Fetal Down Syndrome Brains Exhibit Aberrant Levels of Neurotransmitters Critical for Normal Brain Development
Nigel Whittle, Simone B. Sartori, Mara Dierssen, Gert Lubec and Nicolas Singewald
Pediatrics 2007;120;e1465-e1471; originally published online Nov 12, 2007; DOI: 10.1542/peds.2006-3448

The online version of this article, along with updated information and services, is located on the World Wide Web at: http://www.pediatrics.org/cgi/content/full/120/6/e1465
Fetal Down Syndrome Brains Exhibit Aberrant Levels of Neurotransmitters Critical for Normal Brain Development

Nigel Whittle, MAppSci, Simone B. Sartori, PhD, Mara Dierssen, MD, PhD, Gert Lubec, MD, Nicolas Singewald, PhD

*Department of Pharmacology and Toxicology, Institute of Pharmacy and Center for Molecular Biosciences, University of Innsbruck, Innsbruck, Austria; Neurobehavioral Analysis Laboratory, Genes and Disease Program, Center for Genomic Regulation, Barcelona, Spain and Centre for Biomedical Research on Rare Diseases (CIBERER), Barcelona, Spain; Department of Pediatrics, Division of Basic Sciences, University of Vienna, Vienna, Austria

The authors have indicated they have no financial relationships relevant to this article to disclose.

ABSTRACT

BACKGROUND. In the immature developing fetal brain, amino acids (such as γ-aminobutyric acid, and taurine) and monoamines (serotonin, noradrenaline, and dopamine) act as developmental signals or regulators. In subjects with Down syndrome, dysfunctional brain development is evident from birth as reduction in brain weight, as well as volume reductions in specific brain regions, and an altered number of neurons, dendrites, and dendritic branching is observed. However, mechanisms that underlie the observed dysfunctional brain development in Down syndrome are not clear.

OBJECTIVES. Because diverse amino acids and monoamines are critical for normal brain development, we wanted to determine whether dysfunctional brain development observed in subjects with Down syndrome is associated with altered brain amino acid and/or monoamine levels.

DESIGN/METHODS. We quantified tissue concentrations of diverse amino acids, including γ-aminobutyric acid and taurine, and the monoamines serotonin, noradrenaline, and dopamine in the frontal cortex of fetal Down syndrome tissue at a gestational age of ~20 weeks versus age-matched control aborted fetuses.

RESULTS. Fetal Down syndrome brains showed reductions in the levels of serotonin, γ-aminobutyric acid, taurine, and dopamine in the frontal cortex. No alteration in the levels of arginine, aspartate, glutamine, glutamate, glycine, histidine, serine, or noradrenaline was observed.

CONCLUSIONS. Serotonin, γ-aminobutyric acid, taurine, and dopamine are critical for the acquisition of brain morphologic features, neuronal and glia proliferation, and synapse formation. The detected reductions in the levels of these neurotransmitters may indicate potential mechanisms for the observed dysfunctional neuronal development in the Down syndrome fetal brain.
HARMONIOUS DEVELOPMENT of the mammalian central nervous system (CNS) during embryogenesis not only requires an accurate course of genetic schedules but also depends on appropriate influences by various epigenetic signaling processes.1 Neurotransmitters, such as amino acids (especially γ-aminobutyric acid [GABA]), and the monoamines serotonin, dopamine, and noradrenaline appear at a time before they assume their roles as neurotransmitters in the embryo and have established roles in maturation of the CNS (eg, see review in ref 2). GABA-ergic neurons are detectable in humans from ∼6 to 9 weeks’ gestation and correspond with the onset of the preplate and early cortical plate development.3 During fetal development, taurine is present in high concentrations4 and supports development of 2 to 4 cell human embryos to the blastocyst stage.5 Serotonergic neurons in the raphe are detectable by the fifth week of gestation and develop rapidly through to around the 12th week.6 By the 15th week of gestation, the typical organization of serotonin cell bodies in the raphe nuclei can be observed.7 Dopaminergic neurons have also been detected from the fifth gestational week in the midbrain and develop rapidly from around the eighth gestational week.8 Noradrenergic neurons in the pons are detectable by the fifth week of gestation.6

