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Wistar–Zagreb 5HT rats: a rodent model with constitutional upregulation/downregulation of serotonin transporter

ABSTRACT

By selective breeding for the extreme values of platelet serotonin level (PSL) two sublines of rats with constitutional hyperserotonemia/hyposerotonemia were developed. The velocity of platelet serotonin uptake (PSU), the main determinant of PSL, was used as a further, more specific selection criterion. Directed breeding for its extremes resulted in two sublines of rats with constitutional upregulation/downregulation of platelet 5HT transporter activity, and showed consequent alterations of entire 5HT homeostasis. These sublines, termed Wistar–Zagreb 5HT (WZ-5HT) rats, constitute a genetic rodent model described in this chapter. Besides changes in peripheral 5HT homeostasis, high-5HT and low-5HT sublines of WZ-5HT rats also demonstrate changes in central serotonergic mechanisms. Under physiological conditions, neurochemical differences in the 5HT system between sublines were almost undetectable, but they became evident upon specific pharmacologic challenge, as shown by brain microdialysis study. Differential behavioral phenotypes of 5HT sublines in response to various environmental challenges provide further evidence for differences in their brain functioning. Thus, high-5HT rats exhibit enhanced anxiety-like behaviors while depressive-like behavior and higher alcohol intake co-occur in low-5HT animals. Observed functional and behavioural differences between sublines of WZ-5HT rats strongly indicate that brain serotonergic activity was increased in animals from the high-5HT subline as compared to low-5HT rats. The WZ-5HT rat model may represent an integrative model for serotonin and serotonin transporter research,

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incorporating changes at the genomic/genetic and phenotypic (neurodevelopmental, structural, biochemical, behavioral, etc.) levels and encompassing both central and peripheral 5HT functioning.

INTRODUCTION

The involvement of serotonin (5-hydroxytryptamine, 5HT) in central and peripheral (patho)physiological functions has been evidenced during past decades, but complete understanding of the relation between 5HT system activity and the broad diversity of 5HT effects is still missing.

Animal models, enabling experimental manipulations of brain structures, represent important tools in searching for the role of 5HT in the expression of specific phenotypes. By manipulation of genes encoding the main regulatory 5HT synaptic proteins – transporter, receptors and metabolic enzymes – several models, mostly murine (transgenic, knock-out, antisense oligonucleotides and, most recently, RNA-interfering models) have been generated and phenotypes of these animals have been characterized extensively (for review see [1–6]. In addition to sophisticated molecular-genetic techniques, classical genetic approaches are successfully used in studying the contribution of 5HT signaling to mammal physiology and behavior, such as studies on inbred rodent strains [7, 8] or animals selected for a particular phenotype, for example differences in emotional reactivity, depression-related behaviors, learning ability, differential response to serotonergic or cholinergic agents, etc. [9–12 and references therein].

In designing these breeding models, an individual animal reactivity may serve as a criterion for selective breeding, followed by testing of developed sublines for possible alterations in 5HT system functioning. In an attempt to obtain a model with a constitutive alteration of the 5HT system itself, we have applied an inverse approach – selective breeding of animals for 5HT parameters and than searching selected sublines for physiological and behavioral consequences of breeding. For this purpose, we took advantage of the expression of serotonin transporter (5HTT, SERT), the main controller of free (extracellular) 5HT concentration in both brain and periphery, on readily accessible blood platelets. This unique peculiarity of the mammalian serotonergic system, as compared with other monoamine transmitters, serves as a basis for using platelets as model for serotonergic neurons, which was suggested as long as 40 years ago [13, 14]. These small, anucleated fragments of megakaryocytic cytoplasm, differing from neurons in physiology, morphology,

embryology, and functional role, etc., possess their own serotonin system consisting of vesicles with 5HT densely packed in conjunction with divalent cations and ATP molecules (dense granula), high-affinity membrane 5HT transporter, vesicular monoamine transporter (VMAT2), 5HT receptor (2A subtype) on the plasma membrane and 5HT-degrading enzyme, monoamine oxidase (MAO-B subtype in humans) in the mitochondrial membrane. Biochemical and pharmacological characteristics of all these proteins – transporter, receptor and enzyme – were found to be very similar to those of analogous 5HT proteins in serotonergic neurons, forming the basis of the mentioned platelet model in neurobiology. The subsequent demonstration of the structural and genetic identity of neuronal and platelet 5HT-related proteins [15–17] additionally supported validity of the platelet model. In biological psychiatry, platelet 5HT elements have been studied, with more or less success, as potential trait markers, disease state markers, or pharmacodynamic indicators in the course of treatment with 5HT-related drugs [18–21].

In contrast to humans, collection of larger platelet samples from the rat has been regularly associated with sacrifice of the animal, which considerably hampered experimental studies. We have developed a method for repetitive measuring of platelet serotonin level (PSL) and kinetics of platelet serotonin uptake (PSU) simultaneously in a sample (1 ml) of rat blood [22, 23] and described the fundamental physiology of these two platelet 5HT parameters: frequency distributions, sex and age influences and individual stability over time in a large population of rats of Wistar origin [24–26]. The possibility of ex vivo monitoring of PSL and PSU, as well as findings of stability of their values in the individual animal [24, 26], which clearly indicated their heritability, enabled selective breeding for the extreme values of the above-mentioned platelet 5HT parameters, and permitted development of rat sublines with constitutionally altered 5HT system.

Development of Wistar–Zagreb 5HT (WZ-5HT) sublines

Selective breeding for divergence in platelet 5HT parameters, at first step for extremes in PSL values, has been initiated from a large original outbred population of Wistar rats from the breeding colony of the Rudjer Boskovic Institute, in which approximately 2.5-fold natural variation between extremes of PSL was found [24]. The breeding strategy has been described elsewhere [27, 28]; in brief, males and females with the highest and the lowest PSL, respectively, were mated to generate

