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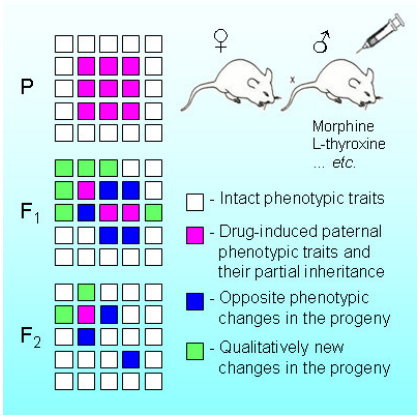

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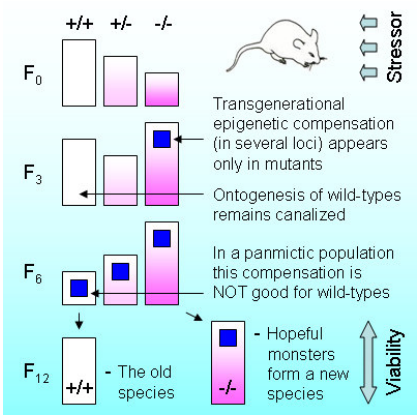
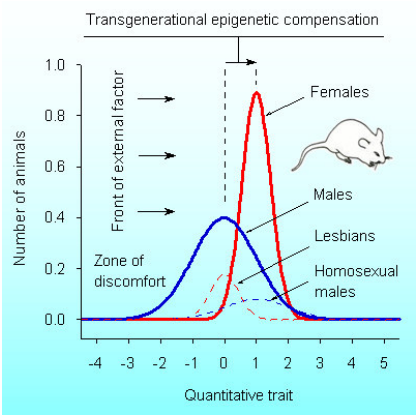
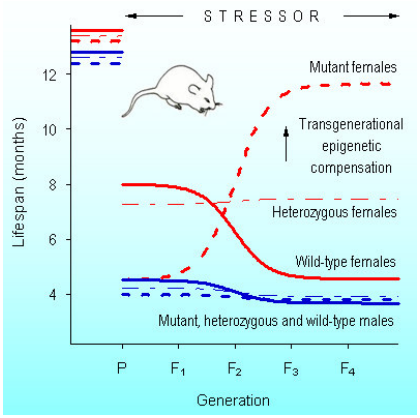


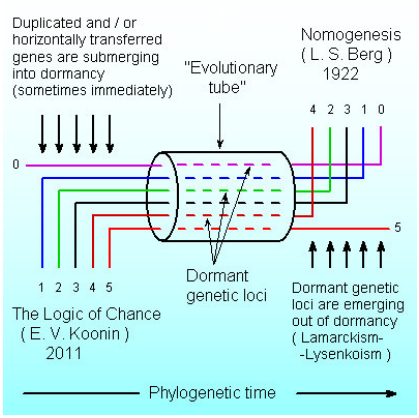
Transgenerational epigenetic compensation in evolution



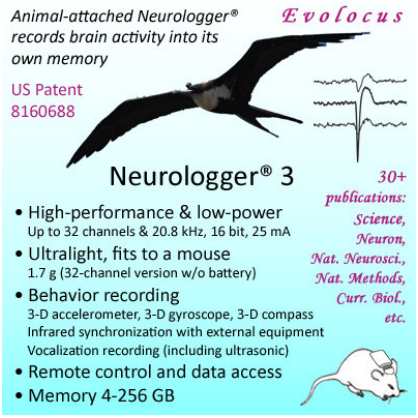
Transgenerational epigenetic compensation and sexual dimorphism



Transgenerational epigenetic compensation and natural selection



Nomogenesis and the logic of chance



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Article

Transgenerational epigenetic compensation

Heritable compensation of disturbed functionality

Dmitri L. Vyssotski[1,2,3]

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.

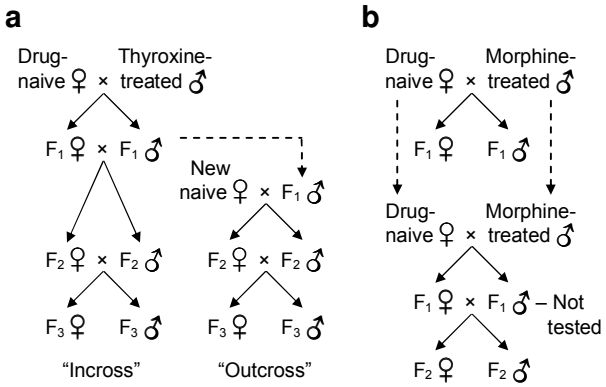

Figure 1 | Breeding paradigms. (**a**) DBA/2J mice, thyroxine study. (**b**) Wistar rats, morphine study. Solid arrows indicate the appearance of progeny, dashed arrows – transition of the same animals.

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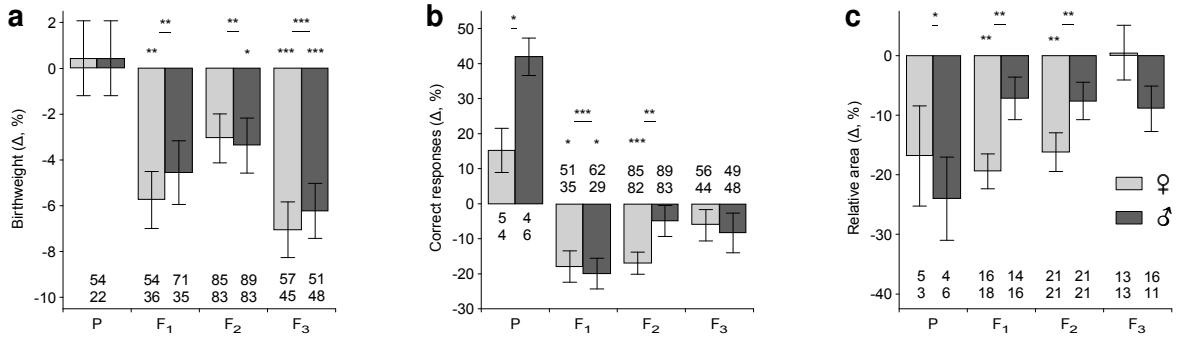

Figure 2 | Phenotype of thyroxine-treated mice and F_1-F_3 descendants. Neonatally thyroxine-treated mice and untreated descendants of thyroxine-treated males. (**a**) Birthweight. (**b**) Two-way avoidance averaged correct responses of 5-day training, 80 trials daily. (**c**) Hippocampal mossy fibers, 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

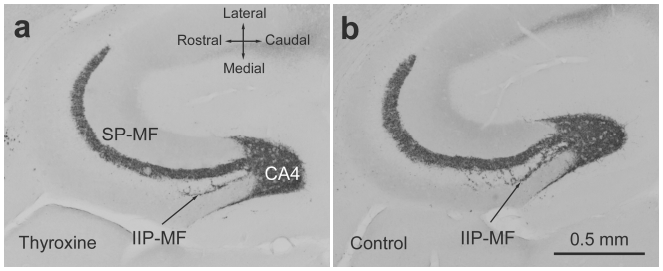

Figure 3 | Hippocampal mossy fiber morphology in the F_3-outcross. Thyroxine study. (**a**) Experimental male mouse. (**b**) Control one. Note the scarce infrapyramidal mossy fiber projection (IIP-MF) in (**a**). Shown samples differ from each other to the greater extent (45%) than mean group values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

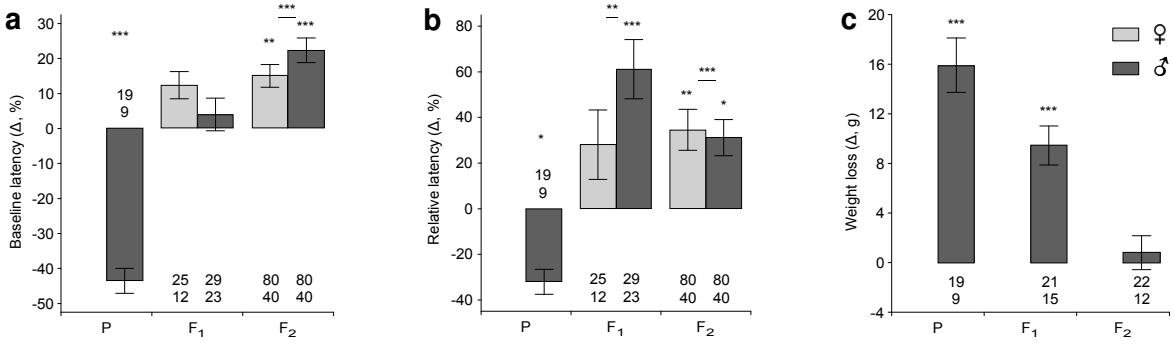

Figure 4 | Phenotype of morphine-treated male rats and F_1-F_2 progeny. (**a**) Pain sensitivity, baseline latency. (**b**) Morphine analgesia, ratio of tail withdrawal latency, measured 30 min after 10 mg/kg morphine administration, to baseline latency. (**c**) Naloxone-precipitated weight loss after 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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Article

Transgenerational epigenetic compensation in evolution

Dmitri L. Vyssotski

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

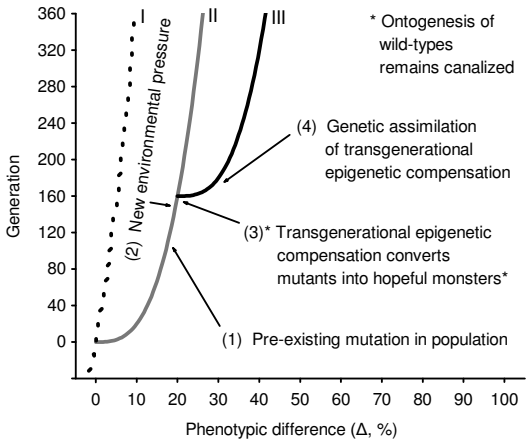

Figure 1 | Transgenerational epigenetic compensation initiates speciation. **I**, **II** and **III** – species or races. Original mutation and its heritable epigenetic compensation are not in the same locus. Speciation demonstrated on hypothetical data.

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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

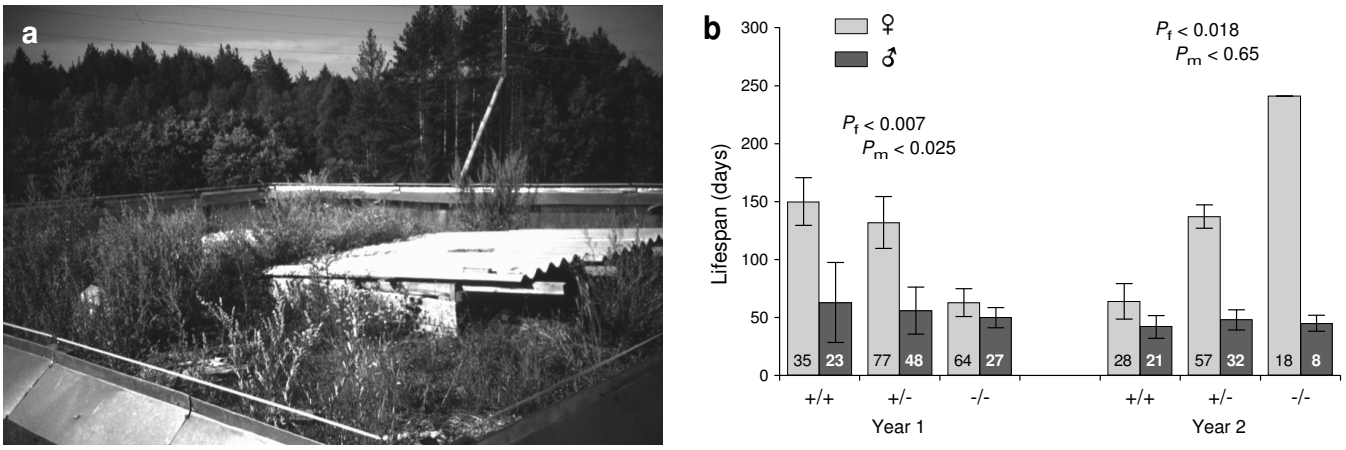

Figure 3 | Lifespan of *Per2*^{Brdm1} mice after release in semi-natural environment. (**a**) Pen 20 × 20 m with two shelters 3 × 2 × 0.7 m each. (**b**) Lifespan (days) after the first release for generations P - F_1 (Year 1) and F_2 - F_4 (Year 2) for all mice that were recorded at least 10 days following 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

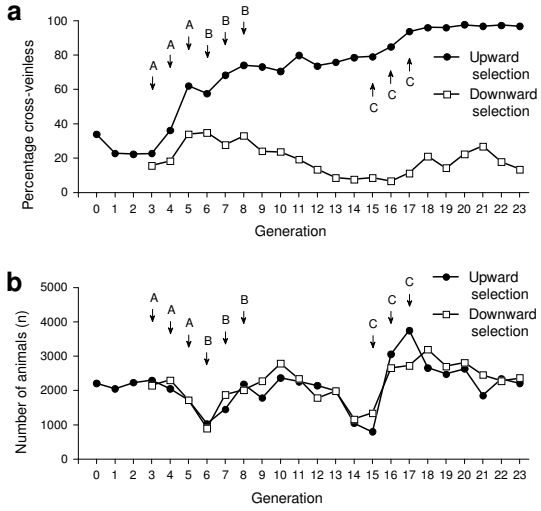

Figure 4 | Transgenerational epigenetic compensation facilitates genetic assimilation. Assimilation of cross-veinless phenotype induced in *Drosophila melanogaster* by heat-shock treatment (40 °C) during 4 hours with onset between 21 and 23 hours after puparium formation. All shown animals (all generations) 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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Article

Transgenerational epigenetic compensation and sexual dimorphism

Dmitri L. Vyssotski[1,2,3]