Down syndrome (DS), trisomy of chromosome 21, is the most common genetic cause of human mental retardation affecting ∼1 in every 800 live births.9 In addition to malformations involving mainly the heart and gastrointestinal tract, abnormalities in brain maturation are evident in DS. It is observed that, up to ∼25 weeks’ gestation, no differences between fetal DS and euploid brain development exist. This is derived from the observations that no differences in neurons and glial cells exist between DS and age-matched “normal” brains in the isocortex, an area containing Cajal-Retzius cells, which are the earliest-generated neurons.10 In addition, no differences in the number of differentiated neurons or alterations in dendritic development are observed in the visual cortex.11 However, from this time point of ∼25 weeks in utero, alterations in neurogenesis and/or differentiation in DS may occur, because at birth, reductions in brain weight, frontal lobe growth, reduction of the anterior posterior diameter fronto-occipital length of the brain hemispheres, flattening of occipital poles, and narrowing of the superior temporal gyri are observed.12 In addition, at birth, patients with DS show neuronal abnormalities, such as decreased numbers and altered distribution of neurons in the cerebral cortex, especially in layers II and IV.13 In addition, decreased basilar dendrites and decreased numbers of dendrites are observed in the visual cortex.11 During the postnatal period, dendritic branching and length are above normal in children with DS who are <6 months old and decrease below what is observed in euploid brains by 2 years of age.14 These observed alterations in DS persist into adulthood, suggesting a morphologic substrate for mental retardation.

To date, no study has investigated the fetal concentration of amino acids and monoamines critical for brain development in subjects with DS. We hypothesized that potential mechanisms for the observed dysfunctional brain development in subjects with DS could result from the altered concentration of amino acids and monoamines critical for brain development. Thus, the aim of this study was to quantify diverse amino acids and monoamines in fetal DS tissue versus age-matched normal control brains.

METHODS

Fetal Brain Samples

Fetal frontal cortex brain tissue of male fetuses with DS (n = 6; mean gestational age: 19.5 ± 1.4 weeks) and aborted male control fetuses (n = 4; mean gestational age: 21.8 ± 2.6 weeks) were used in this study. Brain samples were obtained from the Medical and Molecular Genetics Centre-Institute for Oncology Research, Hospital Duran i Reynals (Barcelona, Spain).

Fetal frontal cortex brain tissue was dissected on a chilled metal plate and was diluted 10-fold (wt/vol) with HCl (0.1 mol/L; prechilled) and tissue ground (2 minutes; in ice slurry). The solution was then centrifuged (14 000 rpm; 10 minutes; 6°C), and the resulting supernatant was filtered (0.2 μm) and immediately stored at −80°C until analysis.

Serotonin Analysis

Determination of serotonin and 5-hydroxyindol-3-acetic acid was performed using high-performance liquid chromatography with electrochemical detection, as published previously.15 Samples of 50 μL were automatically injected by a CMA 200 refrigerated autosampler (CMA, Stockholm, Sweden). Evaluation of serotonin and 5-hydroxyindol-3-acetic acid was conducted by comparing peak heights of samples with a set of external standard solutions using an integrator (SIC Chromatocoder 12; System Instruments, Tokyo, Japan).

Amino Acid Analysis

Determination of aspartate, GABA, glutamate, taurine, glycine, arginine, glutamine, histidine, and serine was performed by high-performance liquid chromatography with fluorometric detection (Merck-Hitachi, Tokyo, Japan) after derivatization with o-phthalaldehyde as described previously.16 Briefly, 50-μL injection volume was used, and quantification of amino acids was achieved by comparison of the peak area of the sample with controlled standard solutions. In addition, the reproducibility of the o-phthalaldehyde derivatization was controlled by the addition of S-carboxymethyl-L-cysteine as an internal standard.
Catecholamine Analysis

Determination of dopamine and noradrenaline was performed radioenzymatically following the method reported previously. Briefly, after incubation with catechol-O-methyltransferase and the methyl donor S-adenosyl-L-[methyl-3H] methionine, the reaction was stopped by cooling (0°C) and adding 1 mol/L of borate buffer (75 μL; pH 8.0). The alkaline aqueous layer (250 μL) was washed with n-hexane (800 μL). After freezing, the organic layer was removed, sodium tetraphenyl borate was added, and the labeled O-methylated products were extracted into diethyl ether. Evaluation of dopamine and noradrenaline was conducted by comparing counts per minute with a set of external standard solutions.

