Nur77 induction and activation are necessary for interleukin-1 stimulation of proopiomelanocortin in AtT-20 corticotrophs

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Received 8 January 2004; revised 3 March 2004; accepted 8 March 2004

First published online 23 March 2004

Edited by Robert Barouki

Abstract Nur77 and Nurr1 are critical for proopiomelanocortin (POMC) regulation by corticotrophin releasing hormone (CRH) in corticotrophs. We analyze the regulation and activity of Nur77 by interleukin (IL)-1 in AtT-20 corticotrophic cells and its consequences on POMC regulation. IL-1 induces Nur77 and not Nurr1 mRNA and shows an increased transcriptional activity on the NurRE site, an effect dependent of p38 protein kinase activity. A NurRE mutation abrogates POMC promoter transcription by IL-1 and a stable AtT-20 clone overexpressing a dominant negative form of Nur77 is unresponsive to IL-1-dependent POMC induction and adrenocorticotrophin (ACTH) secretion. These results demonstrate that Nur77 is essential for POMC stimulation by IL-1 in corticotrophs.

Key words: Nur77; Interleukin-1; Corticotroph; AtT-20; Corticotrophin releasing hormone

1. Introduction

Interleukin-1 (IL-1), among other cytokines, is critically involved in the inflammatory process, and has been shown to affect hormone secretion, acting at different levels of the hypothalamic pituitary adrenal (HPA) axis [1,2]. IL-1 binding sites and receptors have been characterized both in hypothalamic and pituitary glands [3] and in the corticotrophic AtT-20 cell line [4].

IL-1 stimulates the secretion of adrenocorticotrophin (ACTH) and β-endorphin, and induces proopiomelanocortin (POMC) mRNA expression in the pituitary in vivo and in vitro [5,6]. Chronic infusion of IL-1 results in a persistent elevation of ACTH and POMC levels in rats [5,7], and IL-1β stimulates ACTH secretion from human pituitary Cushing tumors in vitro [8]. IL-1β, IL-6, and tumor necrosis factor-α stimulate the POMC promoter and potentiate the stimulatory effect of corticotrophin releasing hormone (CRH) in AtT-20 cells [9]. CRH pretreatment of rats sensitizes the pituitary to IL-1-dependent ACTH secretion [10] possible due to an increment in IL-1 receptor density [11] and IL-1 potentiates β-endorphin secretion stimulated by CRH [12].

Nur77 and Nurr1 are nuclear proteins that belong to the orphan receptor family, and play an important role in POMC induction by CRH. Two major recognition sites have been described in the POMC promoter, the NBRE site located within the nGRE site that binds one of these factors as a monomer [13], and the NurRE site, located at −404 bp from the transcription start site, that can bind these proteins as homodimers or heterodimers [14,15]. The NurRE site was proved to be more relevant for POMC promoter induction by CRH [14]. The third member of the Nurr1 subfamily (NOR1) is less relevant in stimulating the POMC promoter [15].

Stimulation by the gp130-dependent cytokines IL-6, leukemia inhibitory factor (LIF) and IL-11 of ACTH secretion and POMC mRNA expression was also proved [1,2]. In addition, IL-1 stimulates LIF mRNA levels in AtT-20 cells [16]. This implicates that IL-1 may also be part of the auto/paracrine modulation of the pituitary exerted by local production of LIF and IL-6 [1,2].

Further knowledge of IL-6, LIF and IL-11 transduction pathways involved in POMC promoter activation has been gained recently and involves the STAT pathway [17,18]. LIF and IL-11 stimulate the intracellular levels of the inhibitor of cytokine action SOCS-3 and stable overexpression of this protein inhibits ACTH release by these cytokines [19]. Although the signal transduction pathways triggered by the IL-1 receptor have been widely studied [20,21], there have not been advances on the transcription factors involved in IL-1 induction of POMC mRNA.

In this report we analyze the molecular events involved in IL-1 induction of the POMC promoter, analyzing Nur77 and Nurr1 induction and activity.

