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Article

Transgenerational epigenetic compensation

Heritable compensation of disturbed functionality

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The term "epigenetics" defines all meiotically and mitotically heritable changes in gene expression that are not coded in the DNA sequence itself. The ability of environmental factors to reprogram the germ line and to promote transgenerational disease states has significant implications for evolutionary biology. However, the biological function of transgenerational epigenetic inheritance remains unclear. Here we show that epigenetic inheritance promotes transgenerational compensation of disturbed functionality. After chronic morphine treatment of male Wistar rats or neonatal thyroxine treatment of male DBA/2J mice many of the changes discovered in the untreated progeny occurred to be the opposite of those observed in the treated fathers themselves. Phenotypic analysis of the untreated F_1-F_3 generations has revealed several independent epigenetically modified loci. Transgenerational epigenetic compensation was observed in the F_2-F_3 and further generations of transgenic *Per2*^{Brdm1} mice raised under semi-natural outdoor conditions and it was localized not in the same locus as original mutation.

Epigenetically altered patterns of gene expression can occur through several mechanisms those are based on DNA methylation, histone modification and RNA-associated silencing[1-6]. Our increased knowledge of epigenetic reprogramming supports the idea that epigenetic marks are not always completely cleared between generations[6,7]. Incomplete erasure at genes associated with a measurable phenotype can result in unusual patterns of inheritance from one generation to the next. It is also becoming clear that the establishment of epigenetic marks during development can be influenced by environmental factors[3,7]. Transgenerational epigenetic inheritance is often thought to be expressed in phenotypic similarities between parents and descendants[8]. Due to these similarities epigenetic phenomena sometimes can be described as "transgenerational induction"[9].

However under a set of experimental conditions[10-15] it was shown that some of the changes discovered in the untreated progeny tend to be the opposite of those observed in the treated fathers themselves[10]. The opposite changes in drug-treated organisms and their untreated offspring were observed in plants (*Linum usitatissimum*)[11], insects (*Pieris brassicae*)[12] and mammals (Sprague-Dawley rats)[10,13-15]. Exposing male animals to LSD, alloxan, morphine and tolerizing agents makes their descendants not tolerant, but more sensitive to those particular agents[16]. This phenomenon can be referred to as "phenotypic inversion"[17]. Sometimes the opposite changes in the progeny were absolutely unexpected by researchers and just due to this reason they were not considered to be treatment related, despite impressive statistical significance[18].

"Transgenerational induction" and "phenotypic inversion" appear to be contradictive at the phenomenological level. This contradiction entails a question about the main biological function of transgenerational epigenetic inheritance. To resolve this question we investigated transgenerational epigenetic inheritance in 2-3 untreated generations, obtained from drug-treated males and naive females, in different breeding paradigms (**Fig. 1**). We measured developmental, behavioural, neuro-morphological and drug-specific traits in the drug-treated male parents and their untreated F_1, F_2 (incross and outcross) and F_3 offspring. Finally, phenomenological regularities of trans-generational epigenetic inheritance have been discovered. Molecular mechanisms, supporting these regularities, still remain to be investigated.

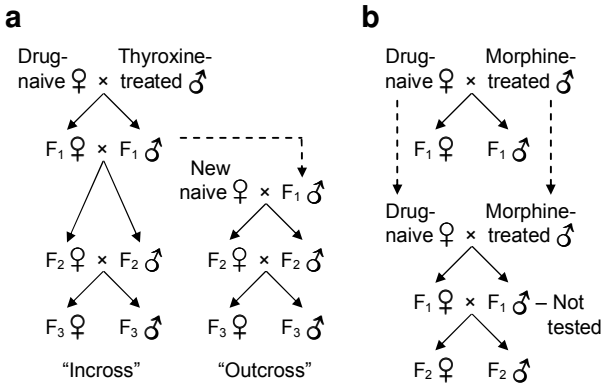

Figure 1 | Breeding paradigms. (**a**) DBA/2J mice, thyroxine study. (**b**) Wistar rats, morphine study. Solid arrows indicate the appearance of progeny, dashed arrows – transition of the same animals.

