Increased NR2A expression and prolonged decay of NMDA-induced calcium transient in cerebellum of TgDyrk1A mice, a mouse model of Down syndrome

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A B S T R A C T
Transgenic mice overexpressing Dyrk1A (TgDyrk1A), a Down syndrome (DS) candidate gene, exhibit motor and cognitive alterations similar to those observed in DS individuals. To gain new insights into the molecular consequences of Dyrk1A overexpression underlying TgDyrk1A and possibly DS motor phenotypes, microarray studies were performed. Transcriptome analysis showed an upregulation of the NR2A subunit of the NMDA type of glutamate receptors in TgDyrk1A cerebellum. NR2A protein overexpression was also detected in TgDyrk1A cerebellar homogenates, in the synaptosome-enriched fraction and in TgDyrk1A primary cerebellar granular neuronal cultures (CGNs). In TgDyrk1A synaptosomes, calcium-imaging experiments showed a higher calcium uptake after NMDA stimulation. Similarly, NMDA administration promoted longer calcium transients in TgDyrk1A CGNs. Taken together, these results show that NMDA-induced calcium rise is altered in TgDyrk1A cerebellar neurons and indicate that calcium signaling is dysregulated in TgDyrk1A mice cerebella. These findings suggest that Dyrk1A overexpression might contribute to the dysbalance in the excitatory transmission found in the cerebellum of DS individuals and DS mouse models.

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Introduction

The motor abnormalities in DS are primarily ascribed to tasks controlled by the cerebellum, including the coordination of voluntary movement, gait, posture and speech. The analysis of single-gene contributions to Down syndrome (DS) have pointed Dyrk1A (Dual-specificity Yak1p Related Kinase) as a good candidate to contribute to specific DS phenotypes. Dyrk1A belongs to a family of dual specificity kinases characterized by their ability to autophosphorylate in tyrosine residues and phosphorylate substrates on serine and threonine residues. Dyrk1A comprises homologous proteins in other organisms such as minibrain (mnb) in D. melanogaster, which was the first member identified. Drosophila minibrain mutants showed brain abnormalities, such as, a reduction in the size of the optic lobes and central brain hemispheres (Tejedor et al., 1995). Later studies in haploinsufficient mice for Dyrk1A (mouse homologue of human Dyrk1A) revealed anomalous brain morphology (Fotaki et al., 2002), suggesting a conserved role of mnb/Dyrk1A in mice and flies.

In the developing central nervous system (CNS) it has been suggested that this kinase is involved in the transition of the neuroepithelial cells from proliferating to neurogenic divisions (Hämmerle et al., 2002). Likewise it has also been suggested that Dyrk1A plays a role in neuronal differentiation. Indeed, exposure of H19-7 hippocampal immortalized cells to FGF results in the Dyrk1A-mediated phosphorylation of CREB (cAMP responsive element-binding protein) and subsequent neuritic outgrowth (Yang et al., 2001). In addition to the involvement of Dyrk1A in CNS development, other functions of this kinase should also be considered, given its prominent expression in the adult CNS, particularly in the areas of the brain controlling motor activity (Okui et al., 1999; Marti et al., 2003). It is similarly worth mentioning that as well as CREB, Dyrk1A is capable of phosphorylating several transcription factors in vitro, such as Forkhead (FKHR) (Woods et al., 2001), Gli1 (Mao et al., 2002) and the nuclear factor of activated T cell (NFAT) family members (Arron et al.,...
All together, the data strongly suggest this kinase has a function in transcriptional regulation, underlying the pleiotropic effect of DYRK1A in CNS physiology. Importantly, DYRK1A maps to chromosome 21q22.2 and is found to be overexpressed in DS brains (Guimerà et al., 1999, Dowjat et al., 2007). Transgenic mice overexpressing DYRK1A (TgDyrk1A mice), previously generated in our laboratory, exhibit some of the pathologic features found in DS individuals, such as neurodevelopmental delay, motor abnormalities and cognitive deficits (Altarfà et al., 2001, Martínez de Lagrán et al., 2004). The learning and memory deficits identified were comparable to those of other reported mouse models of Dyrk1A overexpression (Smith et al., 1997; Ahn et al., 2006), strongly indicating that DYRK1A overexpression alters the normal function of the CNS and can be crucial in determining some of the traits of DS. Since DYRK1A can modulate the activity of a variety of transcription factors, the overexpression of this kinase could affect the activity of the target transcription factor(s), leading to TgDyrk1A mice phenotypes. In this study, cDNA microarray technology was used to assess the possible dysregulation in the expression of genes of relevance in neuronal physiology. Here we show that Dyrk1A overexpression results in a generalized increase of the N-methyl-d-aspartate receptor subunit 2A of glutamate receptors (NR2A), and a more prolonged calcium transient after NMDA stimulation. Taken together, these results suggest that the TgDyrk1A mouse phenotype might be mediated by the dysregulation of NMDA channels, which in turn, alters calcium homeostasis.

