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ORIGINAL ARTICLE

Adenosine, dopamine and serotonin receptors imbalance in lymphocytes of Lesch-Nyhan patients

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Abstract Lesch-Nyhan disease (LND) is caused by complete deficiency of the hypoxanthine-guanine phosphoribosyltransferase enzyme. It is characterized by overproduction of uric acid, jointly with severe motor disability and self-injurious behaviour which physiopathology is unknown. These neurological manifestations suggest a dysfunction in the basal ganglia, and three neurotransmitters have been implicated in the pathogenesis of the disease: dopamine, adenosine and serotonin. All of them are implicated in motor function and behaviour, and act by binding to specific G-protein coupled receptors in the synaptic membrane where they seem to be integrated through receptor–receptor interactions. In this work we have confirmed at protein level the previously reported increased expression of DRD5 and the variably aberrant expression of ADORA2A, in LND PBL respect to control PBL. We have also described, for the first time, a decreased expression and protein level of 5-HTR1A in LND PBL respect to control PBL. If these results were confirmed in the Lesch-Nyhan patients basal ganglia cells, this would support the hypothesis that pathogenesis of neurological manifestations of Lesch-Nyhan patients may be related to an imbalance of neurotransmitters, rather than to the isolated disturbance of one of the neurotransmitters, and this fact should be taken into account in the design of pharmacologic treatment for their motor and behavioural disturbances.

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Introduction

Lesch-Nyhan disease (LND) is a neurogenetic disorder caused by complete deficiency of the hypoxanthine-guanine phosphoribosyltransferase (HPRT) enzyme activity (Lesch and Nyhan 1964; Seegmiller et al 1967; Torres and Puig 2007). HPRT deficiency is inherited as a recessive X-linked trait and it is due to mutations in the HPRT1 gene (Torres et al 2011). HPRT catalyses the salvage synthesis of inosine monophosphate (IMP) and guanosine monophosphate (GMP) from the purine bases hypoxanthine and guanine respectively, using 5'-phosphoribosyl-1-pyrophosphate (PRPP) as a co-substrate.

Neurologically, patients have severe motor disability dominated by dystonia, with occasional choreoathetosis or spasticity (Jinnah et al 2006). Most patients also exhibit recurrent self-injurious behaviour, with unavoidable biting of the lips, tongue or fingers, causing significant self-mutilated lesions (Schretlen et al 2005). The connection between aberrant purine metabolism and these neurological and behavioural characteristics remains largely unknown. LND neurological manifestations prompted to a dysfunction in the basal ganglia region and, as Nyhan pointed out, there is probably an imbalance in neurotransmitters in LND

(Nyhan 2000). Specifically, three neurotransmitters present in basal ganglia have been implicated in the pathogenesis of LND: dopamine (Breese et al 1990; Ceballos-Picot et al 2009; Ernst et al 1996; Jankovic et al 1988; Jinnah et al 1994; Saito et al 1999; Wong et al 1996), adenosine (Bertelli et al 2006; Prior et al 2007; Torres et al 2004) and serotonin (Anders et al 1987; Bavaresco et al 2007; Bertelli et al 2009; Manzke and Gustmann 1989; Saito and Takashima 2000). All of these neurotransmitters are implicated in motor function and behaviour, and act by binding to specific G-protein coupled receptors in the synaptic membrane.

We have previously found that peripheral blood lymphocytes (PBL) predominantly expressed dopamine DRD5 and adenosine ADORA2A receptor (García et al 2009). We also found that dopamine DRD5 and adenosine ADORA2A receptor expression is significantly different between control and LND PBL (García et al 2009). In the present work, we postulated that alterations in adenosine and dopamine receptors could be linked to serotonin receptor disturbances in LND.

The objectives of our study were to explore serotonin receptors in HPRT deficient cells and to confirm adenosine and dopamine receptor disturbances at protein level. We determined serotonin receptors 5-HTR1A, 5-HTR1B y 5-HTR2C expression, whose changes have been associated with psychiatric and mood disorders in pharmacological, post-mortem, imaging and genetics studies (Barnes and Sharp 1999; Gross et al 2002; Heisler et al 2007). As in previous works, we employed LND PBL to test our hypothesis. HPRT-deficient neurons cannot be obtained from living patients, and animal models are not totally satisfactory since animals do not reproduce the neurological manifestations of the disease (Finger et al 1988). PBL obtained from LND patients show the metabolic characteristics of HPRT-deficient cells: accumulation of 5-phosphoribosyl-1-pyrophosphate, enhanced de novo purine synthesis, excessive production and excretion of hypoxanthine and enhanced turnover rate of adenine nucleotides (Brosh et al 1976), and they expressed adenosine, serotonin and dopamine receptors.