2. Materials and methods

2.1. Cell culture and stimulation

AtT-20 cells, obtained from the American Type Culture Collection (Rockville, MD, USA), were cultured in Dulbecco's modified Eagle's medium (DMEM), 10% fetal calf serum (FCS) with glutamine and antibiotics. Cells were treated as indicated for each experiment with human/rat CRH (Bachem, Heidelberg, Germany), IL-1, IL-6 (Roche Molecular Biochemicals, Mannheim, Germany), LIF or IL-11 (RyD system, Inc., Minneapolis, MN, USA). When used, the inhibitors of protein kinases, bisindolylmaleimide-1 and SB203580 (Calbiochem, San Diego, CA, USA) were added 15 min before other stimulatory treatment.

2.2. Northern blot

AtT-20 cells were plated on six-well plates and 24 h later stimulated in serum-free DMEM. RNA was isolated by the guanidine isothio-

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0014-5793/04/$30.00 © 2004 Published by Elsevier B.V. on behalf of the Federation of European Biochemical Societies.
doi:10.1016/S0014-5793(04)00303-5

cyanate followed by the phenol-chloroform method [22]. RNA extraction, denaturing gel electrophoresis, Northern blotting, preparation of radiolabeled probes, and hybridization procedures were performed as described previously [22]. For detection of Nur77 mRNA a *Pvu*II fragment of 1.4 kb from Nur77 cDNA was used [14,23]. For detection of Nurr1 mRNA a *Hin*dIII/*Pst*I fragment of 500 bp was used [24]. A 1-kb *Pst*I cDNA was used as actin probe [25]. The cDNA probes were labeled with a random-primed kit with [α-^{32}P]deoxycytidine triphosphate (dCTP) (Amersham, Buckinghamshire, England, UK). POMC cRNA [26] probe was generated by in vitro transcription using the T3 RNA polymerase in the presence of [α^{32}P]uridine triphosphate (UTP).

2.3. Transient transfection and reporter luciferase, chloramphenicol acetyl transferase (CAT) and β-galactidase (gal) assays

AtT-20 cells were plated on six-well plates and transfected with lipofectamine (Invitrogen, Frederik, MD, USA) as previously described [27] with 500 ng of the reporter plasmids NurRE-luciferase (LUC), NBRE-LUC (that contain three copies of NurRE or NBRE sites coupled to the minimal POMC (−34/+63) promoter respectively), POMC-LUC, POMC(NurREmut)-LUC [14,23], (−480, −336, −281, −163) Nur77 promoter coupled to the CAT gene (Nur77-CAT) [28] or KB-LUC [29], in conjunction with a control RSV-βgal construction (200 ng). After overnight washout, cells were stimulated for 2 or 6 h in serum-free DMEM as indicated. LUC, CAT and β-gal activities were measured as previously described [27]. Results were standardized for β-gal activity.

2.4. Stable transfection

48 h after transfection with the Nur77 dominant negative (pHbNur77ZnCT) expression vector [30], and selection with geneticin antibiotic (300 mg/ml) (Invitrogen, Frederik, MD, USA) for 3 weeks, constitutive expression of the dominant negative Nur77 form was tested by Northern blot using the Nur77 fragment as probe.

2.5. Hormone determination

ACTH was determined by radioimmunoassay (RIA) as we previously described [31].

2.6. Statistical analysis

Statistics were performed using one-factor analysis of variance (ANOVA) in combination with the Scheffe's test. Results are expressed as means ± S.E.M.

3. Results

3.1. Activation and induction of Nur77 by IL-1

IL-1 but not other cytokines that stimulate POMC/ACTH [1,2,5,6,9,18,19], such as IL-6, IL-11 or LIF, stimulates transcription of a construct containing only the POMC NurRE site (NurRE-LUC) (Fig. 1A). This effect is specific for the NurRE site, whereas the NBRE site does not respond to IL-1, but responds, as described [14] to CRH treatment (Fig. 1B).

In correlation with an increased transcription at the NurRE site, IL-1 induces Nur77 mRNA at 1 h returning to basal at 2 h (Fig. 1C). Contrarily, no induction of Nurr1 mRNA was detected at any time (Fig. 1C). This stimulatory effect is due to an increase in Nur77 transcription as (−480, −336 and −281) Nur77 promoter constructs are also stimulated by IL-1. Contrarily, the −163 construct is unresponsive to IL-1 stimulation showing that the −281–163 fragment confers IL-1 sensitivity (Fig. 1D).

