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Article

Transgenerational epigenetic compensation and sexual dimorphism

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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. Transgenerational epigenetic compensation was discovered in the untreated progeny of drug-treated males (rats and mice) as the opposite quantitative phenotypic changes. In natural populations, heritable epigenetic compensation can convert mutants into hopeful monsters, initiates speciation, and therefore determines the route of macroevolution. Transgenerational epigenetic compensation facilitates genetic assimilation of acquired characters in microevolution. The ontogenesis of females is better canalized than that one of males, and natural selection proceeds mainly through selection of males. However the presence of sexual dimorphism in transgenerational epigenetic compensation remains unclear. Here we show that the hereditary basis of transgenerational epigenetic compensation develops mainly in males. However the phenotypic results of this development are more pronounced in their female descendants, starting from F_2. This sexual dimorphism enhances the efficiency of micro- and macroevolution.

"Numerous facts go to show that changes in various sections of the body of a plant or animal organism are not fixed by the reproductive cells with the same frequency or to the same extent." (Trofim D. Lysenko, 1948; p. 535[1]). These words, entirely different from the Lamarckian ones, were written 5 years before the discovery of DNA structure. In 1953 the existence of 5-methylcytosine was considered as a problem for otherwise brilliant theory: "We have considered 5-methylcytosine to be equivalent to cytosine, since either can fit equally well into our structure." (J. Watson & F. Crick, 1953; p. 242[2]). Now, the methylation of cytosine is considered as one of the mechanisms of epigenetic inheritance, those can be used to support Lamarckian process – the inheritance of acquired characters[3]. However the phenotypic results of transgenerational epigenetic inheritance are very far from the Lamarckian expectation: "the modification in the descendants may have no visible likeness to the original one" (Henri Bergson, 1907; p. 83[4]).

Many of the changes discovered in the untreated progeny tend to be the opposite of those observed in the treated parents themselves[5-14]. This phenotypic inversion demonstrates that the main biological function of transgenerational epigenetic inheritance is a transgenerational epigenetic compensation of disturbed functionality[13]. Recently, in the course of 2-year experiment with *Per2*^{Brdm1} mutant mice under semi-natural outdoor conditions[15], it was shown that the transgenerational epigenetic compensation can dramatically increase the lifespan of homozygous mutants, not only in comparison with their initial state, but in comparison with wild-types also[13-14]. This experiment[15] may be the first study in the world in which it is shown how evolution really works, not only "natural selection", but real evolution. In all experiments with paternal or maternal drug treatment, as well as in the above-mentioned experiment with mutant mice in semi-natural conditions, the enormous difference between phenotypes of males and females was observed in the progeny. This gender-related or sex-related difference (sexual dimorphism), observed in the descendants of drug-treated parents, is greater than the difference between males

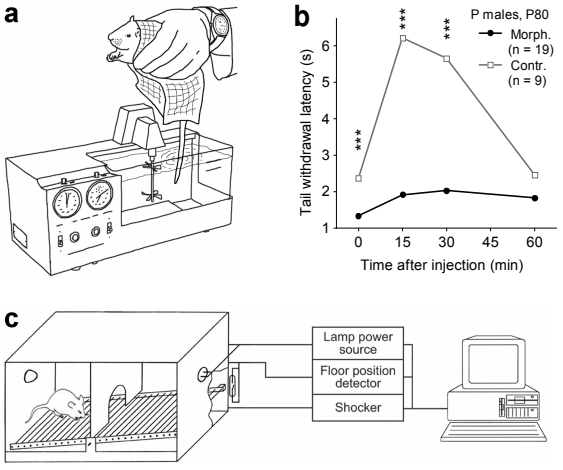

Figure 1 | Tail-withdrawal (**a**) and two-way avoidance (**c**) tests for Wistar rats and DBA/2J mice, respectively. (**b**) Male rats (P) were tested at the age of 80 days in the tail-withdrawal test (56°C), being injected with morphine 10 mg/kg, i.p., after the end of chronic (P42-P79) morphine treatment. Triple asterisk, $P < 0.001$. Mann-Whitney U test. Mean.

