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ARTICLE

THEMED SECTION
Serotonin: New Aspects of its Functions in the Brain

CINP

5-Hydroxytryptophan rescues serotonin response to stress in prefrontal cortex of hyperphenylalaninaemic mice

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Abstract

Adult early treated hyperphenylalaninaemic patients can show specific deficits of prefrontal cortical functions. The development of additional therapeutic strategies for these patients requires the understanding of the mechanisms involved in phenylalanine-dependent impairment of fronto-cortical functions. We tested the hypothesis of phenylalanine interference with aminergic neurotransmission in the prefrontal cortex by evaluating, *in vivo*, amine release in adult Pah^{enu2} mice, the genetic model of phenylketonuria. Mice of healthy background responded to a psychogenic stressor with the classic time-dependent increase of norepinephrine, dopamine and serotonin release from prefrontal cortical terminals. Neither the dopaminergic nor the serotoninergic responses were observable in the Pah^{enu2} mice. Temporary reduction of circulating phenylalanine, by phenylalanine-free diet without amino- acid supplement, promoted recovery of the serotonin response only, demonstrating direct interference with serotonin synthesis in the mature brain. Evaluation of different steps of serotonin synthesis in the prefrontal cortex of hyperphenylalaninaemic mice demonstrated inhibition of cortical tryptophan hydroxylase activity. Finally, systemic administration of 5-hydroxytryptophan, the product of tryptophan hydroxylase activity, allowed frontal cortical serotonin response to stress in hyperphenylalaninaemic mice. Collectively, these results demonstrate that hyperphenylalaninaemia interferes with the ability of the mature prefrontal cortex to respond to psychological challenges, point to serotonin synthesis as the target of phenylalanine interference, and support the use of 5-hydroxytryptophan in lifelong treatment of hyperphenylalaninaemic subjects.

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Introduction

Hyperphenylalaninaemia (HPA) refers to all clinical conditions characterized by excess of circulating phenylalanine (Phe). Brain is most vulnerable to high Phe levels during early postnatal life and early HPA is well known to promote severe mental retardation and neuropathological signs. However, there is increasing evidence of negative effects of high circulating Phe levels in adulthood. Most of these evidences derive from studies on classical phenylketonuria (PKU; McKusick 261600), an inherited metabolic disease caused by a deficiency of the enzyme phenylalanine hydroxylase (PAH; EC 1.14.16.1) necessary to convert the amino acid into tyrosine that leads to accumulation of extremely high levels of circulating Phe (>20 mg/dl). Strict adherence to a Phe-free diet from early post-natal life to early adolescence prevents the severe developmental deficits promoted by HPA. However, even early and continuously treated PKU patients may show specific deficits. Currently adults are encouraged to stay on dietary treatment for as long as possible. However, lifetime dietary therapy is expensive as well as a social burden, and the NIH consensus panel has encouraged research for alternative

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therapies for PKU (National Institutes of Health Consensus Development Panel, 2001).

Prefrontal/executive skills (working memory, abstract reasoning, problem solving, planning ability, sustained attention, inhibitory control, mental flexibility) appear to be deficient in early treated PKU patients that relaxed or stopped the Phe-free diet and deficits correlate with blood Phe concentrations at the time of testing rather than with Phe levels during development (Brumm *et al*. 2004; Channon *et al*. 2004; Diamond *et al*. 1997; Huijbregts *et al*. 2002; Leuzzi *et al*. 2004; Schmidt *et al*. 1994; Smith *et al*. 2000; White *et al*. 2002). These data support the hypothesis that the hyperphenylalaninaemic condition has negative effects beyond development, and suggest that excess Phe interferes with prefrontal cortex (PFC) functioning.

The PFC is innervated by dopamine (DA), norepinephrine (NE), and serotonin (5-HT) afferents and a substantial body of evidence indicates that monoamines in the PFC play a major role in emotion and in cognitive functions (Arnsten & Robbins, 2002; Aston-Jones & Cohen, 2005; Clarke *et al*. 2004, 2005, 2006; Goldman-Rakic, 1999; Lapiz & Morilak, 2006; Walker *et al*. 2008). Although reduced levels of biogenic amines in post-mortem brain tissue (McKean, 1972), low levels of biogenic amine metabolites in cerebrospinal fluid of patients with HPA (Bonafé *et al*. 2001; Butler *et al*. 1981) and reduced brain amine levels and metabolism in PAH^{enu2} mice (ENU2), the genetic murine model of PKU (Pascucci *et al*. 2002, 2008; Puglisi-Allegra *et al*. 2000) have been reported, only cortical DA deficits have been investigated until now. In fact, it has been proposed that Phe interference on cognitive functions depends on impaired DA transmission in the PFC due to the reduced availability of tyrosine hydroxylase, the rate-limiting enzyme for DA production, since DA neurons in the PFC are particularly sensitive to decreases in tyrosine availability (Diamond, 1996). Although this hypothesis is strong and empirically supported, there are conflicting data. Indeed, dietary tyrosine supplement does not promote cognitive improvement in PKU patients (Smith *et al*. 1998), and there are PKU-dependent deficits in frontal functions and processing speed that are nonresponsive to pharmacological modulation of DA transmission (Luciana *et al*. 2004).

Since PFC functioning involves all biogenic amines and previous data reported more severe 5-HT deficits in ENU2 mice (Pascucci *et al*. 2002, 2008; Puglisi-Allegra *et al*. 2000), Phe interference with DA, NE and especially 5-HT cortical metabolism must be investigated.

To test the hypothesis of a Phe-dependent deficit in frontocortical aminergic transmission we evaluated the effect of variable circulating levels of Phe on cortical 5-HT, DA and NE response to an ecologically relevant challenge. This information, unattainable in human subjects, can be achieved in animal models through evaluation of amines release by intra-cerebral microdialysis in specific brain areas of freely moving subjects during exposure to psychological experiences known to affect cortical neurotransmission. This approach can be used in ENU2 mice to identify the mechanism by which excess Phe interferes with cortical aminergic transmission and to directly test its susceptibility to specific pharmacological manipulation. Indeed, excess Phe could influence cortical aminergic transmission by reducing brain availability of the amino-acid precursors, tyrosine and tryptophan or by inhibiting hydroxylase activity. The demonstration of the first mechanism would support lifelong dietary supplementation with large neutral amino acids (Koch *et al*. 2003). The demonstration of the second mechanism would, instead, support therapeutic use of hydroxylated aminergic precursors such as 5-hydroxytryptophan (5-HTP), the main rate-limiting factor in 5-HT synthesis, and the product of tyrosine hydroxylase: 3,4-dihydroxyphenylalanine.

