G-Protein-Associated Signal Transduction Processes Are Restored after Postweaning Environmental Enrichment in Ts65Dn, a Down Syndrome Mouse Model

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Key Words

Cyclic adenosine monophosphate • Phospholipase C • Adenylyl cyclase • Phosphatidylinositol 4,5-bisphosphate • Postnatal development • Environmental enrichment • G protein transduction • Trisomic mouse model • Ts65Dn • Down syndrome

Abstract

Individuals with Down syndrome (DS) present cognitive deficits that can be improved by early implementation of special care programs. However, they showed limited and temporary cognitive effects. We previously demonstrated that postnatal environmental enrichment (EE) improved clearly, though temporarily, the execution of visuospatial memory tasks in Ts65Dn mice, a DS model bearing a partial trisomy of murine chromosome 16; but in contrast to wild-type littermates, there was a lack of structural plasticity in pyramidal cell structure in the trisomic cerebral cortex. In the present study, we have investigated the impact of EE on the function of adenylyl cyclase and phospholipase C as a possible mechanism underlying the time-limited improvements observed. Basal production of cyclic adenosine monophosphate (cAMP) was not affected, but responses to GTP γ S, iso-

prenaline, noradrenaline, SKF 38393 and forskolin were depressed in the Ts65Dn hippocampus. In EE conditions, cAMP accumulation was not significantly modified in control animals with respect to nonenriched controls. However, EE had a marked effect in Ts65Dn mice, in which cAMP production was significantly increased. Similarly, EE increased phospholipase C activity in Ts65Dn mice, in response to carbachol and calcium. We conclude that EE restores the G-protein-associated signal transduction systems that are altered in Ts65Dn mice.

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Individuals with Down syndrome (DS) present cognitive deficits that can be improved by early implementation of special care programs. Positive short-term effects of those early interventions are well recognized, whereas long-term effects are less documented [1–5]. We have previously replicated this approach in an animal model of DS, the Ts(17¹⁶)65Dn (Ts65Dn) [6–9]. Currently, Ts65Dn is the most accepted animal model for human trisomy 21 and includes most of the MMU16 region homologous to HSA21 (from *Mrpl39* to *Zfp295* in the distal telomere) [10]. Trisomic mice show cognitive deficits in spatial

learning and memory, hyperactivity, reduced attention levels and an altered response to novel environments [11–18]. Similar to what has been described in DS, we found in previous studies that postweaning environmental enrichment (EE) modified behavioral performance and learning abilities of Ts65Dn mice, temporarily improving working and reference memories [8]. However, EE was not able to induce long-term structural plasticity in trisomic mice [7] and it produced gender-specific effects so that it improved spatial learning in female but deteriorated it in male Ts65Dn mice [8].

Numerous studies have revealed disrupted post-receptor signal transduction, in particular those mediated by G-protein-regulated adenylyl cyclase (AC) [19–22] and phospholipase C (PLC) [21–24] in DS brains. Consistent changes found in myoinositol and phospholipid composition of fetal and adult DS brain membranes [25–27] may predict deregulation of PLC-linked transduction processes, and our previous studies determined a reduced ability of several brain areas to respond to the indirect (receptor-mediated) and direct stimulation of the PLC [28] and AC [29, 30] signaling systems in Ts65Dn mice. In fact, neurotransmitters using these second messenger systems, such as the serotonergic, noradrenergic, GABAergic, and cholinergic systems, are altered in trisomic mice and DS fetuses [30–37], that may compromise short-term memory and hippocampal function [38]. For example, the serotonin 5-HT_{1A} [39] and 5-HT levels [40] are reduced in DS, and inhibition of 5-HT reuptake with fluoxetine restores cognitive impairments and hippocampal neurogenesis in Ts65Dn mice [41].

Our previous work suggested that the long-lasting adaptation of the AC transduction system was a key element in the effects of neonatal handling in rats [42–44], and clear alterations of AC and PLC signaling systems in DS brains. We here aimed to assess the possible changes in G protein signaling systems upon postweaning EE in Ts65Dn mice. As a target region, we selected the hippocampus, deeply involved in several memory tasks, including spatial memory, and deteriorated in Ts65Dn mice [14, 15, 18, 44] with structural and functional alterations [45–53] similar to those observed in DS patients [54, 55].

