Evolocus

Supplementary Information for

Transgenerational epigenetic compensation

Heritable compensation of disturbed functionality

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Previously, many-many years ago, all transgenerational effects of non-mutagenic external factors were associated with the term "inheritance of acquired characters"[30-34]. Lamarckian expectations were relatively simple: they predicted similarities between treated parents and untreated descendants[33-34]. It is in direct contradiction with complex descendant's phenotype, observed in our experiments (Supplementary Fig. 1).

Above-mentioned schematic representation of our results is based on the following 17 observations.

1. The vast majority of observed changes in the F_1-F_3 untreated offspring of treated males and naive females are opposite of paternal ones, whereas similar changes are relatively rare (**Fig. 2b**, **Fig. 4a**,**b**). (Note contradiction with straightforward expectations – expectations of similar changes in parents and progeny).

2. The most of changes in the F_1-F_2 untreated offspring are very different in males and females (**Fig. 2b**,**c** and **Fig. 4b**).

3. During F_1-F_3 untreated generations the number and expression of abnormalities decrease gradually (**Fig. 2b**,**c** and **Fig. 4b**,**c**).

4. Different abnormalities have different rate of disappearance in successive generations (**Fig. 2b**,**c** and **Fig. 4b**,**c**).

5. Different rate of disappearance of a particular abnormality in successive generations can be seen in males and females (**Fig. 2b**,**c** and **Fig. 4b**).

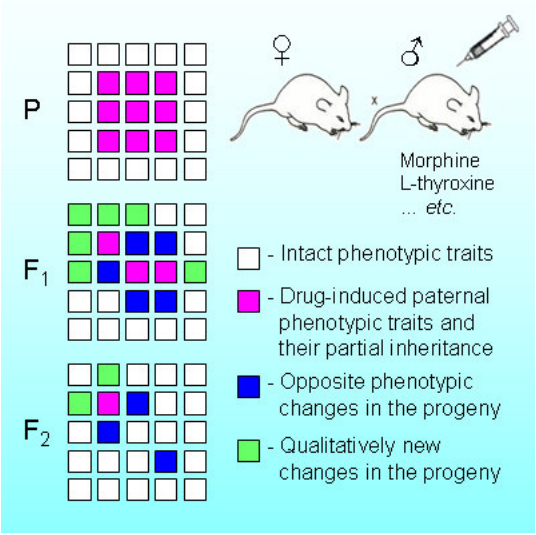
6. Despite general normalization of phenotype in successive generations, some traits, those are normal in F_1, can be abnormal in F_2 (**Fig. 4a**), and some others can be highly abnormal in F_3 only (Fig. S7h[22]), but not in F_2 (Fig. S7f[22]).

7. Epigenetically modified traits persist in the further generations (F_2 and F_3) significantly longer after outcross breeding (new ♀ × F_1 ♂) than after incross one (F_1 ♀ × F_1 ♂) (**Table 1**, **Supplementary Figs. 2b**,**c** and **3b**). (Note the difference with faster "dilution" of a classic mutation in outcross breeding).

8. General normalization of behavioural phenotype in successive incross generations can be achieved by means of supernormal improvement (*i.e.* better, than control) of early learning stages (F_2, Fig. S10a[22], see day 1).

9. The normalization of behavioural phenotype in successive generations can be faster after incross breeding than after outcross one due to improvement of early learning stages, whereas impaired late learning stages remain relatively uncorrected in both incross (F_3, Fig. S11a[22]) and outcross (F_3, Fig. S11c[22], see days 4-5).

10. Within each experimental group of F_1-F_3 progeny, the individual variability of modified traits can be completely uncorrelated (Figs. S17-S19[22], S61-S65[22]); sometimes highly significant correlation in F_1 (Fig. S60b[22]) is followed by the absence of correlation in F_2 (Fig. S60d[22]).



Supplementary Fig. 1 | Summary of the main result**.** Each small square represents schematically one phenotypic trait (quantitative trait). Each cluster of 25 small squares represents the whole body of an organism or its chosen functional system.