Materials and methods

Animals

All animal procedures met the guidelines of the European Community Directive 86/609/EEC, and were approved by the Local Ethical Committee. Same gender littermates were group-housed (up to 4 animals per cage) in standard macaron cages (40 × 25 × 20 cm) under a 12–h light/dark schedule in controlled environmental conditions of humidity (60%) and temperature (22 ± 2 °C) with food and water supplied ad libitum.

Microarray analysis

Microarray experiments were performed with mRNA extracted from the cerebellum of 7-month-old male mice with an mRNA extraction kit (Stratagene). In order to reduce inter-individual variability, each sample consisted of a pool of the RNA from three different mice of each genotype (control, TgDyrk1A L9 and TgDyrk1A L33). Four microarrays were hybridized: TgDyrk1A L9 versus control and TgDyrk1A L33 versus control, each twice. DNA microarrays contained a set of genes involved in neurotransmission (Potier et al., 2002; see the list of genes in Table 1). For expression control, five housekeeping genes were used in this study: actin, Gαd, tubulin, ubiquitin and Hprt (listed in Table 1). Microarrays were hybridized with fluorescent probes obtained by reverse transcription of mRNA from TgDyrk1A and control littermates' cerebella at 60 °C in 3.4× SSC, 0.28% SDS overnight. After washing, Cy3 and Cy5 fluorescence signals were scanned at a 10 μm/pixel resolution on a General Scanning ScanArray 3000 and analyzed with image analysis software Imagene 4.1 (Biodiscovery Inc.). Data were normalized, using either a Lowess fit or linear regression on a set of control genes. Statistical analysis was performed using VARAN, and the error model was generated from control experiments (NT Lowess 0.99, p = 0.01; Gollier et al., 2004).

Quantitative real-time PCR

Total RNA was extracted from the cerebellum of 8-month-old male mice (N = 4 TgDyrk1A and N = 5 control littermates) using TriPure Isolation Reagent (Roche). To avoid genomic contamination RNA samples were treated with DNase (DNase-free, Ambion), as described by manufacturer's protocol. First strand cDNA was synthesized from 200 ng of individual RNA samples using Superscript II reverse transcriptase (Invitrogen) and oligo dT18 (Ambion). qPCR was performed in a LightCycler 480 Real-Time PCR System (Roche Diagnostics) using the LightCycler 480 SYBR Green I Master (Roche). Grin1 and Grin2a transcripts (encoding for NR1 and NR2A subunits, respectively) were amplified by qPCR in independent reactions using the following primers: Grin1-F (5′-CGGTTAAGCCCTGGGAGAA-3′) and Grin1-R (5′-TCTGCTCTACACTTCTTTACCTC-3′), Grin2a-F (5′-GCCGGAGATGTGAGCCTTCC-3′) and Grin2a-R (5′-TCTGGACGACTCTCTCC-3′). Transcript levels were normalized against the reference genes Hprt1 (F: 5′-AGGCTTAAGATGGACCAAG-3′ and R: 5′-TTACTACGAGATGCCCACA-3′) and Actb (F: 5′-GTCACCTCTCCAGCAGTCT-3′ and R: 5′-AGTCCGGCTAAGAACACTTG-3′). All PCR reactions were performed in triplicate. The relative expression levels were analyzed using the Relative Expression Software Tool (REST) established by Pfaffl et al. (2002).

