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Article

Transgenerational epigenetic compensation in evolution

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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 of disturbed functionality was discovered in the untreated progeny of drug-treated fathers as the opposite quantitative phenotypic changes (phenotypic inversion). Epigenetic changes, responsible for heritable compensation, are distributed between several independent loci and these changes disappear gradually and asynchronously during a few untreated generations. The role of hereditary epigenetic compensation in evolution remains unclear. Here we show that transgenerational epigenetic compensation of disturbed functionality converts mutants into hopeful monsters, initiates speciation and facilitates genetic assimilation of acquired characters. The increase of environmental pressure, applied to mutant and wild-type animals, induces heritable epigenetic compensation in mutants (initially less fit), whereas the development of wild-types remains canalized. In a random breeding population this heritable epigenetic compensation increases fitness and lifespan of mutants and decreases lifespan of wild-types.

Hopeful monsters are organisms with a profound mutant phenotype that have the potential to establish a new evolutionary lineage[1,2]. The term "hopeful monster" was introduced by Richard Goldschmidt first in 1933[3] and, then, the detailed theory was provided in 1940[4]. The weakest point of this concept is a requirement that particular mutant should be initially better fit than wild-type. In our article we show that this requirement is not really necessary. Namely, the mutants, those are initially less fit than wild-types, those initially have decreased viability and decreased lifespan, can be converted into hopeful monsters by means of transgenerational epigenetic compensation in a semi-natural population. The canalization of ontogenesis, a concept proposed by Conrad Waddington[5], and the transgenerational epigenetic compensation of disturbed functionality, discovered recently[6], are necessary for understanding of speciation, but they do not provide a solution automatically. The process of genetic assimilation of acquired characters, proposed by Waddington[5], and the process of genetic assimilation of transgenerational epigenetic compensation, discussed in our paper, are important for evolution, but they are too slow to take part in the episode of speciation, which can be extremely fast (**Fig. 1**).

Transgenerational epigenetic compensation of disturbed functionality was observed in the experiments with paternal drug treatment as the opposite phenotypic changes in the untreated progeny (phenotypic inversion)[7]. Such experiments were done with rats and mice using prenatal vinclozolin treatment[8,9], neonatal thyroxine treatment[6,10-12] and young adult morphine treatment[6,12-14]. Phenotypic inversion is evident in the F_1 and F_2 after prenatal plastic mixture treatment[15] (Fig. S4[15] & Fig. 1A[15]), if prenatally-treated rats are numbered as P generation, not as F_1. Previously phenotypic inversion was shown in plants (*Linum usitatissimum*)[16] and insects (*Pieris brassicae*)[17].

Phenomenological properties of transgenerational epigenetic compensation were summarized the following way[6]: 1) only very small portion of all acquired compensatory (and sometimes destructive) changes becomes epigenetically heritable; 2) epigenetic inheritance promotes transgenerational compensation of disturbed functionality and entails the opposite changes in the

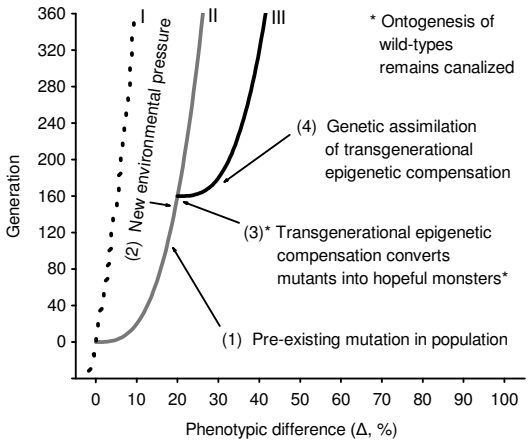

Figure 1 | Transgenerational epigenetic compensation initiates speciation. **I**, **II** and **III** – species or races. Original mutation and its heritable epigenetic compensation are not in the same locus. Speciation demonstrated on hypothetical data.

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untreated progeny; 3) heritable epigenetic changes are distributed in several independent loci and these changes disappear gradually and independently of one another during a few untreated generations; 4) 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[6]. Molecular mechanisms of epigenetic inheritance were discussed elsewhere[18-20].