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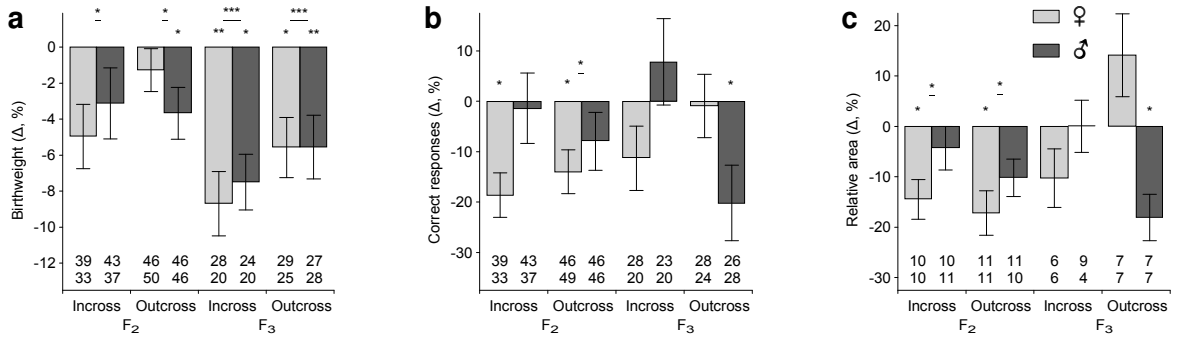


Supplementary Fig. 2 | Phenotype of incross and outcross F_2-F_3 offspring. Untreated descendants of thyroxine-treated male mice. (**a**) Birthweight. (**b**) Two-way avoidance. (**c**) Hippocampal intra- and infrapyramidal mossy fibers. Note significant changes in the F_3-outcross, but not in the F_3-incross (**b** and **c**). Hereinafter: (Δ, %), difference with respect to control (control = 100%). Upper (lower) number near each bar shows exp. (contr.) group size. Asterisk, $P < 0.05$; double asterisk, $P < 0.01$; triple asterisk, $P < 0.001$; asterisk with underline, males and females together. Mann-Whitney U-test. Mean ± SE.

11. Epigenetic inheritance provides possibility for a particular quantitative trait to be decreased or increased in the offspring. Above-mentioned decrease and increase can be achieved using different drugs (**Fig. 2a** *vs*. Fig. S66a,g[22]) or using the same drug, but different protocols of drug administration (**Fig. 2c** *vs*. ref.[35,36]; ref.[3] *vs*. ref.[18]).

12. Weak neonatal (or prenatal) treatment, applied before natural appearance of particular endogenous substance, can induce similar changes in the adult treated males and their untreated offspring (**Fig. 2c**, also ref.[18]), but above-mentioned changes can be the opposite of those observed after strong treatment (ref.[35,36], also ref.[3]).

13. Dominance/recessiveness is a very important dimension of flexibility in transgenerational epigenetic phenomena (for example, the same trait can be dominant in F_1 males and recessive in F_1 females, but after incross breeding it can be recessive in F_2 males and dominant in F_2 females; Figs. S61-S62[22]).

14. Within a lifespan of neonatally drug-treated male its ability to produce progeny with detectable epigenetic changes decreases with increase of time interval between treatment and breeding (Figs. S6[22], S12-S13[22]).

15. Within a lifespan of untreated F_1 descendant the existence of epigenetic changes and/or their detectability decreases with increase of animal age (Fig. S83[22]).

16. Epigenetic changes in the F_1 progeny are more pronounced if drug treatment was applied to younger prospective father (ref.[15] *vs*. Fig. S72c[22]; *i.e.* age 60 days *vs*. 100 days).

17. Several independent heritable epigenetic changes can be formed as a result of neonatal or prenatal treatment. It is quite possible that different heritable epigenetic changes are formed during different periods of paternal ontogenesis.

In the experiment with transgenerational epigenetic compensation and natural selection of a mutant *Per2*^{Brdm1} allele in mice under semi-natural outdoor conditions[25] (**Supplementary Table**) the main conclusion was drawn from the lifespan data. Lifespan data are shown in the **Table 2**. Note that lifespan is shown from the day of release (not from the day of birth). The day of birth remains unknown for the animals, born in field. That is why we have to operate with lifespan, calculated from the day of release.

Observation A. There are impressive gender-related differences: highly significant changes in lifespan were observed in females only, but not in males.

Observation B. P and F_1 homozygous mutant females have decreased lifespan (63 days) in comparison with heterozygous (132 days) and wild-type (150 days) females, however all F_2-F_4 homozygous mutant females have enormously increased lifespan (> 241 days; most of mice were alive at the end of experiment).

Observation C. P and F_1 homozygous wild-type female mice have normal lifespan (150 days), however F_2-F_4 homozygous wild-type females have decreased lifespan (64 days).

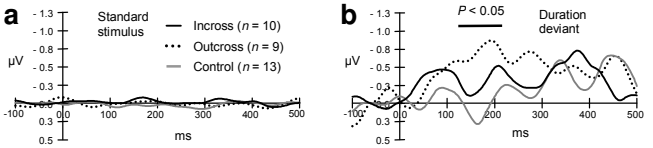
Observation D. Heterozygous females have very stable lifespan: 132 days in P and F_1 and 137 days in F_2-F_4.