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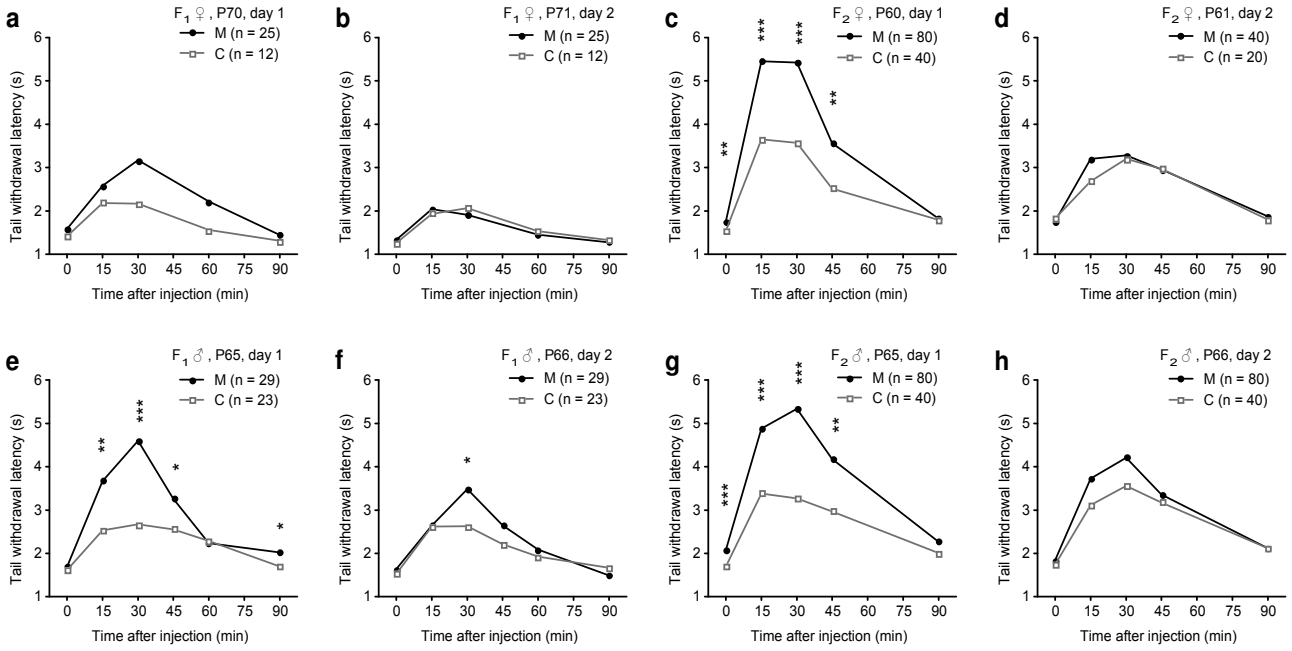

Figure 2 | Tail-withdrawal test in the F_1 & F_2 descendants of morphine-treated male Wistar rats. Each animal was tested twice (days 1 & 2) with the same dose of morphine 10 mg/kg. Morphine was administered i.p. just after the first measurement of tail-withdrawal latency (time "0"). The enhanced analgesic effect disappears at day 2 in the experimental animals, whereas control ones show stable response. In the F_2 generation the enhanced analgesic effect is present not only in males (**e**), but in both sexes (**c**,**g**). There is some difference in the basal pain sensitivity (time "0") in the F_2 generation, but only during the 1-st day (**c**,**g**). M – descendants of morphine-treated males, C – control. Hereinafter: asterisk, $P < 0.05$; double asterisk, $P < 0.01$; triple asterisk, $P < 0.001$. Mann-Whitney U test. Mean (SE or SD is omitted for clarity).

and females, found in wild-type animals as a response to drug application. It means that this sexual dimorphism is a main feature of transgenerational epigenetic compensation, not just some satellite phenomenon. In 1965 it was discovered by Vigen A. Geodakian that the ontogenesis of females is better canalized than that one of males, and natural selection proceeds mainly through selection of males[16-17]. This statement is confirmed by many observations, including recent ones[18], but it is insufficient to explain the enormous sexual dimorphism in the progeny of drug-treated animals.

For our current paper we have chosen several traits (**Fig. 1**) that have demonstrated clear phenotypic inversion in the F_1-F_2 progeny. These results were obtained in the progeny of chronically (P42-P79) morphine-treated male Wistar rats and neonatally (P0-P11) tyroxine-treated male DBA/2J mice. Using these data, together with previously reported results with prenatal (E8-E14) treatments[19-24], we are going to show how the sexual dimorphism in phenotypic expression of transgenerational epigenetic compensation enhances the efficiency of micro- and macroevolution. **Supplementary Fig. 1** summarizes the microevolutionary part of our findings.