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

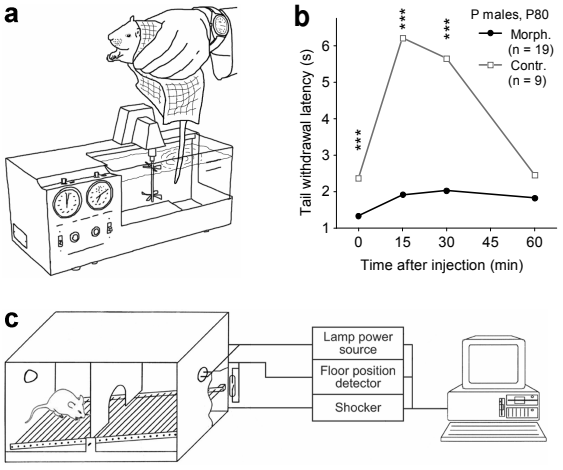

Figure 1 | Tail-withdrawal (**a**) and two-way avoidance (**c**) tests for Wistar rats and DBA/2J mice, respectively. (**b**) Male rats (P) were tested at the age of 80 days in the tail-withdrawal test (56°C), being injected with morphine 10 mg/kg, i.p., after the end of chronic (P42-P79) morphine treatment.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

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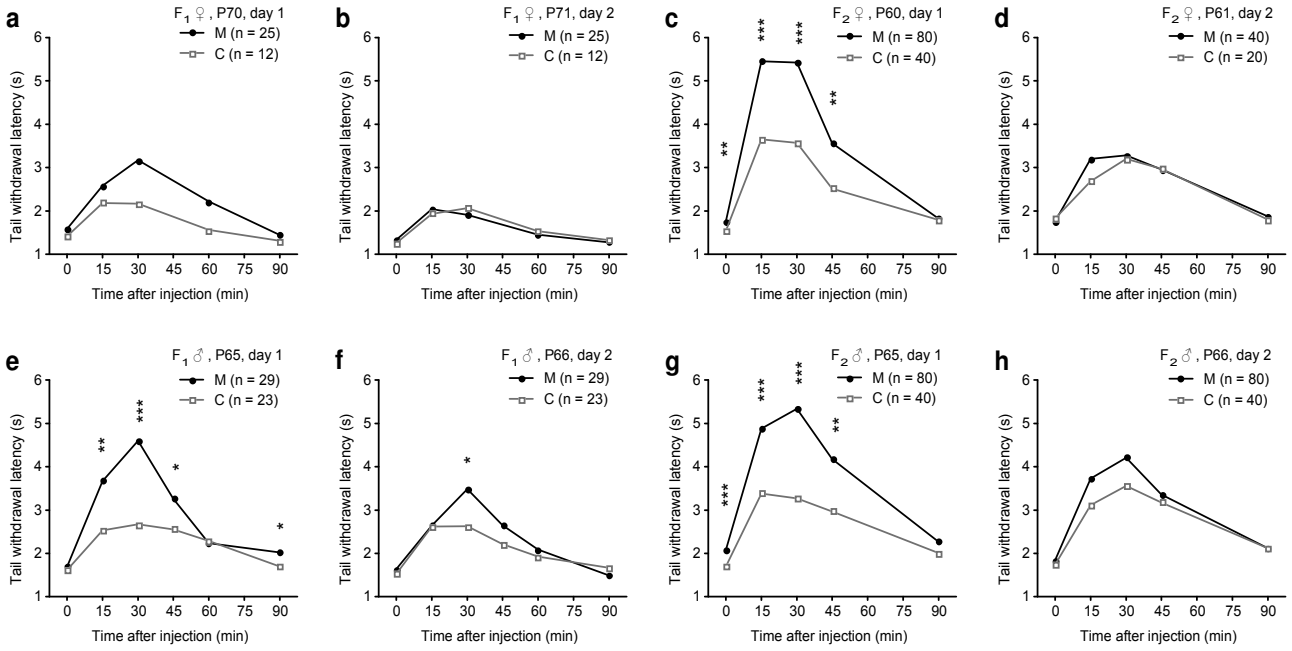

Figure 2 | Tail-withdrawal test in the F_1 & F_2 descendants of morphine-treated male Wistar rats. Each animal was tested twice (days 1 & 2) with the same dose of morphine 10 mg/kg. Morphine was administered i.p. just after the first measurement of tail-withdrawal latency (time "0"). The enhanced analgesic 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

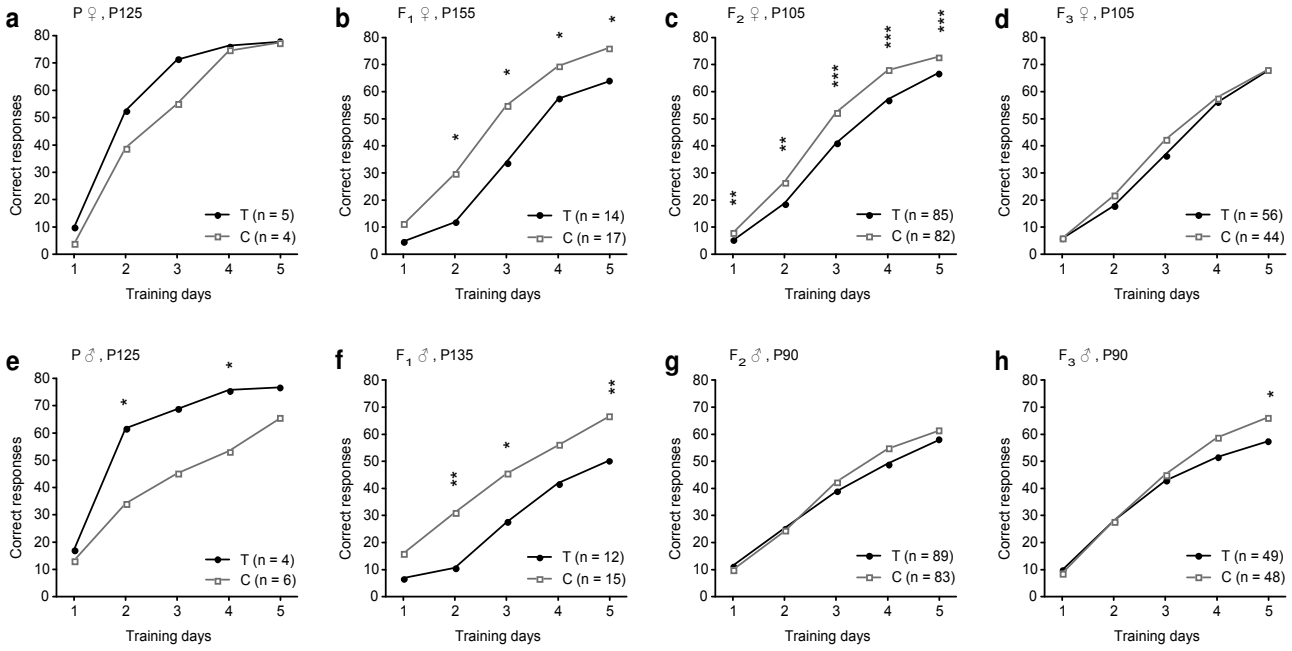

Figure 3 | Two-way avoidance in the thyroxine-treated DBA/2J mice and in the F_1-F_3 progeny of thyroxine-treated males. Note improved performance in the neonatally (P0-P11) thyroxine-treated males (**e**), but decreased performance in their descendants (**b**,**c**,**f**,**h**). Note that the performance 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

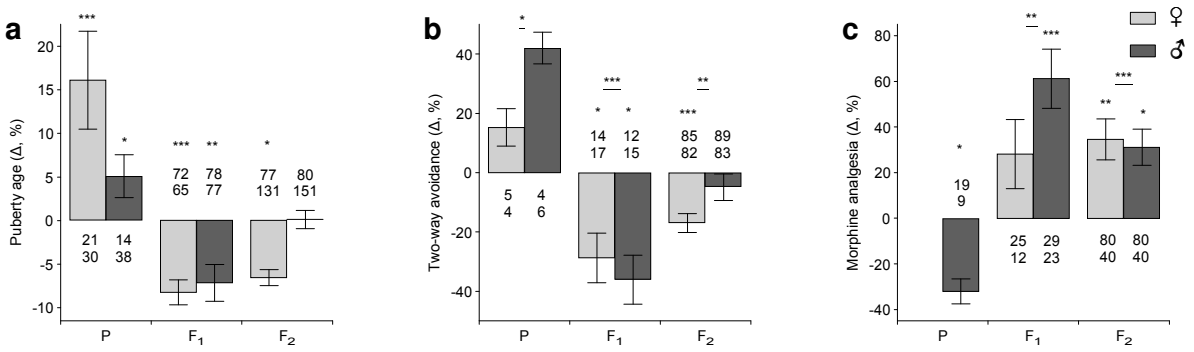

Figure 4 | Phenotypic inversion in the progeny after prenatal (E8-E14), neonatal (P0-P11) and young adult (P42-P79) parental drug treatment. (**a**) Onset of puberty in the experiment of Manikkam and co-authors (2012)[20]: Sprague-Dawley rats (P), both ♀ and ♂, were treated during E8-E14 by i.p. administration 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.

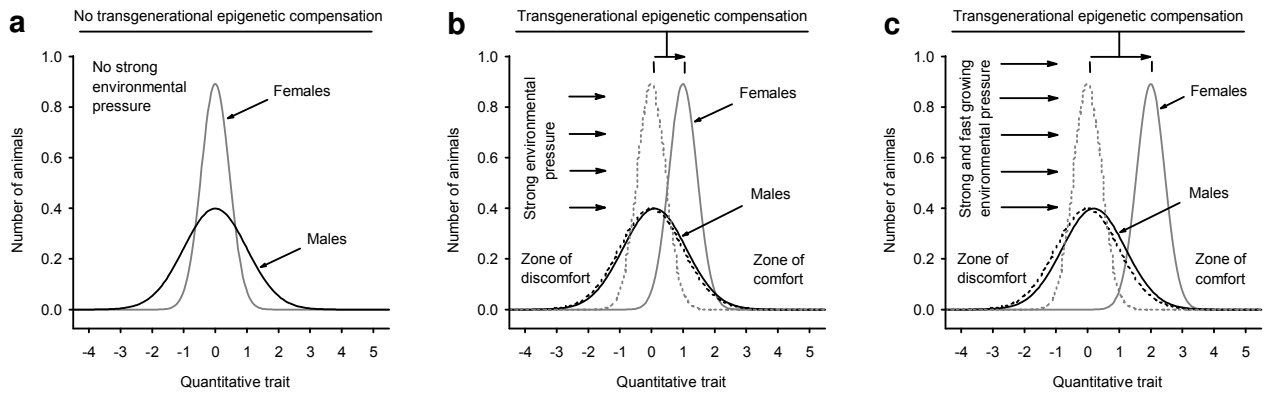

Figure 5 | Distribution of a quantitative trait among females and males in population. Original distribution in males is standard normal distribution. Chosen quantitative trait is functionally linked with given environmental influence (*e.g.*, cold-resistance – low temperatures). Without specific external 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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Additional information

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Article

Transgenerational epigenetic compensation and natural selection

Dmitri L. Vyssotski[1,2,3]

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, the hereditary basis of transgenerational epigenetic compensation develops mainly in homozygous mutant males, but it does not affect their phenotype. In their descendants, being in an independent locus, this heritable epigenetic compensation increases fitness and lifespan of homozygous mutant females and decreases lifespan of wild-type females, starting from F_2. Here we show that this transgenerational epigenetic compensation is a guiding agent of natural selection. Natural selection is not a directing or driving force of evolution anymore. Natural selection needs some guidance. Transgenerational epigenetic compensation can initiate speciation through segregation of mutants and wild-types and/or it can change the selection coefficient of a given mutation.

Transgenerational epigenetic compensation was discovered in the experiments with paternal drug treatment[1-4]. Prenatal, neonatal and adolescent treatment of males leads to observation of inversed phenotype in their F_1, F_2 and F_3 untreated descendants, at least in some traits[1-10].

In this article we will use these experiments with parental drug treatment in order to achieve better understanding of the results of natural selection, observed in the population of laboratory mice, consisted of wild-type, heterozygous and mutant *Per2*^{Brdm1} animals, lived under semi-natural conditions in outdoor pens (**Fig. 1**) during two years[1,11].

Four pens contained four independent populations of mice, at the beginning with 250 animals (in total), Mendelian distribution of genotypes 1:2:1 and equal numbers of females and males. Food and water were supplied by humans and both were constantly placed in two locations inside each pen. Each animal was injected with transponder (Trovan ID100). A square antenna was placed in a horizontal plane around a combination of a food pod with a water bottle, in order to register animals' visits to estimate their drinking and feeding behaviour. All mice were live trapped twice a year and all new mice (born inside pens) were genotyped and received transponders. The lifespan of each mouse was estimated using its visits of food-water places. Food and water consumption could not be analyzed separately, because each of two places contained both food and water.

Pens were protected from terrestrial predators by an electric fence on the top of slate walls. However all local aerial predators had free access to mouse populations. Aerial predators were represented by a tawny owl (*Strix aluco*) [it has been seen many times], a short-eared owl (*Asio flammeus*) [it was possible to hear it sometimes], and other aerial predators could not be excluded. Trovan transponders, injected into mice previously, were found several times in mouse residues in owl pellets, left by birds outside the pens, and this is a direct confirmation of owls' feeding behaviour. All attempts to find transponders from the missing mice inside the pens have brought negative results (practically impossible to find), but the explanation can be different, for example, a transponder can not be read, if it has gone into the wet soil.