It is clear that the outdoor semi-natural conditions in Russia are very harsh for mice (hard to survive) in comparison with laboratory environment. During winter the outside temperature was significantly below 0 °C, see Fig. 3[25]. The combination of a mutant allele with a high environmental pressure has induced transgenerational epigenetic compensation (see Observation B).

Supplementary Table | Number (*n*) of *Per2*^{Brdm1} mice in the field

	Initial release 2005--05-21	Recapture 1 after 116 days 2005-09-14 -2005-09-22		Recapture 2 after 236 days 2006-05-08 -2006-05-13		Recapture 3 after 86 days 2006-08-02 -2006-08-20		Recapture 4 after 282 days 2007-05-11 -2007-05-16	
	P	P	**F_1**	P-F_1	**F_2-F_3**	P-F_3	**F_3-F_4**	P-F_4	**F_4-F_7**
♀ (+/+)	28	11	**19**	12	**20**	8	**21**	5	**24**
♂ (+/+)	26	3	**12**	4	**14**	3	**23**	1	**22**
♀ (+/-)	53	25	**45**	19	**38**	24	**34**	15	**55**
♂ (+/-)	67	7	**38**	8	**20**	2	**27**	3	**31**
♀ (-/-)	40	13	**35**	5	**9**	8	**12**	6	**22**
♂ (-/-)	36	6	**13**	1	**6**	2	**8**	1	**15**
Total	250	65	**162**	49	**107**	47	**125**	31	**169**
% *Per2*^{Brdm1}	54.4	53.8	**55.2**	39.8	**41.1**	48.9	**40.4**	51.6	**47.3**
P		0.79		0.82		0.15		0.54	
Total	250	227		157		172		200	
% *Per2*^{Brdm1}	54.4	54.85		40.71		42.73		48.00	

Initial release at the age of 76 days. New individuals born in the field are indicated in bold type by generations **F_1**, **F_2-F_3**, **F_3-F_4**, **F_4-F_7**; previously marked individuals – P, P-F_1, P-F_3, P-F_4. *P*-values (*Chi*-square, df = 1) show the difference in the number of mutant alleles (-) in the old cohort survivors against new cohorts caught in the same trapping session.



Supplementary Fig. 3 | Auditory evoked potentials in the F_3 incross and outcross. F_3 male descendants of thyroxine-treated males. Mismatch negativity paradigm (MMN)[22]. (**a**) Standard stimulus. (**b**) Duration deviant. Note about twofold greater response to deviant stimulus in Outcross in comparison with Incross (**b**). Bar shows time interval (from 125 ms till 218 ms) with significance $P < 0.05$ "Incross + Outcross" *vs.* Control; t-test for independent samples (two-tailed).

Epigenetically modified loci are supposed to be a part of chromosomal genome. For example, 25 DNA sequences with altered methylation were identified at day P6 in the testis of prenatally treated Fisher rats (P) in vinclozolin study[3]. Hereinafter generation numbering (P, F_1-F_3) is converted towards standards of paternal drug treatment (prenatal, neonatal and adult). Further research of F_1 and F_2 generations, obtained from prenatally vinclozolin-treated male and female parents (Sprague-Dawley rats), has revealed several hundred genes with changed expression (transcription) in the testis at day E16[37]: 2071 (P), 1375 (F_1) and 566 (F_2); however only 202 genes were common for P, F_1 and F_2. Recent research with vinclozolin[38] has revealed several hundred genes with changed expression (transcription) in the brain of adult male and female F_2 descendants of prenatally vinclozolin-treated males and females (P). Different genes had changed expression in hippocampus and amygdala and different genes had changed expression in males and females[38]. However the number of primary heritable epigenetic changes remains absolutely unclear in these experiments, because the most if not all of detected changes are probably secondary changes. This statement is supported by the observed gender-related differences and observed brain-compartment-related differences[38]. In an extreme case even a single heritable epigenetic change in a single locus might be the underlying cause of the observed variety of changes in the above-mentioned studies. Only in the present experiments with morphine and thyroxine it was shown that several primary heritable epigenetic changes are involved into discussed phenomena. It was demonstrated by means of asynchronous disappearance of different modified traits in successive generations and, to the less extent, by means of the absence of individual correlations between different significantly modified traits.

In our independent experiment with hybrid mice and enrichment of housing conditions we have found that cage enrichment during days P21-P60 enhances behavioural hybrid vigour in F_1 mice (B6D2F1), obtained from C57BL/6J females and DBA/2J males. Enhanced hybrid vigour can be observed during the rest of their life in a variety of operant behavioural tasks (Fig. S88[22]). Here postnatal neuroontogenesis creates beneficial behavioural phenotypes on the basis of unexpected heritable changes.