Results

The emergence of a new species (speciation) proceeds through the following 3 stages or steps.

I. The appearance (and further possible long-term existence) of a new mutation in population, with neutral or slightly negative effect in heterozygous organisms and weak negative effect on survival in homozygous ones.

IIa. The application to the population of a new unusual and rather strong environmental pressure immediately induces transgenerational epigenetic compensation in initially less fit homozygous mutants, whereas the individual development of wild-types and heterozygous organisms remains canalized.

IIb. The transgenerational epigenetic compensation, being found in at least one locus which is independent from the locus of mutation, in a panmictic (random breeding) population increases viability of homozygous mutants, has neutral effect on heterozygous organisms and decreases viability of wild-types.

IIc. Any possibility of discrimination between organisms "with" and "without" transgenerational epigenetic compensation will lead to non-random breeding inside this population: mutants will prefer to mate with mutants, wild-types – with wild-types; heterozygous organisms with strong epigenetic compensation will behave more like mutants, the ones with weak epigenetic compensation – more like wild-types.

III. After the formation of a new species on the basis of homozygous mutants (hopeful monsters), transgenerational epigenetic compensation will be slowly, during many generations, replaced by mutations with subtle effects on phenotype, distributed between different regulatory sites of different genes; this replacement is known as "genetic assimilation", but now the process of genetic assimilation is facilitated by transgenerational epigenetic compensation; the transgenerational epigenetic compensation is constantly updated after each episode of genetic assimilation (after each fixation of a new mutation).

Remarks for stages II-III. Sexual dimorphism is an important factor for facilitation of evolution. Transgenerational epigenetic compensation is building up mainly, but not exclusively, in males. It is transmitted through both males and females. Phenotypic effects of transgenerational epigenetic compensation are more pronounced in females (starting from F_2 generation). Genetic assimilation is working mainly through selection of males. Epigenetic compensation and genetic assimilation can start and proceed simultaneously.

The final result of genetic assimilation in morphological evolution, – many subtle-effect single-nucleotide substitutions in regulatory DNA, is described elsewhere[21].

In the **Fig. 1** the following factors are shown. (**1**) Independent appearance of mutant allele in population (some mutations are always present). (**2**) Unusual and strong environmental influence.

P : aaee ♀ × AAEE ♂

F_1 : AaEe ♀ × AaEe ♂

F_2 :

Gamete F_1 ♀ \ Gamete F_1 ♂	AE	Ae	aE	ae
AE	**AAEE**	**AAEe**	AaEE	AaEe
Ae	**AAEe**	AAee	AaEe	Aaee
aE	AaEE	AaEe	**aaEE**	**aaEe**
ae	AaEe	Aaee	**aaEe**	aaee

Figure 2 | Transgenerational epigenetic compensation promotes segregation of mutants and wild-types. **A** – mutant allele, **a** – wild-type allele; **E** – allele of transgenerational epigenetic compensation, **e** – wild-type allele. Black cells contain homozygous mutants with heritable epigenetic compensation, they have enhanced viability. White cells – wild-type animals with heritable epigenetic compensation, they have decreased viability.

(**3**) Heritable epigenetic compensation improves mutant's phenotype – converts homozygous mutants into hopeful monsters. (**4**) Genetic assimilation of heritable epigenetic compensation (facilitated by dynamic flexibility of heritable epigenetic compensation). Note that the ontogenesis of wild-types remains canalized during the whole episode. As a result of panmixia (random breeding), mutant-optimized heritable epigenetic compensation decreases fitness and lifespan of wild-types (**Fig. 2**), like paternal drug treatment decreases fitness of drug-naive descendants. After speciation there are homozygous mutants with heritable epigenetic compensation and wild-types without heritable epigenetic compensation; both avoid breeding with each other (**Supplementary Fig. 1**).