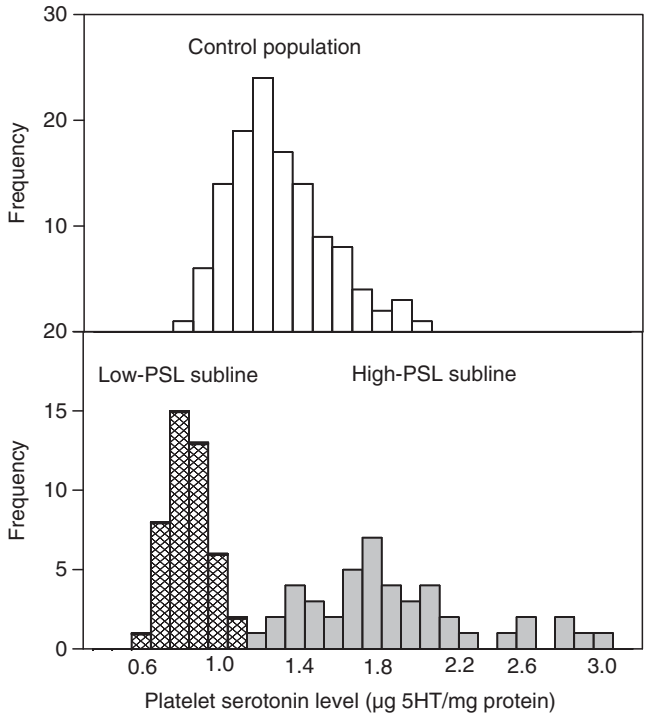

Figure 7.1 Frequency distribution histogram of individual platelet serotonin level (PSL) in control population of rats and in sublines genetically selected for low and high values of this trait (F8 generation) (adapted from [24, 27, 28]).

high and low sublines. For each subline, 12–16 litters were grown at each generation and determination of PSL was performed in offspring when they reached about 100 g of body weight. In selecting breeders for the next generation, mating of brother × sister was avoided as well as mating of animals with extreme platelet counts. A shift in mean PSL values appeared as early as in the F2 generation, while divergence of selected populations was completed by the F8 generation, with mean values stabilized at about 70% (low-PSL subline, hyposerotonemic) and 150% (high-PSL subline, hyperserotonemic) of the mean PSL value of control/unselected population [27, 28] (Figures 7.1 and 7.4a), i.e. the range of PSL values was approximately doubled by directed breeding. Morphometrical analysis performed on electron micrographs of platelets from both sublines revealed that the number of their 5HT storage vesicles (dense granula) has changed proportionally to the 5HT level (Fig. 7.3). This indicates that the volume of platelet 5HT storage vesicles

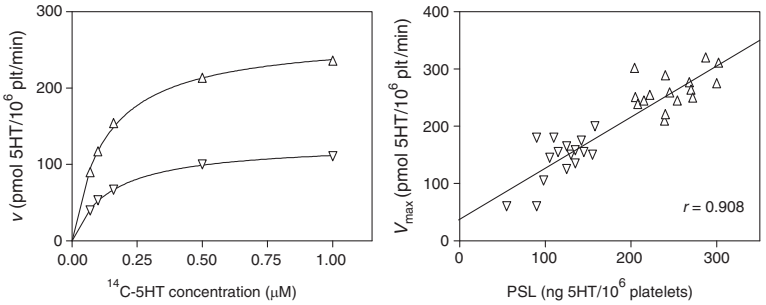

Figure 7.2 Representative saturation curve of serotonin (5HT) transport into platelets of rats from high-5HT and low-5HT subline. Velocities (v) in control animals were in between (not presented) (left). Individual correlation of values of platelet serotonin level (PSL) and maximal velocity (V_{max}) of platelet serotonin uptake in animals from both sublines, r = correlation coefficient (right).

itself is somehow predetermined and that platelets modify their 5HT storage capacity by changing the number, and not the volume, of dense granula (counterpart of synaptic 5HT vesicles).

With regard to individual extremes in PSL values, directed selection resulted finally in more than a fourfold difference in this parameter (Fig. 7.1). From generation F8 on, a frequency distribution of PSL showed a different pattern between sublines: in the low-PSL subline, distribution was narrow and of Gaussian shape; while in the high-PSL subline, the histogram was multimodal, indicating differences in the inheritance pattern between sublines [27, 28].

Platelets, in contrast to neurons, lack the enzymatic potential for serotonin synthesis. Their 5HT content is a result of the transporter-mediated uptake of the amine from the surrounding plasma, so the activity of membrane 5HT transporter (5HTT) appeared as the most probable candidate underlying obtained inherited differences in PSL. Indeed, comparative full-kinetic analysis of ^{14}C-5HT uptake into platelets of animals from selected sublines revealed significant differences in its maximal velocity (V_{max}), reflecting different numbers of 5HT transporter sites on their membranes [29] (Figures 7.2, 7.3). Correlation coefficients between PSL and velocity of PSU were regularly higher than 0.90. On the other hand, the affinity (K_m values) of the transporter was similar in both sublines and not influenced by selective breeding.

Further molecular-genetic studies demonstrated analogous differences in two other platelet 5HTT parameters: transporter protein, measured by Western blot [30], and transporter mRNA, measured by either

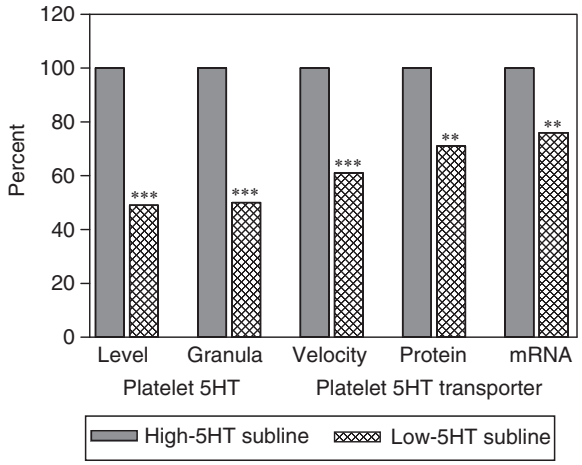

Figure 7.3 Relation among platelet 5HT measures in rats from low-5HT and high-5HT sublines (taken as 100%). High-5HT rats: platelet 5HT level: 2.12 ng 5HT/10^6 platelets; number of dense granules: 192/1000 platelets; velocity of 5HT transporter: 2.45 pmol 5HT/10^6 platelets/min; 5HTT protein: intensity of bands (relative units); 5HTT mRNA: intensity of bands in relation to cyclophylin (relative units); $^{**}p<0.01$, $^{***}p<0.001$ (adapted from [27to32]).

Northern blot or semi-quantitative RT-PCR method [31, 32]. Values of platelet 5HT-related measures were 30–50% lower in animals from the low-PSL subline, depending on the parameter measured (Figure 7.3) It was concluded that selective breeding for the extreme values of 5HT content in platelets lead to a parallel divergence of platelet membrane 5HT transporter protein.

In line with these results, development of a more specific genetic rat model, based on selection of animals for the extremes of PSU activity, was initiated [29], and this model was termed Wistar–Zagreb 5HT (WZ-5HT) rats. The breeding procedure was essentially the same as described for sublines selected on the basis of PSL values, but after first screening of offspring for PSL in each breeding generation, their PSU velocities were measured and used as the main criterion for selecting breeders for the next generation. Again, bidirectional changes in mean values of PSU velocity were produced, paralleled with consequent differences in mean values of PSL.