Thus, the present experiments were designed to elucidate the functioning of the AC and PLC signaling pathways in brain hippocampal membranes of naïve and enriched female Ts65Dn mice, since our previous work showed a deleterious effect of EE on male TS mice cognition related to chronic stress and enhanced aggression under EE conditions [6, 8].

Materials and Methods

Animals

Mice were generated by repeated backcross of Ts65Dn females to C57BL/6Jei × C3H/HeSnJ (B6EiC3) F1 hybrid males. The parental generation was prepared in the research colony at the Jackson Laboratory and mating was performed at the Animal Facilities of the University of Cantabria. The euploid littermates of Ts65Dn mice served as controls. After weaning, the animals were separated by sex and were randomly assigned to and reared in either a standard nonenriched environment (NE) or an enriched environment. Mice were karyotyped after enrichment [56]. Since gender plays a significant role in the response to enrichment [8], our study used only females for Ts65Dn and control groups. Experimental procedures were performed following EU regulations and approved by the Ethics Committee for animal experimentation of the University of Cantabria.

Housing and Enrichment Conditions

The standard environment consisted of Plexiglas cages (20 × 12 × 12 cm); mice were housed 2 or 3 per cage under standard laboratory conditions (20 ± 2°C, 12-hour light/12-hour dark cycle, 15:00 h light on, free access to food and water). For the EE experiments, we followed the protocol previously described [8]. In short, we used 42 × 50 × 20 cm cages with stainless steel ceiling, floor and frontal wall, and plastic lateral and back walls. Eight mice were housed per cage. The cage had two levels connected by a ladder and contained an activity wheel, a wooden swing and various plastic and wooden toys (toilet tissue rolls, blocks, rocks, etc. of different colors) which were changed every 3 days. Food of different tastes was added, with varying degrees of difficulty to get it. EE animals were maintained under the same light/dark cycle and controlled temperature as NE mice. After weaning, mice were reared for 7 weeks in an enriched environment and then returned to standard conditions. Cyclic adenosine monophosphate (cAMP) production was analyzed immediately after completing enrichment.

Materials

For the cAMP assay, 3-isobutyl-1-methyl-xanthine, isoprenaline, cAMP, noradrenaline, forskolin and bovine serum albumin were supplied by Sigma Chemical Company (St. Louis, Mo., USA). Phenoxybenzamine was obtained from Smith Kline & French Laboratories (Philadelphia, Pa., USA). [³H]CGP-12177 and [¹²⁵I]cAMP radioimmunoassay kits were supplied by Amersham (Buckinghamshire, UK). For the inositol phosphate assays, atropine, carbachol, ketanserin, 5-methyltryptamine (5-MT), phosphatidylinositol 4,5-bisphosphate (PIP₂), and ATP were obtained from Sigma Chemical Company, guanosine 5'-O-(3-thiotriphosphate) (GTPγS) from Boehringer Mannheim (Spain), and [³H]PIP₂ (specific activity 6 Ci/mmol) from New England Nuclear (USA). Liquid scintillation Optiphase Hi-Safe II was provided by LKB-FSA (Loughborough, UK).

Preparation of Crude Plasma Membranes

After decapitation, the 42- to 52-day-old mouse brains were removed and placed on ice. The cerebral cortex, the hippocampus, and the cerebellum were dissected free. The three brain regions were frozen on dry ice and stored at –75°C until use. Frozen brain

samples were thawed in ice-cold 20 mM Tris-HCl buffer, pH 7.0, containing 1 mM EGTA (Tris/EGTA buffer) prior to homogenization, then homogenized in 20 volumes of the same hypotonic buffer using a glass homogenizer with a Teflon pestle (20 strokes with a motor-driven pestle at maximum setting). A crude plasma membrane preparation was isolated by repeated centrifugations and rehomogenizations in the hypotonic buffer as described previously for human and rat cerebral cortex [57, 58]. Briefly, the homogenate was centrifuged for 15 min at 40,000 g. The pellet was then resuspended in Tris/EGTA buffer, rehomogenized, and centrifuged again. This procedure was repeated twice. Membranes were aliquots in microcentrifuge tubes and the pellets were kept at -75°C . There is no variation in PLC responsiveness to GTP γ S and agonists for at least 6 months of storage. Protein was measured using the Bio-Rad dye reagent.