In the **Fig. 2** the transgenerational epigenetic compensation is localized in one locus, independent from the mutant one. Epigenetic compensation is useful for mutants and dangerous for wild-types. Homozygous mutants with heritable epigenetic compensation have increased fitness in comparison with all other animals. Wild-type animals with heritable epigenetic compensation have decreased fitness in comparison with both wild-type animals without epigenetic compensation and homozygous mutants with heritable epigenetic compensation. Heritable epigenetic compensation can be dominant, because a lot of abnormalities can be observed in the progeny of drug-naive females and drug-treated males.

If heritable epigenetic compensation is distributed between several independent loci (instead of one main locus), our conclusion remains the same: transgenerational epigenetic compensation enhances viability of homozygous mutants and suppresses viability of wild-types. This is the starting point of speciation: mutant and wild-type subpopulations would like to be separated in order to increase viability of both of them.

Currently our knowledge of molecular mechanisms of transgenerational epigenetic compensation is rather limited. However we are sure that basically the same mechanisms are involved into transgenerational epigenetic compensation of paternal drug treatment (relatively well-known at the

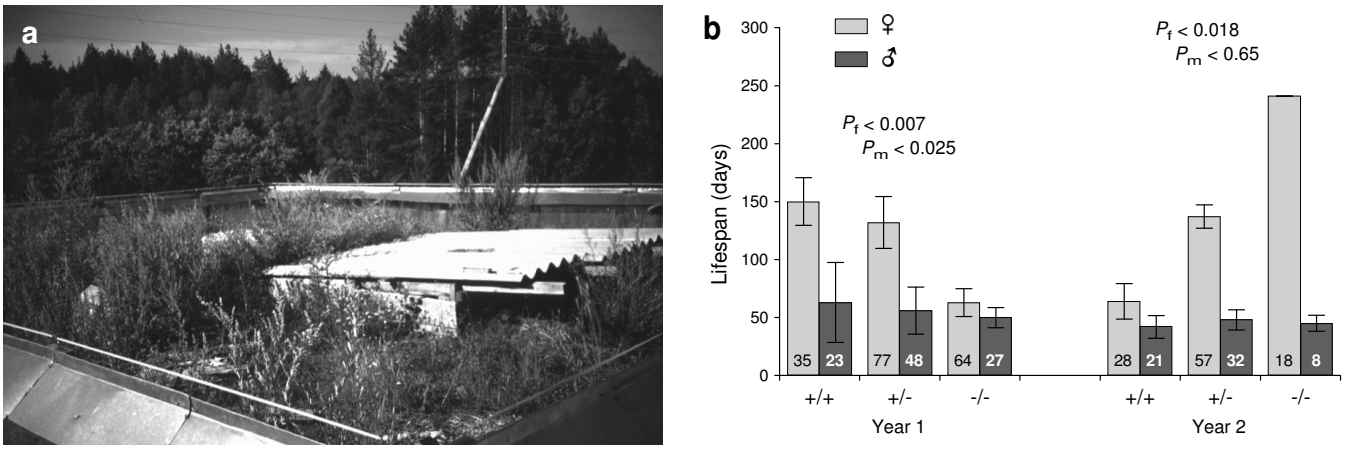

Figure 3 | Lifespan of *Per2*^{Brdm1} mice after release in semi-natural environment. (**a**) Pen 20 × 20 m with two shelters 3 × 2 × 0.7 m each. (**b**) Lifespan (days) after the first release for generations P - F_1 (Year 1) and F_2 - F_4 (Year 2) for all mice that were recorded at least 10 days following release. Wild-type (+/+), heterozygous (+/-) and mutant (-/-) *Per2*^{Brdm1} mice. *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 mutant (-/-) females during Year 2, because the most of these mice were alive at the end of experiment. Data from the experiment of Serge Daan and co-authors (2011)[22].

phenomenological level)[6,12] and transgenerational epigenetic compensation that is building up in homozygous mutants under strong environmental pressure (strong stress)[22].

Transgenerational epigenetic compensation was observed 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[22]. Mutant, heterozygous and wild-type male and female mice (mixed background of C57BL/6 and 129SvEvBrd), initially 250 in Mendelian ratio 1:2:1, were kept outdoors[23] as an isolated population, random breeding inside each of 4 independent pens during 2 years (each pen 20 × 20 m, **Fig. 3a**). Each mouse was individually numbered by 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.