In comparison to the selection of animals for divergence in PSL, two major differences emerged as a result of selection for PSU velocity: firstly, the progress of separation of sublines was faster, completed practically in the F5 generation; and secondly, distribution histograms of 5HT parameters were similar and approximately normal in both

sublines. A final divergence of the mean platelet 5HT parameters between sublines were somewhat higher for PSL than for the PSU (2 to 2.5-fold and 1.5 to 2.0-fold, respectively), with no overlap between sublines in distribution of PSL values and with little overlap in distribution of PSU values. This indicates that factors other than PSU may have contributed, to some extent, to the 5HT content in platelets, e.g. the activity of tryptophan hydroxylase, a rate-limiting enzyme for 5HT synthesis. Namely, higher synthesis and consequently, higher release of 5HT from the gastrointestinal tract into blood plasma could contribute to PSL according to the scavenging function of platelets for free serotonin in plasma [33, 34].

As the selection procedure progressed, the number of pups liveborn became smaller, and there was reduction in fertility and a rise in the occurrence of deaths during early postnatal life. This could be the consequence of inbreeding depression, but given the role played by 5HT in mammalian reproduction [35, 36], it may also have been related to altered 5HT functioning/homeostasis. However, no systematic pattern of these events could be observed across sublines. In parallel with selected sublines, a non-selected (control) line was periodically grown in order to monitor progress of selection as well as natural fluctuations in the population from which selection was started.

CHARACTERIZATION OF WISTAR–ZAGREB 5HT RAT SUBLINES

As a result of bidirectional selective breeding for extreme values of PSU velocity (and consequent PSL) two discrete rat sublines were obtained with differentially affected 5HT homeostasis. These sublines, characterized by high or low velocity of PSU and high or low PSL, were termed the high-5HT and low-5HT sublines, respectively. In this report, a large part of the neurochemical and behavioral outcomes in these rat sublines are summarized; figures are compiled from our published results, preliminary reports and yet unpublished data.

Peripheral 5HT

Besides being present in platelets, 5HT is also present in most peripheral organs, where it mediates the neural and local control of their functioning [34]. Dysregulation of 5HT transporter is thought to be associated specifically with cardiovascular and gastrointestinal disorders [37–40]. Several aspects of the 5HT system have been investigated in WZ-5HT sublines in searching for consequences of selective breeding on the peripheral 5HT homeostasis.

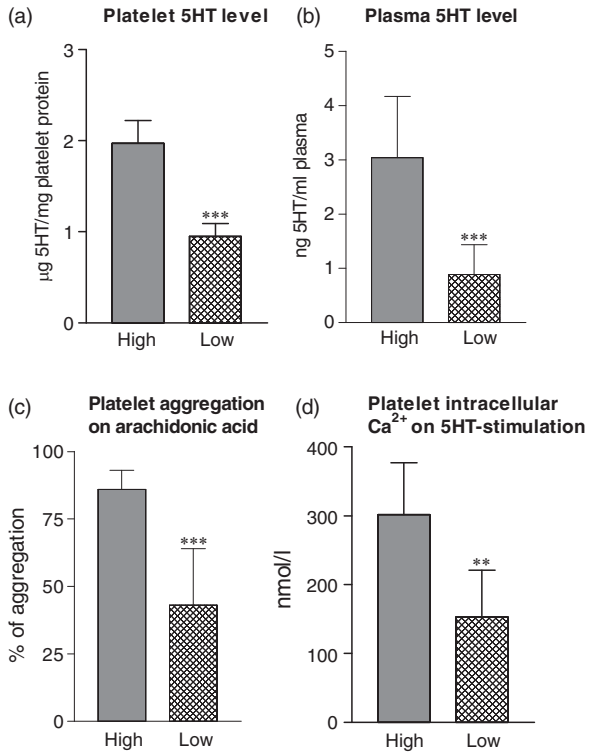

Figure 7.4 Comparison of blood/platelet measures in animals from high-5HT and low-5HT sublines. (a) Platelet 5HT measured fluorimetrically; (b) Plasma-free 5HT measured by HPLC; (c) aggregation measured 2nd minute after addition of arachidonic acid (compare to Figure 7.5); (d) platelet $[Ca^{2+}]_i$ on stimulation with 5 μM 5HT. Mean ± SD, *N* per subline: (a–b) 9; c–d) 6–7. $^{**}p<0.01$, $^{***}p<0.001$. See text for additional explanation (adapted from [29, 51, 54]).

Tissue and plasma 5HT concentration. Tissue 5HT content, measured in several peripheral organs (lungs, spleen, heart, small intestine, cecum, colon) by ion-exchange chromatography-fluorimetry [41], showed higher values in animals from the high-5HT subline, as compared to low-5HT animals. The magnitude of differences was dependent on the organ examined, and ranged from 10–25% in distinct parts of the gastrointestinal tract, through 35% in lungs (having unusually large interindividual variations), to an almost 50% difference in the spleen (probably related to the high content of platelets in this organ).

Intriguingly, extracellular 5HT concentration, measured in platelet-free plasma, was also significantly higher in high-5HT animals (Figure 7.4b). The same results were obtained by using different

analytical methods (HPLC-ED and RIA) and in plasma samples of animals from two consecutive breeding generations, but they still should be taken with some caution. Namely, the concentration of 5HT in plasma is a few orders of magnitude lower than in platelets [33, 42] and, in spite of all methodological attentiveness, activation of highly reactive platelets and consequent release of some 5HT could occur during blood processing. The level of biologically active 5HT in blood plasma results from the tightly regulated equilibrium between its synthesis/release from the gut and inactivation by uptake and enzymatic degradation (lungs, platelets, liver) [33, 34]. The capacity for cleaning plasma from 5HT is approximately twofold larger in platelets (and presumably, and much more importantly, in lungs) from high-5HT rats, so at first glance a lower plasma 5HT concentration might be expected in this subline. The mechanisms leading in the end to the increased level of free 5HT in rats with higher activity of platelet 5HTT is unclear thus far.

Regardless of the mechanisms underlying alterations in peripheral 5HT homeostasis, WZ-5HT sublines may represent a model for studying the role of 5HT in physiology and in somatic disorders, similarly to 5HTT-deficient or transgenic mice models [43, 44]. In addition, animals from the high-5HT subline displaying hyperserotoninemia could contribute to a better understanding of this phenomenon in clinical conditions such as autism [45].

Pharmacodynamic response

Administration of 5HT-related drugs elicited a markedly different response of platelet 5HT parameters: PSL and velocity of PSU, between sublines.

