

# The NMDA receptor antagonist MK-801 abolishes the increase in both p53 and Bax/Bcl2 index induced by adult-onset hypothyroidism in rat

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Hypothyroidism affects neuron population dynamics in the hippocampus of the adult rat, with neuronal damage as the main feature of its effect. This effect is prevented by the blockade of NMDA receptors, which suggests that glutamatergic activity mediates cell death in this condition. Glutamate can also stimulate cell proliferation and survival of newborn neurons, indicating that it can affect different stages of the cell cycle. In this work we measured the expression of specific proteins that control cell proliferation (cyclin-D1), cell arrest (p21), damage (p53) or apoptosis (Bax and Bcl2) in the hippocampus of hypothyroid rats treated with the NMDA receptor (NMDAR) blocker MK-801 during the induction of hypothyroidism. The results show that hypothyroidism increases the expression of markers of DNA damage, cell arrest, and apoptosis, but does not affect the marker of cell proliferation. NMDAR blockade prevents the increase on markers of DNA damage and apoptosis, but does not influence cell arrest or cell proliferation. This suggests that hypothyroidism promotes cell death mainly by an excitotoxic effect of glutamate.

**Key words:** hippocampus, thyroid hormones, cell cycle, neuronal damage

Although it is known that thyroid hormones (THs) are essential for the development of the nervous system during early life, recent reports have provided evidence for their importance in modulating physiological events in the adult nervous system as well (Sala-Roca et al. 2008, Giné et al. 2010). Among these actions, THs are necessary for the maintenance of cell populations in some proliferative regions of the adult brain, particularly the hippocampus (Desouza et al. 2005). We have found that the induction of hypothyroidism in adulthood causes significant neuronal damage in the four CA fields of the hippocampus accompanied by the triggering of the apoptotic pathway and no changes in proliferation (Alva-Sánchez et al. 2004, 2009b). This

suggests that THs maintain neuronal populations mainly by controlling cell death. Besides cell survival, THs also promote cell proliferation, as evinced by the fact that hypothyroidism reduces the survival and differentiation of newborn cells and the number of immature neurons in the adult brain (Ambrogini et al. 2005, Desouza et al. 2005, Montero-Pedrazuela et al. 2006). These TH effects involve the modification of the cell cycle by affecting the expression of regulatory proteins that determine both cell division and cell death by apoptosis (Alva-Sánchez et al. 2009b).

The administration of the NMDA-receptor (NMDAR) antagonist MK-801 during the induction of hypothyroidism reduces the neuronal damage in the pyramidal cell layer suggesting a direct THs/glutamate interaction in the cellular maintenance of the hippocampus (Alva-Sánchez et al. 2009a). It is well known that overactivation of NMDARs causes oxida-

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tive stress and cell death (Hardingham and Bading 2003), but NMDARs could also be involved in the control of cell proliferation, since NMDA antagonists increase neurogenesis in the dentate gyrus of the hippocampus (Nacher and McEwen 2006). This evidence suggests that the effects of hypothyroidism on neuron population survival in the hippocampus could be mediated, at least partially, by the activation of local NMDARs. To assess this possibility we measured the effect of hypothyroidism on the expression of cell cycle-regulatory proteins and the way this expression is affected by NMDAR blockade.