Immunoblotting

Adult mouse cerebella and whole brain homogenates and primary granule cell culture extracts were obtained as described previously (Marti et al., 2003). Briefly, the cerebella were mechanically homogenized in a glass potter, with a lysis buffer containing: 320 mM sucrose, 50 mM Tris–HCl pH 7.4, 10 mM EDTA, 1 mM PMSF and a protease inhibitor cocktail (Complete Mini, Roche). The homogenates were centrifuged 10 min at 800 g at 4 °C and the supernatant was kept for further analysis. Primary cell extracts were collected at DIV7 (day in vitro 7) in 100 μl of lysis buffer (SDS 1% in PBS). Protein concentration was determined using a BCA assay (Pierce). Equal amounts of protein (60 μg) were loaded and separated on a 7.5% SDS-PAGE and transferred onto nitrocellulose membranes (Amersham). Membranes were blocked with 10% skimmed milk in PBS-0.1% Tween-20 (PBS-T) solution and incubated overnight in the same incubation solution with polyclonal antibodies against Dyrk1A (1:250, Martí et al., 2003), NR1 (1:200; Cat. N. AB1516, Chemicon), or NR2A (1:500; Cat. N. M264, Sigma). Protein loading was monitored using a polyclonal antibody against β-Actin (1:3000; Cat. N. A2066, Sigma). Incubation with anti-rabbit or anti-mouse IgG/ HRP (horseradish peroxidase) antibodies (1:2000; Cat. N. P0448 and P0260, respectively; Dako) was performed for 1 h at room temperature. Membranes were rinsed in PBS-T and the immunocomplexes were detected by chemiluminescence with an ECL detection system (Pierce), following the manufacturer's instructions.

Table 1

<table>
<thead>
<tr>
<th>List of the genes contained in the Neurochips</th>
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<tbody>
<tr>
<td>Calcium binding proteins (3)</td>
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<tr>
<td>Enzymes synthesis (8)</td>
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<tr>
<td>Peptides (6)</td>
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<tr>
<td>AMPA receptors (4)</td>
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<td>Kainate receptors (5)</td>
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<td>NMDA receptors (4)</td>
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<td>Metabotropic Glu receptors (8)</td>
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<td>GABA receptors (13)</td>
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<td>Nicotinic receptors (10)</td>
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<tr>
<td>Muscarinic receptors (5)</td>
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<tr>
<td>Dopamine receptors (5)</td>
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<tr>
<td>5-HT receptors (13)</td>
</tr>
<tr>
<td>Adrenergic receptors (9)</td>
</tr>
<tr>
<td>Controls (1)</td>
</tr>
<tr>
<td>Normalizing genes (5)</td>
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</tbody>
</table>
Immunohistochemistry

Adult mice (6–12-month-old) were perfused transcardially with cold PBS, followed by buffered 4% paraformaldehyde in PBS. After fixation, brains were removed from the skulls and immersed in the same fixative overnight. After rinsing in PBS, brains were dehydrated, embedded in paraffin and 5-μm thick sections were obtained with a sliding microtome (Leica).

Sections were processed by the avidin–biotin–peroxidase method (Kit ABC, Dako), with a prior digestion with pepsimin, according to the protocol described by Yamada et al. (2001). Briefly, the sections were washed 3 times in PBS and pre-warmed for 30 min at 37 °C in distilled water, then dipped into a 0.2 N HCl solution with pepsimin at 1 mg/ml, during 15 min at 37 °C. After washing twice with water and with PBS, the endogenous peroxidases of tissue sections were inactivated and incubated with blocking solution (PBS supplemented with 0.2% Triton X-100, 10% Fetal Bovine Serum (FBS) and 0.25% gelatine) for 1 h at room temperature. Sections were then incubated overnight at 4 °C with the primary antibodies anti-NR2A (1:400), anti-NR1 (1:200) or in the absence of primary antibody (for the negative control), in PBS with 0.2% Triton X-100 and 1% FBS. After washing, the sections were incubated 30 min with biotinylated secondary anti-rabbit antibody (1:500), followed by an incubation during 30 min with a Streptavidin–HRP solution. Following washing, peroxidase activity was visualized by incubation of tissue sections with 0.15% diaminobenzidine and 0.01% hydrogen peroxide. The sections were counterstained with hematoxylin–eosin and viewed on a Leica DMR microscope.