1. The increase in PSL following parenteral treatment of rats with 5HT itself, or with its metabolic precursor, 5-hydroxytryptophan (5HTP), was more pronounced in rats with higher activity of 5HTT [46] (Figure 7.5a). Similarly, hypofunctioning of membrane 5HT transporter in low-5HT animals was reflected by attenuated response of their PSL to selective serotonin reuptake inhibitors (SSRIs) fluvoxamine or fluoxetine [46] (Figure 7.5b). On the other hand, no differences in PSL response between 5HT sublines were observed following administration of reserpine, a drug that does not interfere with membrane 5HT reuptake.
2. Similarly to the effect on PSL, serotonin reuptake inhibitors had differential effects on transmembrane 5HT transport

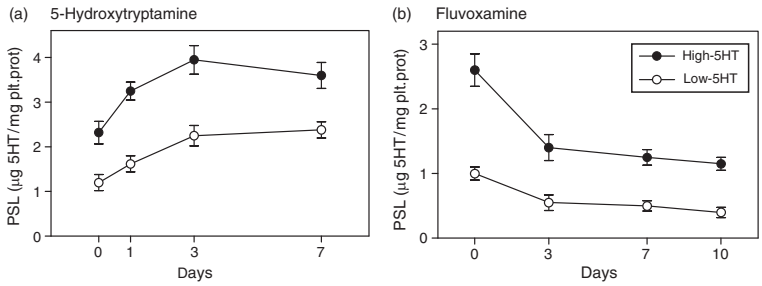

Figure 7.5 Platelet serotonin level (PSL) in animals from high-5HT and low-5HT sublines in the course of treatment with (a) 5-hydroxytryptamine (30 mg/kg/day, 7 days) and (b) fluvoxamine (20 mg/kg/day, 10 days). Daily i.p. injections were given through 7 or 10 days; PSL was measured 24 h after injections indicated on the *x* axis. Basal (pretreatment) PSL values are presented at day 0. Mean ± SD, $N = 6$ (adapted from [46]).

between sublines. In platelets of high-5HT rats, the decrease in 5HT uptake rate was more pronounced during treatment with fluvoxamine, as compared to platelets of low-5HT animals [46]. The observed proportionality of the PSU response to the SSRI treatment to the basal (constitutional) PSU values of the animal may be indicative of analogous central pharmacodynamic effects of SSRIs, and support the claim for personalized approaches to SSRI treatments in humans that was suggested by pharmacogenomic studies [47–50].

Platelet aggregation and intracellular calcium

Platelet response reaction in WZ-5HT sublines was studied by measuring in vitro aggregation of platelets on induction by arachidonic acid. Clear differences between sublines were demonstrated: platelets from high-5HT animals underwent a stronger and faster aggregation response as compared to the later start, slower rise and lower percent of aggregated platelets in the low-5HT animals [51] (Figures 7.4c, 7.6). Because of increased 5HT content in platelets of high-5HT animals, these results accord well with the suggested role of 5HT as a potent amplifier of the platelet aggregation response [52]. The mechanism of amplification is mediated by 5HT-2A receptors on the platelet membrane, whose activation results, finally, in the increase of cytosolic free calcium levels.

A study on measuring intracellular Ca^{2+} concentration in fura-2-loaded platelets revealed significantly higher resting intracellular Ca^{2+}

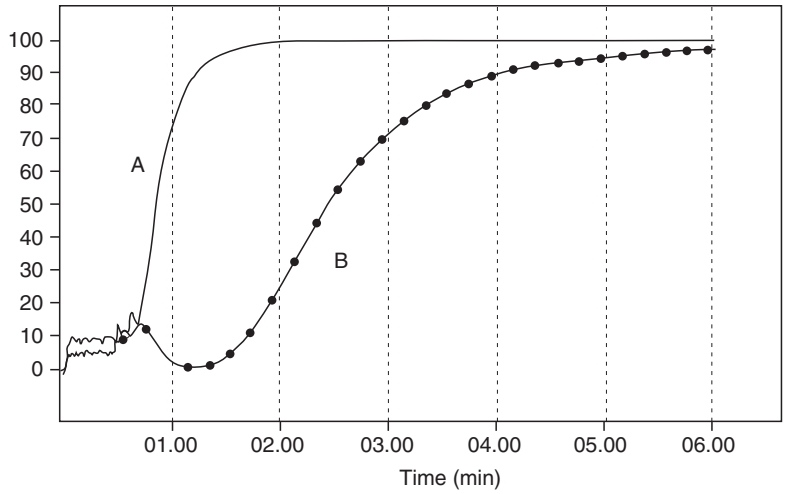

Figure 7.6 Aggregation curves of platelets from high-5HT (a) and low-5HT (b) rat recorded in the course of 5 min period after addition of arachidonic acid to the platelet sample. A typical synchronous aggregometric tracing is shown (compare to Figure 6.1c) (adapted from [51]).

in platelets of animals from the high-5HT subline [53]. Additionally, stimulation of platelets by 5HT in vitro resulted in a significantly enhanced rise in intracellular calcium levels in high-5HT rats [54] (Figure 7.4d). These results provide evidence for functional differences in platelet response reaction between 5HT sublines that may include alteration of intracellular signaling through 5HT-2A receptors and/or calcium signalization. Given the role which platelets have in cardiovascular disorders, specifically atherosclerosis [55, 56], rats with constitutive differences in platelet response may be useful for better understanding clinical pathologies such as myocardial infarction or stroke.

Central 5HT

As mentioned earlier, platelet 5HTT is structurally identical to its neuronal counterpart and encoded by the same gene [17]. Although this does not necessarily implicate functional parallelism between periphery and brain, we hypothesized that selective breeding for extremes of platelet 5HTT activity might result in some correspondence in 5HT neurons. Indeed, neurochemical and behavioral studies provide evidence that selection for peripheral 5HT parameters induced alterations in brain 5HT homeostasis as well.

Physiological conditions

5HT transporter analysis

Neuronal 5HTT has been explored for alterations between 5HT sublines regarding transporter functionality, density of binding sites and gene expression.

1. Kinetics of [^{14}C]-5HT uptake by synaptosomes prepared from the frontal cortex revealed similar saturation curves with no alterations in either V_{max} or K_m values [57]. The absence of differences in brain 5HT uptake rate between sublines is substantially contrasted to findings in platelets [31], indicating different regulation of 5HTT activity in periphery and brain. Our studies were performed by the use of a common radiochemical method, and the possibility that this method was not sensitive enough to detect small changes in brain 5HT uptake velocity [58] should be taken into consideration (although not very likely under our experimental conditions). A radiochemical method did not reveal differences in the 5HT uptake rate between heterozygous 5HTT knock-out (5HTT+/−) and wildtype (5HTT+/+) mice [59], while high-speed chronoamperometry revealed a marked reduction in 5HTT+/− mice [60]. On the other hand, in heterozygous 5HTT+/− rats, V_{max} of 5HT uptake in hippocampal synaptosomes was reduced by only 13% [61]. In our sublines more subtle disturbances in the 5HT system and, consequently, even smaller differences in 5HTT velocity, were expected. We have compared 5HTT activity only in frontal cortex synaptosomes, so the possibility of region-dependent differences in brain 5HTT function, as shown to exist in different rat strains [7], could be considered in future research. The possibility of compensatory 5HT uptake by other monoamine transporters, as has been reported for the 5HTT knock-out mouse [1] should also be taken into consideration.
2. Multiple brain regions of 5HT sublines were explored for divergence in 5HTT protein by saturation-binding assay of [^{3}H]-citalopram [62]. Results demonstrated small (<10%) but significant region-dependent changes in density of [^{3}H]-citalopram binding sites, with higher values observed in high-5HT animals in 9 of 13 brain regions investigated [62].