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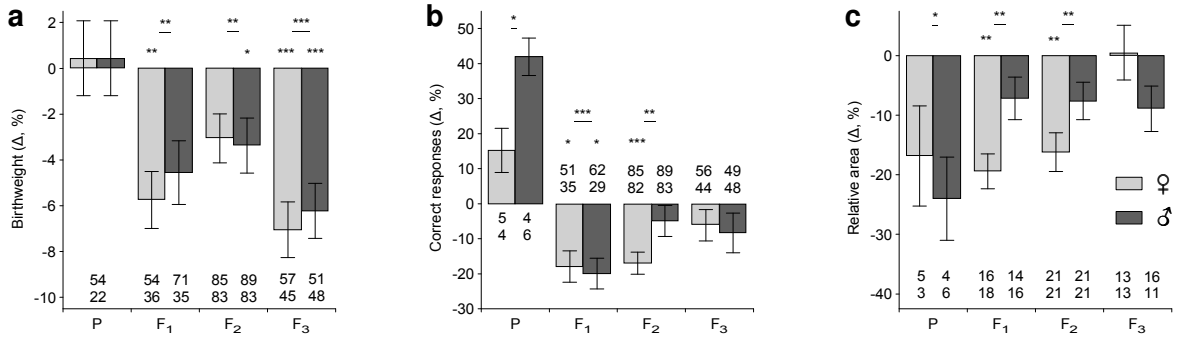

Figure 2 | Phenotype of thyroxine-treated mice and F_1-F_3 descendants. Neonatally thyroxine-treated mice and untreated descendants of thyroxine-treated males. (**a**) Birthweight. (**b**) Two-way avoidance averaged correct responses of 5-day training, 80 trials daily. (**c**) Hippocampal mossy fibers, ratio of intra- and infrapyramidal mossy fiber (MF) fields to suprapyramidal MF. Timm-stained horizontal sections from the mid-septotemporal level (**Fig. 3**). Hereinafter: (Δ, %), difference with respect to control (control = 100%); asterisk, $P < 0.05$; double asterisk, $P < 0.01$; triple asterisk, $P < 0.001$; asterisk with underline, males and females together. Incross and outcross subgroups are pooled in this figure. Mann-Whitney U-test. Mean ± SE.

In this paper we show that epigenetic inheritance promotes transgenerational compensation of disturbed functionality. The terms "precompensation" and "preadaptation" can be used here also. In fact, some elements of the acquired compensation penetrate into several subsequent generations, where they induce partially inversed phenotype in the absence of particular treatment.

We have chosen two different experimental models (**Fig. 1**), known for their positive results with respect to transgenerational effects[16]: morphine treatment of male rats[14,15] and neonatal L-thyroxine treatment of male inbred DBA/2J mice (previously similar studies with L-thyroxine were done using outbred rats[10,13]).

Morphine is known as a classic analgesic which acts *via* binding to cell membrane opiate receptors, which are shown to be on the germ cells also[19,20]. L-thyroxine (T4) – endogenous hormone which is very important for early brain development, it serves as a precursor of hormone triiodothyronine (T3), both T4 and T3 penetrate into the cell nucleus and bind to DNA with a help of nuclear thyroid hormone receptors[21]. Despite the involvement of different molecular mechanisms into morphine and thyroxine action, the epigenetic inheritance patterns occurred to be quite similar.

Results

I. Only very small portion of all acquired compensatory (and sometimes destructive) changes becomes epigenetically heritable.

II. Epigenetic inheritance promotes transgenerational compensation of disturbed functionality and entails the opposite changes in the untreated progeny.

III. Heritable epigenetic changes are distributed in several independent loci and these changes disappear gradually and independently of one another during a few untreated generations.

IV. Only very small portion of all changes in gene expression in the untreated progeny are primary heritable changes; others are the results of secondary adaptation and developmental compensation, initiated by heritable epigenetic changes.

These ideas are summarized in the **Supplementary Fig. 1**.

In the experiments with L-thyroxine and DBA/2J mice we have investigated 813 mice in total: P generation – 76, F_1 – 196, F_2 – 340, F_3 – 201 (**Fig. 2a**). Male DBA/2J mice (inbred strain) were treated as neonates (days P0-P11) with daily subcutaneous injections of L-thyroxine (see **Methods**). Their untreated F_1-F_3 descendants have shown qualitatively new changes (decreased birthweight, **Fig. 2a**), opposite changes (impaired two-way avoidance performance, **Fig. 2b**) and similar changes (decreased intra- and infrapyramidal hippocampal mossy fiber fields, **Fig. 2c**). Note that each bar in this figure represents the difference between experimental and control group (control is taken as 100%). Upper/lower number near each bar represents the size of particular experimental/control group, respectively. Note that decreased birthweight is a very stable trait (**Fig. 2a**). Decreased birthweight is a result of slightly increased litter size (Fig. S7[22]). Decreased two-way avoidance (Shuttle-box) performance exists in both F_1 males and F_1 females, but disappears faster in males (see F_2 and F_3, **Fig. 2b**). Hippocampal mossy fiber projections are decreased in F_1-F_2 female offspring, but not in males.