Materials and methods

Peripheral blood cell isolation and culture

Heparinized blood samples were collected from 12 patients with LND (mean age 8.4 years ±6.4; range 2–20 years) and from 12 control subjects of similar ages (±2 years). LND was diagnosed on the basis of the following characteristics: clinical symptoms and signs characteristic of the enzyme defect, increased plasma and urinary uric acid, hypoxanthine and xanthine concentrations, absent HPRT activity in the hemolysate with a simultaneous increase in adenine phosphoribosyltransferase activity and characterization of the genetic mutation responsible for the deficient enzyme activity (Puig et al 2001). All 12 LND patients presented the classic complete form of HPRT deficiency and are not variant forms. Control subjects were patients who come to La Paz Hospital for minor ambulatory surgery and were in good health. After the Ethics Committee of La Paz Hospital approved the study protocol, informed consent was obtained from patients, control subjects and parents of underage patients.

Isolation of mononuclear cells from peripheral blood was performed by the Ficoll density sedimentation method described by Boyum (Boyum 1964), employing a solution of polysucrose and sodium diatrizoate, adjusted to a density of 1.077 g/ml (±0.001) (Histopaque-1077, Sigma Diagnostic). The isolated mononuclear cells correspond to 88.8% (±1.0) of lymphocytes.

The PBL layer collected was washed twice in erythrocyte lysis buffer (Qiagen GmbH, d-40724, Hilden, Germany) and total RNA was isolated using the QIAamp RNA Blood Mini Kit (Qiagen GmbH, d-40724, Hilden, Germany) for receptor expression.

For protein determination, PBL layer was washed twice in erythrocyte lysis buffer and resuspended in buffer 2:1:1 (HPO_4Na_2 2 mM, DTT 1 mM, EDTA 1 mM; pH 7.4), supplemented with complete protease inhibitor cocktail tablets (Roche Applied Bioscience) and maintained at −20°C until assay.

Serotonin receptors 5-HTR1A, 5-HTR1B y 5-HTR2C mRNA quantification by real-time PCR

Total RNA was reverse transcribed into a first-strand cDNA template using the ImProm-II™ Reverse Transcriptase system (Promega, Promega Corporation, WI, USA) and oligo (dT) 15 mer as primer for RT-PCR. As the genetic architecture of serotonin receptors 5-HTR1A and 5-HTR1B does not allow for the design of an adequate intron-spanning PCR assay, we treated the RNA with DNAse (Ambion, Applied Biosystems) to avoid genomic contamination.

Expression was quantified by real-time PCR in a Roche LightCycler with the use of a relative quantification method. We employed a housekeeping gene, such as β-actin, as a reference gene. A control RNA was reverse transcribed and the cDNA obtained was employed as calibrator. A standard curve for β-actin and for each target was constructed using serial dilutions of this calibrator. The calibrator sample was assigned a concentration value of 100. Concentration was obtained from the standard curve and the results were expressed as gene target concentration/β-actin concentration ratio measured in the same sample material.

Quantification of β-actin expression was determined using LC FastStart DNA Master SYBR Green I (Roche). A

melting curve analysis was used to determine the melting temperature of the amplified products so as to ensure its specificity. Quantification of 5-HTR1A, 5-HTR1B y 5-HTR2C mRNA was carried out by means of a TaqMan detection method. We employed probes from the Universal Probe Library (Roche Applied Science) and primers were designed with the Probe Finder version 2.40 for human software (Roche Diagnostics). We used TaKaRa Premix Ex Taq™ (Perfect Real Time) (TaKaRa BioEurope, France), designed for qPCR using the TaqMan probe detection method. Primers and probes employed are shown in Table 1.