Therefore, the present study aims: (1) to demonstrate altered aminergic transmission in PFC of adult PKU-affected mice and the dependence of this alteration on excess of circulating Phe levels in the mature organism; (2) to discover the mechanism by which excess Phe interferes with frontal cortical amine transmission; (3) to demonstrate the ability of pharmacological manipulations of amine synthesis to rescue frontal cortical amine transmission in the presence of high circulating Phe levels.

Method

Animals

The homozygote (−/−) Pah^{Enu2} (ENU2) and homozygote (+/+) Pah^{Enu2} [wild type (WT)] mice used were obtained by heterozygous mating on BTBR background. Genetic characterization was performed on DNA prepared from tail tissue using the Easy DNA kit (Invitrogen, USA), as previously described (McDonald *et al*. 1990; Shedlovsky *et al*. 1993). At postnatal day 28, animals (sex matched) were housed 2–4 per standard breeding cage with food and water available *ad libitum* on a 12-h light/dark cycle (lights on 07:00 hours). If not otherwise specified, all mice were fed on standard laboratory chow. Experiments

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started when the animals reached age 8 wk. All mice were housed individually 24 h before surgery for microdialysis. Naive animals were used for each experiment. The total number of animals used was 114 (ENU2 72; WT 42).

All experiments were conducted in accordance with European legislation (EEC no. 86/609), with Italian national legislation (DL no. 116/92) governing the use of animals for research, and with the guidelines of the National Institutes of Health for the use and care of laboratory animals.

Drugs

Chloral hydrate, NSD-1015, L-dopa and 5-HTP were purchased from Sigma-Aldrich (USA). NSD-1015 was dissolved in artificial CSF and perfused in probe. Chloral hydrate and 5-HTP were dissolved in saline (0.9% NaCl) and injected i.p. in a volume of 10 ml/kg.

Restraint apparatus

The apparatus was formed by an adjustable neck-blocking support mounted on a Plexiglas base and movable U-shaped metal piece that could be fixed to the base at the level of the animal's hips thus preventing the mouse from turning on its back (Cabib & Puglisi-Allegra, 1991).

In-vivo *microdialysis*

Mice were anaesthetized with chloral hydrate (450 mg/kg i.p.), mounted in a stereotaxic frame (David Kopf Instruments, USA) and implanted unilaterally with a guide cannula (stainless steel, shaft outer diameter 0.38 mm, length 1 mm; Metalant AB, Sweden), fixed with epoxy glue and dental cement, into the medial PFC (AP +2.8, L −0.6; according to the atlas of Franklin & Paxinos, 1998). Mice were allowed to recover in their home cage. The probe (length 2 mm; MAB 4 cuprophane microdialysis probe; Metalant AB) was introduced 24 h before the microdialysis experiments. The mice were lightly anaesthetized with chloral hydrate to facilitate manual insertion of the probe into the guide cannula. Animals were then returned to their home cages. The dialysis probe was connected to a CMA/100 pump (Carnegie Medicine, Sweden) through PE 20 tubing (Metalant AB) and an ultra-low torque dual-channel liquid swivel (model 375/D/22QM; Instech Laboratories, USA) to allow free movement. Artificial cerebrospinal fluid (147 mM NaCl, 1 mM MgCl, 1.2 mM $CaCl_2$, 4 mM KCl) was pumped through the dialysis probe at a constant flow rate of 2 μl/min. On the day of the experiments (48 h after surgery), each animal was transferred to a Plexiglas cylinder provided with microdialysis equipment (Instech Laboratories) and with home cage bedding on the floor. Dialysis perfusion was started 1 h later, then dialysis perfusion commenced; after the start of dialysis perfusion mice were left undisturbed for 2 h before the collection of baseline samples. Dialysate was collected every 20 min. The mean concentration of the three samples collected immediately before any experimental manipulation (<10% variation) was taken as basal concentration.

Only data from mice with correctly placed cannula are reported (Fig. 1*g*). Animals subjected to stress experience were put on restraint apparatus for 120 min. ENU2/Phe-free diet mice were submitted to brain surgery procedure on day 6 of the diet. The microdialysis experiment was started on day 7 of the diet. Dialysate samples (20 μl) were transferred for HPLC analyses.

DA, NE and 5-HT were determined by a HPLC system coupled to a coulometric detector (model 5200° Coulochem II; ESA, USA). For DA and NE detection, the conditioning cell was set at +400 mV, electrode 1 at +200 mV, and electrode 2 at −250 mV; the mobile phase was as described previously (Westerink *et al*. 1998). For 5-HT detection, the conditioning cell was set at +250 mV, electrode 1 at +60 mV, and electrode 2 at +200 mV; the mobile phase was as described previously (Gartside *et al*. 2003). A Nova-Pack C18 column (3.9 × 150 mm; Waters, USA) and a Sentry Guard Nova-Pack C18 pre-column (3.9 × 20 mm) maintained at 30 °C were used. The detection limit of the biogenic amine assay was 0.1 pg.

5-HTP accumulation was assessed following perfusion with Ringer's solution containing 20 μm NSD-1015 (Sigma) pumped through the dialysis probe at a constant flow rate of 2 μl/min. Dialysates were collected at 20-min intervals for 3 h.

5-HTP was determined by HPLC coupled to an amperometric detector (Decade II, ESA). The detector potential was set at +700 mV against an Ag/AgCl reference electrode. The mobile phase was as previously described (Nakahara *et al*. 2000). The detection limit of the 5-HTP assay was 0.1 pg.