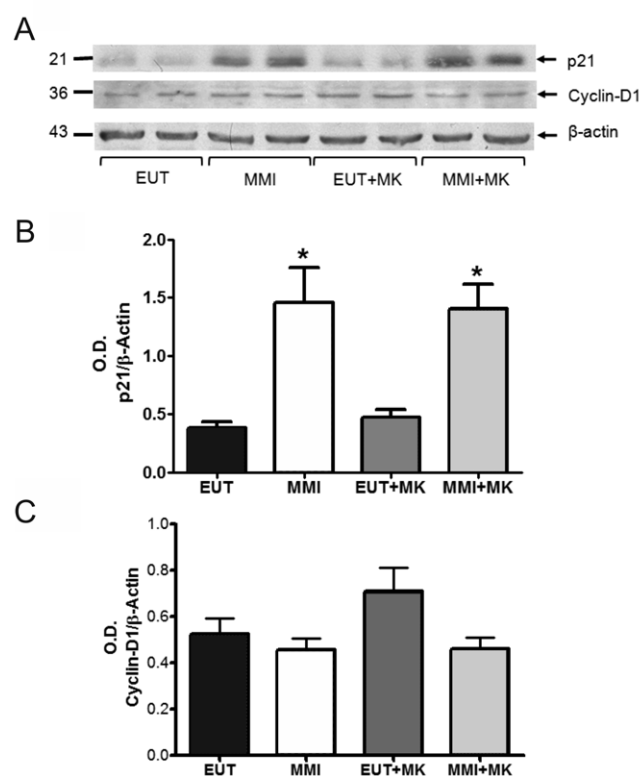


Fig. 1. Effect of NMDA antagonist treatment on protein expression of the main cellular cycle-related molecules – p21 and Cyc D1 – in the hippocampus of hypothyroid adult rat. (A) Western blot analyses for p21, Cyc D1 and β-actin proteins in two representative samples of whole hippocampus corresponding to rats of the four experimental groups. (B–C) Densitometric analysis of three separate experiments after they were corrected for β-actin expression to obtain equal loading. Data are expressed as means ± SEM in experimental groups and presented in a graphic form for p21 (B) and Cyc D1 (C). Comparisons of the individual treatment vs. euthyroid group were analyzed with ANOVA followed by a Newman–Keuls *post-hoc* test.  $n=3$  for each group. \* $P<0.05$ .

Twelve male Wistar rats weighing 250–350 g at the beginning of the experiment were individually housed in a temperature ( $24\pm1^\circ\text{C}$ ) and light (12:12) regulated room, with food and water *ad libitum*. Handling and euthanasia of animals were reviewed and approved by *ad hoc* ethics committees of UNAM and IPN; the experimental procedures in this study complied with the Institutional Animal Care and Use Committee guidelines. All efforts were made to minimize the number of animals used and their suffering. Animals were distributed randomly into 4 groups ( $n=3$ ): two euthyroid groups (EUT) received tap water, whereas the two remaining groups (MMI) received approximately 60 mg/kg of methimazole (MMI; Sigma, USA) dissolved in their drinking water for the induction of hypothyroidism as described before (Alva-Sánchez et al. 2004). One of the hypothyroid groups (MMI+MK) as well as one euthyroid group (EUT+MK) simultaneously received daily injection of the NMDAR antagonist MK-801 (0.5 mg/kg/day; Sigma, USA). Treatments were administered during four weeks, in which colonic temperature, body weight, and water intake were monitored every three days. The MMI concentration was adjusted to water intake and body weight every three days.

Blood samples were taken from the rat's tail before and after treatments in all groups. Serum was obtained by centrifugation ( $3\,500\text{ rpm} \times 15\text{ min}$ ) and stored at  $-20^\circ\text{C}$  until  $T_3$  and  $T_4$  determination. Total  $T_4$  ( $\mu\text{g/dL}$ ) and  $T_3$  ( $\text{ng/dL}$ ) levels were assessed by immunoassay (EIA), using the commercial kit from DSL (USA). At the end of the treatment, rats were sacrificed by decapitation and their brains were quickly removed to dissect the hippocampal formation, which was stored at  $-70^\circ\text{C}$  until processing.