Assuming that [^{3}H]-citalopram binds both membrane and cytoplasmic (non-functional) transporters, the observation that citalopram binding, but not 5HT uptake, differs slightly between 5HT sublines may suggest differences in the cytoplasmic pool of 5HT transporters between sublines. Hypothetically, the total number of neuronal 5HT transporters differs between 5HT sublines, but differences in the number of functional transporters on the cell membrane appear only on stimulation, e.g. membrane depolarization (see below). Redistribution of transporters, resulting in changes in V_{max}, with no alterations in K_m, represents a mode of presynaptic reuptake regulation [63, 64].

3. The possibility of altered 5HTT gene expression in 5HT sublines was explored by measuring mRNA transcripts for 5HTT in midbrain raphe nuclei, by the use of semi-quantitative RT-PCR. A tendency to higher 5HTT mRNA levels in the high-5HT rats (∼15%) was observed, but the difference in the expression of 5HTT between sublines was not significant [31].

Tissue 5HT and 5HIAA concentrations

Tissue levels of 5HT and its major metabolite, 5-hydroxyindoleacetic acid (5HIAA), have been examined by the use of HPLC-ED and ion-exchange chromatograpy-spectrofluorimetry [41] in multiple brain regions (raphe nuclei, frontal cortex, striatum, hippocampus (Figure 7.7a), basal ganglia, amygdala, hypothalamus, thalamus) of 5HT sublines [62, 65]. No significant differences in either 5HT or 5HIAA concentrations were observed in any of the brain regions investigated, but in general, a trend towards a reduction in 5HT and elevation of 5HIAA concentrations could be noticed in high-5HT rats, resulting finally in a significantly higher metabolic ratio (5HIAA/5HT) in most brain regions (∼10%, $p<0.05$) (Figure 7.7b). Results were indicative of slightly increased brain 5HT turnover in animals from the high-5HT subline as compared to the low-5HT animals. Interestingly, a similar increase in tissue 5HIAA/5HT ratio has been observed in 5HTT−/− mice and rats, although the reason for this is not understood [61, 66, 67].

Metabolic enzymes

To explore potential alterations in 5HT metabolism between sublines, indicated by differential 5HT turnover, we have compared

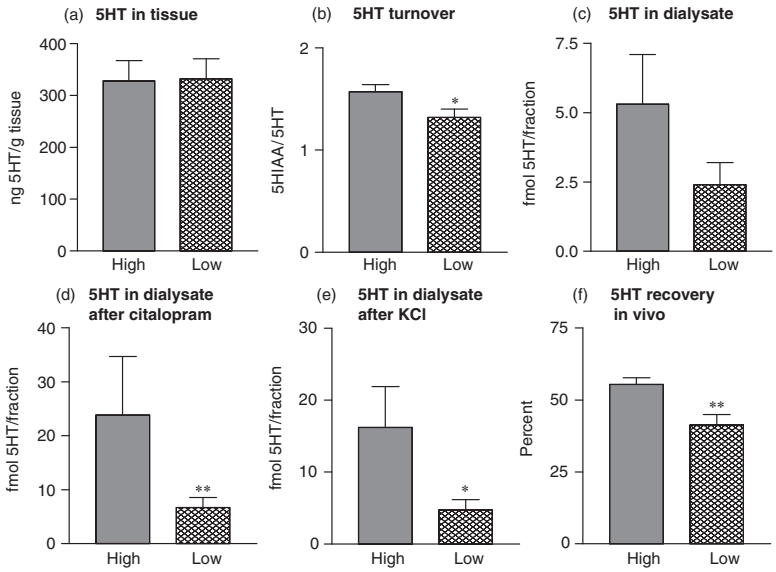

Figure 7.7 Comparison of 5HT measures in hippocampus of animals from high-5HT and low-5HT sublines. (a) Tissue 5HT; (b) Tissue 5HT metabolic rate; (c) basal dialysate 5HT in ventral hippocampus, 20-min fractions; (d) dialysate 5HT in 2nd fraction after infusion of 1 μmol/l citalopram; (e) dialysate 5HT in 1st fraction after infusion of KCl; (f) recovery of exogenous 5HT by hippocampal tissue (100 nmol 5HT/l added through dialysis probe); (a)–(b) mean ± SD, $N = 12$ per subline; (c)–(f) mean ± SEM, $N = 5$–7; $^{*}p<0.05$, $^{**}p<0.01$ (**C–F** estimated based on refs [62, 65]; see text for additional explanation).

activities/expression of their tryptophan hydroxylase (TPH), a rate-limiting enzyme for 5HT synthesis, and of monoamineoxidase (MAO), the major 5HT-degrading enzyme. 5HT is mainly oxidized by MAO-A isoenzyme, but serotonergic neurons (as well as human platelets) contain predominantly the MAO-B isoform.

1. The level of TPH in the raphe nuclei region of 5HT sublines was compared by the use of the immunochemical (Western blot) method. There were no statistically significant differences between sublines in the amount of TPH protein, although a tendency toward lower (∼30%, non-significant) levels was noticed in animals from the high-5HT subline [68].
2. Full kinetic analysis of MAO-A and MAO-B isoenzymes in whole brain homogenates demonstrated no differences in either V_{max} or K_m between 5HT sublines. Further, by the use of

semi-quantitative RT-PCR, mRNA transcripts for MAO-A and MAO-B isoforms were measured in brain cortex and in raphe region of animals from 5HT sublines. With the exception of a noticed tendency toward the increase in MAO-B mRNA in the cortical region of low-5HT rats (∼15%, $p<0.05$), no other differences were found.