Statistical Analysis

Results are expressed as means ± SEM. Between-group differences were investigated by using the nonparametric Mann-Whitney U test. The level of significance was set at P < .05. All of the analyses were performed by using the SPSS 12.0 statistical software package (SPSS, Chicago, IL).

RESULTS

Serotonin

Significant reductions of 41% ± 18% and 43% ± 25% were observed for serotonin (DS versus control: 9 ± 2 vs 14 ± 1 pM/mg tissue; P < .05) and 5-hydroxyindol-3-acetic acid (DS versus control: 17 ± 5 vs 30 ± 4 pM/mg tissue; P < .05) in subjects with DS versus control subjects, respectively (Fig 1A).

Amino Acids

Amino Acids With Suspected Developmental Function

GABA concentration was reduced by 61% ± 17% (DS versus control: 55 ± 10 vs 140 ± 22 μM/mg tissue; P < .05), and taurine concentration was reduced by 25% ± 11% (DS versus control: 584 ± 68 vs 774 ± 32 μM/mg tissue; P < .05) in subjects with DS versus control subjects, whereas no difference was observed for glutamate (DS versus control: 511 ± 52 vs 687 ± 78 μM/mg tissue; Fig 2A).

Other Amino Acids

No differences were observed for arginine (DS versus control: 4 ± 1.7 vs 4 ± 0.6 μM/mg tissue), aspartate (DS versus control: ASP 66 ± 13 vs 71 ± 11 μM/mg tissue), glutamine (DS versus control: 441 ± 61 vs 679 ± 40 μM/mg tissue), glycine (DS versus control: 289 ± 45 vs 326 ± 40 μM/mg tissue), histidine (DS versus control: 49 ± 8 vs 47 ± 12 μM/mg tissue), and serine (DS versus control: 191 ± 26 vs 219 ± 20 μM/mg tissue) in the frontal cortex (Fig 2B).

Catecholamines

A significant reduction of 80% ± 1% was observed for dopamine (DS versus control: 519 ± 207 vs 102 ± 9 pg/mg tissue; P < .005), whereas no difference was observed for serotonin (DS versus control: 9 vs 10 pM/mg tissue; P = 1.0).
observed for noradrenaline (DS versus control: 328 ± 70 vs 152 ± 15 pg) in subjects with DS versus control subjects, respectively (Fig 1B).

**DISCUSSION**

The present study was undertaken to test the hypothesis that altered levels of brain amino acids or monoamines could account for the altered neurodevelopmental process in brains with DS. We observed reductions in serotonin and its metabolite 5-hydroxyindol-3-acetic acid in DS fetal frontal cortex tissue versus control subjects. Reduction of both serotonin and 5-hydroxyindol-3-acetic acid suggests a reduced synthesis in DS subjects that is present from at least the 20th gestational week in the frontal cortex.

Serotonin is present in early fetal CNS tissue serving as a regulator of brain development, and it is also hypothesized that serotonin’s influence continues throughout development. Support for the beneficial role of the serotonergic system affecting neuronal proliferation, differentiation, migration, and synaptogenesis in the developing fetal brain has arisen from studies investigating pharmacologically induced serotonin depletion or enhancement. For example, in embryonic cultures from the rat visual neocortex, treatment with a physiologic concentration of serotonin has a substantial effect on the morphofunctional development rate, as demonstrated by the increase in glia proliferation, neuron differentiation, and synaptogenesis and by an increase in the number of active neurons. Furthermore, in embryonic Helisoma, neuronal cultures serotonin can stimulate neurite outgrowth in nongrowing neurites by raising intracellular calcium.