Western blot detection of ADORA2A, DRD5 and 5-HTR1A receptors

Freeze PBL pellets were lysed by three cycles of freezing and thawing followed by sonication, and centrifuged for 15 min at 14,000 rpm. The supernatants were recovered and used as protein samples. Protein quantification was carried out by Bradford assay (Bradford 1976). SDS-PAGE was performed using the standard laboratory techniques with a discontinuous gradient, 5% (w/v) stacking gel and 8% (w/v) separating gel, in a Mini-PROTEAN III cell (Bio-Rad Laboratories, CA, USA). The gel was then transferred to a PVDF membrane (Roche Diagnostics) in an electroblotter (Bio-Rad Laboratories) for 90 min at 100 V. The membrane was washed three times (20 min each) with phosphate buffered saline (PBS) (Dulbecco, Berlin, Germany) containing 0.5% Tween-20 (PBS-T buffer). After blocking with PBS-T buffer and 2% dry powdered milk, membranes were washed three times for 15 min each with PBS-T and incubated with corresponding primary antibodies (ADORA2A, DRD5 or 5-HTR1A) and β-actin, (all from Santa Cruz, CA, USA) for 1 h at room temperature. After three washes with PBS-T, the membranes were incubated for 1 h with Goat Anti-rabbit IgG HRP secondary antibody (Santa Cruz, CA, USA). The membrane was washed twice (30 min each) with PBS-T buffer. ADORA2A, DRD5, 5-HTR1A and β-actin bands were tested using enhanced chemiluminescence (ECL kit; Amershan Pharmacia Biotech, USA) according to manufacturer instructions. Band densitometry was performed for each receptor and β-actin bands in the same membrane to give a relative quantification of protein as: densitometric β-actin relative ratio.

Data analysis

Analysis of quantification data was performed with LightCycler software. The crossing point (Cp), defined as the cycle numbers where fluorescence levels of all samples are the same, just above background, is automatically calculated by the LightCycler software by the "second derivative maximum method". Data were expressed as a gene target concentration/β-actin concentration ratio and presented as mean ± SD. Statistical tests were performed using the Statview software package (SAS Institute, Inc., USA). $P< 0.05$ was considered significant.

Results

Serotonin receptors 5-HTR1A, 5-HTR1B y 5-HTR2C mRNA quantification by real-time PCR

Appropriate standard curves were constructed for β-actin (slope 3.19, r=1) and for 5-HTR1A (slope 2.66, r=0.94). 5-

Table 1 Primers, probes and amplicon lengths for 5-HTR1A, 5-HTR1B, 5-HTR2C and for housekeeping gene β-actin

Gene	Primer sequence	Universal probe library	Amplicon lenght	RefSeq
5-HTR1A	Forward 5′-GACCGTCAGCTACCAAGTGAT	3	128	NM_000524.2
	Reverse 5′-GATAATTGGCCACGTTCTGC			
5-HTR1B	Forward 5′-GGTCACCGACCTGCTTGT	9	60	NM_000863.1
	Reverse 5′-GTGACAGTGTACATGGTGCTGA			
5-HTR2C	Forward 5′-CCGAGTCCGTTTCTCGTCTA	27	90	NM_000868.2
	Reverse 5′-TCGCGGGTGTTAGCTGAT			
β-ACTIN (ACTB)	Forward 5′-GAGCGGGAAATCGTGCGTGACATT	Sybr Green	76	NM_001101.3
	Reverse 5′-GAAGGTAGTTTCGTGGATGCC			

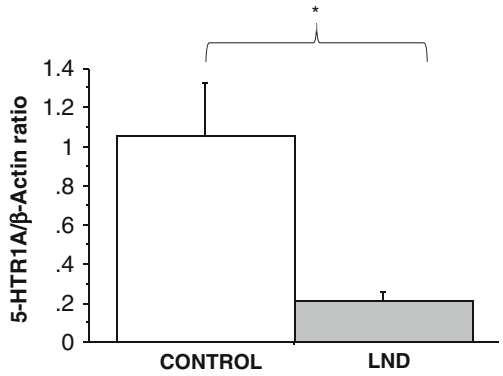

Fig. 1 5-HTR1A expression in LND PBL and in control PBL. Data were expressed as 5-HTR1A concentration/β-actin concentration ratios. *Control vs. LND PBL $P<0.05$

HTR1B and 5-HTR2C expression was detectable in both control and LND PBL but at a very low level. So, not appropriated standard curves could be obtained and mRNA was not quantifiable.

As Fig. 1 shows, 5-HTR1A mRNA levels, expressed as 5-HTR1A/ β-actin ratio, were significantly lower in LND PBL than in control PBL (mean ± SD: 0.21±0.12 vs. 1.05± 0.72; $P<0.05$).