In our electrophysiological experiments with auditory evoked potentials and mismatch negativity recording in inbred, hybrid and mutant mice we have found that local neural circuits can improve organism's profit by selective participation in the processes where their participation makes a difference (Fig. S45[22]). Here neurons self-regulate their own activity looking at its efficiency with a help of local feedback loop.

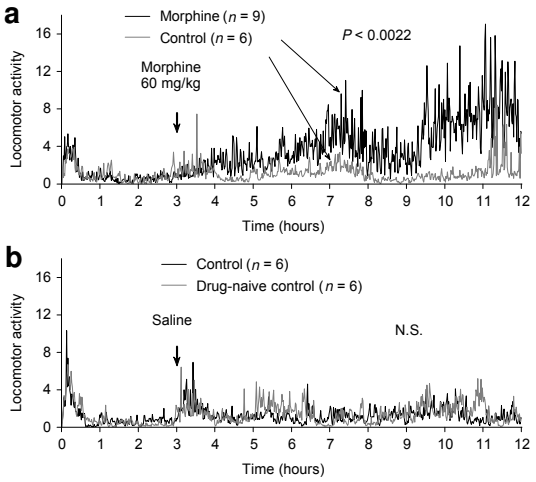
Finally in the progeny of drug-treated males we can see the results of three entirely creative processes: 1) selective transgenerational epigenetic inheritance, 2) creative neuroontogenesis and 3) self-regulated neural activity. They have very different time-scales, but all of them have an impact upon descendant's phenotype. In our paper and SI we discuss only the first one – transgenerational epigenetic inheritance. Others are discussed in SOM[22].

Speaking a little bit idealistic, we can say that from an evolutionary viewpoint the most beneficial evolutionary inventions (both epigenetic and, then, genetic) emerge not only due to paternal creativity (processes inside paternal organism), but, in addition, due to early ontogenetic creativity, early brain development of the next generation. At least 3 successive generations (P, F_1 and F_2) are really necessary here. Processes inside paternal (and, to the less extent, maternal) organism provide only a few raw epigenetic changes to the next generation, whereas further modification of epigenetic heredity and finding of solution for particular evolutionary problem take place during descendant's early ontogenesis and, to a lesser extent, during its further adult brain activity. This distribution of evolutionary efforts between parents and descendants is a nice trick of evolution. It enhances significantly the efficiency of evolutionary process. The consequences of an additional early ontogenetic modification of epigenetic heredity are detectable in F_2 and further generations.

In the experiment with L-thyroxine and DBA/2J mice in the F_1 progeny the opposite changes were observed not only in the two-way avoidance task (**Fig. 2b**), but in the open-field test and in the Morris water maze (Fig. S22d-e,g-h[22]). Thus, the vast majority of observed changes in the progeny are opposite of paternal ones, whereas similar changes are relatively rare. Only morphological trait (intra- and infrapyramidal mossy fibers) was changed in parents and offspring in the same direction (decreased, **Fig. 2c**).

Numerous studies in mice and rats have reported correlations between the extent of the intra- and infrapyramidal mossy fiber (IIP-MF) projection and behaviours thought to be mediated by the hippocampal formation, larger IIP-MF projections being frequently associated with superior performance in spatial memory tasks[36,39] and impaired performance in two-way avoidance task[35]. Our thyroxine-treated animals have shown expected negative correlation between two-way avoidance and IIP-MF projections, although statistically not enough significant ($r = -0.57$, $P < 0.10$; Fig. S17a[22]). However in the progeny such correlations were not detected (Figs. S17-S19[22]).

To represent an unbiased picture, we would like to mention that there are also significantly correlated traits in the experimental F_2. The correlation can be gender-specific, *i.e.* it can be significant in one gender only (*e.g.* females). During experiment with thyroxine it was shown that female-specific correlation does not exist in F_1 (Fig. S20b[22]), but it is highly significant in F_2 (Fig. S20d[22]) and it is near significance level in F_3 (Fig. S20f[22]). This situation looks reasonable, because one of the correlated traits (two-way avoidance performance) has been changed in F_2 in females only (**Fig. 2b**). Although this inheritance pattern can not guarantee the involvement of a single locus (single driving force), it makes this hypothesis highly probable with respect to particular pair of traits.



Supplementary Fig. 4 | Effect of morphine on locomotor activity in F_1 males. Offspring of morphine-treated male rats. (**a**) Locomotor activity, effect of morphine 60 mg/kg administration after 5.5-day morphine treatment and 48 hours after naloxone 2 mg/kg injection. (**b**) Effect of saline administration (control). 1-min averaged values. Each animal was placed in activity-recording cage 3 hours before morphine or saline administration and its individual locomotor activity during these 3 hours was taken as "1". Mann-Whitney U-test; interval 3-12 hours is averaged.