Visualization of probe placement

Probe placement was assessed in slices immunostained for tyrosine hydroxylase, in order to obtain an index of catecholaminergic afference of the area. Indeed, ENU2 mice are exposed to HPA and hyposerotoninaemia from early developmental stages, a

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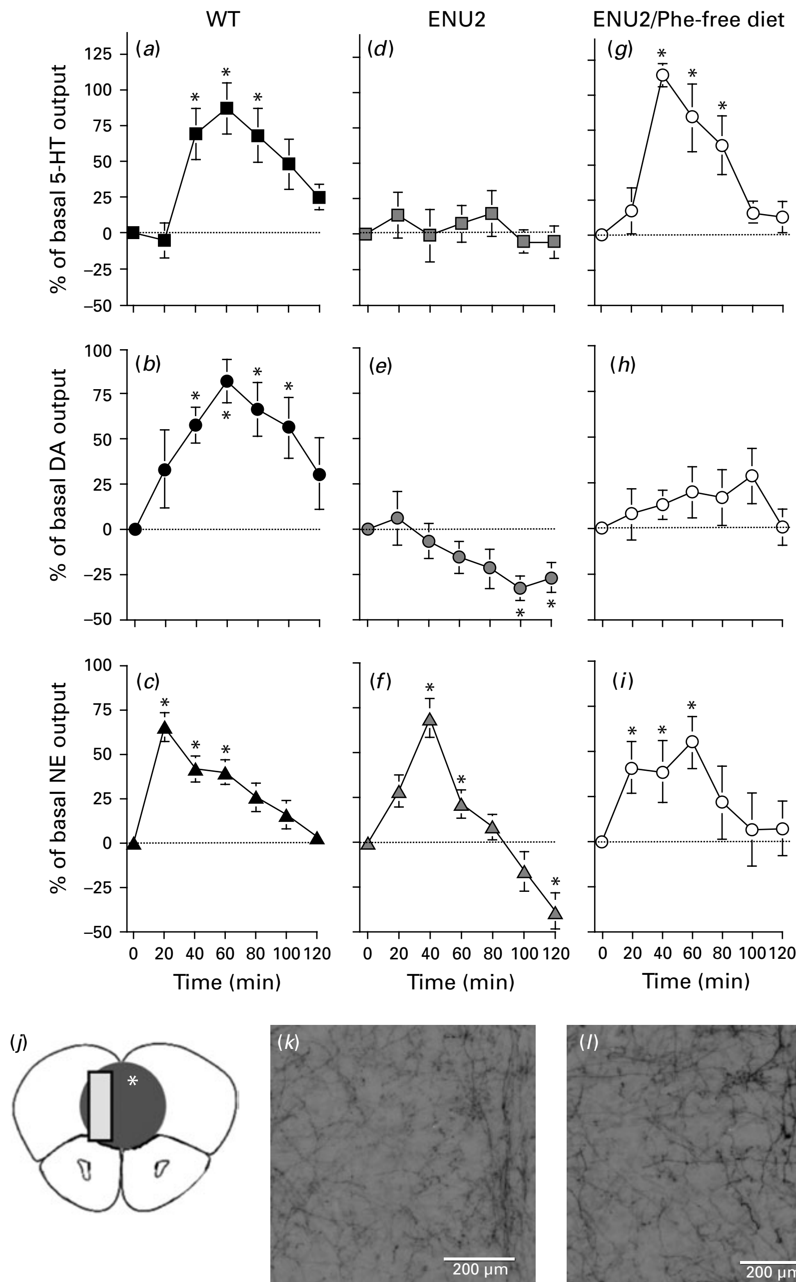

Fig. 1. Aminergic neurotransmission is compromised in the prefrontal cortex (PFC) of ENU2 mice, and deficient prefrontal cortical serotonin (5-HT) transmission is dependent on current blood phenylalanine (Phe) levels. Changes in extracellular levels of 5-HT (*a*, *d*), dopamine (DA) (*b*, *e*) and norepinephrine (NE) (*c*, *f*), are measured by *in-vivo* microdialysis in the PFC of wild type (WT) (*a–c*) and ENU2 (*d–f*) mice during 120-min exposure to restraint stress. Dialysate samples were collected at 20-min intervals. Changes in extracellular levels of 5-HT (*g*), and DA (*h*) and NE (*i*) in the PFC of ENU2 mice exposed to 1 wk Phe-free diet during 120-min exposure to restraint stress. (*g*) ENU2/Phe-free diet mice recover cortical 5-HT response to stress and (*h*) modulate NE response abolishing late fall below basal levels, whereas (*i*) Phe-free diet was ineffective on frontal cortical DA response to stress. Results are expressed as percent change (means ± S.E.M.) from basal values. Statistical analyses were performed on raw data ($n = 8$ mice per group). * $p < 0.05$ *vs.* basal values. (*j*) Schematic representation of microdialysis probe locations (grey rectangle) and punching area for tissue samples (dark grey circle). The numbers indicate millimetres rostral to bregma according to Franklin & Paxinos (1998). Panels (*j*) and (*k*) show no differences in tyrosine hydroxylase-immunoreactive neuropil in the PFC [identified by an asterisk (*) in the graphic representation of panel (*j*) of WT (*k*) and ENU2 (*l*) mice]. Scale bar, 200 μm.

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condition that might have affected PFC maturation. Mice were deeply anaesthetized with chloral hydrate (1 mg/kg i.p.) then immediately perfused through the left ventricle with 0.9% saline followed by cold phosphate-buffered 4% formalin and their brains excised. Brains were post-fixed overnight in the same formalin solution at 4 °C then transferred to a 30% sucrose solution for cryoprotection until they sank. Brains were then frozen with dry ice and cut with a sliding microtome. Transversal sections (40-μm-thick) were collected throughout the forebrain. Immunohistochemistry was performed according to previously published procedures (Conversi *et al*. 2004).

Tissue and blood analysis

Brain and blood analyses were performed as previously reported (Puglisi-Allegra *et al*. 2000). and stored in liquid nitrogen until the day of biochemical assay. Briefly, punches of PFC were obtained from frozen brain slices and stored frozen until analysis. The day of analysis, punches were weighed and homogenized in 0.05 M $HClO_4$. The homogenates were centrifuged at 14000 rpm for 20 min at 4 °C.