Hippocampal formations were homogenized at  $4^\circ\text{C}$  in 2 mL of RIPA buffer (50 mM TRIS-HCl, 105 mM NaCl, 1% Triton X-100, 1% sodium deoxycholate, 0.1% SDS and 2 mM EDTA) containing the proteinase inhibitor cocktail Mini Complete (Roche, Germany). Lysates were centrifuged at  $13\,000\text{ rpm}$  at  $4^\circ\text{C}$  and the supernatants were stored at  $-70^\circ\text{C}$  until use. Protein contents of the extracts were measured by a commercial Bradford assay kit (Bio-Rad, USA). Aliquots containing 50  $\mu\text{g}$  of protein were heated to  $96^\circ\text{C}$  for 5 minutes into a SDS loading buffer (10% glycerol, 2% SDS, 62.5 mM TRIS-HCl, with 5% of 2-mercaptoethanol). Samples were separated in 12% SDS polyacrylamide gels and then transferred to nitrocellulose membranes (0.45  $\mu\text{m}$ , Bio-Rad, USA) using a semi-dry

transfer system. Membranes were washed in TRIS buffered saline (TBS), blocked with 5% non-fat milk for three hours and incubated with the primary antibody at 4°C overnight. The antibodies used were rabbit polyclonal anti-Bax (SC-493) and anti-Cyclin-D1 (SC-718); mouse monoclonal anti-p21 (SC-51689), anti-Bcl-2 (SC-509) and anti-p53 (SC-126); and goat polyclonal anti- $\beta$ -actin (SC-1616), all of them from Santa Cruz Biotechnology (USA), at a final dilution of 1:150 (p53 and CycD1), 1:200 (p21), 1:300 (Bax and Bcl-2) and 1:1 000 ( $\beta$ -actin). Protein bands were visualized with the ECL detection system (Amersham, UK); subsequently images were analyzed with the Scion Image software and normalized to  $\beta$ -actin. Immunoblotting was conducted by triplicate.

Results are expressed as means  $\pm$  SEM. The data on body weight, colonic temperature and thyroid hormone levels were analyzed by repeated-measures two-way ANOVA tests followed by Student-Newman-Keuls *post hoc* tests. Results on immunoblotting were analyzed by one-way ANOVA and Student-Newman-Keuls *post hoc* tests. Values of  $P < 0.05$  were considered statistically significant.

Chronic MMI administration in rats caused signs indicative of a hypothyroid condition. At the end of the treatment both MMI treated groups (MMI and MMI+MK) showed a significant decrease in body weight gain ( $F_{3,8}=26.7$ ,  $P < 0.001$ ; interaction  $F_{3,8}=33.9$ ,  $P < 0.001$ ) and colonic temperature ( $F_{3,8}=4.6$ ,  $P < 0.05$ ; interaction  $F_{3,8}=4.6$ ,  $P < 0.05$ ) as compared to controls (Table I). Of note, the simultaneous administration of MMI and MK-801 caused a lower body weight gain than MMI alone. The hypothyroid status was confirmed by lower  $T_3$  ( $F_{3,8}=7.7$ ,  $P < 0.01$ ; interaction  $F_{3,8}=10.0$ ,  $P < 0.01$ ) and  $T_4$  ( $F_{3,8}=10.5$ ,  $P < 0.01$ ; interaction  $F_{3,8}=10.9$ ,  $P < 0.01$ ) circulating levels at the end of the MMI treatment, as compared to the basal levels and to the control group.

The expression of the cell proliferation marker Cyclin-D1 was not modified by the antithyroid treatment (groups MMI and MMI+MK) nor by the NMDA blocker MK-801 (Figs 1A and 1C). This indicates that cell proliferation in the hippocampus is not altered significantly in these conditions. On the other hand, the marker of cell arrest, p21, was significantly elevated in hypothyroid rats ( $F_{3,8}=9.4$ ,  $P = 0.005$ ; Figs 1A and 1B). The increase in p21 was not prevented by the simultaneous blockade of NMDARs (group MMI+MK). In agreement with previous reports, we found that the hippocampi of hypothyroid rats exhib-

ited increased expression of p53, a marker of DNA damage ( $F_{3,8}=7.4$ ,  $P = 0.011$ ) and higher Bax/Bcl-2 ratio, indicative of apoptosis ( $F_{3,8}=13.3$ ,  $P = 0.002$ ), as compared to controls (Fig. 2). In contrast to p21, the increase in these two markers of cell damage was abolished by the simultaneous treatment with MK-801, so that the values of the MMI+MK group were similar to controls' and significantly lower than those of the MMI group.