Figure 1 | Semi-natural environment for investigation of natural selection. Wild-type, heterozygous and mutant *Per2*^{Brdm1} mice were breeding at will during two years in four pens 20 × 20 m each, each with two shelters[11]. At the beginning of experiment there were 250 mice in total with Mendelian distribution of genotypes 1:2:1 and equal presence of females and males. Tawny owls (*Strix aluco*) were hunting for mice all the year round.

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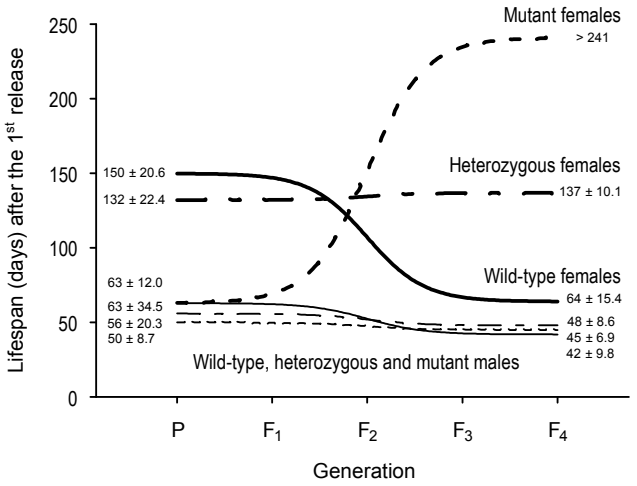

Figure 2 | Lifespan of *Per2*^{Brdm1} mice in pens after the first release[1,11]. All mice were taken out of pens (**Fig. 1**) and released back twice a year. New ones (born during previous half-year) were genotyped. Transponders, bearing individual numbers, were injected into all mice. Antennae, placed around feeding places, were used for registration of behaviour and estimation of lifespan. Lifespan, calculated from the day of 1st release, is shown here. Note an unexpected increase of lifespan in mutant females and simultaneous decrease of lifespan in wild-type females. Median ± SE.

We assume that the presence of Per2 mutant gene in a homozygous state and under harsh semi-natural conditions (*e.g.* temperature conditions) produces the same kind of transgenerational epigenetic compensation as paternal drug treatment (prenatal, neonatal or adolescent).

We know that so different parental treatments as prenatal (E8-E14) vinclozolin treatment and adolescent (P30-P50) maternal morphine treatment tend to produce common gender-specific phenotype in the F_1 and F_2 descendants, observed in the elevated plus-maze. Namely, females, but not males, of generations F_1 and F_2, show decreased time spent on open arms of elevated plus-maze (**Supplementary Fig. 2**). This is an indicator of their increased caution. Pharmacologists usually say that this is an increased "anxiety". However all observations of wild-caught voles, like bank vole (*Clethrionomys glareolus*) and root vole (*Microtus oeconomus*), in laboratory conditions, demonstrate that it is not a correct interpretation of animal behaviour. Wild-caught voles, those do not move at all in many laboratory tasks (due to so-called "freezing" behaviour), demonstrate in fact an increased "caution", but not "anxiety". It is so because the same wild-caught voles outperform any laboratory mouse strain and any laboratory F_1 hybrid, like B6D2F1, in the Morris water maze task[12]. Wild-caught voles are not more "anxious", but they are more "normal" creatures than any laboratory mouse stock.

It is possible that transgenerational epigenetic compensation, being genotype-specific, nevertheless activates some universal mechanisms, those were useful in wild nature, but useless in laboratory conditions during previous more than 100 years. The observed induction of increased caution in females (F_1 and F_2) may have the same level of generalization as general adaptation syndrome, described by Hans Selye[13].

In the **Fig. 2** we can see increased lifespan in the homozygous mutant females (starting from F_2) and decreased lifespan in the wild-type females. Thus, given semi-natural external conditions induced stress in homozygous mutants that resulted in formation of transgenerational epigenetic compensation, expressed in their descendants as increased caution in homozygous mutant females and as disrupted caution in wild-type females. Then, tawny owls have selected the least cautious mice as a source of food.

There is a belief that the main source of mouse losses in these pens is a male-male competition, during which male mice fight with each other up to death. This belief is only partially correct, because, indeed, a fighting mouse is an easy prey for an owl. Note, however, that both strong and weak fighters can be equally good food for an avian predator (an owl has very good hearing abilities and very good vision). The only way to escape from the owl is to avoid male-male fighting in general, and it seems that our laboratory male mice in these pens could not do this. That is why we have very interesting genotype-specific profile of lifespan in females and only low and genotype-non-specific lifespan in males (**Fig. 2**). Note also, that the most intense genotype-specific selection among females took place during summer, when snow was absent and owls could hunt with high efficacy (Supplementary Fig. 3[2]).

Why we are so sure that we are dealing with epigenetic inheritance[14-17] and transgenerational epigenetic compensation, but not with some other factor? Let's look now at the experiments with parental drug treatment and at very-very interesting observations on guinea pigs. We shall move through our data in the following order: 1) mice, 2) rats, 3) guinea pigs.

Neonatal (P0-P11) thyroxine treatment of inbred DBA/2J mice has led to improved two-way avoidance performance in drug-treated animals and to impaired two-way avoidance performance in the F_1 male and female descendants of thyroxine-treated males. In the F_2 animals the impaired two-way avoidance was observed only in females. In the F_3 generation the impaired two-way avoidance was observed only in males of outcross subline (**Fig. 3**).

Other significantly modified traits in all these F_1-F_3 animals, namely decreased birthweight and decreased intra- and infrapyramidal hippocampal mossy fiber projections (shortly: brain morphology), were not correlated with each other and with two-way avoidance performance (no individual correlations)! It was easy to suppose that several independent loci can be involved, but in this case it remains a mystery how all these 3 traits occurred to be recollected together in the F_3-outcross males (Table 1[1] and Supplementary Fig. 2[1]). Only guinea pigs were able to provide insight (several years later). Note that the presence of impaired phenotype in the F_1 and F_3-F_4 males, but not in the F_2 males, was described with respect to humans more than 3000 years ago (see **Supplementary Table 2**).

Adolescent (P42-P79) chronic morphine treatment of male outbred Wistar rats has led to decreased analgesic effect of standard dose of morphine (10 mg/kg) in these treated animals and to increased analgesic effect of standard dose of morphine in their F_1 male descendants. All descendants were tested twice with time interval 24 hours, in tail-withdrawal test (**Fig. 4**). In the F_1 generation, during the first day, F_1 males have shown enhanced analgesic effect, but F_1 females have shown normal phenotype. During the second day all F_1 males and F_1 females have shown normal phenotype. Very high speed, at which abnormal phenotype of F_1 males was converted into normal one, is amazing.

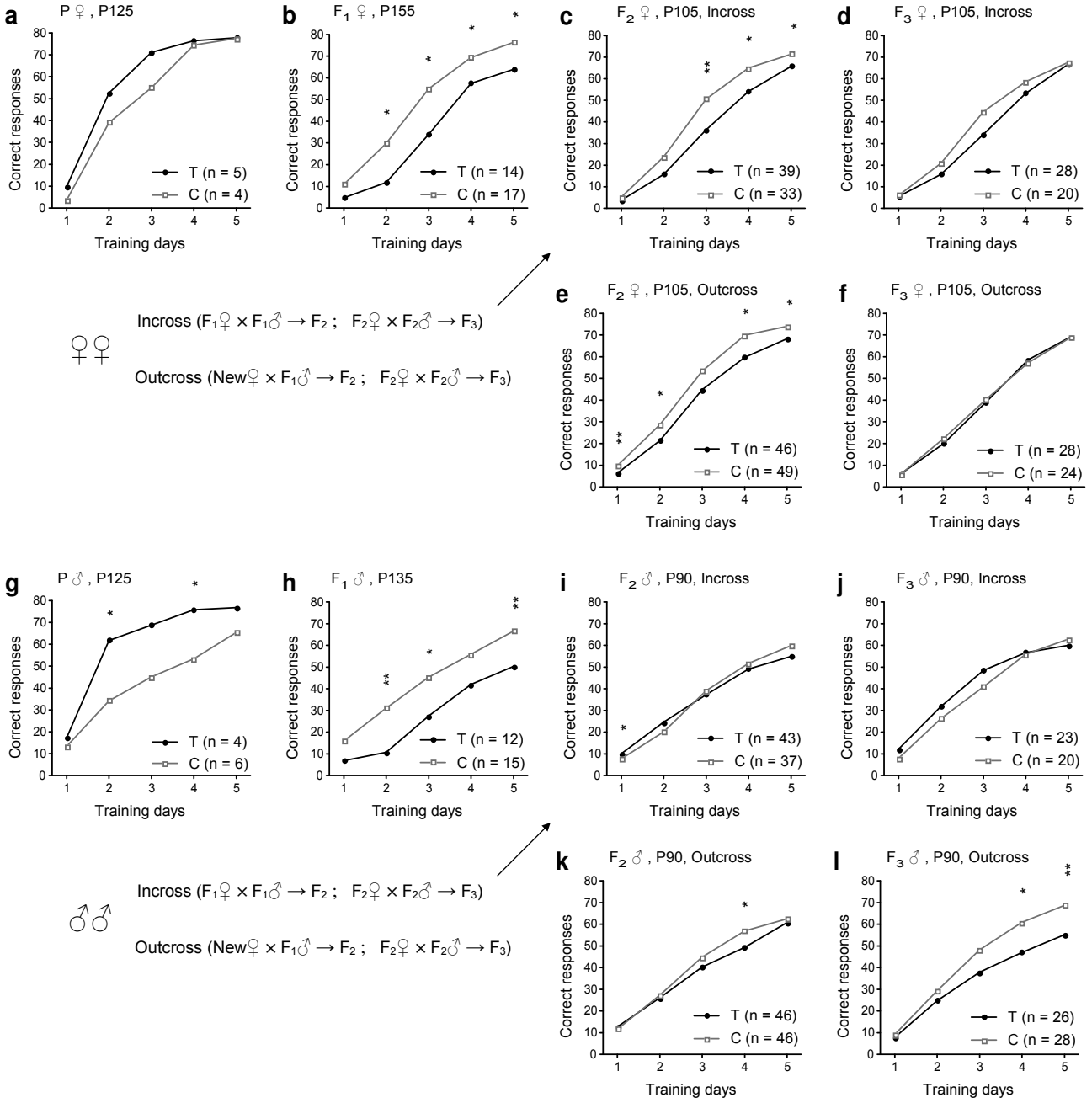

Figure 3 | Two-way avoidance in the thyroxine-treated DBA/2J mice and in the F_1-F_3 progeny of thyroxine-treated males. Note improved performance in the neonatally (P0-P11) thyroxine-treated males (**g**), but decreased performance in their descendants (**b**-**c**,**e**,**h**,**l**). Both Incross and Outcross F_2 females have decreased performance (**c**,**e**). In males the decreased performance was observed in the F_1 (**h**) and in the F_3-outcross (**l**), but not in the F_2 (**i**,**k**). Torah, the Second Commandment (Shemot 20:3-6; Devarim 5:7-10), teaches us that the misbehaviour of fathers (P) leads to problems in their sons (F_1) and problems in the third (F_3) and the fourth (F_4) generations. The second generation (F_2) is not in the original text (**Supplementary Table 2**). T – descendants of treated males, C – control. P125 – postnatal day 125. Asterisk, $P < 0.05$; double asterisk, $P < 0.01$. Mann-Whitney U test. Mean.

In the F_2 generation the vast majority of females have shown enhanced analgesic effect during the first day, but all of them have shown normal phenotype during the second day. In the F_2 generation males the situation is very complex (**Fig. 4**). First, 1/4 (20 males from 80) have shown enhanced analgesic effect during the first day. Second, 1/16 (5 males from 80) have shown enhanced analgesic effect during the second day only – it means that they had normal phenotype during day 1 and abnormal one during day 2. Third, another 1/16 (5 males from 80) have shown enhanced analgesic effect during both day 1 and day 2. Note that one or two such males were present in the F_1 generation, but the total number of experimental males in the F_1 (29 males) was not sufficient to assess whether this is a random mistake or real phenomenon. Note the absence of such strange animals in the control groups. Anyway, the change from "abnormal" to "normal" in the majority of animals and simultaneous change