Extraneuronal 5HT concentration

Extracellular 5HT concentration, assessed in ventral hippocampus (vHPC) by in vivo microdialysis, revealed an approximately twofold difference in baseline 5HT values between sublines [62] (Figure 7.7c). Elevated 5HT levels were observed in high-5HT rats compared to low-5HT rats, while values in control (unselected) animals were intermediate [62]. The difference in extraneuronal 5HT concentration between sublines, although large in magnitude, did not reach statistical significance (likely due to relatively small group sizes and large intergroup variations). However, pronounced divergences in neurochemical and behavioral responses between 5HT sublines, observed in subsequent studies (see below), support the existence of elevated brain 5HT activity in animals from the high-5HT subline in relation to their low-5HT counterparts. Elevated extracellular 5HT concentration in brains of high-5HT rats resembles, in some way, the situation in the periphery in that they also possess a higher extracellular pool of 5HT in the blood, although the responsible mechanisms may be quite different.

Intriguingly, animals selected for higher 5HTT activity seem to resemble 5HTT-deficient mice in having elevated basal extracellular 5HT concentrations, although this elevation in 5HTT−/− mice is comparatively much higher (∼10-fold) [61, 69–71], and, as contrasted to our high-5HT subline, these mice show markedly reduced tissue 5HT and 5HIAA concentrations [66, 67]. On the other hand, heterozygous 5HTT+/− mice, which have moderately reduced 5HT uptake rate, have unchanged tissue 5HT and HIAA levels and no alterations in extracellular 5HT concentrations [58, 66, 70]. Further, transgenic mice having two- to threefold over-expression of 5HTT possess reduced tissue 5HT, unchanged 5HIAA, and decreased extracellular 5HT concentration [72].

(Dis)similarities in neurochemical phenotypes among mentioned models stress the complexity of 5HT regulation mechanisms, and apparently paradoxical situations such, as for instance, antidepressant

clinical efficacy of both drugs which enhance 5HT reuptake (tianeptine; [73]) and drugs which inhibit 5HT reuptake (commonly used antidepressants); or another example, the information that deficiency of 5HT function is associated with an increased risk for anxiety/depression, while pharmacologic blockade of 5HTT has anxiolytic/antidepressant effect [1, 2, 48, 74].

5HT-receptors

Differences in 5HT extraneuronal level and turnover between our sublines, although only moderate, could be expected to induce adaptive changes in the 5HT receptor systems, their signal transducing mechanisms and/or expression of related genes, similarly to changes observed in 5HTT-deficient mice [1, 2, 71, 75, 76].

By the use of RT-PCR, we have measured mRNAs for 5HT-1A, 5HT-1B and 5HT-2A receptors in hippocampus, striatum and cortex, respectively, in animals from high-5HT and low-5HT sublines. No measurable alterations were observed in mRNA levels for the mentioned 5HT receptors in the brain regions investigated. Further, preliminary studies showed similar densities of 5HT-1A and 5HT-2A receptors, as measured by binding of [^{3}H]8-OH-DPAT and [^{3}H]-ketanserin, respectively, in cortical regions of 5HT sublines, and a lack of differences in cortical 5HT-2A protein amount assessed by Western blot method [77–79]. It seems, therefore, that imbalances in brain 5HT homeostasis in our genetically selected animals were not large enough to produce measurable compensatory/adaptational changes in 5HT receptor systems under physiological conditions, at least in the brain cortex. Bearing in mind that adaptational changes in 5HT receptors are region-specific and often small in magnitude, even following complete inactivation of 5HTT [71, 75, 76], the inability to detect alterations in our 5HT sublines was not so unexpected. Research on the expression of regulatory somatodendritic 5HT-1A autoreceptors as well as comparison of their functionality between sublines is in course.

At present, we have not studied the potential consequences of disturbed 5HT homeostasis in WZ-5HT rats on other brain neurotransmitter systems, for instance the noradrenergic or dopaminergic systems, which have strong interactions with the 5HT system. However, no major changes in functioning of several non-serotonergic systems in 5HTT-deficient [3, 61, 70] or 5HTT over-expressing animals [72] have been demonstrated, which makes it unlikely that such changes are present in our model under physiological conditions.

Response to challenges

Neurochemical response

By using in vivo microdialysis, marked differences in response of 5HT neurons to specific pharmacological challenges were observed between 5HT sublines [62], confirming underlying constitutive alterations in functionality of their brain 5HT systems. Thus, an increase in extracellular 5HT concentration in ventral hippocampus (vHPC), provoked by infusion of selective reuptake inhibitor citalopram through the dialysis probe, was much more pronounced in the brains of high-5HT rats (Figure 7.7d). Similarly, K^+-evoked release of 5HT in vHPC, produced by infusion of hypertonic KCl through dialysis probe, was also significantly higher in the same subline (Figure 7.7e).

Observed differences in drug-responsiveness between 5HT sublines may be interpreted as resulting from latent differences in activity of their 5HTT that are not detectable under physiological conditions (although adaptive changes in 5HT release cannot be ruled out as contributing to these observations).

Further evidence for differences in functionality of the brain 5HT system between 5HT sublines was obtained by estimation of in vivo recovery of exogenous 5HT added to the hippocampal tissue, which was also significantly higher in animals from the high-5HT subline [62] (Figure 7.7f). In the light of the results obtained under conditions of pharmacologically challenged 5HTT, it could be speculated that earlier described differences in basal 5HT dialysate between sublines are underestimated.

Demonstration of much stronger responsiveness of brain 5HT system under pharmacological challenges in high-5HT animals, together with elevated extracellular 5HT concentration and 5HT turnover in this subline, speak in favor of an increased serotonergic tone (i.e. hyperactivity of 5HT system) in high-5HT rats, as compared to rats from the low-5HT subline.

Behavioral responses

Given the importance of the 5HT system in integrating sensory processing, motor activities and cognitive functions, we assumed the existence of divergent behavioral phenotypes in 5HT sublines, which was confirmed by further studies.

SEROTONIN SYNDROME

Excessive brain 5HT functioning, induced by administration of various combinations of 5HT-enhancing drugs, leads to development of distinctive, life-threatening, motor behavioral 5HT syndrome in both rodent models and in humans [80–82]. It has been observed that 5HTT knock-out mice, having elevated basal extracellular 5HT concentration, displayed spontaneous serotonin syndrome-like behavior [91].

Behaviors associated with the 5HT syndrome, induced by MAO inhibition and 5HT precursor loading (pargyline + 5HTP), were examined in animals from WZ-5HT sublines. Although not all symptoms were different between the sublines, the response of high-5HT rats was generally more pronounced compared to low-5HT animals [78]. Among behavioral/neuromuscular symptoms, the most prominent differences were observed in body tremor and hyper-reactivity scores, while regarding autonomic phenomena, marked differences were present in the elevation of body temperature [78] (Figure 7.8b). In addition, a clear difference between sublines was noticed in lethal answer to the 5HT syndrome. Although non-specific, this answer could be considered as a valuable indicator of a "final response" of the organism summarizing 5HT-induced dysregulations [78] (Figure 7.8a). In pharmacological studies, using 5HT receptor antagonists, a specific role for 5HT-2A receptors in development of both body tremor and hyperthermia has been documented [83, 84], making it possible that differences in 5HT-2A receptors contributed to the differences in the development of 5HT-syndrome between sublines. In contrast to 5HTT knock-out mice, no spontaneous serotonin syndrome-like behavior was observed in our WZ-5HT sublines.