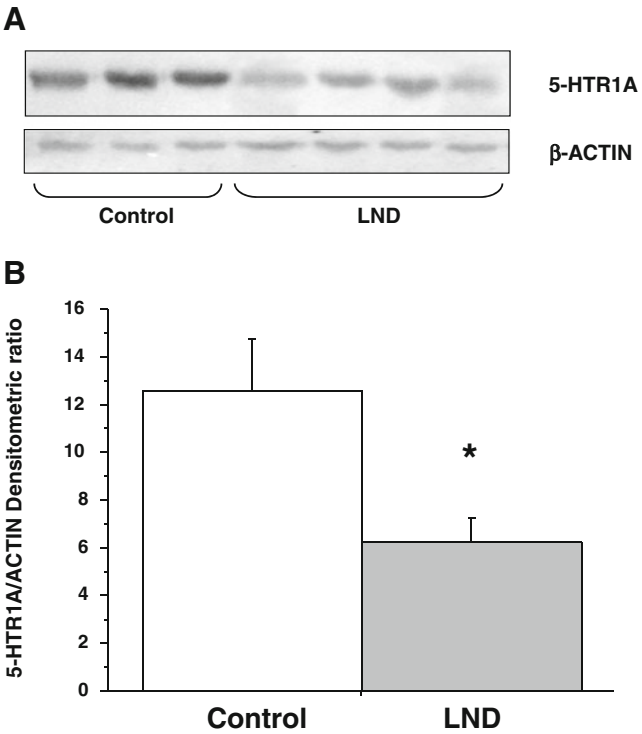

Fig. 2 5-HTR1A protein in control and LND PBL **A** Western blot showing chemiluminescence bands in control and LND PBL for 5-HTR1A (upper line) and β–Actin protein, employed as load control (down lane). **B** Band densitometry results, expressed as densitometric 5-HTR1A/β-actin relative ratio, represented in cell bar chart showing mean + standard deviation, * $P<0.05$

Western-blot detection of 5-HTR1A, ADORA2A, and DRD5 receptors

We detected a statistically significant decrease in the 5-HTR1A densitometric β-actin relative ratio in LND PBL with respect to control PBL (mean ± SD: 6.3±0.99 vs. 12.6± 2.2 densitometric β-actin relative ratio; $P<0.05$) (Fig. 2)

As a mean, there was no statistically significant difference in ADORA2A protein in LND PBL with respect to control PBL (mean ± SD: 4.0±3.4 vs. 6.9±1.9 densitometric β-actin relative ratio). However, there were significant differences between the extracts from different LND patients. So, in the extract from LN1 the ADORA2A densitometric β-actin relative ratio was higher than the control mean while in LN2, LN3 and LN4 ADORA2A densitometric β-actin relative ratio was below-average of control mean (Fig. 3). There were statistically significant differences between the below average group mean (LN2, LN3 and LN4) ADORA2A densitometric β-actin relative ratio and the control PBL (mean ± SD: 6.9±1.9 vs. 2.5± 1.9 densitometric β-actin relative ratio; $P<0.05$) (Fig. 3).

We detected a statistically significant increase in DRD5 protein in LND PBL with respect to control PBL (mean ± SD: 3005±950 vs. 1017±877 densitometric β-actin relative ratio; $P<0.05$) (Fig. 4).