cAMP Assay

The method of Slotkin et al. [59] was modified substituting GTP by GTP γ S. The membrane preparations were diluted 20-fold with 250 mM sucrose, 1 mM ethyleneglycolbis(β -aminoethyl-ether)-N,N'-tetraacetic acid and 10 mM Tris (pH 7.4). Aliquots of membrane preparation containing 40 μg of protein were then incubated for 10 min at 30°C with final concentrations of 100 mM Tris-HCl (pH 7.4), 1 mM adenosine 5'-triphosphate, 10 mM MgCl_2 , 1 mg bovine serum albumin and a creatine-phosphokinase-ATP-generating system consisting of 10 mM sodium phosphocreatine, 8 IU of phosphocreatine kinase and 10 μM GTP γ S in a total volume of 250 μl . The enzymatic reaction was stopped by placing the samples in a 90 – 100°C water bath for 5 min, followed by sedimentation at 3,000 g for 15 min. The supernatant solution was assayed for cAMP using [^{125}I]cAMP radioimmunoassay kits. Preliminary experiments showed that the enzymatic reaction was linear well beyond 10 min and with membrane protein concentration. Concentration of cofactors was optimal, and, in particular, addition of higher concentrations of GTP γ S produced no further augmentation of activity. In addition to evaluating basal activity, the maximal total activity of the AC catalytic unit was evaluated with the response to forskolin (100 μM) plus Cl_2Mn (10 mM). G protein coupling to AC was determined using a nonhydrolyzable analog of GTP, the GTP γ S (10 μM), and β -adrenergic stimulation of activity via Gs using isoprenaline (100 μM) and noradrenaline (100 μM), and D_1 -dopaminergic stimulation using SKF 38393 (10 μM).

PLC Assay

The same membrane preparation used for AC activity assay was also used for basal and stimulated PLC assay. Membrane pellets were resuspended at a concentration of 2.5 mg protein/ml in a cold buffer consisting of 25 mM Tris-maleate, 5 mM ATP, 15 mM MgCl_2 , and 25 mM LiCl, pH 6.8 (adjusted with KOH). Reactions (100 μl total volume) were initiated by adding 40 μl of membranes (100 μg protein) to tubes with 25 mM Tris-maleate, pH 6.8, containing [^3H]PIP $_2$ (final concentration 30 μM), 1 mM sodium deoxycholate, 3 mM EGTA and CaCl_2 necessary to yield different free calcium concentrations (calculated according to Harafuji and Ogawa [60]). The function of PLC was assessed by measuring the hydrolysis of exogenous labeled phosphoinositides, e.g. [^3H]PIP $_2$, and the production of [^3H]inositol phosphates under basal conditions (50 nM calcium free) and after stimulation of neurotransmitter receptors and G protein coupling to PLC with a nonhydro-

lyzable analog of GTP, GTP γ S (10 μM), receptor-driven stimulation of PLC using the muscarinic agonist carbachol (1 mM), the 5-HT $_2$ agonist 5-methyltryptamine (300 μM), and calcium (10 μM).

The reactions were stopped with 1.2 ml chloroform/methanol (1:2, v/v), followed by 0.5 ml of chloroform and 0.25 M HCl to create two phases, and were thoroughly vortexed. The phases were allowed to separate and then, after centrifugation, 1-ml aliquots of the upper aqueous phases containing [^3H]inositol phosphates were mixed with 4 ml of Optiphase Hi-Safe for scintillation counting.