Tissue levels of 5-HT, DA, NE and their metabolites [5-hydroxyindoleacetic acid (5-HIAA), 3-4-dihydroxyphenylacetic acid (DOPAC), homovanillic acid (HVA), and 3-methoxy-4-hydroxyphenylethyleneglycol (MHPG)] were assessed simultaneously in ENU2/Phe-free diet ($n=6$), ENU2/control diet ($n=6$) and WT ($n=6$) mice by HPLC (Puglisi-Allegra *et al*. 2000).

For blood analysis, blood samples were placed in heparinized tubes and centrifuged at 2500 rpm, at 4 °C, for 10 min. An aliquot of supernatant was collected, and transferred to a new tube with 35% 5-sulfosalicylic acid (10:1 v/v), and centrifuged at 8000 rpm, at 4 °C, for 5 min. Aliquots of the supernatant from preparation of brain and blood samples were transferred to HPLC system for biochemical assay. For Western blot analysis, PFC tissues from individual animals (ENU2 and WT) were prepared as previously described (Chamas *et al*. 2004).

Phe, tryptophan, and tyrosine brain and blood levels were assessed by HPLC coupled with a fluorescence detector (Waters 474 model) as previously described (Cabib *et al*. 2003; Pascucci *et al*. 2002).

Experimental procedures

Expt 1. Aminergic neurotransmission in PFC of ENU2 mice

To evaluate DA, 5-HT and NE neurotransmission in PFC of adult hyperphenylalaninaemic mice, one group of ENU2 ($n=8$) mice and one group of WT ($n=8$) were challenged with 120 min of restraint stress. All animals were handled and submitted to the surgery procedure described in the 'In-vivo microdialysis' section. On the day of the experiment all animals were restrained for 120 min after collection of the three samples required to evaluate basal amine outflow.

Expt 2. Effect of blood Phe levels on frontal cortical aminergic metabolism and release

The influence of the hyperphenylalaninaemic status on the content and dynamics of PFC amines was evaluated by *ex-vivo* (tissue concentrations) and *in-vivo* (outflow) assay. The results obtained by the two methods offer different indices of aminergic transmission since the first one evaluates both intracellular and extracellular levels, thus being strongly influenced by synthesis-related factors, while the second is a better measure of extracellular amine levels, modulated by a number of factors unrelated to amine synthesis. Levels of circulating Phe were modulated by temporary elimination of the alimentary Phe.

Ex-vivo experiments were performed in three groups of naive mice. The first two groups were adult ENU2 mice exposed to different feeding conditions: 8 d on a Phe-free diet (Mucedola, Italy) (ENU2/Phe-free diet group, $n=6$), or feeding on the normal diet (ENU2/control diet, $n=6$). The third group was formed by WT mice feeding on the normal diet (WT, $n=6$). All mice were killed by decapitation on day 8 of the diet and brain tissue and blood samples were collected as described in the 'Tissue and blood analysis' section.

Microdialysis experiments were performed in a different group of ENU2 mice exposed to 8 d Phe-free diet (ENU2/Phe-free diet group, $n=8$). All animals were submitted to the brain surgery procedure on day 6 of the diet. The experiment was performed on day 8 of the diet. After collection of baseline samples, all mice were restrained as previously described.

A third group of experiments evaluated influence of the hyperphenylalaninaemic status on two main limiting factors of 5-HT synthesis: brain availability of the amino-acid precursor tryptophan and tryptophan hydroxylation.

Expt 3. Effect of excess Phe on brain availability of the 5-HT amino-acid precursor, tryptophan

To test the relationship between circulating Phe levels and tryptophan access to the brain, blood and brain levels of Phe and tryptophan and their blood/brain

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ratios were evaluated in ENU2 ($n=6$) and WT ($n=6$) male mice.

Expt 4. In-vivo *hydroxylation of tryptophan in PFC of PKU-affected mice*

Hydroxylation of tryptophan was assessed by quantification of the *in-vivo* linear accumulation of 5-HTP following blockade of decarboxylation in the PFC (Carlsson & Lindqvist, 1973). Naive ENU2 ($n=8$) and WT ($n=8$) mice were prepared for microdialysis experiments as previously described. After collection of the basal samples, Ringer's solution was substituted with Ringer's solution containing 20 μm NSD-1015 (Sigma), an amino acid decarboxylase inhibitor. Dialysates were collected at 20-min intervals for 3 h and 5-HTP levels were detected and quantified as previouslydescribed.

In order to rule out the involvement of reduced PFC availability of the enzyme tryptophan hydroxylase (TPH), we quantified the protein in different groups of ENU2 ($n=8$) and WT ($n=8$) by Western blot analysis. Animals were sacrificed by decapitation and PFC tissue was obtained by the punching technique as previously described.

Expt 5. Effect of systemic administration of 5-HTP rescues on prefrontal cortical 5-HT response to stress in PKU-affected mice

A final set of experiments assessed the susceptibility of prefrontal cortical 5-HT stress response in hyperphenylalaninaemic mice to increased availability of 5-HTP.

First, we identified a *per se* ineffective dose of systematically administered 5-HTP by performing a dose–response study. Naive ENU2 ($n=6$) and WT ($n=6$) mice were injected i.p. on two consecutive days with saline or 5-HTP (2.5, 5, 10, 20 mg/kg) and 5-HT *in-vivo* release was evaluated by microdialysis. Each dose was injected in a random order and sufficient time was allowed for neurotransmitter to return to basal levels. However, no more than two 5-HTP doses were administered daily.

The effect of systemic administration of a *per se* ineffective dose of 5-HTP on frontal cortical 5-HT response to stress was evaluated by intracerebral microdialysis. Two groups of naive ENU2 mice were used for two experiments: one group received an i.p. injection of saline (ENU2/Sal group, $n=8$) and a second group received 5-HTP (ENU2/5-HTP 2.5 mg/kg group, $n=8$) before being restrained for 120 min. All mice were prepared for the microdialysis experiment as previously described.