We have seen that behavioural, neuromorphological (**Table 1**, **Supplementary Fig. 2b**,**c**) and even electrophysiological changes (**Supplementary Fig. 3b**) can be more pronounced in the F_3 progeny after outcross breeding than after incross one. Simultaneously, there are some traits which were changed in F_3, obtained from F_2-incross, and F_3, obtained from F_2-outcross, to the same extent: decreased birthweight (**Table 1**, **Supplementary Fig. 2a**) and impaired spatial learning in special home cage – Intellicage™ (Fig. S44[22]).

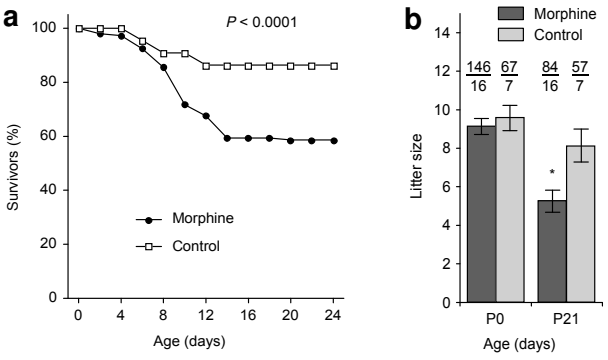
In the experiment with morphine and Wistar rats we have seen that the changes in F_1, those are similar to paternal ones, can be very stable: increased opiate dependence in F_1 males can be detected after 5.5-day morphine treatment (10-60 mg/kg twice daily) as an increased naloxone-induced weight loss (**Fig. 4c**). Here we would like to add that 48 hours after this naloxone administration these animals have shown significant increase (relatively to control) in their locomotor activity as a response to high (60 mg/kg) dose of morphine (**Supplementary Fig. 4a**).

In the experiment with thyroxine it has happened that only neuromorphological trait has been changed in the parents and F_1-F_3 offspring in the same direction (**Fig. 2c**). The same regularity was observed in the experiment with morphine: neuromorphological changes in the F_2 (seen as decreased synaptophysin level in some brain structures, Fig. S60e[22]) were qualitatively similar to particular changes in the initially naive animals after 6 days of morphine treatment and 6 days of morphine withdrawal (this withdrawal is important, because similar changes just after 6-day morphine treatment were not statistically significant, see Table S4[22]).

Above-mentioned data, especially F_2-F_3, for the most part are new. However some other results, obtained in our animals, especially in F_1, reproduce previously reported findings. Postnatal mortality curve in morphine study (**Supplementary Fig. 5a**) precisely coincides with previously published[40]. The same curve was obtained in our F_1 second brood – in the parents of our F_2 generation (Fig. S67b[22]). Enhanced sensitivity to morphine-induced analgesia was reported previously twice (in the F_1 males, but not in females, after paternal morphine treatment)[14,15]. Different standard behavioural tests also have revealed significant deviations in the F_1 progeny of morphine-treated males (Figs. S67d,e[22], S66b[22]). Once again, these deviations were found to be gender-dependent (see, for example, impaired passive avoidance (step-down) performance in males, but not in females; Fig. S67d,e[22]).

There are a lot of changes in the progeny, those are neither similar nor opposite, for example – decreased birthweight. These qualitatively new changes sometimes were classified as "non-specific"[16]. Here we show that it is not necessarily true.

In the available literature there is a notion that the birthweight in the progeny of drug-treated fathers should be decreased[16,41,42]. In our experiments the birthweight was really decreased in the progeny of thyroxine-treated male mice (**Fig. 2a**). However in the progeny of morphine-treated male rats the birthweight was significantly increased (Fig. S66a[22]). This increased birthweight was reproduced in the independent experiment (Fig. S66g[22]). In the previous morphine study[15] the increased birthweight was also reported, but it was not discussed as significant. In our study increased birthweight was not a result of decreased litter size, because litter size was absolutely normal at birth (**Supplementary Fig. 5b**). The difference in litter size appeared later, during days P8-P14, due to increased postnatal mortality (**Supplementary Fig. 5a**). In thyroxine study in all generations (including thyroxine-treated male parents) we did not see increased mortality. Thus, transgenerational epigenetic inheritance can promote both increased and decreased birthweight, the choice is situation-specific. In the experiments with transgenerational epigenetic inheritance there is no direct association between birthweight and postnatal mortality – decreased birthweight can coexist with normal mortality and increased birthweight can coexist with significantly increased postnatal mortality. We can suppose that the most of qualitatively new changes in the progeny, even changed birthweight, can not be classified as "non-specific" *a priori*.