Epigenetic changes disappear gradually from F_1 to F_3. Different traits disappear with different rate. In this experiment the decreased birthweight occurred to be the most stable trait (**Fig. 2a**). The rate of disappearance of other traits is different in males and females. Abnormalities disappear significantly faster or they are initially smaller in males than in females, in this particular experiment with thyroxine (**Fig. 2b**,**c**). However, this statement can not be generalized, because in the experiments

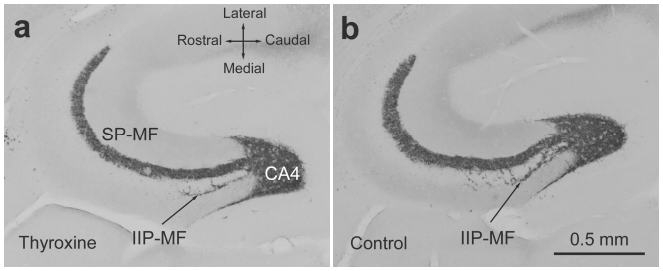

Figure 3 | Hippocampal mossy fiber morphology in the F_3-outcross. Thyroxine study. (**a**) Experimental male mouse. (**b**) Control one. Note the scarce infrapyramidal mossy fiber projection (IIP-MF) in (**a**). Shown samples differ from each other to the greater extent (45%) than mean group values (18%, **Supplementary Fig. 2c**). Scale bar, 0.5 mm.

Table 1 | Statistical significance (*P*) in the incross and outcross

		Birthweight		Shuttle-box		Mossy fibers	
		♀	♂	♀	♂	♀	♂
F_2	Incross	0.13	0.36	0.013	0.96	0.049	0.48
	Outcross	0.47	0.033	0.016	0.30	0.047	0.13
F_3	Incross	0.0050	0.046	0.28	0.60	0.63	0.87
	Outcross	0.030	0.0017	0.85	0.046	0.27	0.025

Descendants of thyroxine-treated males. Comparison with synchronous control, Mann-Whitney U-test. Incross and outcross subgroups have very similar group size (*n*), see **Supplementary Fig. 2**.

with morphine all abnormalities occurred to be significantly greater in male progeny (**Fig. 4a**,**b**).

The most striking result inside thyroxine study – epigenetic deviations in the progeny disappear faster after incross breeding than after outcross one (**Table 1**). It is in contradiction with usually expected behaviour of a classic mutation, which has the longest persistence inside incross-bred subline. However we can see that behavioural and neuromorphological changes can be seen in the F_3 males after outcross, but not after incross breeding. Similar bias can be detected in the F_2 males, but only as a non-significant trend (**Table 1**). The F_3 result is unusual. However behavioural changes in F_3-outcross, but not in F_3-incross, were reported once in descendants of cyclophosphamide-treated male rats[23]. It seems that the incross breeding reinforces some compensatory process, the process which accelerates the normalization of phenotype in the next generation.

In the experiments with morphine and Wistar rats we have investigated 357 rats in total: P_{males} – 28, F_1 – 89, F_2 – 240 (**Fig. 4a,b**). Male Wistar rats (outbred stock) were treated starting from the age of 42 days (body weight 197 ± 20 g, mean ± SD) during 38 days (days P42-P79) with intraperitonial morphine injections twice daily (see **Methods**). Their F_1-F_2 progeny have shown qualitatively new changes (increased birthweight, Fig. S66a[22]), opposite changes (increased reaction latency to high temperature in tail-withdrawal test, *i.e.* increased basal pain threshold, **Fig. 4a**; increased analgesic effect of morphine, **Fig. 4b**) and similar changes (increased opiate dependence after standard morphine treatment, **Fig. 4c**). In addition to effects, observed previously with thyroxine (gender-related differences, gradual disappearance of abnormalities in F_1-F_3), experiments with morphine have revealed other unusual features of epigenetic inheritance.