Extensive literature exists showing the effect of serotonin depletion and the resultant deleterious effect on brain development. It is observed that embryonic serotonin depletion, among other effects, results in a delay in the onset of neurogenesis and reduction in the density of nonserotoninergic synapses (as reviewed in ref 21), as well as a decrease in synaptic plasticity. However, the principle question is whether the reduction of serotonin by 41% observed in this study in DS fetal frontal cortex tissue is sufficient to produce alterations in the brain development of subjects with DS. This reduction in serotonin content in human DS fetal frontal cortex tissue is within the range that chemically induced serotonin depletion induces in a variety of animal models. Reduction of embryonic serotonin tissue concentration by 43% in Helisoma results in the inhibition of neurite and synapse formation, and 50% reduction results in a delay in maturation of thalamocortical pathways. Furthermore, reduction of embryonic serotonin tissue content by 63% is seen to result in reduced cortical thickness; loss of neurons in all layers of the cingular, occipital, and parietal cortex; and reductions in the number of neurons in the raphe nuclei and substantia nigra. In addition, appropriately oriented cells in the hippocampus are also observed. On the basis of these previous findings, we postulate that the observed reduction of serotonin in DS fetal frontal cortex tissue is sufficient to induce alterations in brain development.

As already stated above, concurrent decreases in serotonin and 5-hydroxyindol-3-acetic acid by >40% indicate reduced production but may also additionally suggest a reduced release of serotonin. Similar decreases in cortical serotonin tissue concentration in rodents are associated with reduced extracellular serotonin release (eg, see ref 26). Thus, reduced extracellular release of serotonin may be expected in human fetal DS frontal cortex potentially leading to decreased activation of serotonin receptors. Serotonin-1A receptors are expressed early in embryonic life and have been implicated in various developmental effects in vitro. The serotonin-1A receptor is proposed to modify neuritic branching of developing mammalian cortical neurons, because stimulation of this receptor results in a reduction of neurite branching. An opposite effect is observed in the hippocampus, because serotonin-1A receptor activation stimulates dendritic differentiation, and early postnatal depletion of serotonin reduces the length of dendrites and the number of dendritic spines of hippocampal neurons, an effect that was shown to depend on serotonin-1A receptors. Interestingly, it is observed that the density of the serotonin-1A receptor is decreased in fetuses with DS between the 16th and 22nd week of gestation in both the frontal cortex and the granular layer of the dentate gyrus. Coupled to the fact that there is decreased expression of the serotonin-1A receptor in the frontal cortex in subjects with DS, determined at the same time period as in our study, it seems plausible that the observed shortened basilar dendrites and decreased number of spines with altered morphology observed in subjects with DS (as reviewed in ref 30) may be, in part, attributed to alterations in the serotonergic system.

Our observations that both GABA and taurine levels were reduced in the frontal cortex of subjects with DS may constitute an additional mechanism for abnormal brain development. Extensive investigations have indicated that a rise in intracellular calcium is essential for neuronal growth and differentiation and that this influx of calcium can be produced by the operation of voltage-dependent calcium channels and excitatory amino acid receptors. The main excitatory drive in the mature brain is mediated by glutamatergic synapses. However, in the immature developing brain, glutamatergic synapses are poorly developed because they lack functional AMPA receptors, and, additionally, at resting cell membrane potentials, N-methyl-D-aspartate channels are blocked by magnesium. GABA and GABA-releasing synapses are formed before glutamatergic contacts and provide the principle excitatory drive in developing im-
mature neurons. It is observed that GABA, acting via GABA$_A$ receptor-mediated depolarization, alters resting cell membrane potentials sufficiently to release the voltage-dependent magnesium block on N-methyl-D-aspartate channels to allow calcium influx into the cell. In vitro, GABA, acting on GABA$_A$ receptors, promotes proliferation and differentiation of neurons by increasing the length and branching of neurites in cultured embryonic chick cortical neurons, embryonic rat cerebellum neurons, and embryonic raphe neurons (reviewed in ref 32). In contrast to the trophic actions of GABA, inhibition of GABA$_A$ receptor-mediated calcium influx results in inhibition of neurite outgrowth. In cultured embryonic hippocampal neurons, the presence of GABA$_A$ receptor antagonists results in a reduction in the number of primary neurites and branching points, which results in a concomitant decrease of the total neuritic length. Inhibition of the trophic effects of GABA has been reproduced by agents acting on GABA synthesis, GABA$_A$ receptor blockade, and L-type calcium channel inhibition, which attenuated neurite outgrowth in the embryonic rat neocortex.