During Year 2 the majority of wild-type progeny had heritable epigenetic compensation in one or several loci, but it had not mutant *Per2*^{Brdm1} allele *per se,* – that is why it had decreased lifespan. Simultaneously, the homozygous mutants had heritable epigenetic compensation plus mutant *Per2*^{Brdm1} allele – that is why they had supernormal lifespan (**Fig. 3b**). The supernormal lifespan of 18 mutant females indicates that these homozygous *Per2*^{Brdm1} females are hopeful monsters, the hopeful monsters that were proposed by Richard Goldschmidt many years ago.

The experiment of Serge Daan and co-authors illustrates steps **I**, **IIa** and **IIb** of a speciation episode. We can see that the high number of particular mutants in population (achieved in this case by artificial means, of course) makes possible the observation of initial stages of speciation despite initial low fitness of homozygous mutants. Transgenerational epigenetic compensation has converted homozygous mutants into hopeful monsters. And it was done specifically with females – with the sex that determines the quantity of descendants in the next generation. Initial stages of speciation can be investigated now experimentally. And one of the most important conditions is not only some special features of chosen mutation, but just very high percent of particular mutants in an artificially created population.

Per2^{Brdm1} mice, used in the experiment of Serge Daan and co-authors[22], have significant deviations in opiate system, namely decreased rate of tolerance development in the experiment with morphine-induced analgesia[24]. We know that in rats the paternal morphine treatment leads to enhanced sensitivity to morphine-induced analgesia and enhanced rate of tolerance development in the F_1 and F_2[6,12]. Thus, opiate system can be a common pathway for heritable epigenetic compensation in both situations.

The next step of speciation (step **IIc**), – the discrimination of animals with and without transgenerational epigenetic compensation as potential mates by females, can be illustrated by the experiment of David Crews and co-authors[25], done with Sprague-Dawley rats and vinclozolin. Prospective parents P (both females and males) were exposed to prenatal vinclozolin treatment during E8-E14 (pregnant females received i.p. injections)[25]. We use generation numbering optimized for paternal drug treatment (prenatal, neonatal, young adult, *etc*). Prenatally treated females and males (generation P) were bred with each other to obtain F_1. F_1 females were bred with F_1 males to obtain F_2 generation. Control animals from untreated parents were bred with each other simultaneously with experimental ones. F_2 generation females and males were tested in mate-preference test at P90-P120 (**Supplementary Information**) and, then, F_2 males were tested in odour-salience test at P403 and F_2 females were tested in odour-salience test at P458.

In the odour-salience test males and females investigated 1-inch-round odour-carrying beads during 1 min in their individual home cages. Five beads were exposed to an animal simultaneously, each carrying one of the following odours: 1) vinclozolin subline female; 2) control female; 3) vinclozolin subline male; 4) control male; 5) self-odour.

In rodents, as well as in other mammals and many other dioecious species, including birds, the final choice of mate is produced by a female[26]. Thus, the preference, shown by a female, is the most important.

Females from vinclozolin subline at the age of 458 days have shown significant preference for odour of vinclozolin subline

males ($P < 0.01$). Males from vinclozolin subline at the age of 403 days have shown modest preference for odour of females from control subline ($P < 0.05$). Control females and males did not show significant preferences for control or vinclozolin subline in this test (Fig. 3*B*[25]). Among young animals (P90-P120) in the mate-preference test the opposite pattern was obtained: all females preferred control males ($P < 0.026$, Fig. 2*A*[25]).

In a natural or semi-natural mouse or rat population, if an animal has age of 458 days and it is still alive, this is a very strong indicator that this animal is not a bad one, indeed. Hopeful monsters in the experiment of Serge Daan and co-authors[22] at the end of experiment had age more than 241 days, calculated from the day of release. From the Daan's experiment (**Fig. 3b**) we can see that there is no such a requirement that males, homozygous mutants with heritable epigenetic compensation (*i.e.* hopeful monsters), should have an advantageous phenotype. The advantageous phenotype should exist in females, homozygous mutants with heritable epigenetic compensation, and these females should be able to identify males, homozygous mutants with heritable epigenetic compensation (but may be without advantageous phenotype), as potential mates.