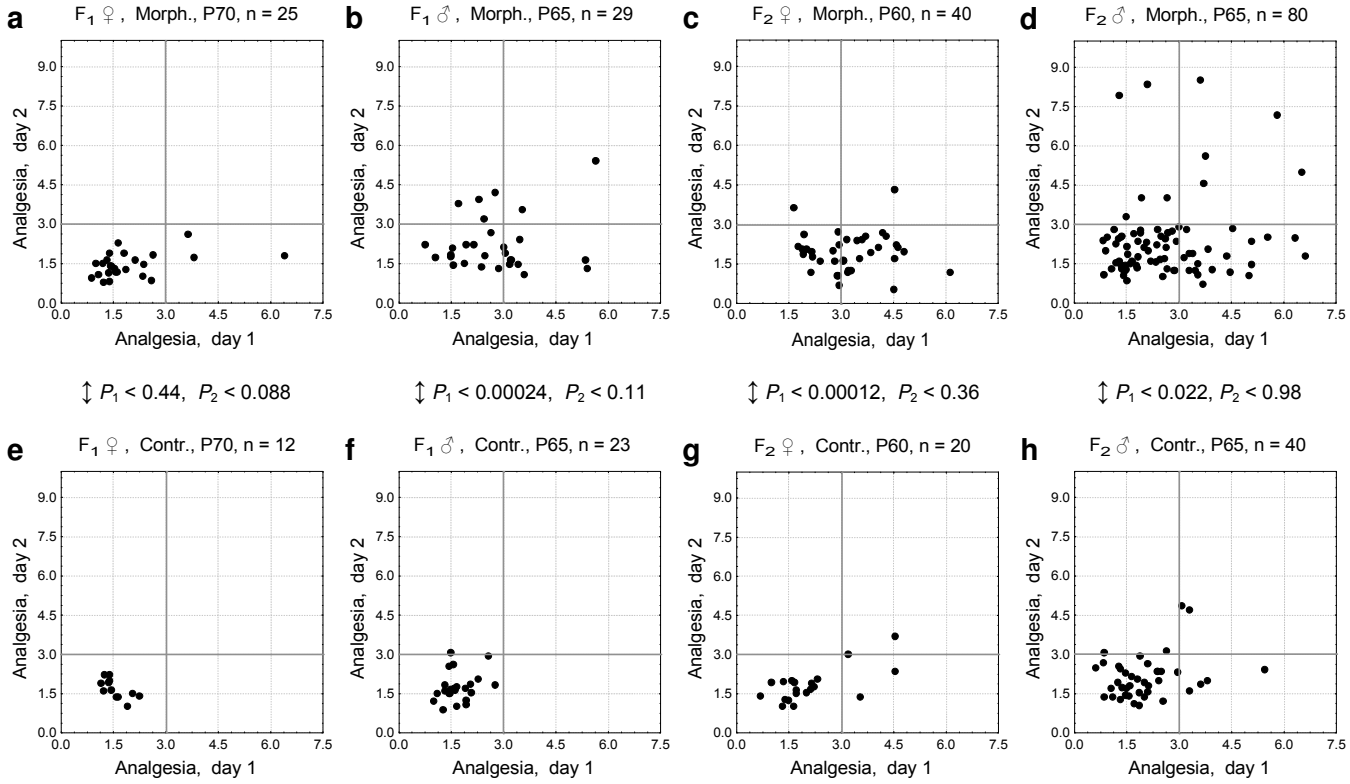

Figure 4 | Tail-withdrawal test in the F_1 & F_2 descendants of morphine-treated male Wistar rats. Each animal was tested twice (days 1 & 2) with the same dose of morphine 10 mg/kg. Morphine was administered i.p. each day after the first measurement of tail-withdrawal latency (baseline latency). Abscissa (day 1) and ordinate (day 2) of each dot (animal) show the ratio of tail-withdrawal latency, measured 30 min after 10 mg/kg morphine injection, to baseline latency. The effect is dominant in F_1 males (**b**,**f**) and F_2 females (**c**,**g**) (day 1), but recessive in F_1 females (**a**,**e**) and F_2 males (**d**,**h**; 1st day – ¼ has effect; 2nd day – 1/8, including 1/16 during both days and 1/16 during exclusively day 2). Heritable changes in two independent loci are sufficient to explain this pattern. P_1 & P_2 – statistical significance between experimental and control groups during day 1 & day 2, respectively. Mann-Whitney U test.

from "normal" to "abnormal" in few ones, during the same 24 hours and treatment procedure, does not have self-evident physiological explanation. At least, it is very unusual, when the second standard dose of morphine produces greater analgesic effect than the first one. Observations on guinea pigs have provided some clue later, more than 10 years after the end of this experiment with rats and morphine.

Once a female animal with unusual phenotype was born among our short-haired multicoloured guinea pigs (*Cavia porcellus*). This female was born in a litter of four (2 females and 2 males; all others with standard phenotype), obtained from multi-coloured female from Elm Hill Labs (Chelmsford, MA; www.elmhilllabs.com) and short-haired multicoloured male with contrasting whorl on its head (so-called "American crested"), obtained from an independent source (hybrid dysgenesis is possible). Video record, taken at postnatal day 1, is available: www.evolocus.com/Video/GuineaPigs2011-09-17.MOV . This video is not absolutely necessary for further understanding of our article, but an experienced observer can extract a lot of non-trivial information from it (all animals, including both parents, are shown). Day of birth is counted as P0 and it is 2011-09-16. At birth, at P1 and during the first several weeks this animal was not recognized as "unusual", despite post-hoc analysis of above-mentioned video record has revealed that this animal was able to demonstrate slightly increased activity already at P1, because it was called "the hard one to get". During her adolescence this female had increased locomotor activity, *e.g.* it was able to move up and down in a 3-level chinchilla's "Super Pet®" cage, using its plastic ramps and being self-motivated. This behaviour was never observed in any other laboratory guinea pig and it is more typical for animals like rats. This female was behaviourally active, but the most interesting its feature was the following: being behaviourally active, it had very low water consumption. Its water consumption, as soon as it was detected, was 3-4-fold lower than daily water consumption of any other guinea pig.

This female with low water consumption and high behavioural activity was crossed with normal male and two pups were obtained in a litter: one was found dead at P0, but another one was considered "normal" until its daily water consumption was measured. This F_1 pup was a female. Water consumption of her mother remained lower than norm during pregnancy and lactation. However water consumption of this F_1 female occurred to be 3-4-fold higher than water consumption of any control animal (**Fig. 5** and **Supplementary Fig. 3**). Increased water consumption was associated with increased urination, occurring in a different location inside the cage. This increased water consumption was stable, it was observed during several months, and it produced an impression that it will be so forever.

On the other hand, it would be interesting to see how this increased water consumption will be normalized and we were

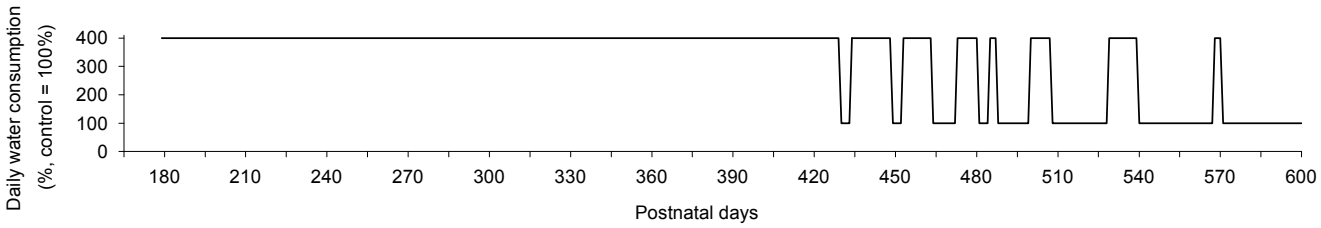

Figure 5 | Water consumption of one female guinea pig, obtained from female with unusually low water consumption (schema). In our heterogeneous outbred stock of guinea pigs (*Cavia porcellus*), one female was obtained that had unusually low (20-25%) water consumption during her adulthood. Contrary to this female, her F_1 female descendant (shown) had enormously increased (300-400%) water consumption (P180-P430). Later (P430+), some periods of normal water consumption appeared, without any intermediate state between "high" and "low" states. There is no physiological reason for the absence of gradual regulation here and, thus, "all-or-none" switch is an intrinsic feature of transgenerational epigenetic compensation.

expecting some smooth curve. We never obtained such smooth curve. At some time point water consumption was normalized abruptly – it has jumped down to the normal level in 24 hours! Water consumption was normal during few days and then it has jumped up as fast as it was jumping down previously **(Fig. 5**). There were only two stable states of this process: normal and high. Any intermediate possibility was absent.

Water consumption had a tendency to switch from "high" to "normal" each time when fresh high quality grass was becoming available on a regular basis (a guinea pig prefers the same species of grass as a white-tailed deer (*Odocoileus virginianus*) in the New York area). And water consumption had a tendency to switch from "normal" to "high" each time when grass quality was going down and, in addition, each time when bedding material in the cage was changed from old and "dirty" to new and "fresh" (we use pine bedding "PetsPick™"). May be, behavioural stress from this change together with temporal unavailability of feces, those are an important source of nutrients for a guinea pig, are the main factors for switching from normal to very high water consumption. It seems that stress of any kind can switch water consumption in this animal from normal level to very high one (**Supplementary Fig. 3**). Note that in normal animals, in both males and females, slight stress leads to slight decrease in water consumption, whereas in this female the same slight stress leads to disproportional increase.

High and abruptly switching water consumption, observed in this female, obtained from female with low water consumption and normal male, indicates that the phenotypic expression of transgenerational epigenetic compensation is not only gender-dependent (see our previous article "Transgeneraional epigenetic compensation and sexual dimorphism"[3]), but it is also stress-dependent, and it is stress-dependent in a very sharp manner in temporal dimension. For such cases Trofim D. Lysenko has introduced the term "unstable, destabilized, heredity" (p. 298[18]).

We have seen very sharp temporal response, very fast switching of transgenerational epigenetic compensation from "off" to "on" state and *vice versa*, and possibility to be "on" during different periods of ontogenesis. It means that, most likely, we do not have here something distributed among many-many independent loci, but we probably have only one change in one locus. Namely, one previously absolutely dormant gene has become transcriptionally active (that is why it is dominant), but the switching of its transcription between "off" and "on" states is heavily gender-dependent (probably, through the effects of sex hormones) and, in addition, the above-mentioned switching is heavily stress-dependent (probably, through the effects of stress hormones). Dormant genetic locus, being brought out of dormancy, becomes open for further regulation of its expression, but not for unconditional presence of its product in the organism.

The idea about dormant genes belongs to Wilhelm Jürgen Heinrich Harms, known as J.W. Harms, and it was proposed by him in 1929[19,20]. At that time it was absolutely unexpected that a re-opened dormant gene can demonstrate so sharp temporal regulation of its expression immediately, during lifespan of a single animal. Similar switching of gene activity, but between generations, was shown for genes *fused* and *star* by Dmitry K. Belyaev and co-authors in 1981[21,22]. It seems that even using 1-bit regulation of the level of expression ("on" or "off"), but having non-trivial temporal structure of this expression during ontogenesis, an organism can achieve a variety of phenotypic results, including a variety of morphological ones, uncorrelated with each other (**Supplementary Fig. 6**).

Dormant genes, being brought out of dormancy by transgenerational epigenetic compensation, are changing the evolutionary landscape faster than natural selection does.

Methods

***Per2*^{Brdm1} mouse experiment.** Mutant *Per2*^{Brdm1} allele is known to compromise circadian organization and entrainment and to cause multiple physiological disturbances[23]. 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 terrestrial predators by slate walls (1 m high and sunk 50 cm into the soil, covered by zinc-plated iron on the top)[11]. Each pen had 2 wooden roofed shelters (3 × 2 m each, 70 cm depth, filled with hay, straw and branches). Inside each pen, but outside of both shelters, 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.

Animals were released into the shelters at the field station Chisti Les (Clear Forest), Bubonizi (Pozhnia, Tvier Region, Western Russia, 56°44'7.99"N; 31°31'34.44"E) on May 21, 2005, at the age of 76 ± 5.4 days (mean ± SD).

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)[24] 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.

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

Guinea pig experiment. Outbred short-haired multicoloured guinea pigs (*Cavia porcellus*) were used. Multicoloured female was obtained from Elm Hill Labs (7 Kidder Rd., Chelmsford, MA 01824; www.elmhilllabs.com) and it was bred with short-haired multicoloured male with contrasting whorl on its head (so-called "American crested"), obtained from Petland Discounts #17 (439 Tarrytown Rd., White Plains, NY 10607). Two females and two males were born 2011-09-16. One female from this litter demonstrated low water consumption being an adult.

We had cages "RB100" (100 × 54 × 44.5 cm) and Super Pet "My First Home Chinchilla Cage Kit" (76 × 45.5 × 76.5 cm; a 2-shelf cage, each shelf 44 × 25 cm, placed at 26 cm and 44 cm from the floor in the opposite parts and connected consequently by two ramps 42.5 × 12 cm each). Bottles 500 ml from LM Animal Farms were refilled daily and their weight was measured at 11:00 PM using electronic scale KS/B-2000 (Max: 2000 g, d = 0.1 g). Pine bedding "PetsPick" and bowls with standard guinea pig food were always in cages. Fresh grass was supplied daily, when available. During snow periods animals received "Kaytee Timothy Hay Ultra" and apples. We kept 1-2 adult animals per cage under normal day-light cycle. Each adult animal had its own plastic house "Super Pet Big Igloo" (D = 24.5 cm (lower), d = 19 cm (upper), H = 16 cm (ext.), h = 13.5 cm (int.); entrance tunnel: L = 6 cm, H = 11.5 cm, W = 10 cm).

Above-mentioned female with low adult water consumption was crossed with normal male (her littermate), and from this cross a female with high adult water consumption was obtained, born 2012-03-09.

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Additional information

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Article

Nomogenesis and the logic of chance

Dmitri L. Vyssotski[1,2,3]

Evolution of available genomes was shown to be proceeding through random changes, the changes that comprise the main modus of evolution (Koonin, 2011)[1]. Morphological evolution of available and extinct *Metazoa* was shown to be going on the basis of law, by means of precession of characters, where characters originally manifested in the young along in the course of time and evolution were displayed also in adult descendants (or supposed descendants) of that organism (Berg, 1922)[2]. This contradiction is obviously solved in nature, where the appearance of any new genetic locus in the genome and its further expression in the phenotype can be separated by unlimited period of time and by unlimited number of generations. The management of dormant genetic loci has come from the previous evolutionary stage, unimaginable today, where organisms were open systems with respect to the flow of genetic elements and were collecting, discriminating and storing genetic elements from the external environment. This was an important period when multiple systems for blocking and unblocking of genetic loci came into being. However even before this stage, it was even more fantastic evolutionary period where replication, transcription and translation were absent and Eigen cycle was not possible, but organisms were collecting randomly available components (proteins, RNA and DNA) by means of action acceptors (Anokhin, 1955)[3,4] – sites of double-stranded DNA mechanically compatible with useful components. Action acceptors themselves were unable to be replicated by modern way (no DNA polymerase!), but they were collecting their pseudo-copies from the environment – the pieces of DNA that were born in the environment and occurred to be compatible by chance with current action acceptors. Action acceptors, – the structures that sense presumably useful results or substances, were directing evolution from the early beginning and they are directing it today through activation and deactivation of dormant genetic loci.