The disappearance of some change in F_1-F_2 can be associated with appearance of some other change (**Fig. 4a**,**b**, see males). Thus, some trait, which is normal in F_1, can be abnormal in F_2 (**Fig. 4a**). It means that transgenerational epigenetic inheritance promotes the penetration of an abnormality from one trait to the other ones.

In addition, an abnormality can penetrate from one gender to another one (in this particular experiment – from males to females). In the F_1 we can see highly abnormal males and normal females, whereas in the F_2 we can see slightly abnormal males and significantly abnormal females (**Fig. 4b**). Similar penetration of modified trait from one gender to another one was observed in the thyroxine study (but from females to males). In fact, in the F_2 we can see the decreased IIP/SP mossy fiber projections in females, whereas in the F_3-outcross this change is more pronounced in males (**Table 1**).

In progeny, different changes have different stability within a lifespan of one generation. Changes in F_1, those are opposite of paternal ones, can be very unstable. For example, the enhanced sensitivity to analgesic effect of morphine in the F_1 males disappears up to non-significant level during 24 hours after single 10 mg/kg morphine injection (Fig. S54a,c[22]). On the other hand, changes in F_1, those are similar to paternal ones, can be relatively stable. For example, increased opiate dependence in F_1 males can be detected after 5.5-day morphine treatment (10-60 mg/kg) as an increased naloxone-induced weight loss (**Fig. 4c**).

Discussion

At present, we can see that epigenetic inheritance can form the following descendant's phenotype (in comparison with paternal one): a few similar changes, a lot of opposite changes and a lot of qualitatively new changes. Whether all these changes were induced by a single epigenetic change in a single locus? If it is so, we should have significant individual correlations between different modified traits in the F_2 generation inside each experimental group. Animals inside an experimental group should be subdivided into "changed" and "unchanged". However it is not the case. Even the traits, those were highly correlated in the F_1 (Fig. S60b[22]), were completely uncorrelated in the F_2 (Fig. S60d[22]). Selected experimental animals with normal behavioural

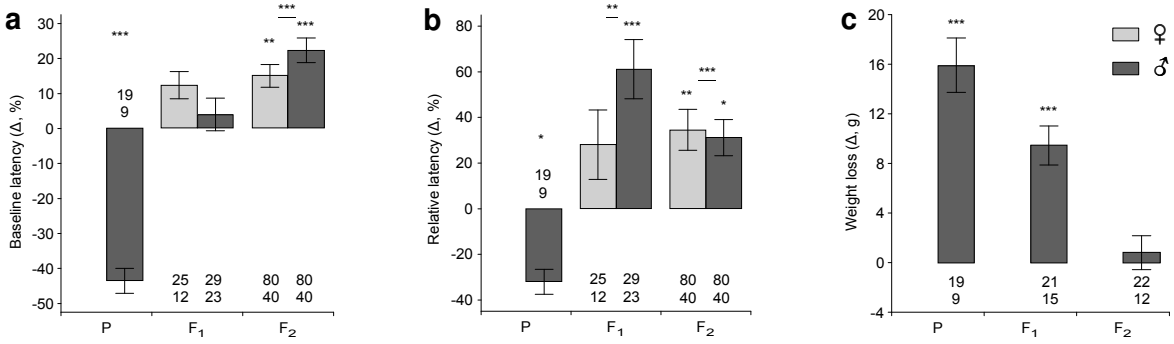

Figure 4 | Phenotype of morphine-treated male rats and F_1-F_2 progeny. (**a**) Pain sensitivity, baseline latency. (**b**) Morphine analgesia, ratio of tail withdrawal latency, measured 30 min after 10 mg/kg morphine administration, to baseline latency. (**c**) Naloxone-precipitated weight loss after 5.5-day morphine treatment (in the F_1 and F_2 offspring) or after 40-day treatment (in the experimental fathers). Mean ± SE.

phenotype (F_2, Fig. S60d[22]) had significant morphological changes in some brain regions (Fig. S60e[22]). The absence of correlations was observed not only in the outbred Wistar rats, but in the inbred DBA/2J mice also (F_2, Figs. S17-S19[22]). It means that there are several (not one) heritable epigenetic changes, which are distributed in several independent loci. The same conclusion can be drawn from the asynchronous disappearance of different modified traits in successive generations (**Fig. 2** and **Fig. 4**).