Supplementary Fig. 5 | Neonatal mortality and litter size in the F_1 progeny. Progeny of morphine-treated male rats. (**a**) Postnatal mortality, males & females together; *Chi*-square (df = 1), P20-P24. (**b**) Litter size at birth (P0) and at P21; Mann-Whitney U-test. Asterisk, $P < 0.05$. Number (n) of pups and litters in a group is shown near each bar. Mean ± SE.

In a lot of studies in rats[43] and mice[35,36] neonatal thyroxine treatment has produced increased intra- and infrapyramidal mossy fiber fields. In rats increased mossy fiber projections can be observed not only after neonatal thyroxine treatment, but also after exposition to ethanol in utero[44] and after being given hippocampal lesions shortly after birth[45]. In the above-mentioned studies with neonatal thyroxine treatment, especially in DBA/2J mice, we can see increased neonatal mortality (up to 50%) and, simultaneously, when several doses were investigated, observed changed in IIP-MF projections were not statistically dose-dependent[35]. For the most common dose (2 µg dissolved in 0.05 ml per pup daily from P0 till P11, subcutaneously) the highest neonatal mortality was observed when L-thyroxine was administered in Sörensen buffer (pH 9.0)[36]. When 0.9% NaCl solution with pH 9.0 was used as a vehicle, we assume that L-thyroxine solution was prepared just before the first administration (as a rule this time interval is not mentioned in the articles).

When in our pilot study we applied time interval about 5-15 min between L-thyroxine solution preparation (in 0.9% NaCl with pH 9.0) and the first injection to P0 pups, we had 100% mortality at day P3 (Table S1[22]). However when this time interval was increased up to 24 hr (exactly) and solution was stored at +4°C, no neonatal mortality was observed (Table S1[22]). This protocol was used throughout our study. It has led to significantly decreased body weight starting from day P9 (Fig. S3a[22]) and decreased adult brain weight and body weight in both males and females (Fig. S2[22]; see legend). Difference between control and experimental groups in body weight was visible starting from day P6 (Fig. S2[22]) and, thus, the first effects of thyroxine administration took place during P0-P5. In DBA/2J mice the endogenous peak of free thyroxine level in serum corresponds to postnatal days P9-P16 with maximum at P14[46].

Thus, our thyroxine treatment has two features: it is relatively weak (in comparison with administration of the same dose in Sörensen buffer) and it takes place significantly before the appearance of endogenous thyroxine peak in serum. The analysis of strain-related differences with respect to endogenous thyroxine levels and IIP-MF projections (SOM[22], pp. 51-53) shows that strong neonatal thyroxine treatment during P0-P11 does not mimic natural effect of thyroxine, but, instead, it produces disruptive effect on particular functional system, the effect which is partially similar to decrease of endogenous thyroxine concentration during its peak period (P9-P16). For example, C57BL/6J strain has large IIP-MF projections (2-fold larger than DBA/2J)[47], but it has low endogenous thyroxine peak (2-fold lower than DBA/2J)[46,48] and this peak appears 2 days later (P14 in DBA/2J and P16 in C57BL/6J)[46]. The enlargement of IIP-MF induced by strong thyroxine treatment during P0-P11 indicates that this treatment produces most likely disruptive or suppressive effect on thyroxine receptive pathway.

Weak thyroxine treatment during P0-P11 leads to decreased IIP-MF projections in parents and offspring (**Fig. 2c**), the result which is compatible with known strain-related differences. Thus, weak thyroxine treatment, applied before the appearance of endogenous thyroxine peak, entails enhanced reaction to endogenous thyroxine in both male parents and their untreated offspring. The details and mechanisms of this process are not clear yet, but temporal distribution of events makes possible the existence of some adjusting process already during development of drug-treated animals. Due to this reason some changes are the same in drug-treated fathers and their untreated offspring. However the effect of weak thyroxine treatment during P0-P4 does not necessarily mean that particular heritable epigenetic change was form during this period. It can be formed significantly later as a consequence or even as a compensation of several non-heritable changes. Note that in this experiment behavioural changes were mainly opposite in parents and progeny and due to this reason we can assume that at least some heritable epigenetic changes were formed after the end of neonatal thyroxine treatment.