In animals like mice, rats and guinea pigs, and also in humans (holocaust survivors and their progeny)[5], the phenomenon of phenotypic inversion can be observed[6-15]. Phenotypic inversion is defined as the opposite quantitative changes in untreated offspring with respect to treated, *e.g.* drug-treated, parents[11]. Phenotypic inversion was also reported in plants[16] and insects[17]. The term was introduced in 2004[18] and it is in use in connection with transgenerational epigenetic compensation[10-15,19-21].

In humans[5] and guinea pigs[15] the phenomenon of phenotypic inversion was registered also in methylation of DNA. Thus, the demethylation of 5-methylcytosine behaves here as a phenotypic trait and not as a heritable basis of transgenerational effects. Very often phenotypic inversion was obtained as a result of paternal drug treatment (prenatal, neonatal and adolescent), using such drugs as morphine[8-14], thyroxine[6,7,10-14] or complex substances like plastic mixtures[22]. However less often it was reported that phenotypic inversion can be expressed during lifespan of a given descendant in a semi-stochastic "all-or-none" fashion[14] (as "unstable, destabilized"[23]).

An example of such "all-or-nothing" expression of phenotypic inversion is shown in the **Fig. 1**, where randomly enhanced water consumption is recorded in female guinea pig, obtained from

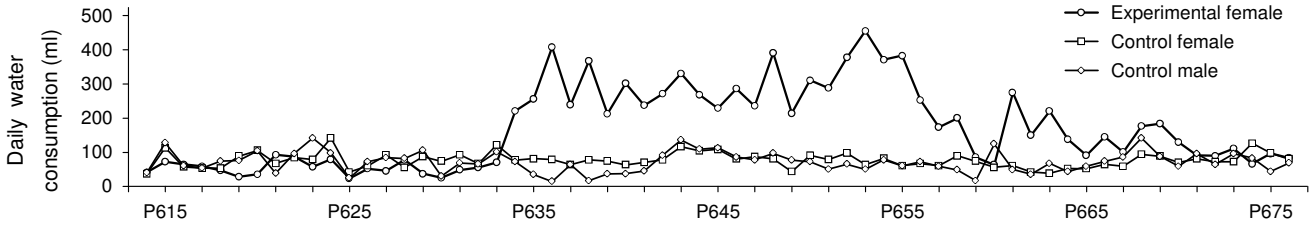

Figure 1 | Randomly expressed increased water consumption in the experimental female guinea pig, obtained from female with low adult water consumption and normal male. Postnatal days P614-P676 are shown. The stochastically increased water consumption in this female is in contradiction with the phenotype of her mother. Her mother was born in a litter of four, among normal littermates. The mother had decreased water consumption and increased locomotor activity and curiosity in home cage, observed during childhood, adolescence, adult life, and during pregnancy and lactation also.

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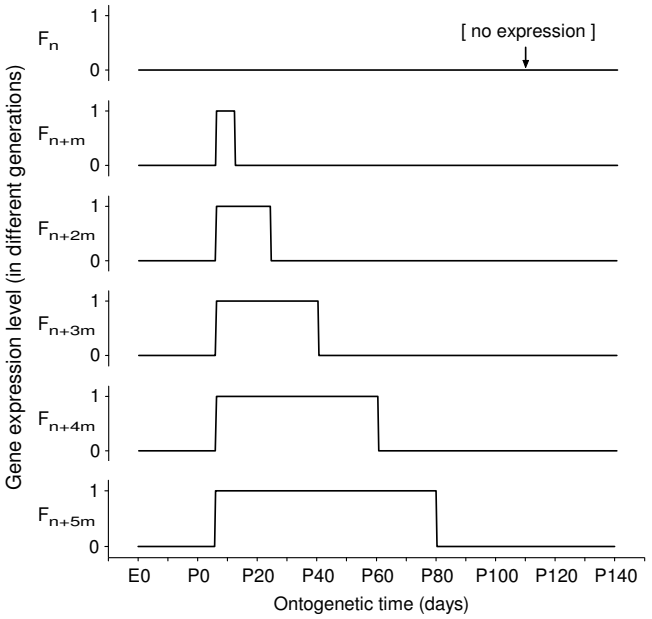

Figure 2 | Expression of one previously dormant genetic locus. Leo S. Berg has described the "precession of characters" in 1922: "… latent characters (factors, *genes*) originally manifested in the young alone… in the course of time and evolution are displayed also in the adult descendants (or supposed descendants) of that organism" [p. 75[2]; the word "*genes*" was italicized by Berg]. Ontogenetic time scale is shown for such animals as rats, keeping in mind experiments with methadone and morphine (Figs. 1[24] and 2[24], Supplementary Fig. 5a[11]). E0 – the first embryonic day, P0 – the first postnatal day.

female with unusually low water consumption. Note the random character of the expression of this phenotypic inversion (see also **Supplementary Figs. 2-3**). Of course, phenotypic inversion is supposed to be a result of compensatory changes[11]. Phenotypic inversion was also registered as an enhanced sensitivity to morphine in the F_2 progeny of chronically morphine-treated male Wistar rats, shown in the **Supplementary Figs. 4-7**. The relative lack of such observations in literature is a consequence of the absence of long-term records (it is thought to be difficult or impractical to monitor all descendants during their lifespan). Such records do exist for daily water consumption in guinea pigs (500 days) and morphine analgesia in rats (25 time points distributed among 7 days). Where long-term records are available, random "all-or-nothing" expression of phenotypic inversion during lifespan of a single animal is usually obvious.

Leo S. Berg has shown that new morphological changes can appear in evolution on the basis of law – by means of the precession of characters (**Fig. 2**). The time scale of shown example is given for the disturbance of opiate system in rats. This relatively new example was not discussed by Berg. The appearance of any new morphological trait, described by Berg, is an "all-or-nothing" response that is non-controllable or poorly controllable in amplitude, but nicely regular in temporal dimension during both ontogenesis and phylogenesis.

In modern experiments with transgenic mice, schematically shown in the **Fig. 3**, the disappearance or attenuation of phenotype in successive generations was observed rather often, but it was not reported so often due to social pseudo-scientific reasons. Both the observations of Berg concerning the appearance of dormant traits in evolution and the modern observations concerning the disappearance of phenotype in successive generations of transgenic mice demonstrate that *Metazoa* have sufficient molecular tools to control dormant genetic loci and to use them purposively.

The evolution of biochemical syntheses, described by Norman H. Horowitz (1945)[25] (**Fig. 4**), implies that any chain of biochemical reactions was developing in evolution from its final result (product). And all further steps were growing from the right to the left (shown as sequence: ד ← ג ← ב ← א), where each new enzyme was introduced by purpose – to provide substrate for previously existing process. Thus, this chain as a whole was build up as a purposive structure, being strictly purposive during each step of its evolution. Each additional step was satisfying the pre-existing action acceptor – the structure that can sense the presence and can use the result of this newly added step. The whole schema of Horowitz is an example of evolution, determined by law, determined by the requirements of pre-existing functional systems.

The law of homologous series in variation, discovered by Nikolai I. Vavilov (1922)[26], also can be used as an illustration of evolution, determined by law. Usually, similar heritable deviations (variations) in different species are explained by mutations in similar important genes that are normally expressed. But if it would be so, such events would be very rare, because such changes would be recessive and observable only in homozygous samples. Contrary to this, similar variations are formed by suddenly expressed dormant genetic loci those are also similar between species. Their sudden expression produces detectable effect in heterozygous individuals, being obviously dominant. Here we would like to repeat that in the experiments with paternal drug treatment[6-14] mothers were always drug-naïve.

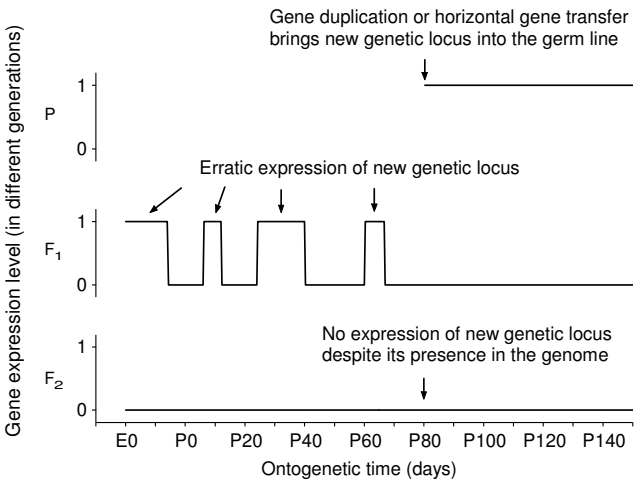

Figure 3 | New genetic locus is submerging into dormancy. In mammals, this process needs at least three shown generations (theoretically, in an idealized situation). In real life, 6-12 generations are required to bring new genetic locus into completely dormant state (many experiments with transgenic animals, mainly mice, are pointing out that this estimation is correct, at least for some genetic loci)[27,28]. Similar results, being frequently obtained, remain typically unpublished (nobody would like to report the disappearance of the phenotype discussed in the previous own article).

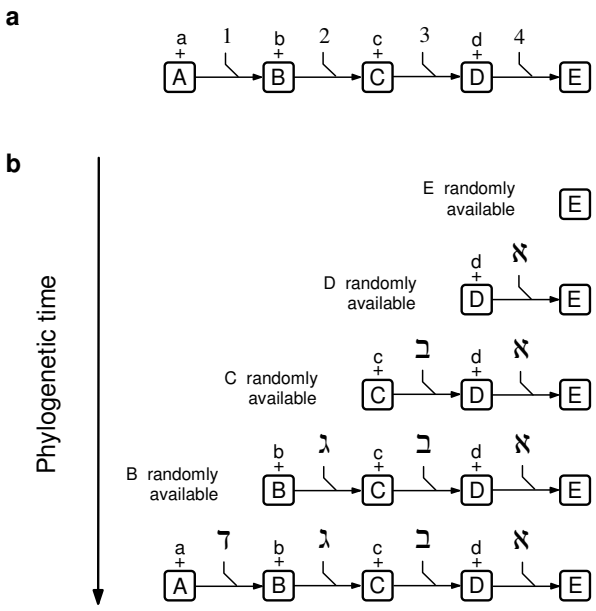

Figure 4 | The evolution of biochemical syntheses by Norman H. Horowitz (1945)[25]. **a**, Chain of biochemical reactions, shown schematically from substrate A to product E, is catalyzed by a set of specific enzymes 1, 2, 3, 4. **b**, In evolution, the order of appearance of specific enzymes is the opposite to the mentioned above and it can be shown as א, ב, ג, ד. Substance, known now as a product, at some point of evolution was randomly available from the environment. At the moment of its partial disappearance from the environment, but under condition that it still could be produced somehow from other available substances, its synthesis was beneficial and specific enzyme came into being.

So, we are dealing with dominant effects in the progeny – with expression of previously dormant genetic loci. Similar results (*i.e.* expression of previously dormant genetic loci) were obtained during domestication of silver foxes by Dmitry K. Belyaev[29,30]. Historically, homologous series of variation were first observed in wheat, which is usually self-fertilized, and later the same regularities were confirmed in rye, a typical cross-fertilized plant (p. 58)[26].

The term "action acceptor" was first introduced by Peter K. Anokhin in 1955[3,4] to describe behaviour of animals, at that time – dogs, as a brain-related feature. However the first action acceptors were present even before the appearance of replication, transcription and translation. Strictly speaking, the action acceptor is the first structure that appears in phylogenetic development of any functional system and this structure can sense and potentially use randomly appearing results, those are born in the external or internal environment by chance. All processes, even so complex as cell division, were appearing in evolution as random events. First – appearing purely by chance. Then – appearing with increased probability during some periods and appearing with decreased probability during some other periods of ontogenesis. Finally – appearing as clearly deterministic and well-controlled processes. Each time the action acceptor was formed before the next evolutionary step, and the next evolutionary step, like the next ferment in a biochemical chain, was found and raised up by the pre-existing action acceptor.

Typically our attention is focused upon the effector parts or production lines that produce "real result". If we see some feedback loop, we have a tendency to accept it as a relatively late addition that just slightly improves this system. However in real life, all feedbacks with their action acceptors were formed in evolution before all currently observable effector parts of given functional systems. It was an action acceptor that was the main acting agent in organization of all effector components from randomly available parts. Each of these parts could be first introduced at any previous evolutionary stage by chance.