Transgenerational epigenetic compensation can be expected in transgenic and "knockout" animals. There are a few published reports[24] (and a lot of unofficial information) about situations when in transgenic and "knockout" animals previously detected phenotype disappears in a few subsequent generations, in spite of undisrupted transgene. Of course, there are known *ad hoc* explanations (disappearance of flanking alleles, subtle differences in background strains, *etc*)[24]. However this phenomenon may be more universal.

Transgenerational epigenetic compensation was observed recently by Serge Daan and co-authors in the F_2-F_3 and further generations of transgenic *Per2*^{Brdm1} mice raised under semi-natural outdoor conditions[25]. Serge Daan and co-authors are the first who have discovered how transgenerational epigenetic compensation of a mutant allele can change the course of natural selection in a semi-natural environment[25]. Mutant, heterozygous and wild-type male and female mice, initially 250 in Mendelian ratio 1:2:1, were kept outdoors in a semi-natural environment[26] as an isolated population, random mating inside each of 4 independent pens during 2 years (see **Methods**). Each mouse was individually numbered by means of subcutaneously injected transponder and all new mice, born in field, were genotyped and numbered twice a year. Transponders were registered by antennas, placed near feeding places. Recording equipment was working 24 hr daily, providing information about feeding activity and, finally, about lifespan of each mouse.

Lifespan data, calculated from the day of release, exist for four cohorts: P, F_1, F_2-F_3 and F_3-F_4. P and F_1 were very similar, but different from F_2-F_3 and F_3-F_4, whereas F_2-F_3 and F_3-F_4 were very similar with respect to all registered aspects of behaviour, including lifespan. Thus, animals were naturally grouped in two categories: P-F_1 and F_2-F_4 (**Table 2**).

It is interesting that F_1 generation, born in field, does not differ from P generation, born in laboratory, with respect to lifespan or any other aspect. Only starting from F_2-F_3 generations, born in the field, transgenerational epigenetic compensation was observed (increased lifespan in mutant (-/-) females, **Table 2**). It means that transgenerational epigenetic compensation was formed during early period of parental ontogenesis. The whole cycle of parental ontogenesis should be under semi-natural conditions, not only some short time interval just before and during breeding period.

The decreased lifespan in the F_2-F_4 wild-type females (**Table 2**) indicates that transgenerational epigenetic compensation is localized not in the same locus as original *Per2*^{Brdm1} mutation. Heritable epigenetic changes are usually distributed in several independent loci (their number is unknown in this outdoor experiment). The majority of F_2-F_4 wild-type progeny has originated from heterozygous parents (**Supplementary Table**). Due to this reason wild-type progeny has heritable epigenetic compensation in one or several loci, but it has not mutant *Per2*^{Brdm1} allele *per se,* – that is why it has decreased lifespan. The majority of F_2-F_4 mutant homozygous mice are descendants of heterozygous animals also (**Supplementary Table**), but they have heritable epigenetic compensation in one or several loci plus mutant *Per2*^{Brdm1} allele – that is why they have normal or even supernormal lifespan. The decreased lifespan in the F_2-F_4 wild-types can not be explained by direct competition with mutants, because there is huge and very stable buffer of heterozygous mice in population (**Table 2**). The effect of transgenerational epigenetic compensation is very gender-specific – it exists here in females only (**Table 2**). It is similar to the F_2 descendants of neonatally thyroxine-treated males – they have behavioural and neuromorphological changes also in females only (**Fig. 2b**,**c**).

Table 2 | Lifespan (days) of *Per2*^{Brdm1} mice after release

	Genotype	Females	*n*	Males	*n*
P - F_1	Wild-type (+/+)	150 ± 20.6	35	63 ± 34.5	23
	Heterozygous (+/-)	132 ± 22.4	77	56 ± 20.3	48
	Mutant (-/-)	63 ± 12.0	64	50 ± 8.7	27
	P	0.007		0.025	
F_2 - F_4	Wild-type (+/+)	64 ± 15.4	28	42 ± 9.8	21
	Heterozygous (+/-)	137 ± 10.1	57	48 ± 8.6	32
	Mutant (-/-)	> 241	18	45 ± 6.9	8
	P	0.018		0.648	

Lifespan after release in the field in P - F_1 and F_2 - F_4 generations for all mice that were recorded at least 10 days following release. *P*-values are given for the effect of genotype (number of mutant *Per2*^{Brdm1} alleles as ordinal variable) according to the Kaplan-Meijer (log rank Mantel-Cox) procedure. Median ± SE. Standard error is not shown for F_2 - F_4 mutant (-/-) females, because the most of these mice were alive at the end of experiment.