Results

I. In the F_1 generation, obtained after prenatal, neonatal or adolescent treatment of male or female parent P, the opposite phenotypic changes in many cases are equally expressed in males and females, and in many other cases they are significantly more pronounced in males.

II. In the F_2 generation, obtained by means of breeding of F_1 female with F_1 male, or breeding of F_1 female with a new naïve male, or breeding of a new naïve female with F_1 male, the above-mentioned opposite (with respect to parent P) phenotypic changes are expressed in females only, whereas males are normal.

III. In the F_3 generation, obtained by means of breeding of any F_2 animal with a new naïve animal, or breeding of F_2 animal, obtained from one new naïve parent, with any other F_2 animal, the above-mentioned opposite (with respect to parent P) phenotypic changes are expressed in males only, whereas all other animals, including males, obtained in line of incross breeding ($F_1♀ \times F_1♂$, $F_2♀ \times F_2♂$), and all females, are normal.

IV. Above-mentioned F_1-F_3 results are already sufficient for mathematical modelling of transgenerational epigenetic compensation in evolution, if we assume that the transgenerational epigenetic compensation is generated only in homozygous mutants, in males and females (or may be mainly in males), in the locus or loci, independent of the locus of mutation. Then, the transgenerational epigenetic compensation is expressed in the consecutive generations like it is described in the **I-III** and it is dominant. Being expressed, the transgenerational epigenetic compensation enhances fitness of homozygous mutants, decreases fitness of wild-types, and probably has no effect on heterozygous animals.

Tail-withdrawal (Water-immersion) test (**Fig. 1a**), in comparison with more common Hot-plate test, can be used without preliminary animal training. Synchronous Hot-plate data are available also (Figs. S54b,d[12], S55b,d[12], S57b,d[12], S58b,d[12]). Chronic morphine treatment of adolescent (P42-P79) Wistar male rats (P) has led to decreased analgesic effect of standard morphine dose 10 mg/kg (**Fig. 1b**) in these animals , but to

