is yet to

be known. CyclicAMP-response element binding protein (CREB) can activate Nurr1 gene transcription [30], while CREB can be activated by calcineurin through CREB regulated transcription coactivator 2 (TORCS2) [26]. Thus, TORCS2 and CREB may be key players of Nurr1 induction downstream of calcium entry via VDCC.

Involvement of calcineurin in cognitive functions and schizophrenia has been reported. Calcineurin-deficient mice showed deficits in cognitive functions [22, 11] and calcineurinA gamma subunit gene was associated with schizophrenia [18, 21]. Thus, our data raise a possibility that defect in the calcineurin gene may exert cognitive dysfunction through suppression of Nurr1 expression.

Conclusion

Nurr1 expression in the CNS neurons is regulated by neural activity, through voltage-dependent calcium channels and calcineurin.

Acknowledgements

We thank André Dietrich and Jean-Marc Bornert for their help in generating mice bearing Nurr1 null alleles, Noriko Ihira for her help in construction of the targeting vector and screening of ES clones. We thank all Ichinose-lab members for critical suggestions and discussions. This work was supported by KAKENHI from MEXT; by The Nakajima Foundation, and by CREST, JST.

References

- [1] I. Ancín, J.A. Cabranes, B. Vázquez-Álvarez, J.L. Santos, E. Sánchez-Morla, M. Alaerts, J. Del-Favero, A. Barabash, NR4A2: effects of an “orphan” receptor on sustained attention in a schizophrenic population. *Schizophr. Bull.* (2013) 555–563.
- [2] Y. Arimatsu, M. Ishida, T. Kaneko, S. Ichinose, A. Omori, Organization and development of corticocortical associative neurons expressing the orphan nuclear receptor Nurr1, *J. Comp. Neurol.* 466 (2003) 180–196.
- [3] B. Barneda-Zahonero, J.-M. Servitja, N. Badiola, A.J. Miñano-Molina, R. Fadó, C. a Saura, et al., Nurr1 protein is required for N-methyl-D-aspartic acid (NMDA) receptor-mediated neuronal survival, *J. Biol. Chem.* 287 (2012) 11351–11362.
- [4] H. Bito, K. Deisseroth, R.W. Tsien, CREB phosphorylation and dephosphorylation: a Ca^{2+} - and stimulus duration-dependent switch for hippocampal gene expression, *Cell* 87 (1996) 1203–1214.
- [5] S. Buervenich, A. Carmine, M. Arvidsson, F. Xiang, Z. Zhang, O. Sydow, E.G. Jönsson, G.C. Sedvall, S. Leonard, R.G. Ross, R. Freedman, K.V. Chowdari, V.L. Nimgaonkar, T. Perlmann, M. Anvret, L. Olson, NURR1 mutations in cases of schizophrenia and manic-depressive disorder, *Am. J. Med. Genet.* 96 (2000) 808–813.
- [6] M. Caiazzo, M.T. Dell’anno, E. Dvoretzkova, D. Lazarevic, S. Taverna, D. Leo, T.D. Sotnikova, A. Menegon, P. Roncaglia, G. Colciago, G. Russo, P. Carninci, G. Pezzoli, R.R. Gainetdinov, S. Gustincich, A. Dityatev, V. Broccoli, M. Teresa, D. Anno, Direct generation of functional dopaminergic neurons from mouse and human fibroblasts, *Nature*. 476 (2011) 224–227.
- [7] Y.Q. Cao, R.W. Tsien, Effects of familial hemiplegic migraine type 1 mutations on neuronal P/Q-type Ca^{2+} channel activity and inhibitory synaptic transmission, *Proc. Natl. Acad. Sci. U.S.A.* 102 (2005) 2590–2595.