Thus, from the early beginning the evolution was proceeding under control of very short and very strong feedback loops – internal feedback loops from the action acceptors. The shortest feedback loop was typically the strongest one. This type of evolution looks teleological and internally purposive. It is teleological and internally purposive – no secret here. For discussion of real teleology and pseudo-teleology of Darwinism we would like to refer to the book of Nikolai Ya. Danilevski,

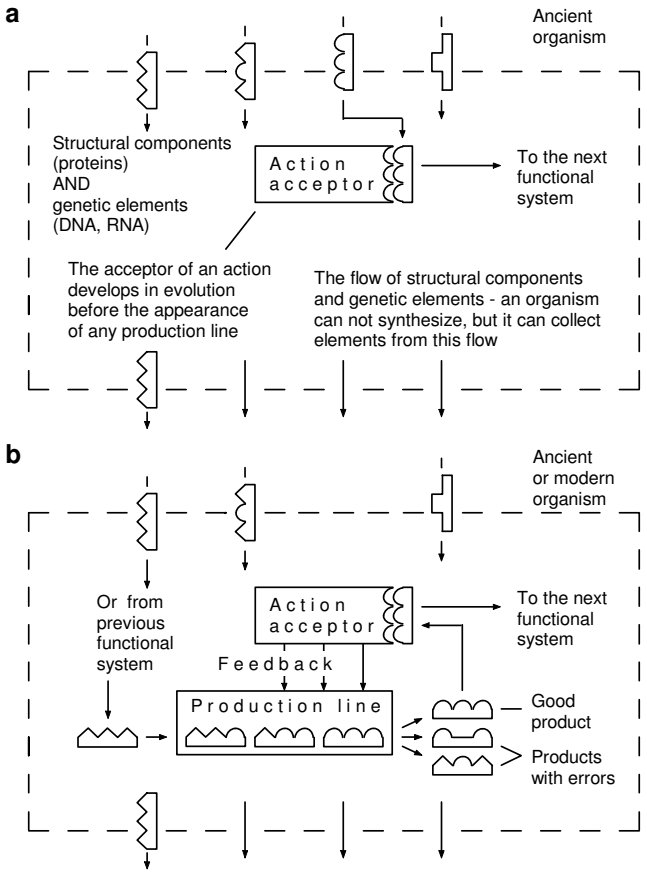

Figure 5 | Action acceptor in evolution. **a**, Early (ancient) organism was an open system not only in terms of energy, but in terms of its structural and genetic components also. It was not able to synthesize, but it was able to collect many components from the environment. The process of collection of components was performed by a set of action acceptors. **b**, Evolution of any production line starts from the acceptor of an action – from formation of potential feedback loop which appears in evolution before the first effector components of given functional system. Functional system is an entity that is searching for or is supporting the existence of some positive (useful) result with a help of feedback loop. The detector of useful result (action acceptor) is the first element in formation of feedback loop, see Fig. 6.11 (p. 241)[4] and Fig. 6.18 (p. 253)[4].

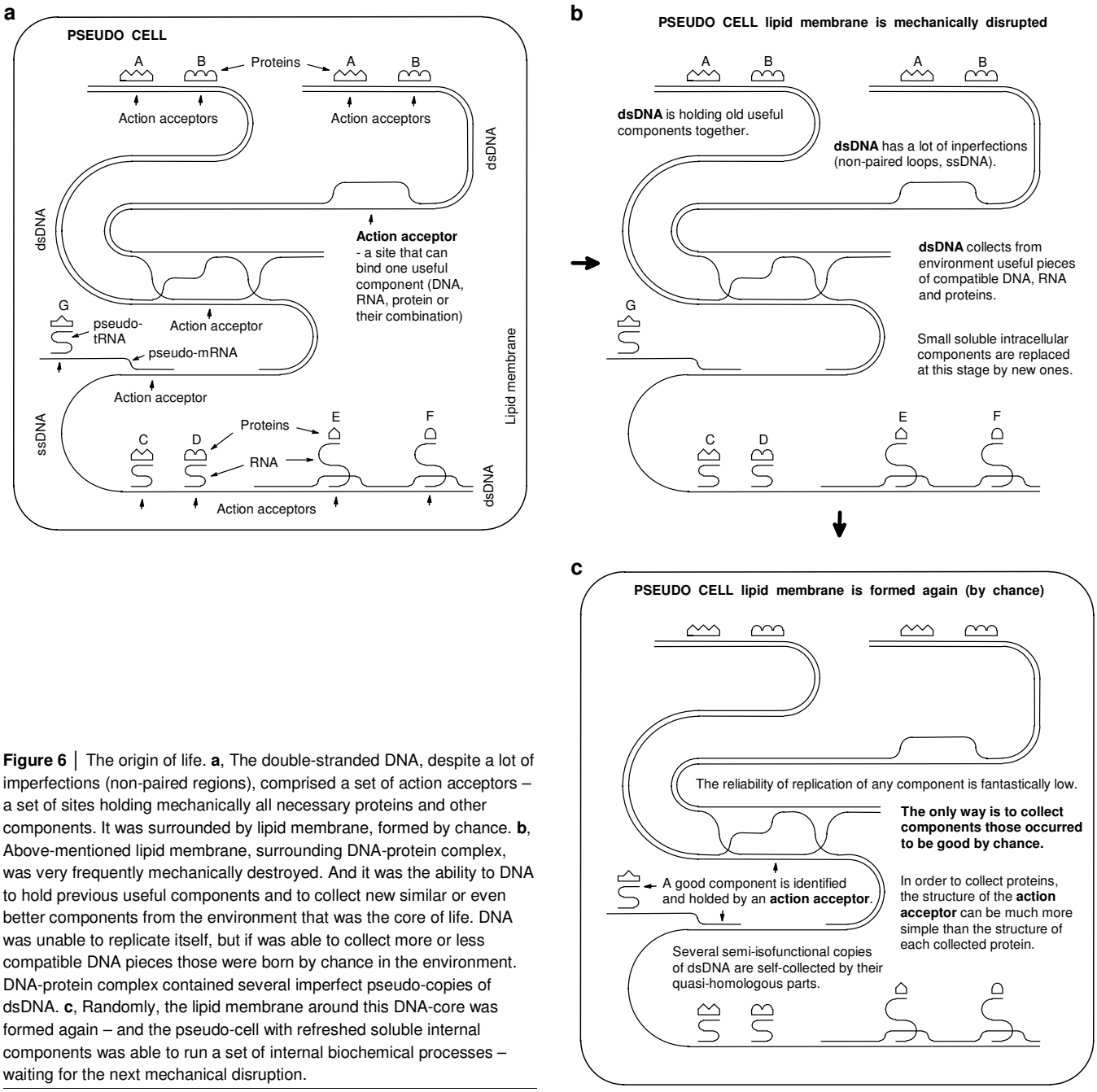

Figure 6 | The origin of life. **a**, The double-stranded DNA, despite a lot of imperfections (non-paired regions), comprised a set of action acceptors – a set of sites holding mechanically all necessary proteins and other components. It was surrounded by lipid membrane, formed by chance. **b**, Above-mentioned lipid membrane, surrounding DNA-protein complex, was very frequently mechanically destroyed. And it was the ability to DNA to hold previous useful components and to collect new similar or even better components from the environment that was the core of life. DNA was unable to replicate itself, but if was able to collect more or less compatible DNA pieces those were born by chance in the environment. DNA-protein complex contained several imperfect pseudo-copies of dsDNA. **c**, Randomly, the lipid membrane around this DNA-core was formed again – and the pseudo-cell with refreshed soluble internal components was able to run a set of internal biochemical processes – waiting for the next mechanical disruption.

published first in 1885[31-33], – it is fantastically important even today. As soon as functional system occurred to be equipped with even weak internal feedback loop – it has information about its own efficiency. And "efficiency" was determined in physiology by Alexander M. Ugolev[34,35] as relation of positive effects to negative ones ("cost factors"). It might be difficult to imagine "ideal organism", but we can always imagine "ideal functional system" – a system that is absent, but its positive result is achieved – this idea was first introduced by Genrich S. Altshuller[36] with respect to technical systems. The increase in complexity, observable in evolution, is not a purpose *per se*, but higher complexity is often, but not always, linked with higher efficiency. Parasitic organisms, evolving towards simplicity, are also good examples of the principle of efficiency.

Thus, any functional system of the organism has an ability, at least theoretically, to evolve towards "ideal functional system" and it can do so using its own internal feedback loops. It would be an error to assume that such feedback loops are good only for relatively simple optimization of the process. Any process exists usually under the pressure of contradictive forces and requirements. An attempt to increase one positive feature typically leads to decrease of another positive feature or to increase of some cost factor. Only the invention that can increase the main positive effect without the increase of the main cost factor would be really important evolutionary step, and this step will be done also with participation of local feedback loops, but the last remark does not mean that this step will be easy to perform.

As shown in the **Fig. 5**, the formation of an action acceptor and the formation of potential feedback loop are preceding in evolution the appearance of effector components of given functional system. The structure that senses the positive result develops in evolution first of all. At the beginning the result can be achieved only randomly – due to pure chance. The effector components will increase the probability of the appearance of positive result only later in evolution.

In modern organism, randomly available genetic and structural components are recruited by the action acceptor into production line in order to achieve qualitatively and quantitatively acceptable final result of this functional system. In modern organisms some action acceptors can be fantastically complex, distributed among multiple cells, but their main function remains the same – to search for and to support the desirable state of the organism or situation (not just to sense more or less good products among products with multiple errors). With respect to genetic components it was necessary not only to collect them, but to put them into domesticated state. The domesticated state means that the organism has an ability to switch given genetic element "on" and "off". The "on-off" switch – presumably reversible genetic change – has appeared in evolution even before the appearance of reliable replication. It means that an ancient organism was unable to reproduce incoming genetic elements, but it was able to switch them "on" and "off" in accordance with requirements of this organism.

As shown in the **Fig. 6**, the life on Earth has started when reliable replication, transcription and translation were absent (everything – below Eigen threshold[1,37]). Trans-membrane transport and trans-membrane potential were absent also. However, double-stranded DNA comprised the core of life. Its task was to collect and hold together all other necessary components (more or less similar DNA, more or less useful proteins and more or less useful RNA – all of them were randomly available from environment – they were developed by pure chance at the beginning of life). RNA was served as an intermediate factor in order to hold useful proteins that were not interacting with dsDNA sufficiently.

The mechanical disruption of this pseudo-cell was not only an analogue of cell division, but it was also an analogue of cell feeding. Whether the above-mentioned collection by dsDNA of more or less similar pieces of dsDNA together with other components could be described as "compositional inheritance as a mechanism of self-reproduction"[38] is an open question. At the beginning of life the mechanical disruption of pseudo-cell was really chance event. Only afterwards the pseudo-cell was able to increase probability of mechanical disruption at some stage of its existence and to decrease probability of mechanical disruption at some other stage of its existence.

Note that proteins that were binding to dsDNA directly, at the next stages of evolution will be "transcriptional factors". Replication, transcription and translation were developed under the control of action acceptors that were collecting only more or less successfully replicated, more or less successfully transcribed and more or less successfully translated components. Action acceptors were (and they remain!) the core elements of life that were able to compensate the fantastically low reliability of replication, the fantastically low reliability of transcription and the fantastically low reliability of translation. All three above-mentioned processes were developed under the control of very

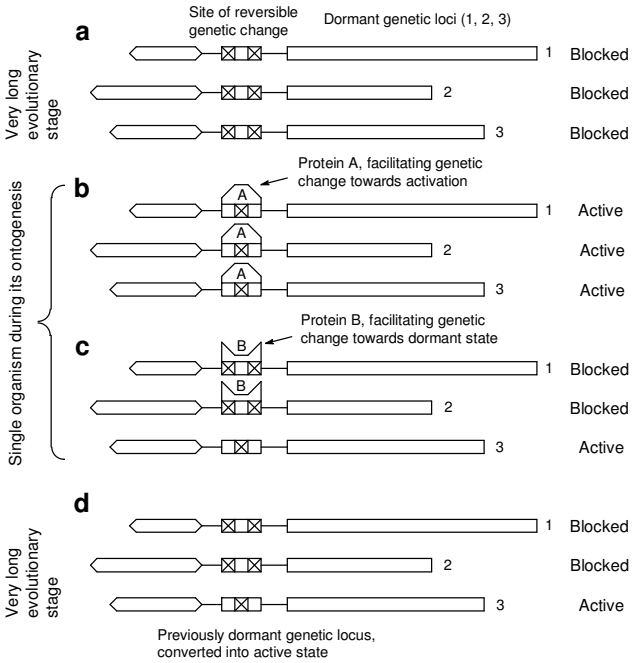

Figure 7 | Activation of previously dormant genetic locus in evolution. **a**, Three dormant genetic loci, each with reversible genetic change in the area of regulatory sites, are shown. **b**, In a deeply stressful situation the specific protein A is expressed, it binds to the site of reversible genetic change and increases the probability of its conversion into active state. **c**, In the exactly the same organism the protein B is expressed, it binds to the same site of reversible genetic change and increases the probability of its conversion into dormant state, but it can not do so with very highly expressed gene # 3. **d**, All previously expressed proteins A and B are finally disappeared, but previously dormant gene # 3 remains in active state (accessible for further regulation of its expression) forever. Similar process was called "orthoselection" in 1934 by J.W. Harms (Harms discussed the transition of vertebrate animals from water to land through multiple attempts, linked with transition of genes from "active" into "passive" state and *vice versa*)[39,40]. See **Supplementary Information**.

local, very short and very strong feedback loops. All proteins, facilitating necessary reactions, were collected together with products of the above-mentioned reactions by dsDNA, even despite any "knowledge" of their interactions were absent in the system (useful components should be held together – that is the principle). Very complex machinery of replication, transcription and translation was formed by means of collection of components that were formed independently and purely by chance. It means that DNA templates and proteins that were later formed of the basis of these templates, at the beginning of life were collected together just because the presence of templates is correlated with the appearance of above-mentioned proteins – both templates and proteins were formed at the beginning of life independently and mainly by chance.

As a short summary we can say that the evolution of the genome of any organism is always random – it is directed only by chance (Koonin, 2011)[1]. Morphological evolution and physiological evolution in general is always determined by law (Berg, 1922)[2]. And it was so even before the appearance of replication, transcription and translation. We can suppose that the very first action acceptors have appeared in evolution also by chance. As soon as the first action acceptors were present and

were able to collect from the environment useful components of different nature, randomly available (DNA, RNA, proteins), the first functional systems were formed and all further evolution was dictated by the requirements of the pre-existing functional systems. This process was and it is internally purposive, however some final goal is not absolutely necessary for its existence. It is sufficient to have local vector of development, each time based on local efficiency of currently present functional systems. This vector sometimes can be erroneous and it can lead to the extinction of the species, but it is always present (just because functional systems with their feedback loops are always present inside given organism).