The frequency of *Per2*^{Brdm1} allele in population has dropped from initial 54% to 40% during the first year (P-F_3), but it has recovered to 48% during the second year (F_3-F_7), due to differential survival (**Supplementary Table**). Thus, transgenerational epigenetic compensation of a mutant allele can completely reverse the course of natural selection. Further investigation of interactions between epigenetic and genetic changes will completely rearrange our understanding of evolutionary theory.

Methods

Thyroxine experiment. DBA/2J mice (P) were treated as neonates during the first 12 days (P0-P11) by subcutaneous injection of a daily dose of 2 μg L-thyroxine dissolved in 0.05 ml 0.9% NaCl made alkaline (pH 9.0) by adding a few drops of NaOH. Solution was prepared once 24 hr before the first administration (kept at +4°C). All pups in a given litter received the same treatment (between 17:00 and 18:00) and were kept in an original litter under their native DBA/2J mother (110-day-old at breeding). Control animals were left undisturbed. Reversed day-light cycle was used (8:00-20:00 – dark, 20:00-8:00 – light). Adult mice were housed individually.

To have F_1, each DBA/2J male (P) at the age of 60 days was housed with 2 or 3 nulliparous 90-day-old naive DBA/2J females during 7 days. At birth pups were numbered and placed under primiparous NMRI foster-mothers to have 4 experimental and 4 control pups in each foster litter. To have F_2-incross, F_1 males at the age of 200 days were housed with F_1 females (2 females × 1 male, incross, but without inbreeding). To have F_2-outcross, F_1 males at the age of 230 days were housed with naive DBA/2J nulliparous 110-day-old females (2 females × 1 male). To have F_3, F_2-incross males at the age of 180 days were housed with F_2-incross females and F_2-outcross males at the age of 150 days were housed with F_2-outcross females (1 female × 1 male), simultaneously. NMRI foster-mothers were used in F_1, F_2 and F_3.

P, F_1, F_2 and F_3 mice were tested in two-way avoidance task ("Mouse Shuttle Box", Campden Instruments Ltd., UK)[27] at the age 90-155 days. Training: 5 days, 80 trials daily. The condition stimulus was light (5 sec), the negative reinforcement was foot-shock 0.15 mA (10 sec), which was supplied together with additional 10 sec of light, but both could be terminated by escaping to another compartment. This termination had a 0.8 sec delay – in order to have optimal DBA/2J training. Inter-trial interval: 5-15 sec. Averaged correct responses of 5 training days are shown in the figures.

For hippocampal mossy fiber (MF) morphometry, the morphometric score for a given individual was taken as a ratio of areas: (intra- and infrapyramidal MF)/(suprapyramidal MF).

Morphine experiment. Male Wistar rats, 42-day-old initially (P42; body weight 197 ± 20 g, mean ± SD), housed in groups 5-10 under normal day-light cycle, were injected intraperitoneally (i.p.) with morphine during 38 days. The first 7 days – twice daily (morning-evening, 8 hr between, mg/kg): 5-10, 15-15, 20-20, 25-30, 35-40, 45-50, 55-60 (10 mg/ml in 0.9% NaCl). Next day – 60 mg/kg in the morning and 6 hr later – injected i.p. with 2 mg/kg of naloxone (2 mg/ml) to induce early in life naloxone-precipitated morphine withdrawal. Next day – injected with morphine 60 mg/kg. The rest 29 days – injected with morphine 60 mg/kg twice daily Monday-Friday, and 60 mg/kg daily Saturday-Sunday. Control males were left undisturbed.

During the last 5 days of morphine treatment P males were housed individually with drug-naive 75-day-old nulliparous Wistar females. To have F_1-2 (F_1, second brood), P males at the age of 175 days (*i.e.* 95 days of withdrawal) were housed individually with familiar females. To have F_2, F_1-2 males at the age of 85 days were bred individually with F_1-2 females (incross, but without inbreeding).

P, F_1, F_2 animals were tested in tail-withdrawal test at the age of 60-95 days. The distal part of the tail of a lightly restrained animal was dipped into circulating water thermostatically controlled at 56 ± 0.2°C. Latency to respond to the heat stimulus, by a vigorous flexion of the tail, was measured to the nearest 0.1 sec, cutoff latency – 15 sec. The test was done once before i.p. 10 mg/kg morphine injection (baseline latency) and 15, 30, 45, 60 and 90 min after. Baseline latency and 30-min latency divided by baseline are shown in the figures.