Synaptosome preparation

Wild type and TgDyrk1A mice cerebella were dissected and homogenized in 5 ml of ice-cold isolation buffer containing 320 mM Sucrose, 1 mM Na-EDTA, 10 mM Tris–HEPES, pH 7.4 and a protease inhibitor cocktail, with a Teflon/glass homogenizer. The homogenates were centrifuged at 600 g for 5 min to obtain a pellet fraction (P1) enriched in cell debris, intact cells and nuclei. P1 was resuspended in a Krebs buffer containing 125 mM NaCl, 5 mM KCl, 0.1 mM MgCl2, 1 mM CaCl2, 10 mM glucose and 10 mM HEPES–NaOH. P1 was centrifuged at 37 °C in a humidified 5% CO2. After 1 day in vitro (DIV 1), one volume of Neurobasal medium (Invitrogen), containing 25 mM KCl and then incubated at 37 °C in humidified 5% CO2. After 1 day in vitro (DIV 1), one volume of Neurobasal medium (Invitrogen), containing 25 mM KCl supplemented with 20 μM cytosine arabinoside (Ara-C, Sigma), was added to the cultures. Three independent cell cultures were established for each genotype.

Determination of intracellular [Ca2+]

Isolated cerebellar granule neurons (CGNs) at 10–12 DIV, plated on poly–l-lysine-coated coverslips, were washed with Krebs-modified buffer (145 mM NaCl, 5 mM KCl, 2 mM CaCl2, 1 mM MgCl2, 10 mM HEPES–NaOH, 11 mM glucose; pH 7.5) and incubated in Krebs buffer with 5 μM Fluo-4-AM (Molecular Probes) for 1 h at 37 °C. The coverslips were mounted on the stage of a Nikon TE-200 inverted microscope equipped with a Nikon 40×, 1.3 numerical aperture, epifluorescence oil immersion lens. Samples were stimulated with a laser line at 488 nm (Melles Griot), and the resultant fluorescence was recorded at 535 nm. Calcium concentration was calculated as described by Kao (1994), considering a Kd for Fluo-4-AM of 400 nM. Fmax values were obtained in the presence of 50 μM ionomycin, and Fmin was determined after addition of 15 mM EGTA. Prior to [Ca2+] measurements, neurons were identified morphologically (differentiated from glial cells), by their relatively small, phase-dark cell bodies with fine processes. If a cell was not clearly identified as a neuron, the subfield was excluded from the analysis. Thereafter, several Regions Of Interest (ROIs) were selected and calcium measurements were performed under basal conditions and following a 10-s pulse of 100 μM NMDA perfusion.

Results and discussion

Transcriptome alterations in the cerebellum of TgDyrk1A mice

Behavioral studies on murine models carrying a Dyrk1A gene dose imbalance, together with the characterization of the expression pattern of Dyrk1A in the CNS, have prompted the suggestion that this kinase may play a role in cerebellar physiology. Transgenic mice overexpressing Dyrk1A (TgDyrk1A) exhibit a delay in the acquisition of mature locomotor activity, possibly related to a defect in motor coordination, an alteration which is maintained in the adult mouse (Altafaj et al., 2001, Martínez de Lagrán et al., 2004). On the other hand, Dyrk1A haplosinsufficiency results in a significant delay in the appearance of cerebellovestibular-dependent reflexes and in adult motor performance (Fotaki et al., 2002, 2004). The possible role of Dyrk1A in cerebellar and motor functions is also supported by strong Dyrk1A immunostaining in the cerebellum and functionally related structures, as well as in central motor nuclei (Martí et al., 2003).