Experiments with vinclozolin and outbred rats

Very similar results were reported recently for prenatal vinclozolin treatment[3,18]. Transient exposure (daily intraperitoneal injection of 100 mg/kg dose) of a gestating female (Sprague-Dawley rat) to vinclozolin between E8 and E15 promotes increased spermatogenic cell apoptosis in the adult (prenatally treated) P males and males in the untreated F_1-F_3 incross and F_2 outcross generations[3]. This is an expected result. However when pregnant Wistar rats were dosed by oral gavage (which is relatively weak treatment, due to lower plasma peak concentration), apoptotic germ cells counts were statistically significantly lower in P, F_1 and F_2 generation males[18]. In this experiment the dose was exactly the same as in previous one and the treatment period has started 2 days earlier (E6-E15 *vs*. E8-E15)[18]. There is some difference in breeding protocols: here prenatally treated P males were mated with untreated females to produce F_1, which were then similarly mated to produce F_2 offspring (*i.e*. F_2-outcross)[18]. In the previous experiment breeding with naive females was used at the F_2 level only (F_2-outcross)[3]. The onset of androgen receptor expression takes place during sexual differentiation at gestational day E15 in Sprague-Dawley and E17 in Wistar rats (in fetal reproductive tissue, mesenchymal cells surrounding the differentiating wolffian duct)[49,50]. Thus, weak vinclozolin treatment applied before the appearance of maximum of androgen receptor functional activity leads to some adjustment (precompensation) inside particular functional system and entails decreased apoptosis in both prenatally treated males and their untreated F_1-F_2 male offspring. However other traits demonstrate different behaviour. Sperm number and sperm forward motility were significantly decreased in drug-treated animals and their progeny after strong paternal prenatal vinclozolin treatment[3], but these traits were not changed in treated males and their offspring as a result of weak treatment[18].

Obtained results can not be explained by any unidimensional model (in terms, for example, decreased-increased drug sensitivity). To show this we can choose 3 traits in each experiment and represent them as coordinates (x, y, z) in a 3-dimensional space [for simplicity "1" will be "increase", "-1" – "decrease", "0" – "no change", "Ø" – "no data"]. For our morphine experiment: basal pain threshold (x); analgesic effect (y), opiate dependence (z): P ♂ (-1, -1, 1), P ♀ (0, 0, 0), F_1 ♂ (0, 1, 1), F_1 ♀ (0, 0, Ø), F_2 ♂ (1, 1, 0), F_2 ♀ (1, 1, Ø). The effect "F_1 ♂ (0, 1, Ø), F_1 ♀ (0, 0, Ø)" was also observed after treatment of 2-month-old Sprague-Dawley male rats (prospective fathers) by a single large morphine injection 24 hr before mating[15] and after administration of morphine in a liquid diet during 8 days with 5-day drug-free period before mating[14]. However in the experiment

with maternal morphine treatment[41], where prospective mothers received morphine injections twice daily during 5 days with 5-day drug-free period before mating, the results were different: F_1 ♂ (Ø, -1, Ø), F_1 ♀ (Ø, -1, Ø). For our experiment with neonatal thyroxine treatment of DBA/2J male mice: birthweight (x), two-way avoidance (y), mossy fibers (z): P ♂ (0, 1, -1), P ♀ (0, 0, 0), F_1 ♂ (-1, -1, 0), F_1 ♀ (-1, -1, -1), F_2 ♂ (-1, 0, 0), F_2 ♀ (-1, -1, -1), F_3 ♂ (-1, 0, 0), F_3 ♀ (-1, 0, 0). Strong thyroxine treatment[35] had different effect: P ♂ (0, -1, 1), P ♀ (0, -1, 1). In the experiments with prenatal vinclozolin treatment: apoptosis in the testis (x), sperm number (y), sperm motility (z). For strong treatment[3] [i.p. injections]: P ♂ (1, -1, -1), F_1 ♂ (1, -1, -1), F_2 ♂ (1, -1, -1), F_3 ♂ (1, -1, -1). For weak treatment[18] [oral gavage]: P ♂ (-1, 0, 0), F_1 ♂ (-1, 0, 0), F_2 ♂ (-1, 0, 0).

As we can see here, prenatal, neonatal and young adult paternal pharmacological treatments induce complex multidimensional response in the untreated F_1-F_3 generations. With respect to some traits this response looks like preadaptation (see morphine, weak thyroxine and weak vinclozolin treatments). In some experiments with very acute influence just before mating[15] it is possible that post-meiotic (pre-zygotic) selection of germ cells with pre-existing epigenetic changes can be an important factor. In the outbred animals post-meiotic (pre-zygotic) selection can be expected concerning not only epigenetic variation, but genetic variation also. However the most important observation is independent from these local (and mainly still unknown) mechanisms. The majority of heritable epigenetic changes in the F_1-F_3 generations are the results of primarily adaptive processes, the results which can not be predicted using simple mechanistic rules. These processes are entirely creative, they are distributed between several consecutive generations and they take into account not only something simple like "drug sensitivity", but many multidimensional factors.