Opiate dependence was investigated in P, F_1, F_2 males at the age of 70-95 days. To have detectable morphine dependence in the offspring, F_1 and F_2 males (both experimental and control) were injected i.p. during 5.5 days (morning-evening, 12 hr interval, morphine, mg/kg): 10-10, 20-20, 30-30, 40-40, 50-50; next day – 60 mg/kg in the morning and 6 hr later – injected i.p. with 2 mg/kg of naloxone. Weight of each animal was measured to the nearest 1 g before naloxone administration and 24 hr later. Weight loss was taken as an indicator of opiate dependence.

The influence of 60 mg/kg morphine injection on locomotor activity was investigated in F_1 males 48 hours after above-mentioned naloxone administration (12-hr record: 3 hr before and 9 hr after injection).

Mann-Whitney U-test was used as a basic method for data analysis.

***Per2*^{Brdm1} mice experiment.** Mutant *Per2*^{Brdm1} allele is known to compromise circadian organization and entrainment and to cause multiple physiological disturbances[28]. Male and female animals (1/4 homozygous mutants, 2/4 heterozygous and 1/4 wild-types; 250 mice in total; mixed background of C57BL/6 and 129SvEvBrd) were individually numbered by means of injected transponders, which can be read by an external antenna, and were placed in 4 independent (20 × 20 m each) open outdoor pens, isolated from each other and ground predators by slate walls (1 m high and sunk 50 cm into the soil, covered by zinc-plated iron on the top)[25]. Each pen had 2 wooden roofed shelters (3 × 2 m each, 70 cm depth, filled with hay, straw and branches). A photograph of similar experimental setup can be seen in the Fig. 2a[26]. Inside each pen, but outside of a shelter, there were two feeding places (food + water), each equipped with antenna, which allowed monitoring of animal visits during 2 years in a non-stop manner. The end of feeder visits provided precise information about lifespan of each animal. All animals were live trapped and new (born in field) animals were genotyped and injected with transponders twice a year.

Original animals were released into shelters at the field station (Tvier Region, Western Russia) on May 21 at the age of 76 ± 5.4 days (mean ± SD) – this is P generation. 116 days later all animals were live trapped and released back. At this time point all animals born in the field during preceding 116 days were genotyped and injected with transponders – all of them were F_1 generation. Subsequent recaptures 2, 3 and 4 were done as shown in the **Supplementary Table**. Starting from the second recapture, generation numbering (F_2-F_3) was not absolutely precise due to natural temporal birth distribution.

Additional method-related details can be found in the ref.[22] for thyroxine and morphine experiments and in the ref.[25] for *Per2*^{Brdm1} mice experiment.

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Supplementary Information for

Transgenerational epigenetic compensation

Heritable compensation of disturbed functionality

Dmitri L. Vyssotski[1,2,3]

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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**).

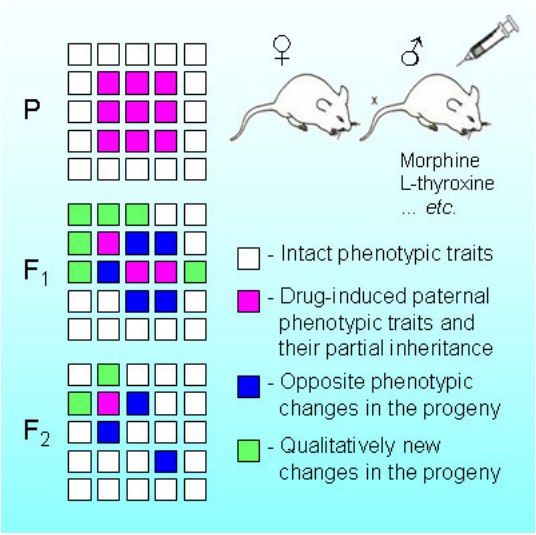
6. Despite general normalization of phenotype in successive generations, some traits, those are normal in F_1, can be abnormal in F_2 (**Fig. 4a**), and some others can be highly abnormal in F_3 only (Fig. S7h[22]), but not in F_2 (Fig. S7f[22]).