Since Dyrk1A is able to modulate a number of transcription factors, modifications in cerebellar function in TgDyrk1A might be related to changes in the transcriptional profile. Thus, possible alterations in gene expression in the cerebellum were examined with cDNA microarrays containing a set of genes including most of the neurotransmitter receptor subunits (Table 1). Many other important
genes that were not present in the microarrays were not evaluated in this study. Two independent transgenic mice lines (L9 and L33), showing a similar behavioural phenotype, (Altaj et al., 2001) were analyzed at adult stages. The analysis of the microarray data of both transgenic mice lines showed a significant overexpression of three out of the 96 genes analyzed. The differentially expressed genes encode for: N-methyl-D-aspartate (NMDA) receptor subunit 2A (NR2A), serotonin receptor subunit 1E/F (5HT1E/F) and Gamma-aminobutyric receptor type A subunit γ3 (GABA-γ3) (data not shown). The fundamental role of NMDA receptors in excitatory neurotransmission, together with their modulatory role in cerebellar-dependent motor coordination (reviewed in Sánchez-Pérez et al., 2005), and the alterations in motor coordination found in TgDyrk1A mice, prompted us to further characterize the overexpression of the NR2A subunit in TgDyrk1A mice cerebella. With respect to internal standards, microarray analysis showed that the NR2A transcripts were overexpressed in transgenic mice lines 9 and 33, with ratios of 1.96 and 2.0, respectively.

NMDA heteromeric receptors are mainly composed of a structural subunit (NR1) and an NR2 subunit (NR2A-D) that modulates the biophysical properties of the NMDA receptor. In the adult cerebellar cortex, NR2A is one of the major subunits expressed and requires assembly with NR1 in order to be functional (Thompson et al., 2000; Yamada et al., 2001). An increase of NR2A subunit expression raises the possibility of disturbing the stoichiometry between NR1 and NR2 subunits, or of increasing the density of NR1/NR2A heteromers and so altering the synaptic properties of TgDyrk1A neurons. The latter would imply an increase of NR1 subunit levels, similar to what has been previously described in PC12 cells (Saito et al., 2003). Since an NR1 probe was not present in the microarray, the analysis of Grin1 transcript level (encoding for the NR1 subunit) was evaluated by quantitative real-time PCR of cDNAs obtained from 5 wild type and 4 TgDyrk1A mice cerebella. In this analysis, Grin2a transcripts (encoding for the NR2A subunit) were also studied. This analysis showed that Grin2a, with respect to the reference genes Hprt1 and β-Actin, was upregulated with a factor of 1.33 and 1.41, respectively. The statistical analysis showed that this upregulation was statistically significant (p = 0.0015 and p = 0.001, respectively), thus confirming the previous transcriptome analysis. The analysis of Grin1 relative expression showed an upregulation factor of 1.61 and 1.71 (using Hprt1 or β-Actin, respectively), that was statistically significant (p = 0.041 and p = 0.029). This result indicated that Grin1 was also upregulated in the cerebellum of TgDyrk1A mice.

These results indicate that Dyrk1A might participate in the activation of Grin1 and Grin2a promoters. The characterization of the transcription regulatory regions of both genes has identified the presence of CRE/NRSE cis-acting sequences and a potential CRE and CRE-like elements controlling the expression of NR1 and NR2A (Zarain-Herzberd et al., 2005; Desai et al., 2002). The latter is of particular interest since CREB transcription factor has been described as a substrate of Dyrk1A (Yang et al., 2001). Therefore, an overexpression of Dyrk1A could modulate CREB phosphorylation status, promoting phosphoCREB binding to the CRE and CRE-like element, thus enhancing Grin1 and Grin2a promoter activation. Moreover, the involvement of additional transcription factors targeting different regions of those promoters is a possibility that cannot be ruled out.