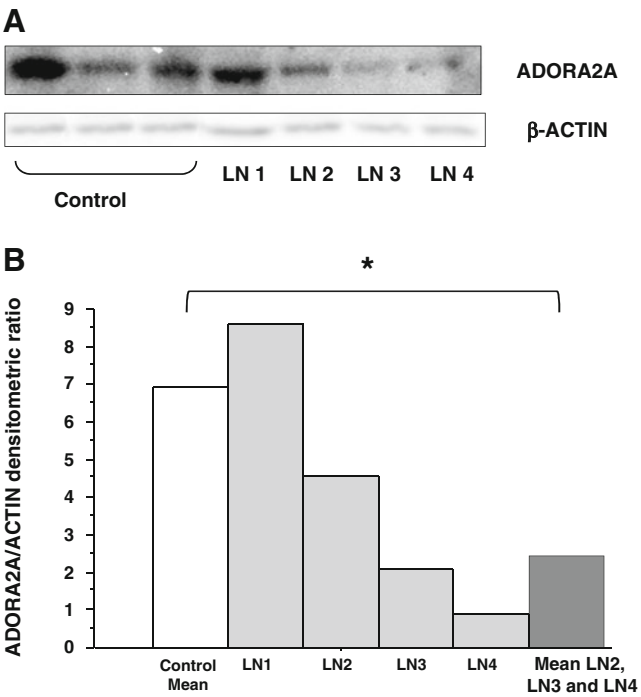

Fig. 3 ADORA2A protein in control and LND PBL **A** Western blot showing chemiluminescence bands in control and LN1, LN2, LN3 and LN4 PBL for ADORA2A (upper line) and β–Actin protein, employed as load control (down lane). **B** Band densitometry results, expressed as densitometric ADORA2A/β-actin relative ratio, represented in a cell bar chart showing mean value for control PBL, individual values for LND PBL, and mean for LN2, LN3, and LN4. Control vs. LN2, LN3 and LN4 group * $P<0.05$

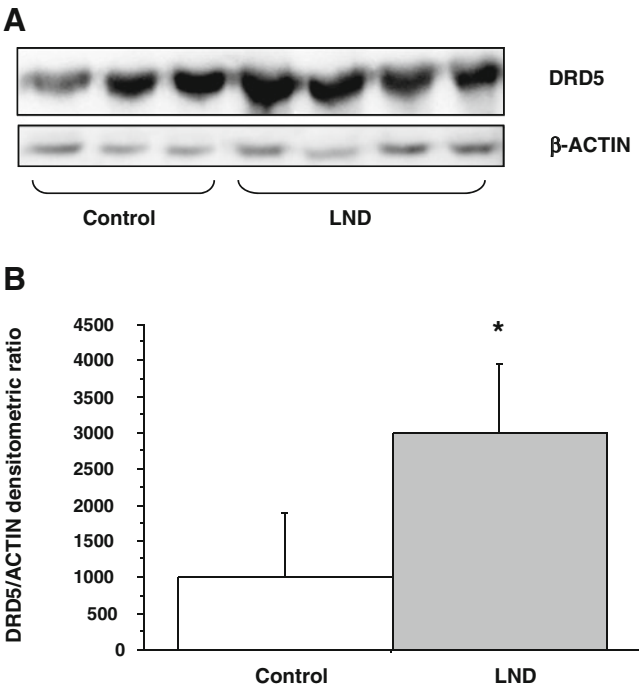

Fig. 4 DRD5 protein in control and LND PBL **A** Western blot showing chemiluminescence bands in control and LND PBL for DRD5 (upper line) and β–actin protein, employed as load control (down lane). **B** Band densitometry results, expressed as densitometric DRD5/β-actin relative ratio, represented in a cell bar chart showing mean + standard deviation, * $P<0.05$

Discussion

In this study, we described, for the first time, a decreased 5-HTR1A expression in LND PBL with respect to control PBL. This fact, together with the previously described abnormal expression of ADORA2A and the increased expression of DRD5 (García et al 2009) in LND PBL implies the coexistence of several neurotransmitter protein G coupled receptors alterations in LND. As is the case for DRD5 alterations (Ferrari et al 2008), disturbances in 5-HTR1A expression in peripheral blood cells have been previously linked to mood disorders (Wang et al 2010).

According to our results, 5-HTR1A, ADORA2A and DRD5 seem to be the preferred expressed serotonin, adenosine, and dopamine receptors in PBL so, in this study we have analyzed 5-HTR1A, ADORA2A and DRD5 protein receptors by western blot in LND and control PBL. We found that variations at the mRNA level match the receptor protein quantity. Thus, decreased 5-HTR1A expression in LND PBL with respect to control PBL is accompanied by a minor 5-HTR1A protein in LND extracts versus control extracts. Also, protein levels for both DRD5 and ADORA2A are also in accordance with the mRNA expression previously quantified for each of them by real time PCR (García et al 2009). We have previously reported (García et al 2009) that out of 12 LND patients, eight showed ADORA2A expression