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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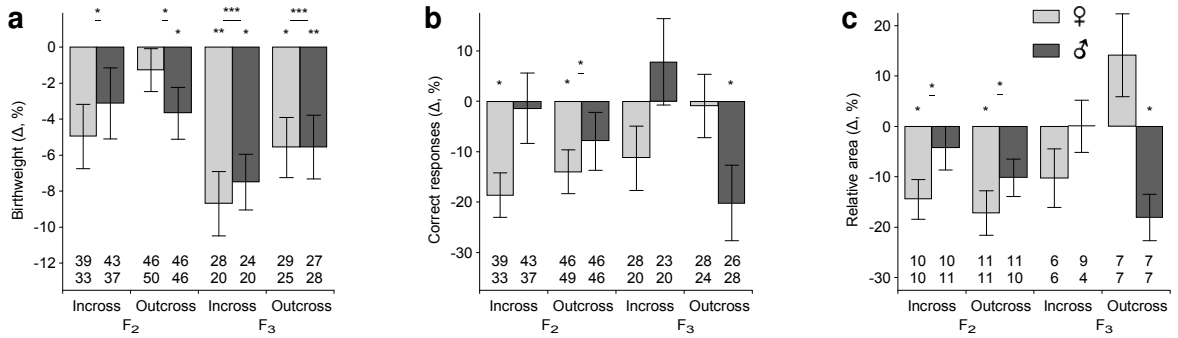


Supplementary Fig. 2 | Phenotype of incross and outcross F_2-F_3 offspring. Untreated descendants of thyroxine-treated male mice. (**a**) Birthweight. (**b**) Two-way avoidance. (**c**) Hippocampal intra- and infrapyramidal mossy fibers. Note significant changes in the F_3-outcross, but not in the F_3-incross (**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).

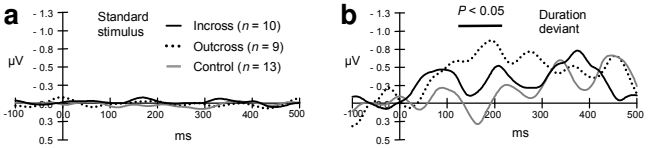
Observation D. Heterozygous females have very stable lifespan: 132 days in P and F_1 and 137 days in F_2-F_4.

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.

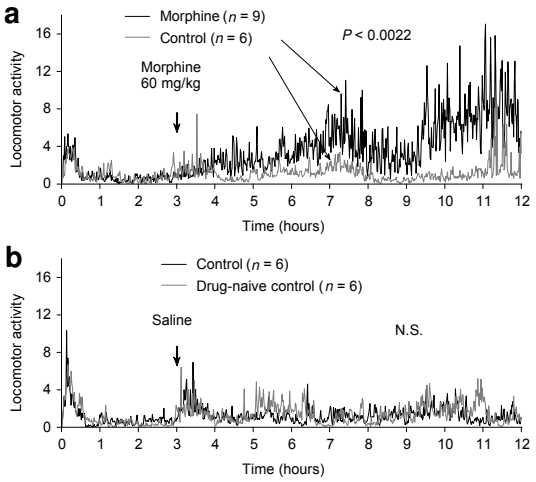
Finally in the progeny of drug-treated males we can see the results of three entirely creative processes: 1) selective transgenerational epigenetic inheritance, 2) creative neuroontogenesis and 3) self-regulated neural activity. They have very different time-scales, but all of them have an impact upon 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]).

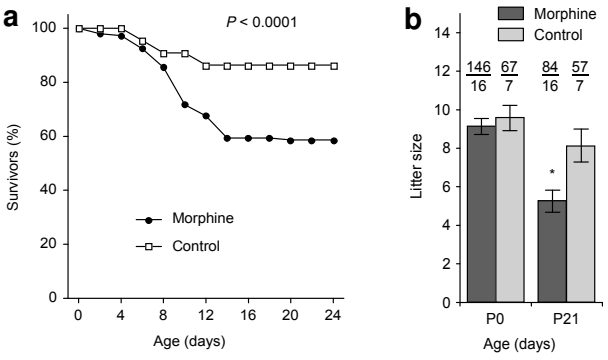
In the experiment with morphine and Wistar rats we have seen that the changes in F_1, those are similar to paternal ones, can be very stable: increased opiate dependence in F_1 males can be detected after 5.5-day morphine treatment (10-60 mg/kg twice daily) as an increased naloxone-induced weight loss (**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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