Table 1. *In-vivo* baseline values of 5-HT, DA and NE in the PFC of wild-type (WT) and ENU2 mice

	Group	
	WT	ENU2
5-HT	1.63 ± 0.13	1.81 ± 0.28
DA	0.95 ± 0.14	1.05 ± 0.14
NE	2.04 ± 0.24	1.19 ± 0.19[a]

Values are expressed as means ± S.E.M.
In-vivo basal release of 5-HT, DA and NE from PFC of WT and ENU2 mice (pg/20 μl). ANOVAs revealed significant effect of genotype factor for NE basal levels ($F_{1,14}=7.39$, $p<0.05$ *vs.* WT).
[a] $p<0.05$ *vs.* WT mice.

Statistics

The effect of stress on aminergic cortical release was analysed in each group (ENU2 and WT mice) by repeated-measures analysis of variance (ANOVA) (time, seven levels, 0, 20, 40, 60, 80, 100 and 120 of restraint) for each amine. Statistical analyses were performed on raw data (concentrations of pg/20 μl) and data were presented in figures as percent change from baseline level. Simple effects were assessed by one-way ANOVA for each time-point. Baseline levels are reported in Table 1.

The effects of Phe-free diet on basal levels of 5-HT, DA, and NE were analysed by one-way ANOVA (group, two levels = ENU2/Phe-free diet and ENU2/control diet). The effects of Phe-free diet on aminergic cortical release in ENU2 mice subjected to restraint were evaluated by ANOVA (time, seven levels, 0, 20, 40, 60, 80, 100 and 120 of restraint).

Comparison of levels of cortical tissue amines and metabolites and blood Phe among groups (WT, ENU2/Phe-free diet and ENU2 control) was made by one-way ANOVA followed by Duncan's *post-hoc* test for multiple comparisons. Results concerning cortical tissue levels are reported in Table 2.

The effects of genotype (WT and ENU2) on Phe and, tryptophan and tyrosine brain and blood levels and on blood/brain ratios were evaluated by one-way ANOVA. The effects of genotype on 5-HTP accumulation in PFC were analysed by repeated-measures ANOVA with one between factor (genotype, two levels, WT and ENU2) and one within factor (time, ten levels, 0, 20, 40, 60, 80, 100, 120, 140, 160 and 180). The effect of genotype (WT and ENU2) on TPH protein levels was evaluated by one-way ANOVA. The effect of 5-HTP on 5-HT frontal cortical release

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Table 2. Effect of phenylalanine-free diet on cortical tissue levels of 5-HT, DA, NE and their metabolites

	Group		
	WT	ENU2/control diet	ENU2/Phe-free diet
5-HT	947±120	193±30[a]	739±32[b]
5-HIAA	185±16	19±3[a]	173±13[b]
DA	182±22	92±16[a]	108±14[a]
DOPAC	29±3	12±1[a]	17±1[a]
HVA	126±10	91±9[a]	97±8[a]
NE	551±54	154±27[a]	386±19[a,b]
MHPG	26±3	14±3[a]	23±1

Values are expressed as means±S.E.M.
Effect of 1 wk of phenylalanine-free diet on tissue levels of 5-HT, DA, NE and their metabolites in PFC (ng/g wet weight±S.E.M.). ANOVAs revealed a significant effect of group factor for three amines (5-HT: $F_{2,15}=27.68$, $p<0.0001$; DA: $F_{2,15}=8.20$, $p<0.01$; NE: $F_{2,15}=30.06$, $p<0.0001$) and their metabolites (5-HIAA: $F_{2,15}=55.46$, $p<0.0001$; DOPAC: $F_{2,15}=14.57$, $p<0.001$; HVA: $F_{2,15}=4.68$, $p<0.05$; MHPG: $F_{2,15}=6.411$, $p<0.01$).
[a] $p<0.05$ *vs.* WT mice.
[b] $p<0.05$ *vs.* ENU2/control diet mice ($n=6$ for group).

was analysed in ENU2 mice by ANOVA (time, seven levels, 0, 20, 40, 60, 80, 100 and 120). Comparisons at each time-point were performed by one-way ANOVA. The effect of 5-HTP treatment on 5-HT release in PFC of ENU2 mice subjected to restraint was analysed by repeated-measures ANOVAs with one between factor (treatment, two levels, saline and 5-HTP2.5) and one within factor (time, seven levels, 0, 20, 40, 60, 80, 100 and 120).

Results

Expt 1: Aminergic neurotransmission is compromised in the PFC of ENU2 mice

Release of biogenic amines was evaluated by intracerebral microdialysis in the PFC of freely moving PKU-affected mice (ENU2, $n=8$) and unaffected background (WT, $n=8$), in basal condition and under environmental challenge (restraint stress).

Basal aminergic outflow from PFC of mice from the two genotypes was evaluated following prolonged habituation to the testing cage as the mean of three stable samples. The two genotypes did not differ for either 5-HT or DA basal outflow while NE outflow from the PFC of ENU2 mice was significantly reduced (Table 1). It should be noted that examination of tyrosine hydroxylase-immunoreactive neuropil did not indicate a reduced catecholaminergic afference in the prefrontal cortical area targeted by the microdialysis probes in mice of the ENU2 genotype as shown in Fig. 1(*k*, *l*) for WT and ENU2 mice, respectively. Schematic representation of microdialysis probe location (grey rectangle) and punching area for tissue samples (darker circle) are presented in Fig. 1 *j*, where the asterisk indicates location of the immunostained samples.

All animals were then restrained and 5-HT, DA and NE outflow in the PFC was assessed throughout 120-min stress. Mice of the WT strain responded to the stressful experience with a significant time-dependent increase of 5-HT ($F_{6,42}=5.78$, $p<0.001$; maximal increase at 60 min: ~80%; Fig. 1*a*), DA ($F_{6,42}=4.46$, $p<0.01$; maximal increase at 60 min: ~80%; Fig. 1*b*) and NE ($F_{6,42}=11.79$, $p<0.001$; maximal increase at 20 min: ~70%; Fig. 1*c*) release from PFC terminals. PKU-affected mice did not show any increase of prefrontal cortical 5-HT or DA release (Fig. 1*d*,*e*). Indeed, DA outflow decreased below basal levels from 100 min onwards ($F_{6,42}=3.49$, $p<0.01$; Fig. 1*e*). Instead NE outflow showed time-dependent changes characterized by a late activation (maximal increase at 40 min ~70%), and a reduction below basal levels after 100 min ($F_{6,42}=12.52$, $p<0.001$; Fig. 1*f*). These data demonstrate that in PKU-affected organisms prefrontal cortical 5-HT and DA systems are unable to sustain the neurophysiological response to stress while the NE system retains this ability but undergoes rapid exhaustion.