Data Analysis

All experiments were carried out 4 times (4 biological samples), each in triplicate and with every experimental group being submitted to the experimental procedures (EE vs. NE) at the same time (each experiment containing a pool of 2 animals from each experimental group) ($n = 4$ – 6).

cAMP levels were calculated as picomoles of cAMP formed per milligram of protein, and the breakdown of [^3H]PIP $_2$ by PLC was calculated as picomoles of IP $_3$ formed per milligram of protein per minute. Data are expressed as means $\pm$ SEM. Significance of the overall effect of the drugs was determined by two-way ANOVA followed by Duncan's multiple range tests and comparisons between groups were evaluated using Student's *t* test (two-tailed). The level of significance was set at $p < 0.05$. Statistical analysis was performed using SPSS for Windows v. 9.0.

Results

Effects of EE on cAMP Production

Basal production of cAMP after inhibition of phosphodiesterase activity by IBMX (1 mM) was similar in the hippocampus of Ts65Dn mice and wild-type littermates (fig. 1a). However, upon G protein stimulation by using GTP γ S, even though cAMP accumulation was significantly increased compared to the basal state in both groups (euploid, $p < 0.001$, Ts65Dn, $p < 0.05$), significant differences among the groups were detected so that the values attained in the Ts65Dn group were significantly smaller compared to controls after stimulation with GTP γ S ($t = 5.39$, $p < 0.0001$; fig. 1a). When the production of cAMP was stimulated at the level of β -adrenergic and D_1 receptors with isoprenaline (10 μM), noradrenaline (100 μM) and SKF 38393 (10 μM), significant differences in the responses between groups were observed, though a significant increase compared to basal levels was induced in both groups of mice ($p < 0.05$). The cAMP level attained was significantly lower in Ts65Dn than in control mice upon stimulation with isoprenaline ($t = 8.65$, $p < 0.0001$), noradrenaline ($t = 6.5$, $p < 0.0001$) and forskolin ($t = 3.62$, $p < 0.01$).

Fig. 1. Mean $\pm$ SEM of basal cAMP accumulation and after stimulation with GTP γ S, isoprenaline (ISO), noradrenaline (NA), SKF 33893 and forskolin in the hippocampus of female nonenriched (NE) euploid versus Ts65Dn (a), enriched (EE) euploid versus Ts65Dn (b), euploid nonenriched versus enriched (c) and Ts65Dn nonenriched versus enriched (d) mice. * $p < 0.05$; ** $p < 0.01$; *** $p < 0.001$ (Student's t test).

EE had differential effects in both genotypes. In female euploid mice, EE reduced cAMP activity (fig. 1c) after stimulation with GTP γ S ($t = 5.4$, $p < 0.0001$), isoprenaline ($t = 7.71$, $p < 0.0001$), noradrenaline ($t = 4.34$, $p < 0.001$) and forskolin ($t = 2.27$, $p < 0.05$). However, in Ts65Dn females, EE significantly increased cAMP activity (fig. 1d) after stimulation with GTP γ S ($t = 4.01$, $p < 0.001$), isoprenaline ($t = 4.81$, $p < 0.0001$), noradrenaline ($t = 5.79$, $p < 0.0001$) and forskolin ($t = 4.26$, $p < 0.001$ (fig. 1c). Ts65Dn enriched females showed an increase in cAMP accumulation with respect to enriched euploid females after stimulation with GTP γ S (98.5 ± 1.9 vs. 125.4 ± 6.4 in enriched control and Ts65Dn mice, respectively; $t = 4.0$, $p < 0.001$; fig. 1b), isoprenaline (111.2 ± 1.6 vs. 137.5 ± 6.6 in enriched control and Ts65Dn mice, respectively; $t = 3.8$, $p < 0.01$), noradrenaline (117.3 ± 3.4 vs. 145.2 ± 7.4 in enriched euploid and Ts65Dn mice, respectively; $t = 3.42$, $p < 0.01$) and forskolin ($400.23 \pm$

9.4 vs. 507.1 ± 31.4 in enriched euploid and Ts65Dn mice, respectively; $t = 3.26$, $p < 0.01$).