Protein levels of NMDA receptor subunits are altered in TgDyrk1A mice

We next studied whether the upregulation on Grin1 and Grin2a transcripts was associated to an increase in NR1 and NR2A protein contents. Western blot experiments showed an increase of NR1 and NR2A subunits expression in TgDyrk1A cerebella (Fig. 1A). In addition, immunoblot analysis performed in TgDyrk1A whole brain protein extracts also showed NR1 and NR2A overexpression, indicating that the upregulation was not only restricted to the cerebellum (Fig. 1A).

Fig. 1. Study of NMDA receptor subunits NR1 and NR2A expression in the adult brain of wild type and TgDyrk 1A mice. (A) Western blot showing specific overexpression of NR2A and NR1 subunits in the cerebellum (left panel) and in whole brain (right panel) protein homogenates. Actin was used as an internal loading control. (B) Immunohistochemical analysis of NR1 and NR2A subunit expression in paraffin-embedded coronal sections of adult cerebellum (WT, TG). Haematoxylin and eosin were used for counterstaining. Thick arrows point to Purkinje cell somas and arrows indicate the glomeruli in the granule cell layer. M: Molecular layer; P: Purkinje layer; G: Granular layer.

To determine the cellular localization of the altered amounts of NMDA receptor subunits, we performed the immunohistochemical analysis of NR1 and NR2 subunits from wild type and TgDyrk1A mice cerebella. The immunostaining pattern was similar for both subunits, showing a strong signal in the granules of the granular layer and a light to moderate immunoreactivity in the molecular layer and the somas of some Purkinje cells (Fig. 1B). Despite the overexpression of NR1 and NR2A subunits, no major changes in their immunostaining pattern were detected in TgDyrk1A mice cerebella.

Subcellular localization of NMDA receptor subunits in TgDyrk1A cerebellar synaptosomes

To perform their function, NMDA receptors need to target and dock in the plasma membrane. Thus we decided to further analyze
the subcellular localization of NMDA receptors in TgDyrk1A neurons. After mechanical homogenization of cerebella from either wild type (N=6) or TgDyrk1A mice (N=6), we purified the synaptosome-enriched fraction and analyzed the subcellular distribution of the NR2A receptor subunits by immunoblotting. The analysis of the post-nuclear fraction (S1) confirmed the presence of increased amounts of NR2A and Dyrk1A in the cerebella of TgDyrk1A mice (Fig. 2A). After centrifuging the S1 fraction, a cytosol-enriched fraction S2 (enriched with calretinin, a cytoplasmic calcium-buffering protein) and a membrane-enriched fraction P2 (enriched with SNAP25, a protein associated to the presynaptic plasma membrane) were obtained. Immunoblot analysis showed that Dyrk1A post-nuclear fraction was mainly cytoplasmic, with a small amount being present in the synaptosomal fraction, as described by Aranda and collaborators (personal communication). The NR2A subunit was predominantly detected in the membrane fraction (Fig. 2A). Despite the increase of NR2A and Dyrk1A levels, the pattern of their distribution was not altered in TgDyrk1A synaptosomes, indicating that the excess NR2A subunits accumulated in the plasma membrane, where they can exert their calcium channel function.

We next investigated whether the increase of the NR2A subunit could have functional consequences in terms of NMDA-induced rises of [Ca^{2+}]. We studied NMDA-induced calcium influx in wild type (N=7) and TgDyrk1A (N=7) cerebellar synaptosomes. We determined the rise in intrasynaptosomal calcium concentrations [Ca^{2+}]i, triggered by either 100 μM NMDA or 100 μM glutamate, in the presence of 10 μM glycine. Activation of the Fura-2-AM-loaded synaptosomes resulted in a rapid increase in the F340/F380 ratio, eliminated completely by the addition of 1 mM EGTA (data not shown). The administration of 100 μM NMDA, induced an increase of intrasynaptosomal calcium concentration [Ca^{2+}]i in wild type synaptosomes (11%±7.0). Interestingly, in TgDyrk1A synaptosomes, NMDA induced a stronger [Ca^{2+}]i rise (24%±5.0), shown to be statistically significant (p=0.014) (Fig. 2B). However, the addition of glutamate induced a similar intracellular calcium rise in both wild type and TgDyrk1A synaptosomes (30%±7.8 vs. 36%±3.1, respectively). These experiments showed that NR2A subunit overexpression is associated with an increase of NMDA-induced calcium uptake in TgDyrk1A synaptosomes. Moreover, the absence of a differential glutamate response suggests that this effect is triggered specifically by the activation of NMDA-type receptors, but not by other members of the glutamate-activated calcium channel family. This is in agreement with the absence of transcriptional changes in the non-NMDA glutamate receptors (Table 1) analyzed in our microarray experiments.