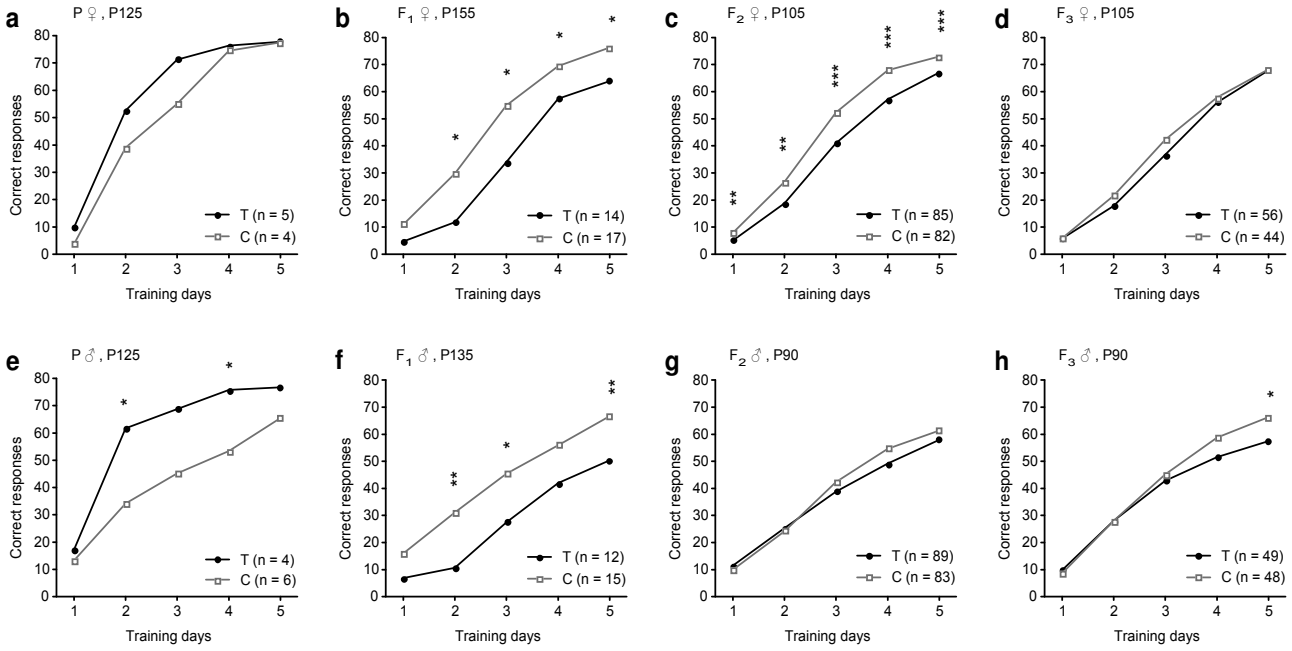

Figure 3 | Two-way avoidance in the thyroxine-treated DBA/2J mice and in the F_1-F_3 progeny of thyroxine-treated males. Note improved performance in the neonatally (P0-P11) thyroxine-treated males (**e**), but decreased performance in their descendants (**b**,**c**,**f**,**h**). Note that the performance of the F_2 males is absolutely normal (**g**), whereas the F_2 females demonstrate deviation with very high statistical significance (**c**). In the F_3 generation this significance disappears, but look at the last day in males (**h**) and see **Supplementary Fig. 3** for differences between Incross and Outcross subgroups. T – thyroxine-treated animals (generation P) or descendants of thyroxine-treated males (F_1-F_3), C – control.

enhanced analgesic effect in their F_1 male (**Fig. 2e**), but not female (**Fig. 2a**), offspring. The enhanced analgesic effect in F_1 males in Hot-plate test after paternal morphine treatment was reported previously[9-10]. Recently, the enhanced analgesic effect in the F_1 male, but not female, offspring was observed in the Hot-plate test after adolescent (P30-P40) maternal morphine treatment (Fig. 3[25]). We did a replication of our Tail-withdrawal test with all our animals 24 hours later and have found that the previously enhanced analgesic effect was attenuated up to normal level in the experimental animals (**Fig. 2b,f**). This attenuation is equal to the enhanced rate of tolerance development. The enhanced rate of tolerance development was reported in the F_1 males, but not females, after adolescent (P30-P40) maternal morphine treatment (Fig. 4[25]). Thus, both paternal and maternal adolescent morphine treatment lead to the same phenotype in the F_1 offspring: enhanced analgesic effect of morphine in males, but not in females, and enhanced rate of tolerance development in males, but not in females.

In the F_2 generation, obtained in our experiment by incross (F_1♀ × F_1♂), the enhanced analgesic effect and the enhanced rate of tolerance development was observed in both F_2 males and females (**Fig. 2c-d,g-h**). Thus, contrary to the F_1, the F_2 females are significantly affected as well as F_2 males.

In the experiment with adolescent (P30-P40) maternal morphine treatment[26], the F_2 generation was obtained through F_1 female outcross (F_1♀ × new♂), but only males were tested[26]. The effect of repeated quinpirole (D2/D3 dopamine receptor agonist) injections on locomotor activity, namely enhanced locomotor activity, occurred to be similar for F_1 and F_2 males (Fig. 2a,b[26]). In the previous experiment with adolescent (P30-P50) maternal morphine treatment[27], the effect of morphine injection on locomotor activity after preliminary 7-day morphine treatment and 7-day abstinence was observed in the F_1 males (Fig. 3[27], *Bottom panel*, $P < 0.01$), but not females (Fig. 4[27], *Bottom panel*, N.S.); the increased locomotion was observed. In our experiment, the effect of morphine injection on locomotor activity after 5.5-day morphine treatment and naloxone-precipitated withdrawal was similar – the increased locomotion in F_1 males, whereas females were not tested (Supplementary Fig. 4a[13], $P < 0.0022$).

Thus, transgenerational epigenetic compensation can be formed by paternal or maternal adolescent morphine treatment. In the F_1 it is expressed mainly in males, even if only females in the previous generation were morphine-treated during their adolescence (P30-P40). In the F_2 the transgenerational epigenetic compensation is expressed equally in both males and females.