The stimulatory effects of IL-1 on NurRE-dependent transcription and Nur77 mRNA induction are blocked by SB203580, a specific inhibitor of p38, and not by bisindolylmaleimide, a specific inhibitor of protein kinase C (PKC) (Fig. 2A and B). This implicates p38 activation as essential for IL-1 stimulation.

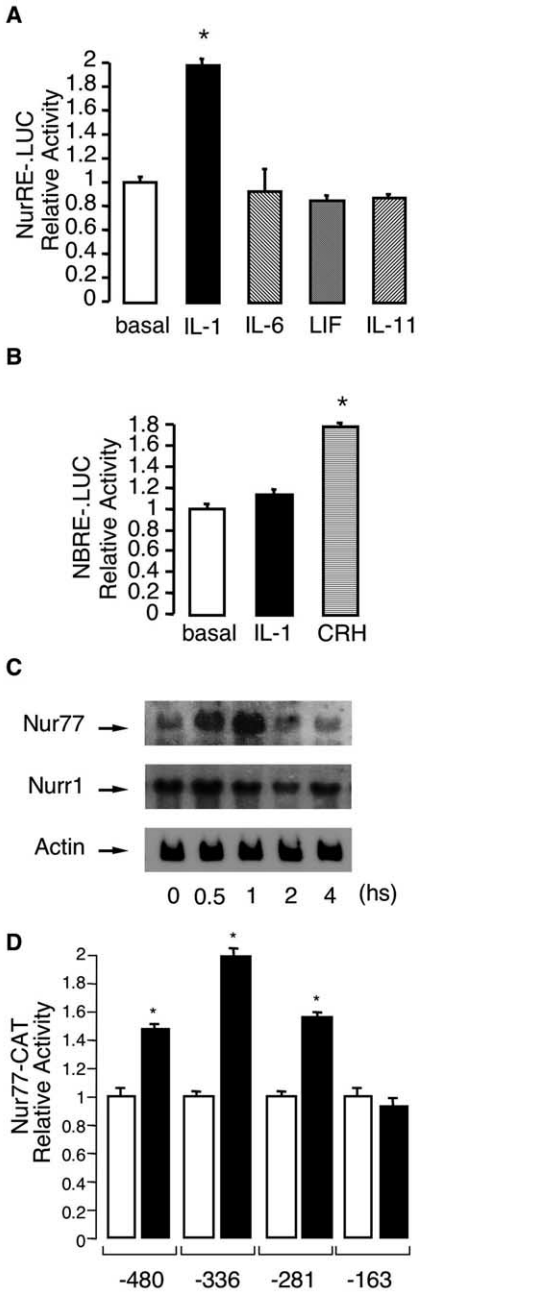

Fig. 1. IL-1 stimulates Nur77 and NurRE activity in AtT-20 cells. Relative activity of NurRE-LUC (A) or NBRE-LUC (B) in transfected AtT-20 cells under IL-1 (100 U/ml), IL-6 (2000 U/ml), LIF (20 ng/ml), IL-11 (10 ng/ml), or CRH (100 nM) stimulation. Means ± S.E.M. of four independent experiments ($n = 3$ each, $^*P < 0.001$ with respect to corresponding basal levels). C: Northern blot analysis of AtT-20 cells stimulated at the indicated times with IL-1 (100 U/ml). One representative out of three independent experiments with similar results. D: Relative activity of −480, −336, −281 and −163 promoter Nur77-CAT constructs in transfected AtT-20 cells unstimulated (white bars) or stimulated for 1 h with IL-1 (100 U/ml) (black bars). Means ± S.E.M. of two independent experiments ($n = 3$ each, $^*P < 0.001$ with respect to the corresponding basal levels).

3.2. IL-1 and CRH synergize on Nur77 activity

The enhancement of POMC induction [9] and ACTH secretion [12,32] by CRH, induced by IL-1 pretreatment (Fig. 3B) is observed also on Nur77 activity (Fig. 3A).

3.3. The NurRE site is essential for POMC transcription by IL-1 stimulation

POMC stimulation by IL-1 depends on the NurRE site, as a construct with a mutation on NurRE (POMC(NurREmut)-LUC) that does not bind Nur77 [14] is not stimulated by IL-1, contrarily to the wild-type POMC-LUC construct (Fig. 4).