Prolonged NMDA-induced calcium transient in TgDyrk1A cerebellar granule neurons

To evaluate the relevance of these biochemical changes in a cellular context, we established primary cultures of granular neurons (CGN) from the cerebella of wild type and TgDyrk1A mice. The analysis of mature cell cultures (with differentiated neurons, at DIV7) showed the presence of NR1 and NR2A subunits, as previously reported (Janssens and Lesage, 2001). Cell extracts from the CGNs showed a significant increase of Dyrk1A (2.3-fold) and NR2A (2-fold) protein levels in TgDyrk1A primary cell cultures (Fig. 3A). However, no differences were detected in the NR1 content of the CGN obtained from the cerebella of both genotypes. The absence of the co-overexpression of NR1 and NR2A subunits in this cellular model raised the issue of whether an excess of the NR2A subunit alone could have any functional consequences with respect to the NMDA response. This was evaluated by performing calcium imaging of the CGNs. Intracellular calcium measurements were monitored in differentiated CGNs (at DIV12), after a 10-s-pulse application of 100 μM NMDA (Fig. 3B). The NMDA-induced calcium influx peak and amplitude showed no significant differences between the CGNs obtained from wild type or TgDyrk1A mice (Fig. 3C). This result is in agreement with recent studies of transient transfection of NMDA receptor subunits in CGNs (Przybylewski et al., 2002). In their study, the authors show that an overexpression of the NR2A subunit increases the number of cell surface receptors, without affecting either the amplitude of miniature excitatory postsynaptic currents (mEPSC) or single-channel conductance.

Despite the fact that there were no changes in the peak and amplitude of the NMDA-induced calcium rise in TgDyrk1A CGNs, the time constant τ, corresponding to the recovery of resting [Ca^{2+}], was found to be significantly increased in TgDyrk1A mice (26.6 ms±2.2 vs. 13.4 ms±0.5; p=0.009; Figs. 3C and D), indicating that NMDA promoted a prolonged calcium transient. This prolonged decay observed in TgDyrk1A CGNs was associated with a significantly higher [Ca^{2+}]i signal integral after NMDA stimulation (1.08±0.03±2.2 vs. 1.71±0.09; p=0.02; Figs. 3C and E). These results suggest that Dyrk1A overexpression might impair the calcium-buffering capacity of cerebellar granule neurons. Interestingly, a slower decay in [Ca^{2+}], after the
activation of ionotropic receptors has been reported in cell lines established from the cerebral cortex of trisomic Ts65Dn mice (Cárdenas et al., 1999). Ts65Dn mice, the most accepted model for Down syndrome, has a segmental trisomy of mouse chromosome 16 (MMU16) region synthenic to human chromosome 21 (HSA21). Dyrk1A is located in the triplicated region and is overexpressed in the Ts65Dn mouse brain (Dowjat et al., 2007). Therefore, the similar NMDA-induced calcium kinetics observed in these two murine models, suggests that Dyrk1A overexpression might contribute to the proposed impairment of calcium-buffering capacity in the cortical