Transgenerational epigenetic compensation can be transmitted from F_1 to F_2 through females, by means of F_1 female outcross, as it was shown in the above-mentioned experiment of John Byrnes and co-authors (2013)[26] with adolescent (P30-P40) maternal morphine treatment, and it can be transmitted from F_1 to F_2 through males, by means of F_1 male outcross, and this result was obtained in our experiment with neonatal (P0-P11) paternal L-thyroxine treatment. Concerning morphine treatment we have to add that the basal pain sensitivity was not affected in the F_1, but it was slightly, but significantly, decreased in the F_2 in both males and females (**Fig. 2c,g**), and this effect was eliminated after the first morphine injection (**Fig. 2d,h**).

Two-way avoidance (Shuttle-box)[28] test is a fully automated operant task where an animal learns to move to the opposite (dark) compartment as a response to light stimulus presentation (**Fig. 1c**). Training consists of 80 light presentations daily, during

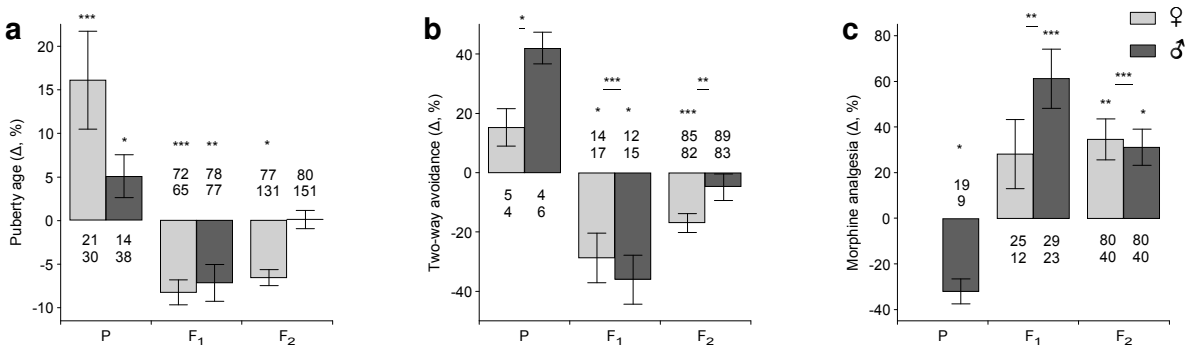

Figure 4 | Phenotypic inversion in the progeny after prenatal (E8-E14), neonatal (P0-P11) and young adult (P42-P79) parental drug treatment. (**a**) Onset of puberty in the experiment of Manikkam and co-authors (2012)[20]: Sprague-Dawley rats (P), both ♀ and ♂, were treated during E8-E14 by i.p. administration of plastic mixture to a pregnant female. (**b**) Thyroxine experiment, 2-way avoidance averaged correct responses of 5-day training, **Fig. 3**. (**c**) Morphine experiment, ratio of tail-withdrawal latency, measured 30 min after 10 mg/kg morphine injection, to baseline latency, **Figs. 1b** & **2a,c,e,g**. Each bar (Δ, %) represents the difference with respect to control (control = 100%). Underline – males and females together (for **b** & **c**). Mean ± SE.

5 consecutive training days (**Fig. 3**). Neonatal (P0-P11) L-thyroxine treatment of males and females leads to improved performance in these animals (**Fig. 3a,e**), slightly more pronounced in males (probably due to better canalization of ontogenesis in females, as usual). In the next generation (F_1), obtained from thyroxine-treated males and drug-naïve females (**Supplementary Fig. 2b**), the phenotypic inversion in the form of decreased performance is equally expressed in males and females (**Fig. 3b,f**). Thus, transgenerational epigenetic compensation can be equally expressed in the F_1 males and females. Note, however, that morphological traits, which typically do not show phenotypic inversion, but show Lamarckian inheritance, can be more deeply changed in F_1 females, than in F_1 males (Fig. 2c[13]).

In the F_2 generation, obtained by both incross (F_1♀ × F_1♂) and outcross of F_1 males (new♀ × F_1♂), the decreased performance in two-way avoidance task was observed exclusively in females (**Fig. 3c**, **Supplementary Fig. 3a-b**). Thus, in the F_2 generation, the transgenerational epigenetic compensation is expressed in females, but not in males.