Taurine is postulated to be a neurotrophic factor in brain development, because proliferation and differentiation of neurons are increased in pure cerebral fetal human brain cultures containing taurine. Furthermore, abnormal kitten cortex development is observed in association with maternal dietary taurine deprivation, mainly because cortex neuroblasts are aggregated both at the ventricular and pial zones, having failed to migrate and differentiate normally. In addition, only a few pyramidal and nonpyramidal neurons are found that exhibit heavily spined dendritic processes indicative of poor arborization in maternal taurine deprivation. Furthermore, it is observed that taurine triggers inward currents and induces membrane depolarization resulting in elevation in intracellular calcium concentrations in the rat embryonic cortex.

Taken together, these observations suggest that GABA and taurine act to increase calcium influx into the neuron providing the main excitatory drive within fetal brain development. Because inhibition of GABA$_A$ receptor-mediated transmission and reduction in taurine concentration results in dysfunctional brain development, the reductions in GABA and taurine that we observed in this study may constitute another important mechanism for the altered brain development in subjects with DS. We observed no alteration in the concentration of arginine, aspartate, glutamate, glutamine, glycine, histidine, or serine in DS fetal frontal cortex brains. Unaltered tissue concentration of these amino acids demonstrates that the reduction in tissue concentration observed for serotonin, GABA, taurine, and dopamine was specific for fetal brains of subjects with DS.

It has been suggested that dopamine plays a role in establishing synaptic contacts, because it is observed in rodents that depletion of dopamine via lesions of the ventral tegmental area results in the reduction of cortical thickness. However, a caveat to this experiment is that it is impossible to exclude a combined effect with serotonin, because it is very difficult to thermally lesion the ventral tegmental area without damaging the serotoninergic fibers projecting to the neocortex. It might be interesting to investigate the expression of D1 receptors, because they are highly expressed in the developing brain and are involved in working memory performance later in life.

It would be very important to determine whether the observed reductions in neurotransmitters are a cause or consequence of the observed malformed brain development in subjects with DS. As indicated in the Introduction, there are only specific brain regions with reduced volume, including the frontal cortex, observed from birth in subjects with DS. This observation potentially indicates that downregulation of neurotransmitters in fetal brains may be a cause of malformed brain growth. On the other hand, if downregulation of neurotransmitters is a consequence of a malformed brain, it is likely that reductions in all of the neurotransmitters would be observed. The fact that we found specific alterations in some neurotransmitters but not others would argue against the observed changes being a consequence of a malformed brain. Follow-up studies determining the time course of both brain development and neurotransmitter levels are necessary to clarify this question.

**CONCLUSIONS**

Normal brain development depends on a series of precisely timed events governed by genetic and epigenetic events. Serotonin, GABA, taurine, and dopamine, among other compounds, are critical for brain development, because they promote the detailed wiring within the developing fetal brain. In vitro studies have demonstrated that decreases in the concentration of these compounds induce alterations in signaling processes resulting in dysfunctional brain developmental events, such as generation, migration, and differentiation of neurons and glia cells. In the brain with DS, abnormalities in brain development are evident from birth, however, the cause of developmental failure still remains unclear. In the present study we have observed specific reductions in serotonin, GABA, taurine, and dopamine in the frontal cortex of fetal brains with DS. The observed reduction in serotonin tissue concentration in DS seems to persist into adulthood and may be involved in behavioral symptoms, including anxiety, compulsive behaviors, self-injury, and aggression, which have been shown to be sensitive to serotonin-increasing treatments, such as selec-
tive serotonin reuptake inhibitors.41–43 Treatments directed against the other neurotransmitters observed to be altered in fetal DS may result in additional viable treatment strategies. We conclude that alterations in serotoninergic, GABA-ergic, and dopaminergic signaling circuitry during critical developmental periods may contribute to dysfunctional brain development in DS.

REFERENCES
17. Singewald N, Philippu A. Catecholamine release in the locus coeruleus is modified by experimentally induced changes in haemodynamics. Naunyn Schmiedebers Arch Pharmacol. 1993;347:21–27
38. Flint AC, Liu X, Kriegstein AR. Nonsynaptic glycine receptor


Fetal Down Syndrome Brains Exhibit Aberrant Levels of Neurotransmitters Critical for Normal Brain Development

Nigel Whittle, Simone B. Sartori, Mara Dierssen, Gert Lubec and Nicolas Singewald

Pediatrics 2007;120:e1465-e1471; originally published online Nov 12, 2007;
DOI: 10.1542/peds.2006-3448