Regardless of the underlying mechanisms, functional differences between 5HT sublines indicate that the basal serotonergic tone of the individual plays a critical role in the intensity of response/symptoms to 5HT-enhancing drugs. An increased behavioral sensitivity to these drugs has been shown also in mice with deletion of 5HTT function [82], suggesting the possibility of their use as a model for humans at greater risk for developing 5HT-syndrome [82]. Our high-5HT subline may serve a similar purpose regarding this clinical condition.

ANXIETY-RELATED PHENOTYPE

In line with the current, although oversimplified, hypothesis that increased anxiety-related behaviors are related to increased 5HT neurotransmission [5, 50, 85, 86], we hypothesized that animals from 5HT sublines could display differences in anxiety-related behaviors.

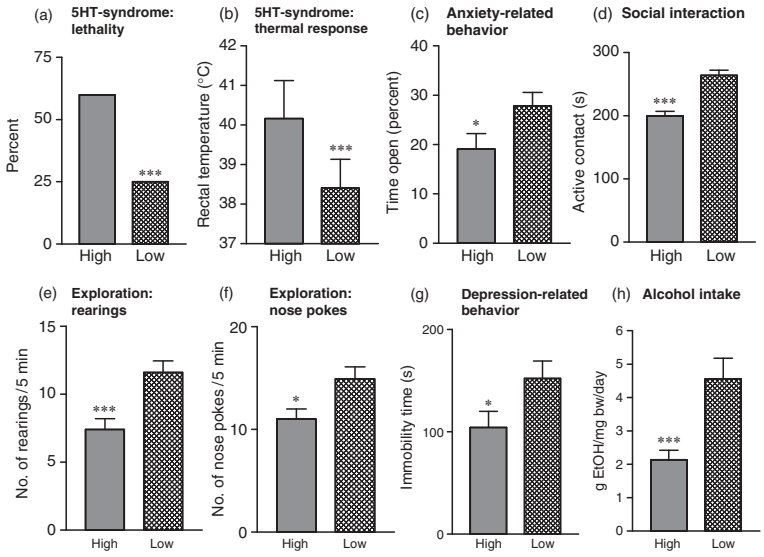

Figure 7.8 Behavioral measures in animals from high-5HT and low-5HT subline. (a),(b) 5HT syndrome induced with 50 mg/kg pargyline + 5 mg/kg 5HTP; lethality recorded 24 h after 5HTP injections, temperature measured 90 min after 5HTP injection; (c),(e) 5-min elevated plus maze; (d) 10-min open field, 300 lx; (f) 5-min hole-board; (g) 5-min forced swimming; (h) two-bottle choice paradigm, average intake of 12% alcohol during 12 days in females; mean ± SEM, *N* per subline: (b),(g),(h) 6–7; (d),(f) 12; (a),(c),(e) 20–23; $^{*}p<0.05$, $^{***}p<0.001$. See text for additional explanation (adapted from [54, 88, 89, 107]).

Their anxiety-like phenotype was tested in several common paradigms using animals from three breeding generations. Results were partly obtained in an independent laboratory (Seville, Spain; [89]), providing additional strength to the findings.

Overall, significant differences were observed between sublines, with high-5HT animals displaying a higher level of anxiety-related behavior and lower level of exploration, which are in line with the view that avoidance and novelty seeking share common mechanisms [87]. Specifically, high-5HT rats spent less time in the open arms of the elevated plus maze (Figure 7.8c). In the social interaction test they spent less time in active contact with conspecifics [88] (Figure 7.8d) and displayed a narrower spectrum of social behaviors [88]. High-5HT animals also showed lower levels of exploratory activity across three different anxiety-related tests (zero-maze, elevated-plus maze, hole-board) [88] (Figures 7.8e, f). The most prominent difference between

5HT sublines was considerably enhanced freezing behavior in high-5HT animals when compared to their low-5HT counterparts and to control animals [89]. Since the exposure of a rat to a novel environment represents a mild stressor, there is a possibility that different emotionality in our sublines might be related to their different response to stress. On the other hand, it is unlikely that increased anxiety-related behaviors were a consequence of altered locomotion, as 5HT sublines did not differ in the number of squares entered [88] or distance traveled in the open field test.

Enhanced anxiety-related behaviors in animals from the high-5HT subline accord well with presumed over-activity of their 5HT system. Again, findings in high-5HT animals (with upregulated 5HTT) resembled, surprisingly, that of mice with deleted 5HTT gene, which consistently displayed heightened anxiety-like behaviors (and are hyperserotonergic) [2, 67, 87, 87, 90, 91]. It is also in line with the low-anxiety phenotype displayed in 5HTT-over-expressing mice [72].

DEPRESSION-RELATED PHENOTYPE

As opposed to the excessive 5HT neurotransmission in anxiety, deficient 5HT transmission has usually been associated with depression [92, 93] and enhancement of 5HT availability has been thought to underlie the therapeutic effects of conventional antidepressants [94]. However, the situation seems much more complex: thus, drugs that inhibit the 5HTT have both anxiolytic and antidepressant potential, symptoms of anxiety and depression are often comorbid and functional gene variants in the 5HTT are associated with greater risk for depression as well as anxiety disorders [50, 86, 95]. Making a distinction between anxiety- and depression-like behaviors in animal models [96] is not easy, either.

WZ-5HT rats were tested for depression-related behaviors in the forced swimming test (FST), one of the most widely used models of depression [97]. Results showed significantly increased immobility of low-5HT rats, being an indicator for a depression-like phenotype (Figure 7.8g). Three distinct types of active behavior (climbing, swimming and diving), which are thought to be affected differently by various classes of antidepressant drugs [98, 99], were scored separately, showing that between 5HT sublines the climbing behavior was most different.

Given that depressive behaviors are related to deficient brain 5HT transmission, increased immobility in low-5HT rats may refer to the presumed hypofunctioning of their 5HT system, while decreased immobility in high-5HT rats may reflect an antidepressant-like coping

reaction mediated by increased 5HT transmission. Intriguingly, an increased level of depression-related immobility has been observed in 5HTT knock-out mice [67, 100], a finding that is opposite to what might be expected from their hyperserotonergic tonus. However, there are also situations/paradigms where 5HTT-deficient mice failed to demonstrate depression-like behaviors [101, 102] indicating that these mice may not be suitable for studying baseline depression-like behaviors. Recently a higher depression-like state in 5HTT knock-out rats has also been suggested based on their increased immobility in FST and reduced sucrose consumption [103].