Expt 2: Deficient prefrontal cortical 5-HT transmission is dependent on current blood Phe levels

To identify if disturbances of PFC aminergic transmission were strictly dependent on plasmatic Phe levels, we exposed adult ENU2 mice to 1 wk Phe-free diet (ENU2/Phe-free diet, $n=8$). The alimentary regimen reported ENU2 plasmatic Phe to WT levels ($F_{2,15}=437.15$, $p<0.0001$; ENU2/Phe-free diet: 8.82±1.20; ENU2 control: 319.14±15.26; WT: 15.64±2.63 ng/μl) but it did not recover DA tissue levels in the PFC (Table 2) nor PFC DA response to stress (Fig. 1*h*). Instead, the temporary reduction of Phe dietary intake increased PFC tissue levels of 5-HT (Table 2), thus eliminating differences between PKU-affected and non-affected mice, and recovered time-dependent 5-HT response to restraint stress in PFC of PKU-affected mice ($F_{6,42}=9.08$, $p<0.0001$; Fig. 1*g*; maximal increase at 40 min: ~100%). Finally, Phe-free

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Table 3. Blood and brain levels and blood/brain ratios of amino acids in wild-type (WT) and ENU2 adult mice

	WT			ENU2		
	Blood	Brain	Ratio (blood/brain)	Blood	Brain	Ratio (blood/brain)
Phenylalanine	146 ± 9	3.4 ± 0.4	0.024	3936 ± 250[a]	61.2 ± 8.5[a]	0.015[a]
Tryptophan	301 ± 48	1.7 ± 0.3	0.006	315 ± 29	1.4 ± 0.2	0.004

Values are expressed as means ± S.E.M.
Amino acids levels (ng/10 ml) in blood and brain samples, and blood/brain ratios in WT and ENU2 adult mice.
[a] $p<0.05$ *vs*. WT ($n=6$ for group).

diet modulated NE response abolishing late fall below basal levels (Fig. 1*h*) and partially restored NE tissue levels.

These results indicate that only deficits of 5-HT transmission were dependent on circulating Phe levels.

Expt 3: Excess Phe does not reduce brain availability of the 5-HT amino-acid precursor tryptophan

To determine if high Phe levels inhibit tryptophan transport across the blood–brain barrier of ENU2 mice, we compared Phe and tryptophan blood and brain levels in ENU2 and WT mice (Table 3). Phe concentration either in the blood or in the brain of ENU2 mice was much higher than in WT mice [blood (~2700%): $F_{1,10}=205.1$, $p<0.001$; brain (~1800%): $F_{1,10}=29.40$, $p<0.001$] as expected for a mouse model of PKU; however, there were no differences between the two genotypes for tryptophan blood or brain levels. Moreover, Phe blood/brain ratio was significantly reduced in ENU2 mice while tryptophan blood/brain ratio was identical in the two genotypes. These results do not support the hypothesis that excess Phe interfere with tryptophan access to the brain.

Expt 4: **In-vivo** *hydroxylation of tryptophan in PFC is deficient in PKU-affected mice*

The two genotypes did not differ for 5-HTP basal outflow (WT: 0.4 pg/20 μl + 0.14; ENU2: 0.39 pg/20 μl + 0.07. In WT mice blockade of aromatic L-amino acid decarboxylase promoted a time-dependent increase of frontal cortical 5-HTP outflow that became significantly higher than basal levels after 60 min, reached a peak increase (185% of basal levels) after 100 min and remained stable thereafter. ENU2 mice showed a very limited increase of 5-HTP levels (70% of basal value) after 100 min of constant infusion with NSD-1015. Statistical analysis showed significant genotype × time interaction for 5-HTP accumulation ($F_{10,140}=5.23$, $p<0.0001$) indicating marked differences between the dynamics of 5-HTP accumulation in the PFC of the two genotypes (Fig. 2*a*). To determine if the observed reduction of cortical TPH activity was dependent on reduced availability of the enzyme, we measured TPH protein levels in PFC of ENU2 and WT mice by Western blot analysis. The results did not reveal any significant difference between the two genotypes (Fig. 2*b*,*c*). These results further support disturbances of TPH activity in the HPA brain.

Expt 5: Systemic administration of 5-HTP enables prefrontal cortical 5-HT response to stress in the HPA brain

First, we determined 5-HTP dose–response curves in freely moving ENU2 mice ($n=6$) injected i.p. with saline or 5-HTP (2.5, 5, 10, 20 mg/kg) by evaluation of 5-HT *in-vivo* releases by microdialysis. Each dose was injected in a random order and sufficient time was allowed for neurotransmitter return to basal levels. No more than two daily doses were administered. ENU2 mice showed a dose-dependent increase of frontal cortical 5-HT outflow. The dose of 2.5 mg/kg 5-HTP failed to affect 5-HT outflow while the maximal effect was observed following administration of 20 mg/kg (Fig. 3*a*,*b*).