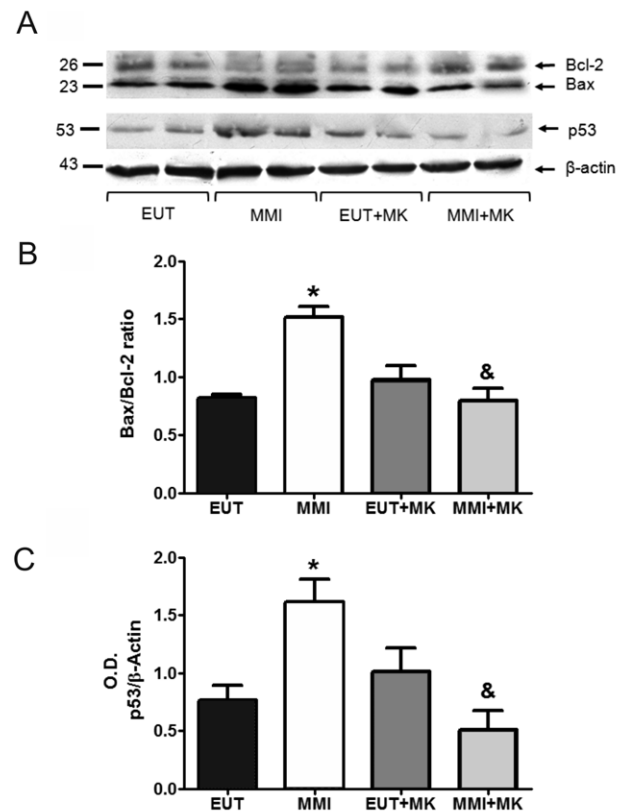


Fig. 2. Effects of NMDA antagonist treatment on the Bax/Bcl-2 ratio and p53 expression in the hippocampus of hypothyroid adult rat. (A) Western blot analyses for p53, Bcl-2, Bax, and  $\beta$ -actin proteins in two representative samples of whole hippocampus corresponding to rats of the four experimental groups. (B) The Bax/Bcl-2 ratio was calculated from densitometry absorbance values of three separate experiments after they were corrected for  $\beta$ -actin expression to obtain equal loading. Bax/Bcl-2 ratios from experimental groups are presented in the graph as means  $\pm$  SEM. (C) p53 densitometry analysis of three separate experiments after they were corrected for  $\beta$ -actin expression to obtain equal loading. Data are expressed as means  $\pm$  SEM and presented graphically. Comparisons of the individual treatment vs. euthyroid group were analyzed with an ANOVA followed by a Newman-Keuls *post-hoc* test.  $n=3$  for each group. \* $P < 0.05$  vs. EUT group; & $P < 0.05$  vs. MMI group.

Table I

Thyroid status for the Euthyroid, Hypothyroid, and MK-801 supplemented groups								
	EUT (n=3)		MMI (n=3)		EUT+MK (n=3)		MMI+MK (n=3)	
	Basal	4 <sup>th</sup> week	Basal	4 <sup>th</sup> week	Basal	4 <sup>th</sup> week	Basal	4 <sup>th</sup> week
Body weight (g)	292±6	358±9	300±2	284±5*	296±9	360±10	301±7	234±11**
Colonic temperature (°C)	38.0±0.1	38.0±0.2	38.2±0.1	36.9±0.3*	38.3±0.5	37.9±0.2	38.1±0.1	36.5±0.2*
T <sub>4</sub> (µg/dL)	12.5±1.6	14.0±1.9	11.2±1.2	2.4±1.3*	11.5±1.0	12.9±1.2	13.2±1.1	2.9±1.4*
T <sub>3</sub> (ng/dL)	40.4±5.7	39.3±5.5	41.8±4.3	9.2±3.2*	37.9±4.8	41.1±3.1	40.5±3.9	8.7±2.9*