Effects of EE on PLC Activity

In the hippocampus, Ts65Dn mice showed no differences in the accumulation of hydrolyzed PIP₂ in basal and GTP γ S- or calcium-stimulated conditions. However, a reduction in PLC activity was observed after stimulation with 5-MT and carbachol, the latter reaching statistical significance ($t = 2.7$, $p < 0.01$; fig. 2a). After EE, euploid mice showed a reduction in PLC activity after stimulation with carbachol ($t = 2.72$, $p < 0.01$; fig. 2c), whereas Ts65Dn mice did not show changes in PLC activity under enriched conditions (fig. 2d). Thus, the differences observed upon carbachol stimulation between euploid and Ts65Dn mice in NE conditions disappeared after EE (fig. 2b). Contrary to euploid females, in trisomic mice EE produced a slight increase in PLC activity after stimulation

Fig. 2. Mean $\pm$ SEM of basal PIP₂ activity and after stimulation with GTPγS, 5-MT, carbachol and calcium in the hippocampus of female nonenriched (NE) euploid versus Ts65Dn (a), enriched (EE) euploid versus Ts65Dn (b), euploid nonenriched versus enriched (c) and Ts65Dn nonenriched versus enriched (d) mice. *p < 0.05; **p < 0.01 (Student's t test).

with 5-MT, carbachol and calcium, which, although not statistically significant, led to a significant difference in PLC activity after stimulation with calcium when compared to enriched euploid mice (159.07 ± 26.22 vs. 340.5 ± 88.9 ; $t = 1.96$, $p < 0.05$; fig. 2b).

Discussion

Special care programs are one of the most successful therapeutic interventions used in DS. However, their beneficial effects are limited in time. We aimed here at understanding how EE contributes to cellular mechanisms underlying learning and memory formation in DS. Our data show impairments in AC and PLC in Ts65Dn mice and suggest that EE has differential effects on these signaling systems depending on the genotype. Since our previous work demonstrated a lack of struc-

tural plasticity upon enrichment in trisomic mice, these results may suggest that these transient biochemical effects are not translated into long-lasting structural changes.

Basal activity of AC was not affected in the hippocampus of trisomic mice, since cAMP accumulation was similar compared to wild-type littermates in both NE and EE conditions. However, when the system was challenged by receptor stimulation of β -adrenoceptor, or using GTPγS, a nonhydrolyzable analog of GTP, or forskolin to directly activate the catalytic subunit of the enzyme, an impaired response was clearly detected in the trisomic mice. Our present results confirm the deficits we reported in the frontal and hippocampal cortices of young adult male Ts65Dn mice [29, 30], and in DS individuals [21], where we observed a severe deficiency in AC activity, with a very poor response to various stimulatory signals, including forskolin [21]. In the present work, this reduction was also

detected in the hippocampus of Ts65Dn females that could be related to their learning deficits. In fact, long-term memory in a step-down active avoidance task was improved upon direct infusion of forskolin into the hippocampus [61].

Deregulation of the cAMP cascade is known to have an important role in neurogenesis [62] and synaptogenesis [63]. cAMP also regulates spine density by gating brain-derived neurotrophic factor signaling, a neurotrophin implicated in dendrite formation [64]. Several of these features are altered in DS. Furthermore, the reduced AC activity may lead to decreased activities of downstream PKA and some of its substrates, such as synapsin I, CREB or ARPP-19 (cAMP-regulated phosphoprotein) that are related to specific learning deficits associated with hippocampal function [53–55, 65].

However, no alterations in cAMP accumulation could be detected upon dopaminergic (D_1) receptor stimulation with the D_1 agonist SKF 38393 in either NE or EE rearing conditions in trisomic mice. This is in contrast to our previous data in human postmortem tissues [21], and may be seen as contradictory to previous reports showing reductions in dopamine and its acidic metabolites DOPAC and HVA, in DS fetal and adult brains [35, 40, 66]. Our study suggests that, even if dopamine levels are reduced, the D_1 -coupled postreceptor mechanisms may not be affected in the hippocampus of trisomic mice, contrary to the effects observed at the cortical or cerebellar levels [21, 28].