Then, two groups of ENU2 mice were submitted to different i.p. treatments: saline (ENU2/Sal groups, $n=8$) or 5-HTP 2.5 mg/kg (ENU2/5-HTP2.5 group, $n=8$) and subjected to stress procedure in microdialysis experiments to assay 5-HT release in PFC. ENU2 mice pre-treated with the ineffective dose of 5-HTP but not mice injected with vehicle showed a time-dependent increase of 5-HT outflow in the PFC when exposed to the restraint procedure (significant treatment × time interaction: $F_{6,84}=4.07$, $p<0.01$; Fig. 3*c*). A significant increase in cortical 5-HT outflow

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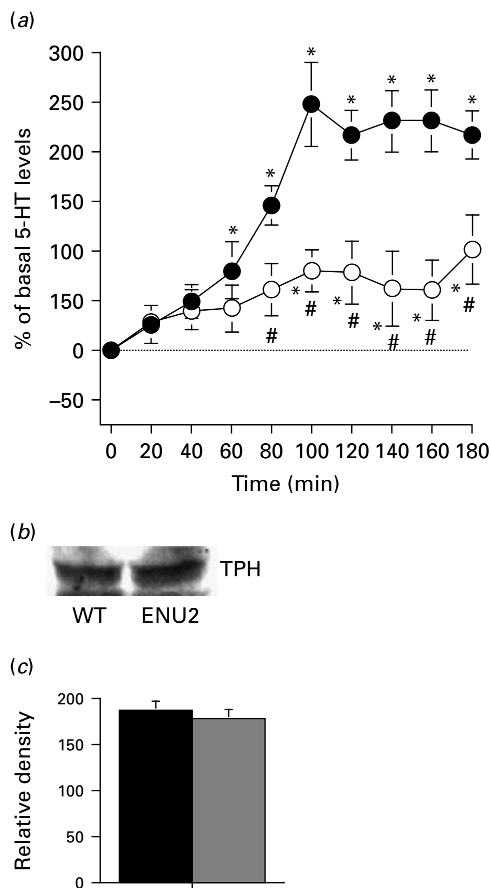

Fig. 2. *In-vivo* hydroxylation of tryptophan in prefrontal cortex (PFC) is deficient in PKU-affected mice. (*a*) Prefrontal cortical enzymatic activity of tryptophan hydroxylase (TPH) is determined measuring accumulation of transient intermediate 5-HTP *in vivo* during continuous infusion of 20 μM NSD-1015 (*m*-hydroxy-benzyl-hydrazine). Dialysates were collected at 20-min intervals. Results are expressed as percent change (means $\pm$ S.E.M.) from basal values. Statistical analyses were performed on raw data. In comparison with WT mice (–●–), the time-course of changes in extracellular levels of 5-HTP in PFC of ENU2 mice (–○–) reveals severe reduction in 5-HTP accumulation in ENU2 mice, significant from 80 min onwards ($n = 8$ mice per group). * $p < 0.05$ *vs.* basal values; $^{\#}$ $p < 0.05$ compared to WT mice. (*b*) Western blot of TPH protein obtained from PFC of ENU2 and WT mice and (*c*) quantification of protein using chemiluminescence (mean $\pm$ S.E.M.). Comparison of Western blot quantification of TPH in PFC of ENU2 and WT mice did not indicate reduced enzyme availability in mutant mice.

was evident following 60 min of stress experience and reached maximal increase (~60% from basal value) at 100 min, compared to saline-treated ENU2 mice (Fig. 3 *c*).

Discussion

The present results demonstrate that excess in circulating Phe directly interferes with the ability of the mature PFC to respond to a psychogenic stressor, point to 5-HT synthesis as the target of Phe interference, and support the use of 5-HTP in lifelong treatment of hyperphenylalaninaemic subjects.

Psychogenic stressful experiences, such as restraint, are known to promote increase in amine release in the PFC of experimental animals, a response relevant for cognitive, emotional and behavioural coping (Cuadra *et al*. 2001; Dalley *et al*. 1996; Feenstra *et al*. 2000; Finlay *et al*. 1995; Goldstein *et al*. 1996; Jedema *et al*. 1999; Kawahara *et al*. 1999; McQuade *et al*. 1999; Matuszewich *et al*. 2002; Page & Lucki, 2002; Pascucci *et al*. 2007). In agreement with these studies, healthy mice respond with enhanced 5-HT, DA and NE release to the experience of being held in the restraining apparatus. In contrast, although basal frontal cortical outflow of DA and 5-HT was unaffected and basal release of NE was reduced, when PKU-affected mice were submitted to restraint, 5-HT outflow did not change while DA outflow was even reduced. Thus, our results suggest specific impairment of 5-HT and DA cortical transmission in PKU-affected mice. Moreover, these results, as well as being the first observation of frontal cortical aminergic transmission deficits in phenylketonuric mice, also suggest the presence of compensatory mechanisms involved to maintain suitable DA and 5-HT basal outflow, necessary to hold basic physiological functions. However, these mechanisms were extremely fragile and limited, and unable to sustain the activation solicited by relevant physiological stimulations, such as stress. Nevertheless, ENU2 mice showed a largely spared cortical noradrenergic response to restraint stress.

Comparison between basal and stress-induced DA and NE outflow suggests a dynamic relationship between the two catecholamines within the PFC. Indeed, DA is the NE precursor and the absence of parallel changes in their outflow when tyrosine (DA precursor) is dramatically reduced further supports the relative independence between brain amine synthesis and release. In basal conditions compensatory mechanisms appear to reduce DA availability for NE synthesis while under stress challenge the latter is favoured. One possible explanation of this shift in priority could be the role of frontal cortical NE transmission in decision-making, and in the 'resetting' of ongoing activities in the face of a relevant change of environment. Indeed, rapid direction of

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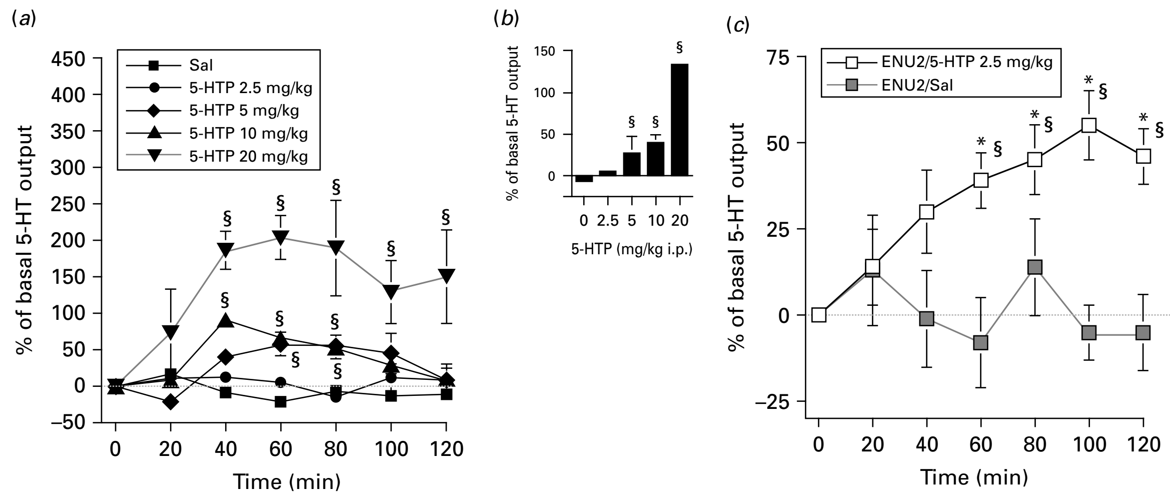