\* $P < 0.05$  vs. Euthyroid group at the same treatment time; \*\* $P < 0.05$  vs. MMI group at the same week

Thyroid hormones play a major role in the maintenance of the hippocampal cell population. THs have been shown to affect both proliferative and apoptotic rates in proliferative niches (Puzianowska-Kuznicka et al. 2006), thus suggesting that these hormones regulate the progression of cell cycle in the proliferative cell populations. In line with this, we have previously shown that the thyroid status of the individual affects the expression of protein markers that signal the stages of the cell cycle (Alva-Sánchez et al. 2009b).

Glutamatergic activity is necessary for neuronal survival and migration but it can also cause cell death (Hardingham and Bading 2003). In particular, glutamatergic NMDARs are involved in the control of both neurogenesis (Nacher and McEwen 2006) and cell death in the hippocampus (Camacho and Massieu 2006), and seem to mediate some of the hippocampal effects of hypothyroidism (Alva-Sánchez et al. 2009a). In this work we assessed the participation of NMDARs in the alterations of the cell cycle in the hippocampus caused by hypothyroidism.

We induced hypothyroidism to a group of rats. This condition resulted in a reduction in body weight gain and colonic temperature, which is in line with the known actions of THs on metabolic rate (Silva 2006), release of growth hormone (Laron 2003), and food intake (Kong et al. 2004). The thyroid status of the rats was further confirmed by the reduction in serum T<sub>3</sub> and T<sub>4</sub> levels. As expected, the treatment with MK-801 did not cause any effect on these variables when administered alone, nor did modify the induction of hypothyroidism when administered along with the antithyroid treatment. We then measured the expression of proteins indicative of specific stages of

the cell cycle in hippocampus extracts of these rats. Cyclin-D1 promotes the transition from G1 phase to S phase in response to mitogenic signals, and thus it promotes cell replication (Sherr and Roberts 1999). In this study, cyclin-D1 was not altered by hypothyroidism, which indicates that cell proliferation is not affected in this condition. This agrees with previous results showing that T<sub>3</sub> treatment, but not hypothyroidism, modifies the expression of cyclin D1 in the hippocampus (Alva-Sánchez 2009b). The administration of MK-801 increased slightly the expression of cyclin-D1 but kept it unmodified in hypothyroid animals. Based on the fact that MK-801 prevents neuronal damage provoked by hypothyroidism, the present results indicate that this form of neuroprotection does not depend on the activation of cell proliferation.

When DNA is damaged in the S phase, p53 tumor suppressor protein induces p21 increase for cycle arrest, allowing DNA repair. p53 can also induce apoptosis by raising Bax levels and leading to caspase activation (Miller et al. 2000). The increase in Bax relative to the antiapoptotic protein Bcl-2 implies that the cell is engaged in a process of cell death by apoptosis (Yuan and Yankner 2000). In our results, hippocampi of hypothyroid animals showed increased expression of p53 and p21 along with a rise in the Bax/Bcl-2 ratio, which correspond to a situation of generalized DNA damage and cell cycle arrest together with an increase in cell death rate by apoptosis in the hippocampus. This is consistent with previous reports and accounts for the increased neuronal damage seen in the hippocampus of hypothyroid rats (Desouza et al. 2005, Alva-Sánchez et al. 2009b).