In the F_3 generation all effects are absent in females, but in the F_3 males, those were obtained after outcross breeding (new♀ × F_1♂, F_2♀ × F_2♂), the transgenerational epigenetic compensation was observed (**Supplementary Fig. 3h**). Thus, transgenerational epigenetic compensation can be transmitted from F_1 to F_2 through males, and, furthermore, in the F_3 generation it is expressed only after outcross breeding. This difference between incross and outcross was observed in our experiment in many traits, not only in the Shuttle-box, namely: birth weight, hippocampal mossy fiber morphology, electrophysiological response – auditory evoked potential in the frame of mismatch negativity paradigm (Table 1[13] and Supplementary Fig. 3b[13]). The decreased Shuttle-box performance in the F_3-outcross (F_1♀ × F_1♂, new♀ × F_2♂), but not in the F_3-incross (F_1♀ × F_1♂, F_2♀ × F_2♂), was reported previously in male, but not in female, descendants of cyclophosphamide-treated male rats (Fig. 12[29]). Thus, transgenerational epigenetic compensation is expressed in the F_3 males only after outcross breeding, and it is absent in all F_3 females.

In the experiment with prenatal (E8-E14) plastic mixture treatment, conducted by Mohan Manikkam and co-authors (2012)[20], this treatment has led to delayed onset of puberty in prenatally-treated male and female rats (Fig. 4a[20]). The effect was more pronounced in females, but the sex ratio was significantly disturbed in this generation and some males probably were not born or were not born alive (Fig. S1[20]). In the next generation (F_1) the accelerated onset of puberty was observed in both males and females, but in the following generation (F_2) the accelerated onset of puberty was evident only in females (**Fig. 4a**). In the experiment of Michael Skinner and co-authors[30] with prenatal (E8-E14) vinclozolin treatment, the F_2 generation females had 1301 genes with changed expression in hippocampus (at P450) *vs.* 92 genes in males (at P360).

Fig. 4 shows that prenatal (E8-E14), neonatal (P0-P11) and adolescent (P42-P79) paternal treatments lead to the same pattern of transgenerational epigenetic inheritance: F_1 effects are equal in males and females or they are more pronounced in males, but all F_2 effects are present mainly in females.

Discussion

The F_1 and F_2 results are obtained in several independent studies with very different protocols of drug administration and animal testing and, thus, they look reliable. We can not say the same about the F_3 results, due to the lack of data. The F_4 and further results are not available at all now.

However the available data allow us to describe the following *modi operandi* of micro- and macroevolution.

The main modus of microevolution

1. In a stable random bred population, without any unusual external influence, typical quantitative trait is distributed normally among males and females, with higher variability among males (**Fig. 5a**).

2. After application of a strong environmental pressure, functionally linked with above-mentioned quantitative trait, the transgenerational epigenetic compensation will shift the mean value of female phenotype towards better adaptation (**Fig. 5b**).

3. Further increase of environmental pressure (**Fig. 5c**) will increase above-mentioned sexual dimorphism so that all females will be out of the zone of discomfort, but natural selection will be working among males; and through natural selection of males the genetic assimilation[31] of a given acquired trait will be achieved in this population.