- [8] W.A. Catterall, Voltage-gated calcium channels, *Cold Spring Harbor Perspectives in Biology* 3 (2011) 1-23.
- [9] Y. Chen, M. Tsai, C. Shaw, C. Chen, Mutation Analysis of the Human NR4A2 Gene , an Essential Gene for Midbrain Dopaminergic Neurogenesis, in Schizophrenic Patients, *Am. J. Med. Genet.* 757 (2001) 753–757.
- [10] W.I. Colón-Cesario, M.M. Martínez-Montemayor, S. Morales, J. Félix, J. Cruz, M. Adorno, L. Pereira, N. Colón, C.S. Maldonado-Vlaar, S. Peña de Ortiz, Knockdown of Nurr1 in the rat hippocampus: implications to spatial discrimination learning and memory, *Learn. Mem.* 13 (2006) 734–744.
- [11] J.R. Cottrell, J.M. Levenson, S.H. Kim, H.E. Gibson, K.A. Richardson, M. Sivula, B. Li, C.J. Ashford, K.A. Heindl, R.J. Babcock, D.M. Rose, C.M. Hempel, K.A. Wiig, P. Laeng, M.E. Levin, T.A. Ryan, D.J. Gerber, Working Memory Impairment in Calcineurin Knock-out Mice Is Associated with Alterations in Synaptic Vesicle Cycling and Disruption of High-Frequency Synaptic and Network Activity in Prefrontal Cortex, *J. Neurosci.* 33 (2013) 10938–10949.
- [12] M. Crispino, G. Tocco, J.D. Feldman, H.R. Herschman, M. Baudry, Nurr1 mRNA expression in neonatal and adult rat brain following kainic acid-induced seizure activity, *Brain Res. Mol. Brain Res.* 59 (1998) 178–188.
- [13] J. Hawk, T. Abel, The role of NR4A transcription factors in memory formation, *Brain Res Bull.* 85 (2011) 21–29.
- [14] J. Hawk, A. Bookout, NR4A nuclear receptors support memory enhancement by histone deacetylase inhibitors, *J. Clin. Invest.* 122 (2012) 3593-3602.
- [15] T.G. Hazel, D. Nathans, L.F. Lau, A gene inducible by serum growth factors encodes a member of the steroid and thyroid hormone receptor superfamily, *Proc. Natl. Acad. Sci. U.S.A.* 85 (1988) 8444–8448.

- [16] C. Jiang, X. Wan, Y. He, T. Pan, J. Jankovic, W. Le, Age-dependent dopaminergic dysfunction in *Nurr1* knockout mice, *Exp. Neurol.* 191 (2005) 154 – 162.
- [17] B. Kadkhodaei, T. Ito, E. Joodmardi, B. Mattsson, C. Rouillard, M. Carta, S. Muramatsu, C. Sumi-Ichinose, T. Nomura, D. Metzger, P. Chambon, E. Lindqvist, N.G. Larsson, L. Olson, A. Björklund, H. Ichinose, T. Perlmann, *Nurr1* is required for maintenance of maturing and adult midbrain dopamine neurons, *J. Neurosci.* 29 (2009) 15923–15932.
- [18] C. Kyogoku, M. Yanagi, K. Nishimura, D. Sugiyama, A. Morinobu, M. Fukutake, K. Maeda, O. Shirakawa, T. Kuno, S. Kumagai, Association of calcineurin A gamma subunit (PPP3CC) and early growth response 3 (EGR3) gene polymorphisms with susceptibility to schizophrenia in a Japanese population, *Psychiatry Res.* 185 (2011) 16-19.
- [19] B.Y.H. Lam, W. Zhang, N. Enticknap, E. Haggis, M.Z. Cader, S. Chawla, Inverse regulation of plasticity-related immediate early genes by calcineurin in hippocampal neurons, *J. Biol. Chem.* 284 (2009) 12562–12571.
- [20] S.W. Law, O.M. Conneely, F.J. DeMayo, B.W. O'Malley, Identification of a new brain-specific transcription factor, *NURR1*, *Mol. Endocrinol.* 6 (1992) 2129–2135.
- [21] C.M. Liu, C.S. Fann, C.Y. Chen, Y.L. Liu, Y.J. Oyang, W.C. Yang, C.C. Chang, C.C. Wen, W.J. Chen, T.J. Hwang, M.H. Hsieh, C.C. Liu, S.V. Faraone, M.T. Tsuang, H.G. Hwu, *ANXA7*, *PPP3CB*, *DNAJC9*, and *ZMYND17* genes at chromosome 10q22 associated with the subgroup of schizophrenia with deficits in attention and executive function, *Biol. Psychiatry* 70 (2011) 51–58.
- [22] T. Miyakawa, L.M. Leiter, D.J. Gerber, R.R. Gainetdinov, T.D. Sotnikova, H. Zeng, M.G. Caron, S. Tonegawa, Conditional calcineurin knockout mice exhibit multiple