Fig. 3. Systemic administration of 5-hydroxytryptophan (5-HTP) rescues prefrontal cortical serotonin (5-HT) response to stress in ENU2 mice. (*a*, *b*) Dose-dependent effects of 5-HTP (2.5, 5, 10 and 20 mg/kg i.p.) on 5-HT outflow in the in prefrontal cortex of unstressed ENU2 mice. Results are expressed as percent change (means ± S.E.M.) from basal values during 120- min post-injection (*a*) or as mean percent change over 120 min (*b*). § $p<0.05$ *vs*. saline group. Drug was administered to time 0. (*c*) Recovery of prefrontal cortical 5-HT response to stress by ENU2 mice submitted to 120-min restraint following systemic administration of 2.5 mg/kg 5-HTP. Dialysates were collected at 20-min intervals. Results are expressed as percent change (means ± S.E.M.) from basal values. Statistical analyses were performed on raw data ($n=8$ mice per group). * $p<0.05$ *vs*. basal values; § $p<0.05$ compared to vehicle-injected mice.

attentive resources to novel events and increased NE release has been reported in response to all known experimental stressors (Dayan & Yu, 2006; Doya, 2008).

ENU2 mice are a genetic model of classic untreated PKU, therefore deficits in prefrontal cortical aminergic transmission could result from disturbances of neural development. However, deficits in 5-HT were totally dependent on the levels of circulating Phe. In fact, ENU2 mice temporarily exposed to a Phe-free diet showed a normal response to stress in terms of enhanced prefrontal cortical 5-HT release and a complete recovery of PFC tissue levels of 5-HT and of its metabolite 5-HIAA. Interference of DA transmission in ENU2 mice was insensitive to Phe reduction. In fact, ENU2 mice with normalized blood Phe levels did not respond with enhanced cortical DA outflow to stress, nor did they show an increase in tissue levels of DA or DA metabolites, but showed a significant reduction of basal DA outflow. The lack of effect of the Phe-free diet on cortical dopaminergic transmission could be explained by absence of tyrosine supplement in the diet. When tyrosine concentrations are abnormally low and Phe levels are very high, as in a PKU-affected organism, Phe can substitute tyrosine as substrate for DA synthesis (De Pietro & Fernstrom, 1994; Fernstrom & Fernstrom, 2007; Kufami *et al*. 1990; Milner *et al*. 1986). Therefore, the reduction of DA basal outflow in the PFC of Phe-restricted ENU2 mice may be explained by elimination of all substrates for DA synthesis. In line with this hypothesis, previous results indicated recovery of normal DA synthesis following a Phe-free regimen of the same duration as the one used here but supplemented with tyrosine (Joseph & Dyer, 2003).

The complete dependence of serotoninergic cortical synthesis on blood Phe levels suggests that cortical serotoninergic transmission could be compromised in adult early treated PKU patients who are off-diet, thus offering support to the role of 5-HT in HPA-dependent prefrontal cortical dysfunctions. Therefore, we looked for the mechanism involved in Phe-dependent deficit of 5-HT transmission in PFC. Phe excess could interfere with 5-HT metabolism by reducing brain availability of its amino-acid precursor: tryptophan. Indeed, one influential hypothesis suggests that in HPA brain, Phe excess saturates blood–brain barrier carriers interfering with the access of other amino acids to the brain (Pietz *et al*. 1999). This hypothesis supports lifelong dietary supplementation with tryptophan (Koch *et al*. 2003). However, present and previous (Pascucci *et al*. 2002, 2008) data do not show evidence of reduced tryptophan availability in the HPA brain. Moreover, present data showed no difference in tryptophan blood/brain ratio between PKU-affected and -unaffected mice, indicating no disturbances of tryptophan access to the HPA brain.

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Excess Phe can also interfere with amine synthesis by inhibiting hydroxylase activity (Curtius *et al*. 1981; Ogawa & Ichinose, 2006). Such mechanism would reduce availability of 5-HTP, the main rate- limiting factor in 5-HT synthesis. Thus, we evaluated tryptophan hydroxylation by measuring *in-vivo* 5-HTP accumulation in the PFC of freely moving mice during pharmacological blockade of aromatic L-amino acid decarboxylase and demonstrated a severe deficit in PKU-affected mice, in agreement with previous results of reduced tissue levels of 5-HTP in the brain of adult and developing ENU2 mice (Pascucci *et al*. 2002, 2008).

In the last experiment, administration of a *per se* ineffective dose of 5-HTP allowed 5-HT response to stress in PFC of PKU mice. These data strongly support the effectiveness of 5-HTP treatment of HPA-dependent hyposerotoninaemia. In this regard, it should be pointed out that our results question the use of tryptophan in HPA patients. In fact, dietary supplementation of tryptophan may lead to supraphysiological concentrations that may cause increase of its peripheral catabolism and not lead to increased 5-HTP and 5-HT levels (Turner *et al*. 2006). Therefore, the results of the present study suggest the introduction of 5-HTP in lifelong treatment of HPA. Indeed, severe deficits in frontal cortical 5-HT transmission, such as those observed in the animal model of PKU in the present study, can be responsible of cognitive disturbances (Clarke *et al*. 2004, 2005, 2006; Goldman-Rakic, 1999; Walker *et al*. 2008). On the other hand, 5-HTP is commercially available, has been used clinically for over 30 years, can be easily administered as dietary supplement and is well tolerated (Turner *et al*. 2006). Finally, the data reported here indicate that the drug is effective on brain 5-HT transmission even in the presence of extremely high Phe concentrations.

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Statement of Interest

None.

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