Thus, evolution is a purposive process, and each its step is based on local efficiency. These are no analytical means that could distinguish between the results of the above-mentioned process and the results of evolution, directed by God, if our understanding of God is provided by Orthodox Judaism. In both cases all local decisions are solutions of contradictions between local positive effects and local cost factors. Thus, both descriptions have equal relation to the observable universe.

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Additional information

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Evolocus

Advertising Feature

Neurologger 3 and its history

Neurologger has been originally designed to record EEG, local field potentials and neuronal activity in freely moving animals in their natural environments. Later its spectrum of applicability was extended to EMG and ECG recording. Recently Neurologger has been applied to study of auditory communication in animals and, thanks to increased up to 200 kHz sampling rate in single-channel mode, to study of ultrasonic echolocation in bats. Today Neurologger is represented by two versions: Neurologger 2A/2B and Neurologger 3. Neurologger 2A/2B remains our lightest version (1.3 g without battery) and it is capable to record up to 4 channels at 33.3 kHz, 10-bit into soldered on board memory (1 or 2 GB). Neurologger 3 is slightly heavier (1.7 g without battery), it records up to 32 channels at 20.8 kHz, 16-bit into microSDXC card (4-256 GB) and it has remote control and data access through BlueTooth communication with Windows 10 machines.

The first version of the device engineered in 2002 was capable to record up to 8 EEG channels at 500 Hz or up to 2 neuronal channels at 10 kHz (Vyssotski *et al*., 2006[1]). The data were stored at Secure Digital (SD) memory card with the capacity up to 32 GB. However, because of its size (66 × 36 × 10 mm) and weight (22 g) the logging unit was attached at the back of the animal and was connected to the head with the cable. The last was not really convenient. For this reason the first Neurologger version is currently used only with the large animals attached to the head (Lesku *et al*., 2011[6]; Lyamin *et al*., 2012[10]).

To have easy recording of EEG and neuronal activity in small animals, the second miniature version of Neurologger has been designed in 2005. Significant decrease of size (22 × 15 × 5 mm) and weight (2 g) allowed us to attach the unit directly to the head of laboratory mice and flying homing pigeons (Vyssotski *et al*., 2009[3]). Neurologger 2 was capable to record up to 4 channels at sampling rate up to 9.6 kHz in its soldered 256 MB memory. This version has been successfully used in the set of studies (Rattenborg *et al*., 2008[2]; Pang *et al*., 2009[4]; Brankack *et al*., 2010[5]). Starting from 2009 the next modification of the logger called Neurologger 2A has been developed. Standing on successful concept of Neurologger 2, the novel version has got a set of new features. One of them is precise real time infrared (IR) synchronization of the record in the logger with the external events. Synchronizing labels can be sent manually by an operator or automatically by a computer. Specialized unit for sending these labels is called "Neurologger Synchronizer". Its features are described in separate documentation (see **Supplementary Information**). The second feature is recording of 3-D acceleration. The default sampling rate of accelerometer is 400 Hz – it has been found sufficient for most cases. However, the sampling rate can be increased up to 1 kHz if needed. In

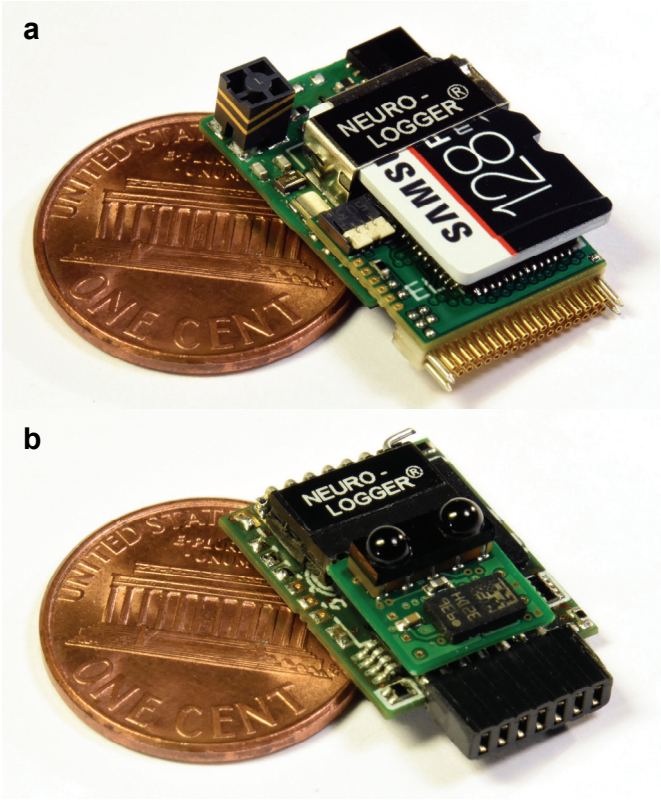

Figure 1 | Neurologger 3 (**a**) and Neurologger 2A/2B (**b**). Shown Neurologger 3 (**a**) has 32 channels and 128 GB Micro SDXC card (Samsung). Both Neurologger 3 and Neurologger 2A/2B have infrared (IR) sensor to receive information from external equipment, sent by infrared (IR) emitter (*e.g.* processed signal from a video camera, for example – animal "track"). Two black "eyes" on the top of Neurologger 2A/2B (**b**) are optical elements of IR sensor. Neurologger 2B differs from Neurologger 2A by increased sampling rate (33.3 kHz vs. 19.2 kHz) and several added modes, including single-channel 200 kHz mode.

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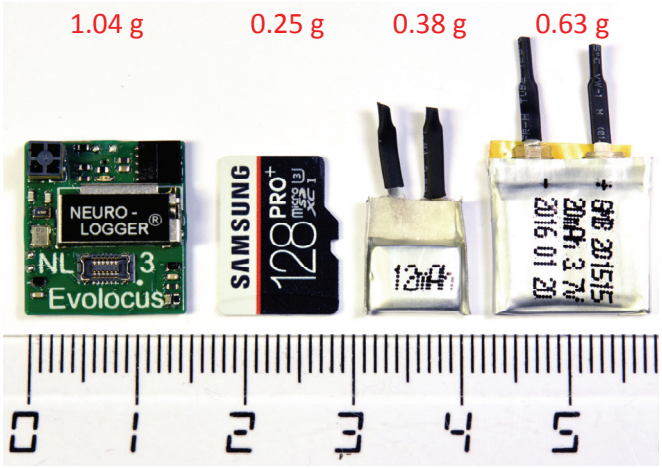

Figure 2 | Neurologger 3, an example of its memory (Samsung 128 GB Micro SDXC) and two examples of its battery. Batteries of different capacities can be used depending on desired record duration. Two examples of batteries of different weight/capacity are shown. The logger needs only one battery. Note that the duration of record is usually limited by the battery and not by the memory size.

addition, memory capacity has been increased up to 1 GB (2 GB by request) and maximal electrophysiological data sampling rate was increased up to 19.2 kHz, 4 channels. The last allowed us to use this unit for studying vocal communication in birds (Anisimov *et al*., 2014[22]). Version 2A is the most popular Neurologger version that was used in 20 publications including publication in *Science*[9].

The next version Neurologger 2B form 2015 is enhanced version of Neurologger 2A. The maximal sampling rate of all four channels was increased up to 33.3 kHz (from to 19.2 kHz in the Neurologger 2A). This was done for recording of vocal communication in some animals whose vocalization spectrum exceeds 9.6 kHz. In addition, special modification of Neurologger 2B records single channel data with frequencies up to 200 kHz. This feature was added for studying echolocation in bats, but it also can be used for investigation of ultrasonic communication in rodents (mice and rats).

The necessity to record multichannel neuronal data leaded to manufacturing the third version of Neurologger in 2016. Neurologger 3 has been designed to record 16 or 32 neuronal channels having size and weight similar to the second version. However, neuronal activity usually should be correlated with animal behavior. To record vocalization of the animal, an audio cascade with a microphone capable of recording frequencies up to 100 kHz (200 kHz sampling rate) has been added.

Basically, an idea to record ultrasonic vocalizations has been inherited from the previous version of the Neurologger 2B. However, contrary to the previous model, the novel version allows us to record ultrasound simultaneously with 32-channel neuronal activity.

In addition, animal behavior can be tracked by an array of inertial sensors (3-D accelerometer and 3-D gyroscope) complemented by 3-D magnetic compass. All sensors can be polled with the frequencies up to 600 Hz, simultaneously with recording of neuronal and ultrasonic data.

The Neurologger 3 is designed to record neuronal activity, LFPs or EEG from up to 32 electrodes. The maximal sampling rate is 20.833 kHz per channel in 32-channel version and 25 kHz in 16-channel. The frequency band of electrophysiological activity recording is freely configurable in 32-channel version and factory configurable in 16-channel version. Neuro-recording part is based on Intan RHD2132 and RHA2116 chips (32- and 16-channel versions respectively). These chips are known to be the best in the market. Sound recording is normally realized by 12-bit 200 ksps ADC of the microcontroller of the Neurologger. However, one also can use one channel of 16-bit Intan chip to record sound, but its frequency band will be limited by Intan settings common for all channels. One should note that if maximal number of channels (32) and maximal neuronal sampling rate 20.833 kHz are used, sampling rate of audio channel can't exceed 125 kHz. To sample the microphone channel with the maximal frequency 200 kHz one has to decrease neuronal sampling rate to 15.625 kHz (in 32-channel mode). The inertial sensors and magnetometer (3-D accelerometer + 3-D gyroscope + 3-D compass = "9-D" motion sensors) can be sampled in the background of all these modes with the sampling rate about 600 Hz. Resolution of all sensors is 16 bit. Ranges are software configurable. Resuming, the following two modes can be recommended:

1) 32 neuronal channels 20.833 kHz, sound 125 kHz, motion sensors 579 Hz. Dataflow to memory: 1.77 megabytes per second.
2) 32 neuronal channels 15.625 kHz, sound 200 kHz, motion sensors 625 Hz. Dataflow to memory: 1.60 megabytes per second.

The logger consumes about 25 mA from 3.7 V Lithium-polymeric battery in these modes.

Weights of the logger parts without neuronal recording board are shown in the **Fig. 2**. The scale is in centimeters.

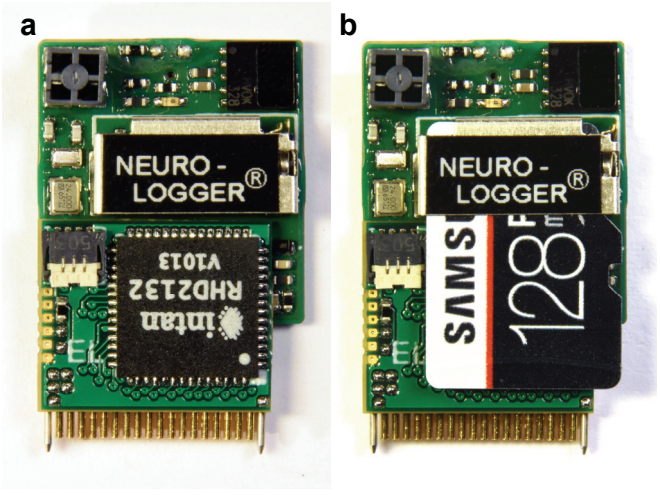

Figure 3 | Neurologger 3 with its headstage and memory. To record neuronal activity the neuronal headstage should be attached to the processor module how it is shown in this picture (**a**). The 32-channel headstage with Hirose connector is placed at the bottom side (invisible). Then, the memory card is placed above the headstage (**b**). This combination weighs 1.73 g.

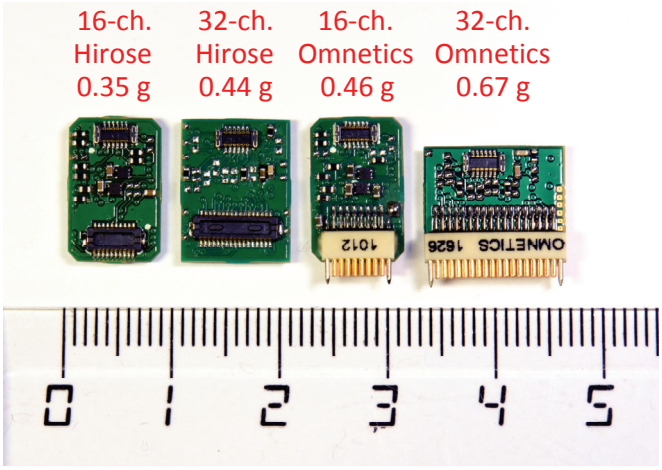

Figure 4 | Four neuronal headstages, available for Neurologger 3. From the left to the right: 16-channel Hirose, 32-channel Hirose, 16-channel Omnetics and 32-channel Omnetics. Both Omnetics and Hirose types are pin compatible with Neuronexus silicon probes. Please see Neuronexus Internet side for the pin layouts. Omnetics connectors are larger and they are more widespread. Many companies produce electrodes with this type of connectors. Their benefit is the mechanical strength; no additional fixation of the logger on the head is needed if Omnetics connectors are used. Hirose connectors are smaller, but they need a special clamp that would push two opposite parts to each other for reliable fixation.

One of the most important parameters of neuronal recording system is internal noise of the amplification cascade that should be as small as possible. Also, no disturbances should penetrate to the high-impedance electrode inputs. The following sample of record shows signal recorded by the Neurologger 3 with 32-channel headstage. In this test all channels except one were connected to the signal generator producing 10 µV rectangular pulses with frequency 5 Hz. The last top-most channel was connected to the microphone to record environmental noise. The recording cascade was configured for the frequency band 1-7500 Hz (band-pass filter). As one can see, 10 µV steps are clearly visible (**Fig. 5**). The internal noise of the amplification cascade is about 2 µV RMS.