The experiment of David Crews and co-authors[25] provides necessary evidence for non-random breeding in population consisted of animals with and without transgenerational epigenetic modification. Adult mutant females with successful transgenerational epigenetic compensation prefer to mate with adult mutant males with transgenerational epigenetic compensation. Such animals will try to be an isolated subgroup.

Temporal geographic isolation, proposed by the theory of punctuated equilibrium of Niles Eldredge and Stephen Gould[27], will work for evolution only if the hopeful monsters will be concentrated in the isolated subpopulation, not just some randomly chosen individuals from the original population.

The next evolutionary step (step **III**) is a genetic assimilation of transgenerational epigenetic compensation (**Supplementary Fig. 2**). It is similar in principle to the genetic assimilation of an acquired character, described by Conrad Waddington[5]. The process of evolutionary development of an adaptive phenotype was represented by Waddington as several stages or steps: 1) development of quasi-proportional reaction to external influence, *i.e.* sub-optimal adaptive reaction, which is genetically fixed; 2) development of optimal reaction to external stimulus, quasi-independent from the magnitude of external influence, this canalized reaction is genetically fixed also; 3) development of replacement of external influence by internal factors or stimuli, and this replacement is also genetically fixed. Finally, previously ontogenetically acquired phenotype becomes a classic genetically fixed feature, the feature which is independent under normal conditions from the external environment, and this feature is very well canalized[5].

With respect to the genetic assimilation, the hereditary epigenetic compensation plays two roles: 1) it facilitates genetic assimilation (for example, genetic assimilation of an acquired character); 2) hereditary epigenetic compensation itself can be genetically assimilated.

Mutations in regulatory sites with subtle effect on phenotype can be easily selected (natural selection) only if the matching functional system[28], which is waiting for them, already exists. This matching functional system[28] can be developed as an acquired character during ontogenesis as a result of external environmental pressure. However in many cases, when an external pressure is applied, ontogenetic plasticity is very limited, because it happens at relatively late stage of ontogenesis. In the frame of classic genetic assimilation, without the involvement of epigenetic compensation, mutations which affect early stages of ontogenesis can exist in population, but they will not be selected, because suitable functional system, which can get benefit from them, will not exist, because it can not be developed as an acquired character under external influence.

Only heritable epigenetic compensation can develop expected functional system at earlier stages of ontogenesis in the next generations. Heritable epigenetic compensation with very high probability will disturb early ontogenetic stages in descendants. This disturbance will elicit the next wave of heritable epigenetic compensation. Finally, during several generations very efficient functional system can be developed. And each collected useful mutation will rearrange heritable epigenetic compensation further, in a way that some other, additional set of mutations will become preferable. Thus, it is some kind of a self-corrected search for mutations in a particular population.

Genetic assimilation of an acquired character, facilitated by transgenerational epigenetic compensation, can be illustrated by the experiment of Conrad Waddington (1953)[29]. In this experiment cross-veinless phenotype was induced in *Drosophila melanogaster* by heat-shock treatment. Epigenetic inheritance systems in *Drosophila melanogaster* are not the same as in mammals, especially with respect to methylation, which is practically absent in *Drosophila*[19]. However we need high numbers of animals in order to distinguish a classic genetic assimilation from its possible transgenerational epigenetic facilitation. It was found that when pupae of a wild Edinburgh strain, S/W5, were given a temperature shock (4 hours at 40 °C) starting at 21 to 23 hours after puparium formation, a fair number of crossveinless wings developed, although none appeared under normal conditions. It was decided to use this as the character to be selected. There is, of course, no reason to believe that the phenocopy would in nature have any adaptive value, but the point at issue is whether it would be eventually genetically assimilated if it were favored by selection, as it can be under experimental conditions. It was decided to concentrate on this effect, and to set up two separate selection lines. In one, only those flies which showed the crossveinless effect after treatment were bred from ("upward" selection, which should increase the frequency of response), while, in the other, the crossveinless flies were rejected, and only those still showing normal wings were used to carry on the line ("downward" selection)[29].