ALCOHOL PREFERENCE

An inverse relationship between brain serotonergic tone and alcohol consumption has been demonstrated repeatedly [104, 105], with accumulating evidence indicating abnormal 5HTT function in alcoholic patients and respective animal models [107].

Voluntary alcohol consumption, studied in WZ-5HT rats using a two-bottle choice paradigm, showed significant differences between sublines. Low-5HT rats demonstrated much higher alcohol preference defined in terms of both alcohol intake (g EtOH/kg/day) and percentage of total fluid consumed [108] (Figure 7.8h). Alcohol consumption in females exceeded that of males in both 5HT sublines and, in females, differences between sublines were even more pronounced [108]. The amount of alcohol consumed by low-5HT females (4–5 g/kg/day) fell near the range of alcohol intake in sublines of animals specifically bred for alcohol preference [109]. Conversely, high-5HT males avoided alcohol virtually completely in the regimen applied. Low-5HT rats also consumed a larger amount of alcohol in the forced drinking paradigm, when alcohol was given to animals as the only drinking fluid [110]. We suggest that the low-5HT subline of WZ-5HT rats could represent an additional animal model for studying inter-relations between 5HT and alcohol.

Given the inverse relationship between brain 5HT activity and alcohol consumption, the observed higher alcohol intake/preference in low-5HT animals are in line with the supposed hypoactivity of their brain 5HT system. Correspondingly, lower alcohol consumption was shown in hyperserotonergic 5HTT knock-out mice in comparison to wildtype mice [111, 112].

In the end, differential behavioral phenotypes of 5HT sublines in response to various challenges provide further evidence for differences in their brain functioning. High-5HT rats exhibit enhanced anxiety-like behaviors while depressive-like behavior and higher alcohol intake

co-occur in low-5HT animals. This relationship confirms, in a novel model, that these behavioral features may result from the constitutive alterations in brain 5HT functioning. With considerable simplifications, hyperactivity of the 5HT system has been generally related to anxious behaviors [50, 85, 86] and 5HT hypoactivity has been related to the pathogenesis of depression and alcoholism [92, 93]. Thus, the pattern of behavioral divergence between 5HT sublines on challenge could be used as evidence for hyper- and hypofunctioning of brain 5HT system in high-5HT and low-5HT sublines of WZ-5HT rats, respectively. It is possible to speculate that WZ-5HT rats may be suitable to model human 5HT-ergic dysfunctions associated with 5HTT genetic polymorphisms. The fact that our rats selected for high 5HTT activity resembled in several aspects 5HTT-deficient animals was opposite to our expectations; it seems challenging to discern the full range of processes/behaviors disrupted by the selection of animals for the extreme activities of 5HTT.

Finally, in the course of developing of both our models – hyperserotonemic/hyposerotonemic sublines (selection for PSL) and WZ-5HT rats (selection for PSU) – various other aspects of their reactivity were also tested. Pronounced differences in their immune reactivity were demonstrated [113, 114], preliminary studies of their learning ability [89] and pain sensitivity [115] were performed and gender-related differences in responsiveness to challenges between sublines were studied. These results, however, fall beyond the scope of this chapter.

CONCLUDING REMARKS

By selective breeding for the extreme values of platelet serotonin level (PSL) two sublines of rats with constitutional hyperserotonemia/hyposerotonemia were developed. The velocity of platelet serotonin uptake (PSU), the main determinant of PSL, was used as a further, more specific selection criterion, and directed breeding for its extremes resulted in two sublines of rats with constitutional alteration of platelet 5HT transporter activity, and consequent alterations of entire 5HT homeostasis. The mentioned sublines, termed Wistar–Zagreb 5HT rats (WZ-5HT rats), constitute a genetic rodent model described in this chapter. Besides changes in peripheral 5HT homeostasis, high-5HT and low-5HT sublines of WZ-5HT rats also demonstrate changes in central serotonergic mechanisms.

Under basal conditions, neurochemical differences in the 5HT system between sublines were almost undetectable, indicating its large

potential to resist continuous directed genetic pressure. However, upon an additional challenge (pharmacological, environmental), adaptation equilibrium is exceeded and latent differences between 5HT sublines become evident. Given the results of neuropharmacologic (in vivo microdyalisis) and behavioral studies, it could be argued that the brain serotonergic activity is increased in rats from the high-5HT subline as compared to low-5HT rats. As such, WZ-5HT rats might provide an appropriate model to address the biological and behavioral impact of altered regulation of the 5HTT gene, with its main characteristics summarized as follows.

1. The model has been generated by directed breeding for naturally occurring extremes of specific biochemical phenotypes and, as such, it resembles the physiological situation more closely than models developed by genetic engineering techniques (although continuous selective pressure finally escapes the field of genuine physiology).
2. The range of divergence of phenotypic measure under selection – 5HTT activity – is only moderate when compared with the original population. However, pronounced differences in 5HTT activity exist between sublines themselves, with expected divergence in their 5HT homeostasis.
3. Dysregulation of 5HTT is constitutional, i.e. present during the ontogeny, and thus could influence developing serotonergic neurocircuitry with consequent alterations in brain 5HT system functioning.
4. Generation of the model is a stable and reproducible process as evidenced by restarting selective breeding ab ovo several times during the last 15 years with essentially the same results.
5. Bidirectional selection offers the possibility of simultaneous studies on animals with virtually identical constitution, except for dysregulation (upregulation/downregulation) of a parameter under selection – 5HTT activity (although the possibility of some non-specific co-selections could not be completely excluded).
6. WZ-5HT rats represent an integrative model for 5HTT (and serotonin in general) research, incorporating changes at genomic/genetic and phenotypic (neurodevelopmental, structural, biochemical, behavioral, etc.) levels and encompassing both central and peripheral 5HT functioning.
7. An important outcome of model development is the demonstration of a close inter-relation between peripheral and

central components of the 5HT system in the rat – namely, by changing the platelet we have changed the rat brain (there is no intent to make a direct analogy to humans in this respect).

8. The WZ-5HT rat model may be relevant for investigating central/peripheral 5HT functioning in physiological, pathological, pharmacological, neurodevelopmental, etc., conditions.

Further neurochemical, behavioral and molecular characterizations of WZ-5HT sublines are currently underway, including neurodevelopmental studies (somatosensory barrel fields), studies of the effects of dysregulated 5HT transmission on spatial memory, as well as sequencing of the 5HTT gene in search of potential polymorphic gene variants linked to the particular subline.

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