3.4. The AtT20N77 cell line is unresponsive to NurRE-dependent transcription, POMC mRNA induction and ACTH secretion by IL-1

As an additional approach for the significance of the NurRE site for POMC transcription, we generated and functionally checked a stable transfected cell line that constitutively expresses a dominant negative form of the Nur77 protein [30], denominated AtT20N77 (Fig. 5A). AtT20N77 cells are less responsive (5- vs. 25-fold) to CRH stimulation and do not respond to IL-1 stimulation of NurRE-LUC (Fig. 5B). This effect is specific for Nur77 activity as both AtT20N77 and AtT-20 cells increase nuclear factor (NF)-κB activity to similar levels as by IL-1 stimulation (data not shown).

Contrarily to IL-1 stimulation of POMC mRNA levels and

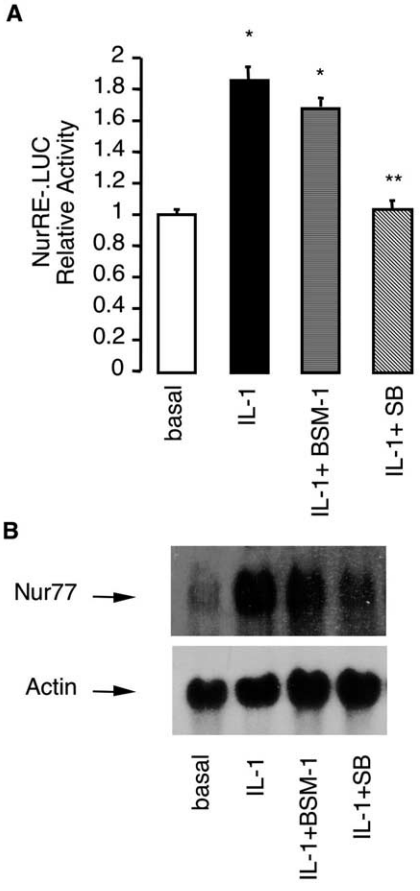

Fig. 2. p38 activity mediates IL-1 stimulation of Nur77. Relative activity of NurRE-LUC in transfected AtT-20 cells (A) or Northern blot analysis of AtT-20 cells (B) stimulated with IL-1 (100 U/ml), IL-1+bisindolylmaleimide-1 (BSM-1) (10 mM) or IL-1+SB203580 (SB) (10 mM). A: Means ± S.E.M. of three independent experiments ($n = 3$ each, $^*P < 0.001$ with respect to basal levels; $^{**}P < 0.001$ with respect to IL-1 levels). B: One representative out of three independent experiments is shown.

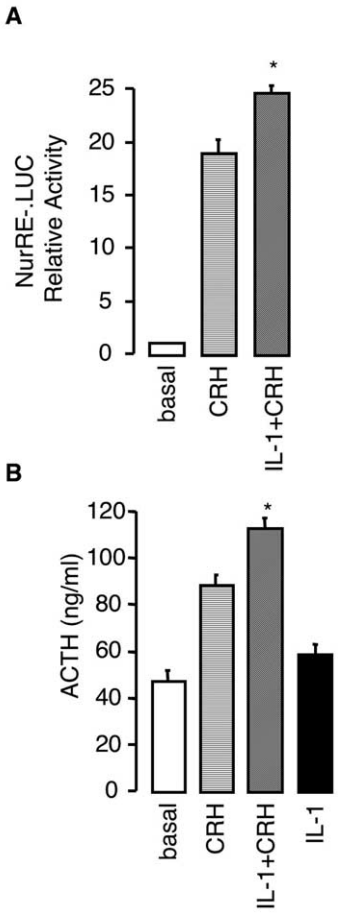

Fig. 3. IL-1 increases CRH induction of NurRE activation. Relative activity of NurRE-LUC in transfected AtT-20 cells (A) or ACTH secretion in AtT-20 cells (B), after 16 h pretreatment with IL-1 (100 U/ml), washout, and further 6 h (A) or 1 h (B) stimulation with CRH (100 nM). A: Means ± S.E.M. of two independent experiments ($n = 3$ each, $^*P < 0.05$ with respect to CRH levels). B: One representative, out of three independent experiments ($n = 4$ each, $^*P < 0.05$ with respect to CRH treatment).