Observed cross-veinless phenotype, induced by heat-shock treatment, is considered by us as an indicator (direct or indirect) of some physiological adaptation to heat-shock treatment. This indicator is not adaptive *per se*, of course. Transgenerational epigenetic compensation is trying to play its role in the process of adaptation. That is why it facilitates selection in upward direction and inhibits selection in downward direction (**Fig. 4a**).

Initially this experiment has started with upward selection line only and with relatively wide window of heat-shock treatment onset (17 to 23 hours after puparium formation). Afterwards, starting from the third generation, the downward selection line was added and the time window of heat-shock treatment onset

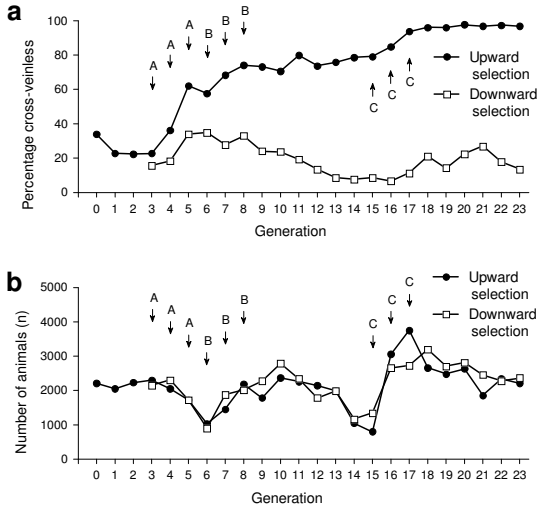

Figure 4 | Transgenerational epigenetic compensation facilitates genetic assimilation. Assimilation of cross-veinless phenotype induced in *Drosophila melanogaster* by heat-shock treatment (40 °C) during 4 hours with onset between 21 and 23 hours after puparium formation. All shown animals (all generations) are heat-shock treated. (**a**) Percentage of animals with cross-veinless phenotypes. (**b**) Number of investigated animals. A, B and C – episodes with probable transgenerational epigenetic compensation. Other time intervals – episodes with pure classic genetic assimilation. Data from the experiment of Conrad Waddington (1953)[29].

was narrowed to 21 to 23 hours after puparium formation. We can see the impressive increase in the percentage of cross-veinless phenotype in both upward and downward selection lines (**Fig. 4a**, episode A), and this is a result of transgenerational epigenetic compensation. Note also episode C (**Fig. 4**). Before episode C we can see that the number of animals in all groups was rather low during two preceding generations (14 and 15, **Fig. 4b**) and we can suppose that a combination of this treatment with some environmental factors was rather stressful for population. This stress can be a reason of transgenerational epigenetic compensation seen in both upward and downward selection lines (**Fig. 4a**, episode C). Look next at the episode B (**Fig. 4**). Stress during episode B has induced transgenerational epigenetic compensation in upward selection line only. Between episodes B and C (generations 8 - 13) we can see the expected very regular progress in both upward and downward direction (**Fig. 4a**) and during the same period the number of animals in both lines is very stable (**Fig. 4b**). We suppose that the role of transgenerational epigenetic compensation during this time interval (generations 8 - 13) is close to zero and we can see here a classic genetic assimilation[5].

Thus, real experiment with genetic assimilation can deal with both classic genetic assimilation and transgenerational epigenetic compensation of disturbed functionality, and, furthermore, genetic assimilation can be significantly facilitated by transgenerational epigenetic compensation.

Discussion

What can we say about macroevolution and microevolution? Microevolution, or evolution of a species without speciation, usually consists of genetic assimilation of acquired characters and genetic assimilation of heritable epigenetic compensation. Different stochastic and neutral changes of heredity belong to microevolution also. Macroevolution, or the appearance of a new species, usually consists of a systemic mutation in Goldschmidt's sense[4], which is in our terms a combination of a key mutation with its heritable epigenetic compensation.

Heritable epigenetic compensation is not only "heritable epigenetic compensation of a key mutation", but it is heritable epigenetic compensation of a complex, consisted of: (a) key mutation; (b) strong environmental influence. The origin of mutation is not specified. The requirement is that this mutation should be present in population in detectable quantity. Thus, initially it should not have too deep negative impact upon fitness and survival. Later, the enhanced fitness of homozygous mutants can be formed by transgenerational epigenetic compensation, induced by environmental pressure.