However, the system also should be capable to record signals from the high-impedance sources. To test this capability of our recording system, we provided 10 µV signal from the signal generator to the logger though resistors of nominal 10 kΩ, 100 kΩ and 1 MΩ. The following three charts show examples of records obtained with the listed above impedances of neuronal recording electrodes (**Fig. 6**). One can see that the record with 10 kΩ resistor is practically indistinguishable from the signal recorded from the low-impedance signal source directly. An increase of the source impedance up to 100 kΩ and 1MΩ increases the background noise as expected. However, even in the case of 1 MΩ source the 10 µV steps are clearly visible in the record. This is a good prerequisite for high-quality neuronal recording, because typical spike size recorded from 1 MΩ electrodes is usually about 100 µV or more.

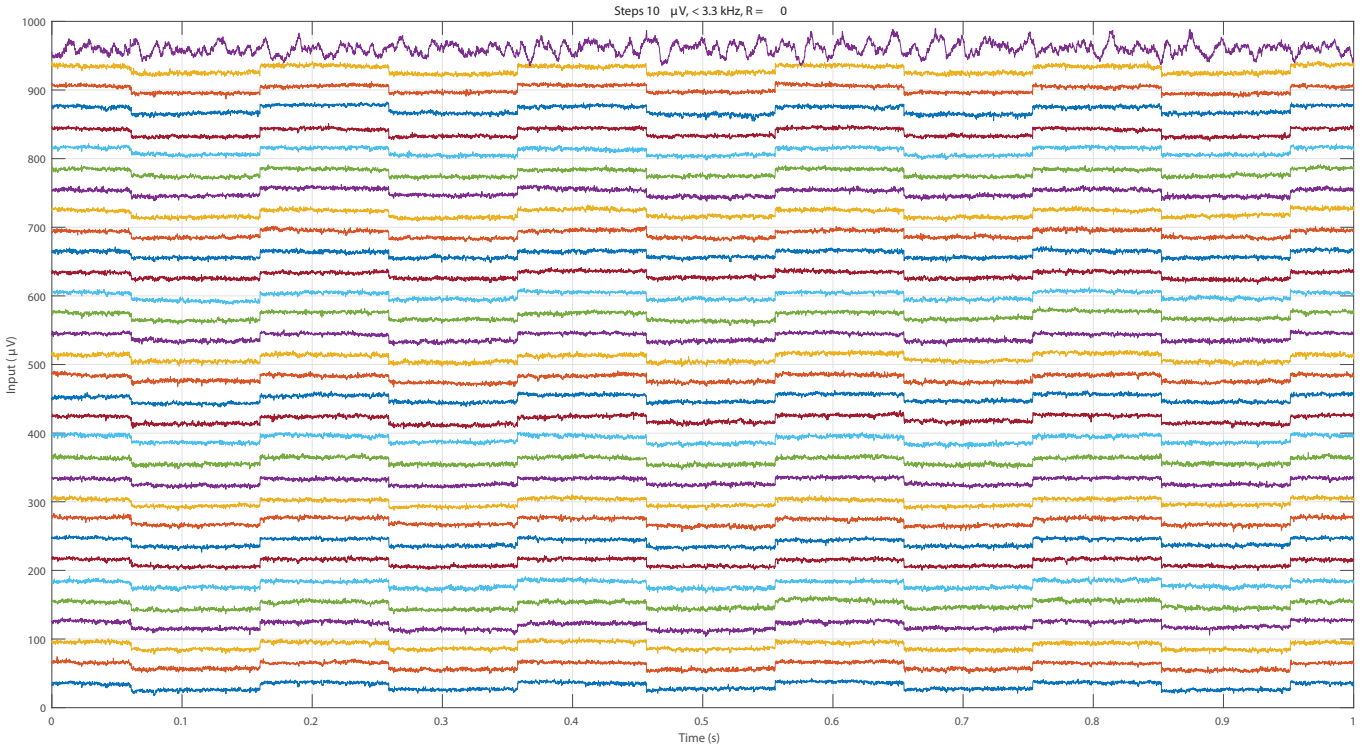

Figure 5 | Noise and electromagnetic disturbances in the Neurologger 3. Neurologger 3 has extremely low noise and electromagnetic disturbances in its frequency range *F* = 1-3300 Hz. The original signal recorded in the frequency band 1-7500 Hz was additionally low-pass filtered at 3300 Hz frequency. The duration of shown fragment is 1 second. A sequence of 10 µV peak-to-peak rectangular pulses was given to the input of the logger either directly (chart above) or through 10 KΩ, 100 KΩ or 1 MΩ resistors (**Fig. 6**). The last (top) channel was connected to the microphone to record environmental sounds. The 10 µV step is clearly visible in all channels, except the last (top) one.

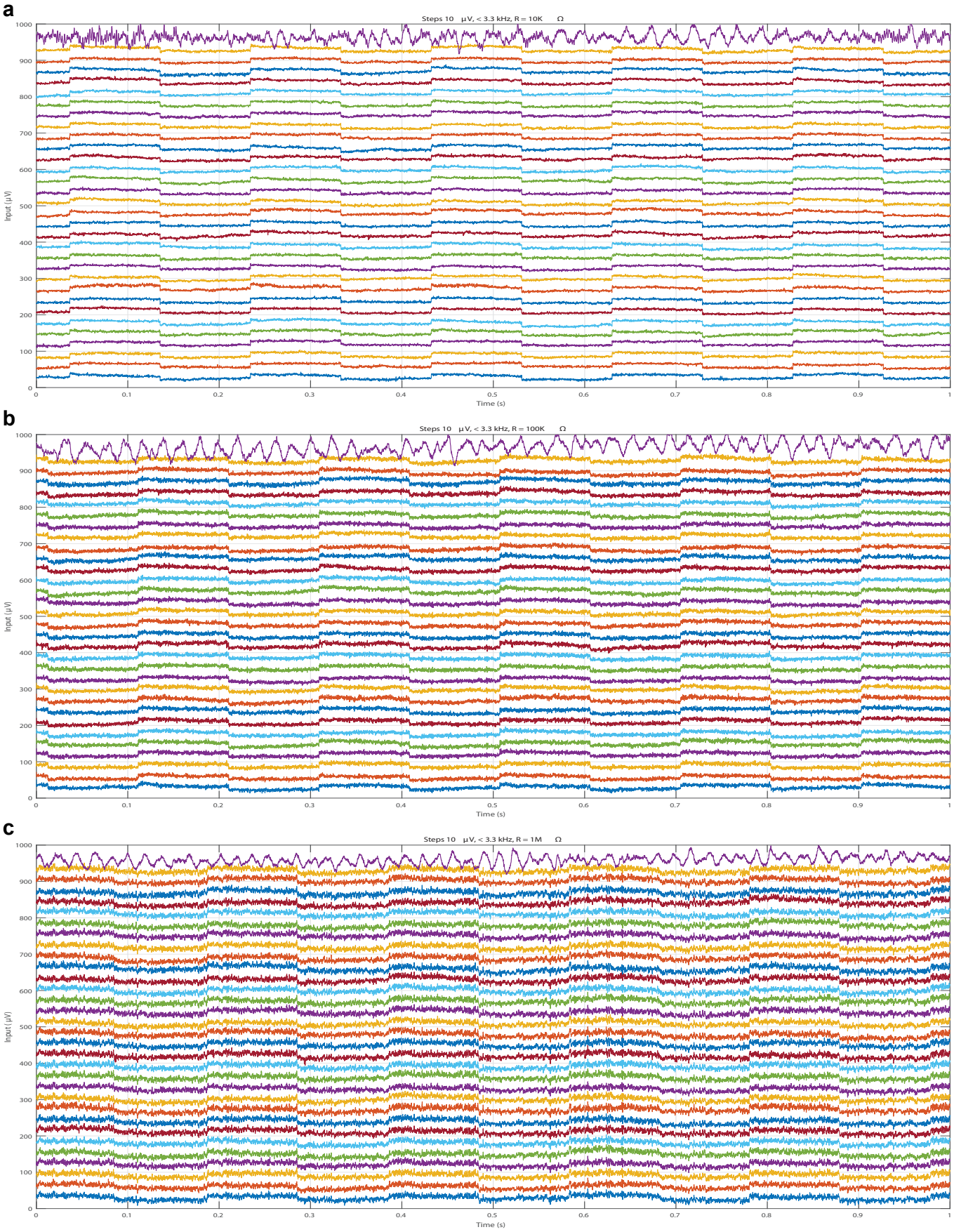

Figure 6 | Noise and electromagnetic disturbances in the Neurologger 3 when a sequence of 10 µV peak-to-peak rectangular pulses was given to the input of the logger through 10 KΩ (**a**), 100 KΩ (**b**) or 1 MΩ (**c**) resistors. All other conditions are identical to the conditions of the record in the **Fig. 5**.

Table | Comparison of three generations of Neurologger®

	Neurologger 1	Neurologger 2A/2B	Neurologger 3
Primary usage	EEG, EMG and LFPs recording in large animals: marine mammals, ruminants	EEG, EMG and LFPs recording in mice and larger animals; ECG, vocalization recording including ultrasonic	Multichannel neuronal recording in mice and larger animals; vocalization recording including ultrasonic
Number of channels	**8 differential channels**	**4 channels** freely assigned to two referent electrodes	**16 or 32 channels** with one referent electrode; or 16 differential channels
ADC resolution	10 bit; 2x oversampling in low-frequency modes	10 bit; 2x-8x oversampling in low-frequency modes	16 bit
Sampling rate	8 channels up to 800 Hz; or 2 channels up to 10 kHz; or 1 channel 20 kHz; higher sampling rates by request	**Version 2A:** 4 channels up to 19.2 kHz **Version 2B:** 4 channels up to 33.3 kHz; or 1 channel up to 200 kHz	**32-channel version:** 32 channels up to 20.8 kHz; free selection of channel sequence; selected channels can be sampled more often than others **16-channel version:** Fixed sequence of 16 channels up to 25 kHz
Locomotion recording	Optional analog 3-D accelerometer occupies three channels	Optional 3-D accelerometer	3-D accelerometer, 3-D gyroscope, 3-D magnetic compass
Vocalization recording	-	Optional microphone and contact microphone are connected to neurophysiological channels; optional dynamic range expansion	Dedicated 12-bit 200 ksps microphone ADC works simultaneously with neuronal 16-bit ADC; optional dynamic range expansion; attachment of a microphone to one 16-bit channel is also possible
Expansion possibilities	Asynchronous serial bus up to 1.5 Mbps (UART) and digital input/output lines at the main CPU can be custom programmed by request.	Dedicated communication controller with different peripheral interfaces is connected to 8 Mbps synchronous bus (SPI). It can be custom programmed by request.	Inter-integrated circuit (I2C) communication bus 400 kbps makes possible chained connection of multiple custom-developed peripheral devices. Development of the following peripheral units is planned or will be done by request: optical and electrical brain stimulators, motorized microdrive, GPS. 32-ch version has 3 auxiliary analog inputs and one digital output.
Data memory	Micro-SD high-capacity (4-32 GB) memory card	Soldered memory chip 1-2 GB	Micro SD high-capacity (4-32 GB) or extended capacity (64-256 GB Micro SDXC) memory card
Maximal memory filling speed	30 kBps (2 channels, 10 kHz)	300 kBps (1 channel, 200 kHz)	1.77 MBps (32 channels 20.8 kHz, sound 125 kHz, motion sensors 580 Hz)
Maximal recording duration	Limited by the battery	1 GB will be filled when 4 channels are sampled with the frequency: 100 Hz: 20 days 17 h 400 Hz: 5 days 4 h 1600 Hz: 1 day 7 h 9.6 kHz: 5 h 10 min 19.2 kHz: 2 h 35 min 33.3 kHz: 1 h 29 min One channel at 200 kHz: 59 min 39 s 3D accelerometer increases volume by 50% in low-frequency modes.	In most cases limited by the battery. 128 GB card is sufficient for recording during 20 hours in the highest data rate mode listed above. If only 32 channels are sampled with the frequency 15.625 kHz, 128 GB is sufficient for 31 hours.
Current consumption	~5.5 mA in EEG mode	**Version 2A:** 1.5 - 4.3 mA, mode-dependent; In EEG mode with 3D accelerometer. 2.0 mA **Version 2B:** in high-frequency modes (≥33.3 kHz): 6.0 - 11.7 mA, mode-dependent	11-25 mA, mode-dependent; All neuronal modes ~25 mA.
Logger size (w/o battery)	36 x 31 x 6 mm	From 18 x 15 x 3 to 22 x 15 x 8 mm	From 20 x 15 x 6 mm to 24 x 15 x 8 mm
Logger weight (w/o battery)	**5.31 g**	**0.95 - 1.71 g**, version-dependent	**1.29 – 1.96 g**, version-dependent
Recommended batteries and their weights	Lithium-polymeric 3.7 V 240 mAh 9.0 g rechargeable battery will be sufficient for 1 day 19 h. Non-rechargeable 3.6 V 1200 mAh 8.9 g LS14250 will be sufficient for 9 days.	A couple of non-rechargeable Zn-Air 1.4 V batteries ZA 10 (75 mAh), ZA312 (175 mAh), ZA13 (305 mAh) with the weights per pair 0.635, 1.02, 1.66 g respectively will be sufficient for 1, 2 and 4 days of EEG recording. Lithium-polymeric batteries, for instance 3.7 V 12 mAh 0.38 g GM300910, also can be used.	Lithium-polymeric 3,7 V 20 mAh, 0.63 g 40 mAh, 1.05 g 50 mAh, 1.58 g Will provide duration of neuronal recording of 15 min, 1 h 15 min and 2 h 15 min respectively.

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Additional information

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Competing financial interests: Neurologger is a trademark, registered in the USA, #3776356. Neurologger 2A/2B and Neurologger 3 are protected by U.S. patents #8,160,688 and #9,492,085 (both patents are applicable to Neurologger 2A/2B and to Neurologger 3). Other patents are pending.

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