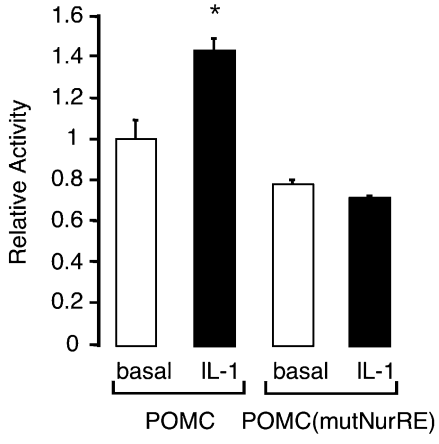

Fig. 4. NurRE confers IL-1 stimulation of POMC promoter. Relative activity of POMC-LUC or POMC(NurREmut)-LUC in transfected AtT-20 cells, stimulated 2 h with IL-1 (100 U/ml), as indicated. Means ± S.E.M. of two independent experiments ($n = 3$ each, $^*P < 0.01$ with respect to basal levels).

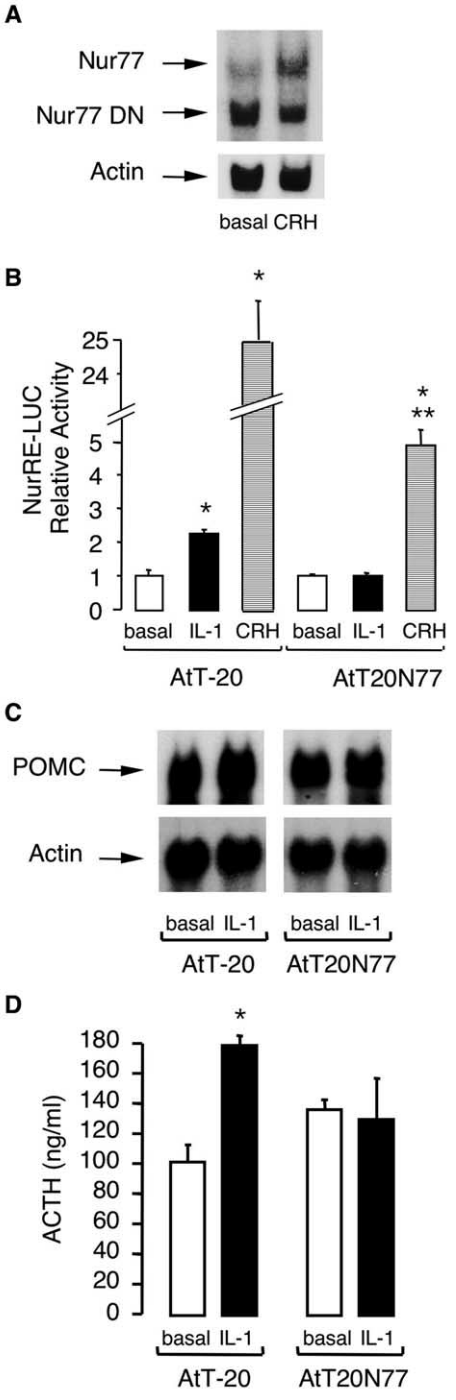

Fig. 5. Nur77 is essential for POMC mRNA induction and increased ACTH secretion by IL-1. A: Northern blot analysis of the AtT20N77 clone under basal and CRH (100 nM)-stimulated condition for detection of constitutive dominant negative Nur77 expression (Nur77 DN). B: Relative activity of NurRE-LUC in transfected AtT-20 and AtT20N77 cells under IL-1 (100 U/ml) or CRH (100 nM) stimulation. Means ± S.E.M. of three independent experiments ($n = 3$ each, $*P < 0.001$ with respect to the corresponding basal levels, $**P < 0.001$ with respect to CRH-stimulated AtT-20 cells). Northern blot analysis (C) or ACTH secretion (D) of AtT-20 and AtT20N77 cells stimulated for 18 h with IL-1 (100 U/ml). C: One representative out of three independent Northern blots is shown. D: One representative out of two independent experiments ($n = 4$ each, $*P < 0.01$ with respect to basal levels).

ACTH secretion in AtT-20 cells [6], IL-1 is unable to stimulate AtT20N77 cells (Fig. 5C and D).