When we analyzed the effects of EE in euploid mice, we could detect reduced cAMP production under basal conditions and after stimulation with a variety of signals: GTP γ S, β -adrenergic and dopaminergic agonists, and forskolin. This effect is comparable to those previously reported in rats, where environmental stimulation produced long-lasting reduction of central noradrenergic responsiveness [67], and postnatal handling induced short- and long-lasting depression of AC functioning [42, 43]. This may be explained since EE, when provided early in the life of normal animals, may also serve as a factor capable of moderating stressing responses, thus enabling the animals to cope more properly with learning challenges.

Interestingly, EE had opposite effects on TS mice, since it increased the production of cAMP upon G protein stimulation by using GTP γ S and stimulation via Gs by the β -adrenergic agonists noradrenaline and isoprenaline, but again did not modify the response to the D_1 dopaminergic agonist SKF 38393. In fact, contextual learning failure in Ts65Dn mice can be reversed by using

a prodrug for norepinephrine called l-threo-3,4-dihydroxyphenylserine, or xamoterol, a β_1 -adrenergic receptor partial agonist [68]. We previously demonstrated beneficial effects of EE on cognition in female Ts65Dn mice [6, 8]. However, the cognitive beneficial effects were not accompanied by structural plasticity changes in trisomic mice [7]. It is well documented that the AC system is a main determinant for neural structural plasticity [69–72] and derangement of this system has been argued to explain the cognitive and plasticity deficits in DS [72, 73]. However, it is not clear how these changes correlate to cognitive changes after environmental stimulation. In our study, EE downregulated the AC system in euploid mice and increased the activity of this system in TS female mice while improving cognition in mice of both genotypes. The cAMP system is known to be involved in mnemonic processing and angiogenesis. However, both, increases and decreases in cAMP activity have been reported in different syndromes with altered cognition. cAMP is reduced in fragile X syndrome [74] but enhanced in autism [75, 76]. It has been proposed that in autism elevated cAMP may reflect a compensatory mechanism to increase low cAMP levels.

Furthermore, other molecular pathways have been shown to respond differently in Ts65Dn and euploid mice after pharmacological treatments. Siddiqui et al. [77] demonstrated that while in euploid mice the chr21-encoded proteins, TIAM1 and DYRK1A, and phosphorylation of AKT, ERK1/2 and the transcription factor ELK are involved in the locomotor stimulatory response of the NMDA receptor channel blocker MK-801, in Ts65Dn mice, levels of phosphorylation are constitutively elevated in naïve, unstimulated mice, and the MK-801-induced changes in TIAM1 and DYRK1A and in phosphorylation are either absent or abnormal, with both genotype- and brain region-specific patterns, emphasizing the complexities of the pathway perturbations that arise with segmental trisomy. Our data provide first direct evidence of restoration of the severe disturbances in the AC-signaling pathway using an environmental manipulation.

Regarding PLC functionality, basal and calcium- or GTP γ S-stimulated PIP₂ hydrolysis was preserved in the hippocampus of Ts65Dn mice under NE rearing conditions. However, we detected a reduction of PLC activity that reached statistical significance after muscarinic receptor stimulation, confirming our previous results in the cerebellum of male Ts65Dn mice [28]. This reduction points to a deficient cholinergic system at the hippocampal level that may explain some of the memory deficits present in Ts65Dn mice [78].

EE has been associated with increases in the functional efficiency of several neurotransmitter systems including the serotonergic and cholinergic systems and increases protein kinase C levels [79–85]. Our data suggest that EE may compensate the ACh depletion in the hippocampus of Ts65Dn mice [78], by increasing the efficiency of their signal transduction system [86], but is also increasing calcium-stimulated PIP₂ hydrolysis thus suggesting that the impact of EE could be broader increasing signaling through other neurotransmitter systems as well.

In conclusion, we here demonstrate that EE was able to increase G-protein-associated signal transduction processes in Ts65Dn mice. This effect might be partially dependent on a regulatory action on AC and PLC themselves since forskolin- and calcium-induced accumula-

tion of cAMP and PIP₂ were recovered. These changes, however, may not be effective in initiating structural plasticity that creates a stable trace over a long period of time [7].

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