- abnormal behaviors related to schizophrenia, *Proc. Natl. Acad. Sci. U.S.A.* 100 (2003) 8987-8992.
- [23] M.A. Pearen, G.E.O. Muscat, Nuclear hormone receptor 4A signaling: implications for metabolic disease. *Mol. Endocrinol.* 24 (2010) 1891–1903.
- [24] P. Rojas, E. Joodmardi, Y. Hong, T. Perlmann, S.O. Ogren, Adult mice with reduced *Nurr1* expression: an animal model for schizophrenia, *Mol. Psychiatry.* 12 (2007) 756–766.
- [25] K. Saijo, B. Winner, C.T. Carson, J.G. Collier, L. Boyer, M.G. Rosenfeld, F.H. Gage, C.K. Glass, A *Nurr1*/CoREST pathway in microglia and astrocytes protects dopaminergic neurons from inflammation-induced death, *Cell.* 137 (2009) 47–59.
- [26] R.A. Screaton, M.D. Conkright, Y. Katoh, J.L. Best, G. Canettieri, S. Jeffries, E. Guzman, S. Niessen, J.R. 3rd Yates, H. Takemori, M. Okamoto, M. Montminy, The CREB coactivator TORC2 functions as a calcium- and cAMP-sensitive coincidence detector, *Cell.* 119 (2004) 61–74.
- [27] T. Sekiya, I. Kashiwagi, R. Yoshida, T. Fukaya, R. Morita, A. Kimura, H. Ichinose, D. Metzger, P. Chambon, A. Yoshimura, Nr4a receptors are essential for thymic regulatory T cell development and immune homeostasis, *Nat. Immunol.* 14 (2013) 230–237.
- [28] H. Tokuoka, Y. Goda, Myosin light chain kinase is not a regulator of synaptic vesicle trafficking during repetitive exocytosis in cultured hippocampal neurons, *J. Neurosci.* 26 (2006) 11606–11614.
- [29] H. Tokuoka, Y. Goda, Activity-dependent coordination of presynaptic release probability and postsynaptic GluR2 abundance at single synapses, *Proc. Natl. Acad. Sci. U.S.A.* 105 (2008) 14656–14661.
- [30] N. Volakakis, B. Kadkhodaei, E. Joodmardi, K. Wallis, L. Panman, J. Silvaggi, B.M. Spiegelman BM, T. Perlmann, NR4A orphan nuclear receptors as mediators of

CREB-dependent neuroprotection, *Proc. Natl. Acad. Sci. U.S.A.* 107 (2010) 12317–12322.

- [31] S. Vuillermot, E. Joodmardi, Schizophrenia-relevant behaviors in a genetic mouse model of constitutive Nurr1 deficiency, *Genes. Brain. Behav.* 10 (2011) 589–603.
- [32] Q. Xiao, S.O. Castillo, V.M. Nikodem, Distribution of messenger RNAs for the orphan nuclear receptors Nurr1 and Nur77 (NGFI-B) in adult rat brain using in situ hybridization, *Neuroscience*. 75 (1996) 221–230.
- [33] Z.Z.B. You, T. Perlmann, C. Bäckman, B.J. Hoffer, Elevated locomotor activity without altered striatal dopamine contents in Nurr1 heterozygous mice after acute exposure to methamphetamine, *Behav. Brain Res.* 143 (2003) 95–100.
- [34] R.H. Zetterström, L. Solomin, L. Jansson, B.J. Hoffer, L. Olson, T. Perlmann, Dopamine neuron agenesis in Nurr1-deficient mice, *Science* 276 (1997) 248–250.