It has been demonstrated that hippocampal neurons exposed to glutamate show increased Bax protein levels dependent on p53, presumably as an excitotoxic effect of this neurotransmitter (Xiang et al. 1998). In the rat hippocampus *in vivo*, the neuronal damage caused by hypothyroidism is prevented by blocking the NMDAR-mediated glutamatergic stimulation (Alva-Sánchez et al. 2009a), thus suggesting that cell damage in hypothyroid animals is related to excitotoxicity by glutamate. The present results confirm this view by showing that the treatment with the NMDA blocker MK-801 prevents the rise in both p53 and Bax/Bcl-2 index caused by hypothyroidism. This can be interpreted as the following concatenation of events: the hypothyroid condition promotes the NMDAR activation, which in turn increases p53 expression, probably by increasing DNA damage, and triggers apoptosis leading to cell death.

The relationship between THs, NMDA activity and cell fate is puzzling, however. Controversial data show, on one hand, increased glutamate-associated excitability in adult-onset hypothyroidism (Giné et al. 2010), thus implicating excitotoxicity as a main process in this condition; but, on the other hand, it has been recently reported that the release of glutamate in the CA3-hippocampal region is reduced in hypothyroid rats *in vivo*, without effect on high-affinity glutamate transporters (Sánchez-Huerta et al. 2012). Moreover, the stimulation of NMDARs is known to promote neuron survival by stimulating the expression of CREB-dependent protective proteins (Hardingham and Bading 2003) and BDNF (Jiang et al. 2005). These paradoxical effects of NMDARs on cell fate could be related to the different actions of these receptors according to their location on the target cell: synaptic NMDARs seem to be associated with neuroprotection, whereas the extra-synaptic NMDARs may be related to neurodegeneration (Ivanov et al. 2006).

It has been demonstrated that THs can modify glutamatergic neurotransmission in hippocampus by nongenomic actions (Caria et al. 2009). However, hypothyroid animals show reduced glutamate release (Sánchez-Huerta et al. 2012), which suggests that the neuronal damage caused by hypothyroidism is not mediated by an increased stimulation of the NMDARs. Alternatively, an effect of the THs on the NMDAR activity could be postulated. It has been proposed that the NR2A subunit of the NMDAR is implicated in neuroprotection whereas the NR2B subunit medi-

ates neuronal damage (Liu et al. 2007). These two subunits are expressed differently in synaptic and extrasynaptic NMDARs (Liu et al. 2007), so that glutamate could have opposite effects on cell survival according to the location of its receptors. Finally, it has been shown that the thyroid status of the individual modifies the expression of the NR2B subunit in the hippocampus of the adult rat (Lee et al. 2003, Kobayashi et al. 2006). On this basis we hypothesize that neuronal damage in our study could be due to an increased activity of the extrasynaptic NMDARs promoting apoptosis. This possibility deserves further investigation.

Besides apoptosis, the rise in p53 in the present work caused cell arrest by increasing p21 expression. Accordingly, hypothyroid animals showed increased levels of both p53 and p21. However, the blockade of NMDA activity prevented the rise in p53 but had no effect on p21 levels, which implies that hypothyroidism affects the expression of these two proteins by independent mechanisms. Independent regulation of p53 and p21 has been documented in other tissues (Macleod et al. 1995). Moreover, it has been proposed that p21 could act not only to arrest the cell cycle during DNA damage but also to maintain a status of quiescence in proliferative cells in order to delay replication and to keep a population of proliferative cells throughout the life span of the individual (Kippin et al. 2005). Thyroid hormones could be involved in the control of p21, since mutant cells lacking functional p53 show increased p21 and cell arrest when T<sub>3</sub> is restrained (Toms et al. 1998). On this basis, our data suggest that the hypothyroid condition stimulates cell arrest in the hippocampus by increasing p21 expression independently of p53, probably as a compensatory mechanism to maintain cell populations when DNA damage increases.

In summary, our results suggest that NMDAR-mediated glutamatergic activity is involved in cell damage caused by hypothyroidism, by promoting DNA damage and apoptosis. This effect could be due to changes in the distribution of NMDARs in the target neurons. Further studies with other experimental models are necessary to investigate this possibility.

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