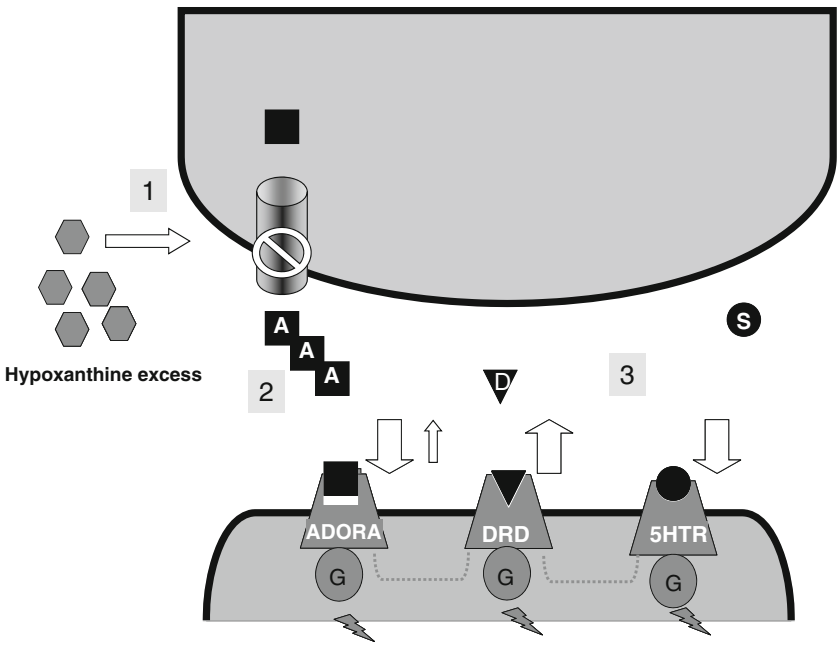

Fig. 5 Hypothesis: Link between HPRT deficiency and dopamine and serotonin dysfunction could be mediated by altered purinergic neurotransmission due to hypoxanthine excess. 1) Hypoxanthine excess due to HPRT deficiency causes a decrease in adenosine transport, competing with its transporters. 2) A defective reuptake of adenosine after binding to its specific receptor would cause an increase in the amount of extracellular adenosine that can bind to the receptor, promoting a decrease in the expression of adenosine receptors. 3) Adenosine, dopamine and serotonin receptors seem to be integrated through intermembrane receptor–receptor interactions, and adenosine receptor disturbance may be followed by alterations in dopamine and serotonin receptor

significantly lower than control, meanwhile four LND patients presented significantly higher ADORA2A expression. When differences between patients with high and low ADORA2A expression were analysed, there was no clinical, biochemical, enzymatic, molecular or treatment-related difference between both groups (García et al 2009). These results were confirmed in the present study at protein level, showing that most of LND patients (3 out of 4 LND patients) present a lower ADORA2A protein than control subjects in their PBL. We also confirmed, at protein level, the previously found increase of DRD5 expression in PBL from LND patients (García et al 2009). This fact is in agreement with the increase in dopamine receptors demonstrated by immunohistochemical methods in the *post-mortem* analysis of the putamen, and the caudate nucleus of two LND patients (Wong et al 1996) and supports the hypothesis of dopaminergic hypersensitivity in HPRT deficiency by using cells from living patients. We can therefore conclude that LND PBL shows an alteration in serotonin, adenosine and dopamine receptor expression and receptor protein level.

Of course, to reach physiopathological relevance for neurological manifestations of LND, further studies are necessary to confirm these results in HPRT deficient neuronal cells. To explain these receptors imbalance in LND we have hypothesized that the link between HPRT deficiency, dopamine, and serotonin dysfunction could be mediated by altered purinergic neurotransmission due to hypoxanthine excess (Fig. 5). Recently, Erdorf et al have postulated, in rat B103 neuroblastoma cells, that HPRT deficiency is associated with abnormal purinergic signaling, encompassing P2X and P2Y receptors and nucleotidases (Erdorf et al 2011). Hypoxanthine excess, found in the CSF of LND patients (Jankovic et al 1988), causes a decrease in adenosine transport, since it would compete with its equilibrative transporters (Torres et al 2004; Prior et al 2007). A defective reuptake of adenosine after binding to its specific receptor would cause an increase in the amount of extracellular adenosine that can bind to the receptor, promoting a change in the expression of adenosine receptors. Adenosine, dopamine and serotonin receptors belong to the G-protein coupled superfamily. They seem to be integrated through intermembrane receptor–receptor interactions and one of their agonists can modulate the response to the agonist of another in an integrative mechanism for signalling (Fuxe et al 2010). Therefore, in HPRT-deficient cells, adenosine neurotransmitter disturbance may be followed by alterations in dopaminergic and serotonergic neurotransmission.

Unfortunately, there is no effective pharmacologic treatment for LND neurological manifestations, and the most promising, levodopa, has proven not to be useful (Visser et al 2011). If receptors imbalance were confirmed in the LND basal ganglia cells it should be taken into account in the design of pharmacologic treatment for the motor and behavioural disturbances in patients with LND.

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