4. Discussion

Recently, the molecular pathways involved in CRH induction of POMC mRNA were clarified, and involve the orphan receptor subfamily proteins Nur77, Nurr1 and NOR1 [13–15,23].

In this paper we demonstrate that IL-1 effects on POMC mRNA induction involve also Nur77 (but not Nurr1) induction and activity. Stimulation at the NurRE site by IL-1 is dependent on p38 protein kinase activity. We demonstrate that Nur77 is essential for the function of IL-1 in corticotroph cells: (a) the POMC promoter with the mutated NurRE site is unresponsible to IL-1 stimulation and (b) the induction of POMC mRNA, stimulation of ACTH secretion and increased transcriptional activity at the NurRE site are blocked in AtT20N77 cells, which express a Nur77 dominant negative form. The functional relevance of Nur77 induction by IL-1 is underlined by the fact that the IL-1–CRH synergism on ACTH secretion correlates with the synergism in Nur77 transcriptional activity.

The insensitivity of the NBRE site to IL-1 correlates with a preponderant role of NurRE to CRH stimulation of POMC [14] and may be a consequence of different responsiveness of each site, as CRH produces a 20-fold increase in NurRE-LUC, and only a 2-fold increase in NBRE-LUC [14].

IL-1 stimulates Nur77 and not Nurr1 mRNA. Nur77 homodimeric binding may be involved in NurRE-dependent activation by IL-1 treatment, although we can not rule out possible heterodimeric formation among Nur77 and NOR1 or preexisting Nurr1 protein. The strict dependence of Nur77 is also confirmed, as overexpression of a dominant negative form in a stable transfected cell line abolishes NurRE-dependent transcription, POMC mRNA induction and ACTH secretion to IL-1 treatment.

Mutual modulatory action of CRH and IL-1 at the pituitary for control of β-endorphin and ACTH secretion was described. CRH and IL-1 produce a higher increase in β-endorphin and ACTH secretion with respect to CRH alone [9–12].

IL-1 has been shown to modulate CRH receptors in the pituitary [5] and CRH treatment affects IL-1 sensitivity of pituitary in vivo [10] and in vitro [11]. We demonstrate that IL-1 and CRH synergize to increase transcription at the NurRE site, this effect provides a molecular basis for the synergism between IL-1 and CRH on ACTH secretion and POMC mRNA induction.

IL-1 stimulation triggers different signal transduction pathways (PKC, JunK and p38 protein kinase pathways, among others) upon interaction of the IL-1 receptor with accessory proteins such as the IL-1 receptor accessory protein (IL-1RAcP) and TRAF proteins [20]. We show that the p38 protein kinase but not the PKC activity is necessary for NurRE-dependent transcription. Contrarily, the inhibitory effect of p38 on Nur77 mRNA was less marked, implicating that some other signal is involved in Nur77 mRNA increase. Direct stimulation of Nur77 transcriptional activity by p38 cannot been ruled out. In concordance, several phosphorylation sites, present in the Nur77 protein, may be the target for p38 regulation. IL-1 treatment of AtT-20 cells stimulates the phos-

phorylation of a protein with the same molecular weight than Nur77 [32]. Further studies are required to identify whether p38 modulation is exerted by direct phosphorylation of Nur77 as is the direct phosphorylation of Nur77 by extracellular signal-regulated kinase (ERK)2 [27].

This work unravels for the first time the molecular pathways involved in pituitary POMC mRNA induction and ACTH-stimulated secretion by IL-1, and demonstrates a strict Nur77 dependence for IL-1 effects in corticotrophic cells.

Acknowledgements: We thank Dr. J. Drouin (Montreal, QC, Canada) for providing us with the NurRE-LUC, the NBRE-LUC and the POMC(NurREmut)-LUC constructs, Dr. Malcom Low (Portland, OR, USA) for the POMC-LUC construct, Dr. N. Ohkura (Tokyo, Japan) for the Nurr1 cDNA, Dr. A. Winoto (Berkeley, CA, USA) for the Nur77-CAT constructs and the Nur77 dominant negative expression vector, Dr. M. Bell (Rochester, MN, USA) for the KB-LUC plasmid. This work was supported by grants from the University of Buenos Aires, the Max Planck Institute of Psychiatry, the Argentine National Research Council (CONICET) and Agencia Nacional de Promoción Científica y Tecnológica-Argentina.

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