Fig. 3. NMDA-induced [Ca\textsuperscript{2+}] transient in cerebellar granular neurons (CGN) established from wild type and TgDyrk1A cerebella. (A) Western blot analysis and relative quantification of Dyrk1A, NR1 and NR2A protein levels of CGNs (at DIV7), established from wild type and TgDyrk1A mice cerebella. Actin was used as an internal loading control. Values represent means±SEM of three independent experiments. *p<0.05; Student’s t-test. (B) Representative fluorescence images of Fluo-4-AM-loaded wild type CGNs (DIV12) at resting state (basal), during a 10 s pulse of 100 μM NMDA perfusion and after wash (recovery). (C) Time course of fluorescence changes (F/F₀) in region of interest after 10-s pulse of 100 μM NMDA perfusion in CGNs established from wild type (continuous line) and TgDyrk1A (dotted line) cerebella. Data represent the mean of four independent experiments. (D) Statistical analysis of time constant (τ) resulting from simple-exponential fit: f(t)=Σ Ai e ^ (-t/τi) + C in wild type (white bars) and TgDyrk1A CGNs (black bars), after 100 μM NMDA perfusion. (E) Statistical analysis of [Ca\textsuperscript{2+}] signal integral after a 10-s pulse of 100 μM NMDA in wild type (white bars) and TgDyrk1A CGNs (black bars). [Ca\textsuperscript{2+}] signal integral quantifies calcium influx and was calculated in the interval between [Ca\textsuperscript{2+}]-peak and recover to resting [Ca\textsuperscript{2+}]. Data are expressed as arbitrary units (A.U.) of the mean±S.D. of three independent experiments per genotype (30–40 ROIs analyzed in each experiment). Student’s t-test was performed to statistically evaluate the differences between TgDyrk1A and wild type CGNs. *p<0.05, **p<0.01.
neurons of trisomic mice. Moreover, a prolonged recovery of calcium levels has also been reported after brief glutamate applications in Ts16 hippocampal neurons (Schuchmann et al., 1998). Interestingly, in the Ts16 mice, a model of Down syndrome with a complete trisomy of MMU16, NR2A subunits have been found to be overexpressed (Klein et al., 2001).

The experiments measuring calcium dynamics show that the increase in NMDA-induced calcium rise observed in TgDyrk1A synaptosomes is not detected in terms of peak and amplitude in TgDyrk1A cerebellar granule neurons. This could be explained by the inability of synaptosomes to reestablish resting calcium levels whereas, in a cellular context (CGNs), calcium influx initiated by NMDA activation would be balanced by intracellular mechanisms allowing cells to recover resting calcium levels. Despite the overall compensation of the calcium rise, the prolonged time to recovery of [Ca\textsuperscript{2+}] to basal levels suggests that in TgDyrk1A CGNs, calcium may alter the neuronal signalling pathways that involve calcium as a second messenger, as occurs in DS individuals and DS mouse models (Ruiz de Azúa et al., 2001; Lumbéras et al., 2006) thus contributing to the phenotypic alterations observed in TgDyrk1A mice. According to this hypothesis, restoring NMDA receptors to normal physiological activity could correct NMDA-dependent phenotypes in TgDyrk1A mice. Along such lines, it has recently been described that the normalization of NMDA receptor (NMDAR) functionality in a murine model of Down syndrome (Ts65Dn mice), improves behavioral alterations (Costa et al., 2008). The authors showed that the acute administration of the competitive NMDA antagonist memantine led to the recovery of the performance deficits displayed by Ts65Dn mice in a fear conditioning test, thus supporting the theory that NMDA receptor dysfunction could play a role in the pathogenesis of DS. According to the authors, NMDAR dysfunction might be due to the overexpression of RCAN1 (a gene present in HSA21, with its murine homologue located in MMU16), an inhibitor of calcineurin, causing an increase in NMDAR mean open time and opening probability. The data obtained in our study suggest that Dyrk1A overexpression might also contribute to the alterations of NMDA receptors detected in trisomic mice. Based on the recently described synergy between DYRK1A and RCAN1 in cardiac tissue (Arron et al., 2006), a cooperative mechanism of both HSA21 gene products altering NMDA receptor functionality can be hypothesized.

In the present paper we describe for the first time that Dyrk1A overexpression leads to alterations in the transcriptome of TgDyrk1A mice. These changes affect the NMDA-type glutamate receptors and overexpression in Down syndrome (Ts65Dn mouse) models of Down syndrome on a fear conditioning test. Neuropharmacology 2003, 162–1632.


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