Experiments reported by Ivan P. Pavlov (1923)

The intensity of treatment and the age of treated animals are very important not only for pharmacological influences. Obtained results provide insight into widely discussed in the past very unusual experiments with the inheritance of "conditioned reflexes", reported by Ivan P. Pavlov on November 9, 1923[51]. White outbred mice of 5 consecutive generations (both males and females) were trained to form conditioned reflex to electro-mechanical bell, so that the animals were trained to run to their feeding place on the ringing of bell. The following results have been obtained. The 1-st generation required 298 training trials. Three hundred times was it necessary to combine the feeding of the mice with the ringing of the bell in order to accustom them to run to the feeding place on hearing the bell ring. The 2-nd generation required, for the same result, only 114 trials, the 3-d – 29, the 4-th – 11, the 5-th – 6. Experiment was prolonged, but the next 6-th and 7-th generations did not show further improvement (*i.e.* 5-7 trials were necessary)[52].

These experiments were conducted by Nikolai P. Studentsov between October 1, 1920 and May 17, 1923[53]. Animals of F_1-F_3 generations were trained starting from the age of 21 day (P21), F_4-F_5 generations – from 30 days (P30). Mice were trained in the long (about 1.5 m) and relatively narrow box with one glass wall. At the beginning of each training trial animals were placed into the box, in its one side. In the opposite side the feeder with milk-wet oats was placed, separated from the mice by glass sliding door. During each trial animals were exposed to sound during 2 min 5 sec in total. During the first 5 sec the glass door was closed, then it was opened and mice had access to food during 2 min, being exposed to ringing bell. Electro-mechanical bell was rather loud. Sound level in dB SPL is not known today, but the same electro-mechanical bell was sufficient to induce audiogenic seizures in 25-40% of mice of the same outbred stock at the age of 26-30 days as a result of 60-90 sec exposure in slightly different experimental setup (without any walls between mice and bell)[51]. Both experiments were organized by N. P. Studentsov in the laboratory of I. P. Pavlov[54]. Audiogenic clonic and tonic seizures were not reported in the above-mentioned transgenerational experiment, but sound-induced wild running was observed[53]. Seizure susceptibility time window is not known for particular white mice. However, for example, DBA/2J mice have time window of audiogenic seizure susceptibility from P16 till P42 with maximum at P21[55]. High-frequency pure tone (12 kHz, 120 dB SPL) induces audiogenic seizures in 53% of DBA/2J at the age of 30 days (P30)[55]. Genomic imprinting was shown for audiogenic seizures using DBA/2J and epilepsy-prone mice[55] and, thus, epigenetic inheritance can play some role in this phenomenon. All known further experiments which were trying to reproduce Studentsov's findings have missed both high sound level and young age of trained animals[52]. Now we can suppose that transgenerationally transmitted epigenetic suppression of audiogenic seizure susceptibility might be a primary cause of performance improvement in Studentsov's experiments.

P.S.

In a biomedical research very frequently the importance of a biological phenomenon is associated with the level of danger, the danger of particular physiological deviations. Concerning transgeneratinal epigenetic effects we have found that these effects are detectable and statistically significant. However the last does not mean that all of them should be described as potentially dangerous, terrible, *etc.* Typical transgenerational epigenetic deviation, detectable in experimental animals, does not exceed extreme values, given by different inbred strains. It means that despite above-mentioned deviation can be described as abnormal with respect to particular strain, it should be considered as non-dangerous in view of all known inbred strains and outbred stocks. The last remark is important for possible projections of discovered phenomena to human population. Not all consequences of a paternal pharmacological treatment should be obviously bad for the next generation.

P.P.S.

Very often the reproducibility of experimental results is considered as an indicator of their quality. Experiments with transgenerational epigenetic inheritance are about fourfold less reproducible than typical single-generation experiments (*e.g.* with similar pharmacological treatments). This estimation takes into account both a quantity of modified traits and their statistical significance level. Experiments with prenatal vinclozolin treatment, neonatal L-thyroxine treatment and young adult morphine treatment have about the same level of reproducibility and it is not very high. Poor reproducibility in multi-generational experiments is not a result of poor experimentation, technical

problems or inappropriate statistical analysis. The observed level of reproducibility is an essential feature of particular biological system. This is a part of natural biological reality. The outcome of a transgenerational epigenetic experiment is dependent on many factors those are not so important for a single-generation experiment. For example, there are very sharp age-dependency and very sharp dependency on drug administration pattern in transgenerational experiments. Many old expectations turned out to be wrong in this field. And expectation of "normal" reproducibility is one of them.

Supplementary References (references 1-29 are given in our main paper)

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