If mutation is not present in population in detectable quantity, the population will respond to a new strong environmental pressure without speciation. Initial reaction of population to external influence will be quasi-Lamarckian: transgenerational epigenetic compensation will be formed during a few generations. Afterwards, if above-mentioned environmental pressure will be still present, the epigenetic hereditary changes will be replaced by genetic changes (mutations) during relatively slow process of genetic assimilation.

Natural selection remains a part of evolutionary theory, just because it is a part of evolutionary process. Genetic assimilation proceeds through natural selection, especially through natural selection of males. However natural selection is not a "driving force" or "directing force" of evolution, because the efficacy of transgenerational epigenetic compensation determines the direction of natural selection during each evolutionary episode (during any episode with or without speciation).

Sexual dimorphism was found to be important for evolution in the frame of classic genetics by Vigen Geodakian[26,30]: females have better canalization of their ontogenesis, smaller variability in natural populations, and mutations and harmful external influences have lesser impact on their phenotype and survival; whereas the ontogenesis of males is less canalized, mutations have more direct projections to their phenotype, males have higher variability in natural populations; and, as a consequence, natural selection is working mainly in males, whereas females promote sufficient quantity of descendants in each generation.

Transgenerational epigenetic compensation was shown to be highly significant in the progeny after paternal drug treatment – after treatment of males. And it is extremely interesting to see that in their progeny the results of this treatment are more pronounced in females than in males. It is not so evident in the first generation (F_1): there are experiments with equal changes in F_1 males and females (Fig. S4[15], Fig. 2b[6]) and there are experiments with even more pronounced changes in F_1 males (Fig. 4b[6]). However in the second generation (F_2) all changes are more pronounced in females: here we have experiments with prenatal treatment with plastic mixture (Fig. 1A-B[15]), neonatal treatment with L-thyroxine (Fig. 2b[6]) and young adult treatment with morphine (Fig. 4b[6]). The enhanced transgenerational epigenetic compensation in females can be observed despite better canalization of their ontogenesis, typical for all females.

In the experiment of Serge Daan and co-authors[22], with mutant mice in semi-natural environment, all hopeful monsters were

exclusively females. Transgenerational epigenetic compensation is in the process of its development mainly in the organisms of males, but the phenotypic results of this process are more beneficial for their female offspring. This distribution of evolutionary functions between males and females allows to have practically adapted females (as a result of transgenerational epigenetic compensation) and males, those are still working for further improvement of transgenerational epigenetic compensation and/or working for its genetic assimilation (which will be a result of natural selection, active among males only). In a natural population the transgenerational epigenetic compensation, more beneficial for females, and the canalization of ontogenesis, more pronounced in females, are working for the same final goal: to have maximum quantity of females, suitable for breeding. These females will be bred with a few the most advanced males, those are the best in production of transgenerational epigenetic compensation and are the best with respect to mutations, useful for genetic assimilation of the above-mentioned transgenerational epigenetic compensation.

Methods

Methods for *Per2*^{Brdm1} mice experiment are given in the refs.[6,22]. Methods for mate preference experiment are provided in the ref.[25]. Methods for genetic assimilation experiment can be extracted from the ref.[29], but it should be noted that the description given in the ref.[29] can produce false impression that the narrowing of the time interval of the onset of heat-shock treatment from 17-23 hr to 21-23 hr after puparium formation was introduced at Generation 5. Indeed, Generation 5 was chosen as the first generation for demonstration in the Fig. 2[29]. However the data from the Table 1[29], namely identical changes during Generations 3-5 in the "upward" and "downward" lines, shown in our **Fig. 4**, indicate that the above-mentioned narrowing of the time interval was introduced synchronously with the introduction of "downward" selection line at Generation 3. There is no legal contradiction between this statement and the description, provided by Waddington, because 21-23 hr time interval is completely included into the officially declared for these Generations 3-4 time interval 17-23 hr.

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