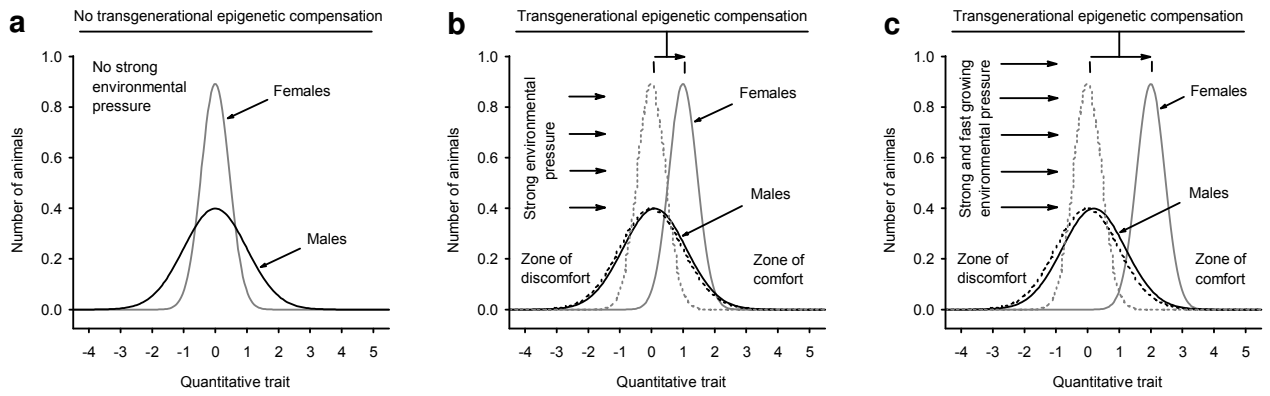

Figure 5 | Distribution of a quantitative trait among females and males in population. Original distribution in males is standard normal distribution. Chosen quantitative trait is functionally linked with given environmental influence (*e.g.*, cold-resistance – low temperatures). Without specific external pressure (**a**) the variability in males is wider than in females (the ontogenesis of females is better canalized). After application of a new environmental pressure (**b**), at least during 2-3 generations, the transgenerational epigenetic compensation will shift female distribution towards comfort zone, whereas males will remain in the zone of discomfort. The transgenerational epigenetic compensation is mainly dormant in males, it is not detectable in male phenotype, and therefore it is not helpful for their survival. If given environmental pressure will be increased further (**c**), males will proceed to develop transgenerational epigenetic compensation for females to remove them from the discomfort zone. But the number of males, suitable for breeding, will be decreased. The natural selection among males will lead to genetic assimilation of transgenerational epigenetic compensation in population.

The main modus of macroevolution

1. The appearance of a new mutation in population, its presence in some individuals (the presence of mutation is necessary for further events, even if the phenotypic results of this mutation are purely behavioural, because the biologically important behaviour is very well canalized also).

2. The application to the population of a strong environmental pressure will lead to development of transgenerational epigenetic compensation in homozygous mutants only, but not in heterozygous and wild-type animals; the loci of epigenetic compensation and mutation are usually independent.

3. In the further generations (starting from F_2), the transgenerational epigenetic compensation will be expressed mainly in females and with the following interaction with genotype: it will increase fitness of homozygous mutants; it will have no effect on fitness of heterozygous animals; it will decrease fitness of wild-types. (This was observed in the *Per2*^{Brdm1} mutant mice; Fig. 3b[14]).

4. There is a point of bifurcation here:

a) The result of transgenerational epigenetic compensation can be the accelerated replacement of wild-type allele in population by mutant one – no speciation in this case; the process starts with low selection coefficient, then the selection coefficient is increased by transgenerational epigenetic compensation, and, finally, it is low again when previous wild-type allele is completely replaced and transgenerational epigenetic compensation is significantly attenuated; this process can be helpful for genetic assimilation – for fast genetic fixation of a weak-effect mutation in population;

b) Transgenerational epigenetic compensation can lead to non-random breeding in population, namely: "wild-type ♀ × wild-type ♂" and "homozygous mutant ♀ × homozygous mutant ♂", because such breeding schema is beneficial for all animals in this population; the population will be self-separated into two independent populations: new mutant population and old wild-type population (Supplementary Fig. 1[14]). Remark: Due to the sexual dimorphism in expression of transgenerational epigenetic compensation, the beneficial phenotype will be expressed in homozygous mutant females, but not in homozygous mutant males, however, nevertheless, these females will choose homozygous mutant males (with transgenerational epigenetic compensation) as potential mates (similar result was obtained with rats and vinclozolin; Fig. 3*B*[23]).

5. After the appearance of two species (new and old), in the new species the transgenerational epigenetic compensation will be slowly replaced by weak-effect mutations through genetic assimilation; and during genetic assimilation the multiple episodes similar to the described one in the **4a** will take place.

6. After the completion of genetic assimilation there will be two species. They will avoid breeding with each other under normal conditions. However their hybrids (F_2 and further generations) will not have lack of viability, because transgenerational epigenetic compensation will be absent in both populations.

Further details can be found in the **Supplementary Information** and in our previous publication "Transgenerational epigenetic compensation in evolution"[14].

Methods

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). See **Supplementary Fig. 2a**.

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. This testing was repeated 24 hours later to assess acute tolerance.

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. See **Supplementary Fig. 2b**.

P, F_1, F_2 and F_3 mice were tested in two-way avoidance task ("Mouse Shuttle Box", Campden Instruments Ltd., UK)[28] 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.

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

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