Legends to figures

Fig. 1 Induction of Nurr1 protein levels by neural activity

- (A) Validation of Nurr1 immunocytochemical detection. Cultured cerebral cortical neurons were prepared from *Nurr1*^{+/+} and *Nurr1*^{-/-} fetuses 16 days post coitum. At 14 DIV, neurons were stimulated for 4 hr by 20 mM KCl and 20 mM bicuculline, and immunostained for Nurr1 and NeuN. Scale bar, 50 μ m.
- (B) Validation of Nurr1 immunodetection by Western blot. P0 mouse brains were homogenized and subjected to Western blot for detection of Nurr1 protein. Note that the Nurr1 signal is absent in extracts from brains of P0 *Nurr1*^{-/-} mice.
- (C) Cultured hippocampal neurons at 14 DIV were stimulated by 20 mM KCl and 20 mM bicuculline for 3 hr, and immunostained for Nurr1 and NeuN. Scale bar, 100 μ m.
- (D) Quantification of (C), showing Nurr1 signal intensity, and the ratio of Nurr1-positive cells to NeuN-positive cells. N = 3 coverslips for each condition, and n = 75 to 140 Nurr1-positive cells per coverslip. * p<0.05, t-test.
- (E) Western blot analysis of Nurr1 induction. Cultured cerebral cortical neurons were stimulated by 20 mM KCl and 20 μ M bicuculline for 3 hr, and Nurr1 expression is analyzed by Western blot. Right, quantification of Western blot, showing increase in Nurr1 protein level by stimulation.

Fig. 2 Neural activity blockade decreases Nurr1 levels in hippocampal neurons

- (A) Immunocytochemical detection of Nurr1 and NeuN in cultured hippocampal neurons in which neural activity was blocked by 1 mM TTX. Scale bar, 100 μ m.
- (B) Quantification of (A). N = 3 coverslips for each condition, and n = 38 to 77 Nurr1-positive cells per coverslip. * p<0.05, t-test.

Fig. 3 Neural activity blockade decreases Nurr1 levels in hippocampal neurons

- (A) Cultured hippocampal neurons were stimulated by 20 mM KCl and 20 mM bicuculline for 3 hr, with or without 100 mM CdCl₂, or 10 mM CNQX, 100 mM DL-APV, and 1 mM TTX. Cells were fixed and stained for Nurr1 and NeuN. Scale bar, 100 μm.
- (B) Neurons were pretreated for 5 min with or without specific inhibitors for VDCCs, and stimulated by 20 mM KCl and 20 mM bicuculline for 3 hr. Cells were fixed and stained for Nurr1 and NeuN. The following inhibitors were used: 10 mM nimodipine (L-type VDCC), 1 mM W-conotoxin-MVIIIC (P/Q-), 0.5 mM W-conotoxin-GVIA (N-), and 0.1 mM SNX-482 (R-). Scale bar, 100 μm.
- (C) Neurons were pretreated with indicated inhibitors, and then stimulated by 20 mM KCl and 20 mM bicuculline for 3 hr, followed by Western blot analysis for Nurr1. Effects of VDCC inhibitors were replicated by Western blot.

Fig. 4 Nurr1 induction is mediated by calcineurin, but not CaMK

- (A, B) Neurons were pretreated with or without 1 mM Cyclosporin A (CsA), 100 nM FK506 (both are calcineurin inhibitors), or 1 mM KN-93 (CaMK inhibitor) for 1 hr, then stimulated by 20 mM KCl and 20 mM bicuculline (K⁺/bic) for 3 hr. Expression of Nurr1 was analyzed by immunocytochemistry (A) or Western blot (B). Scale bar, 100 μm (A).

Fig 1

Fig 2

Fig 3

Fig 4

A

B