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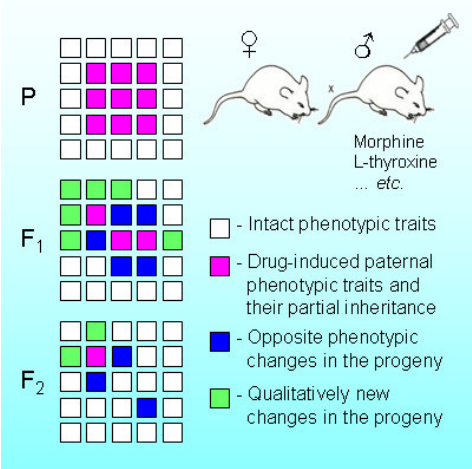

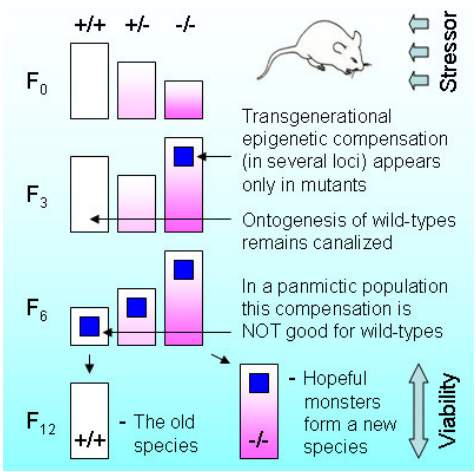

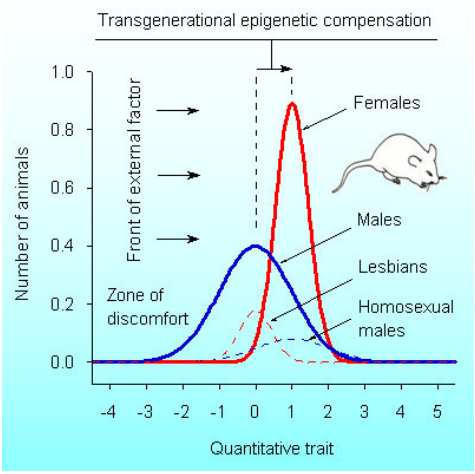

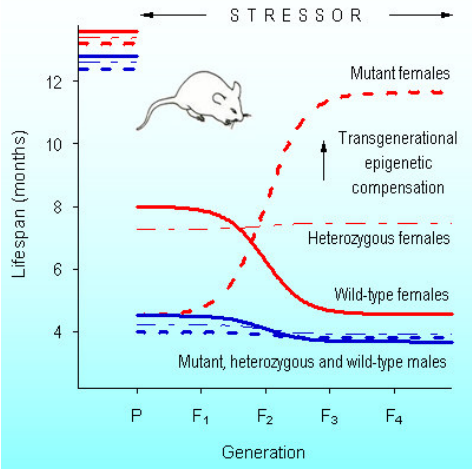

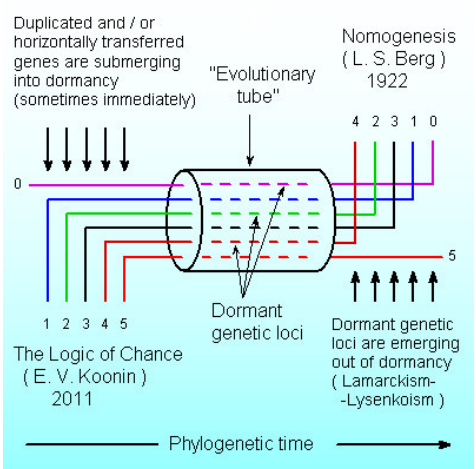

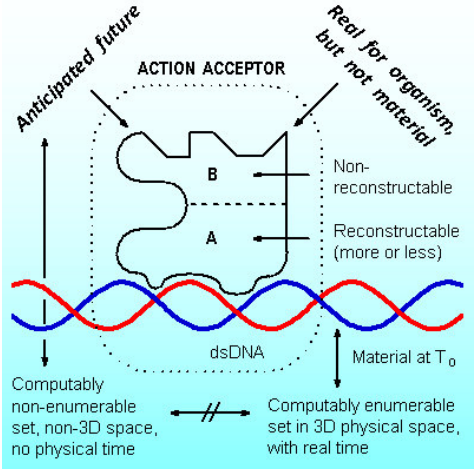

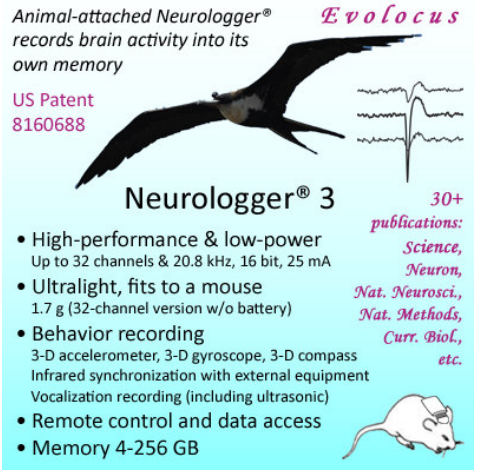

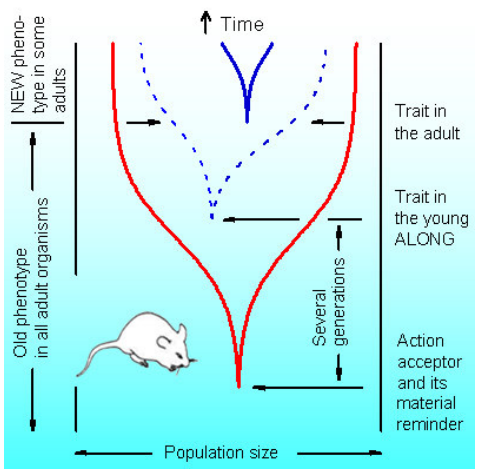

Special Printed Issue for Neuroscience 2024 Conference

Table of Contents

Articles

Application Notes

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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.

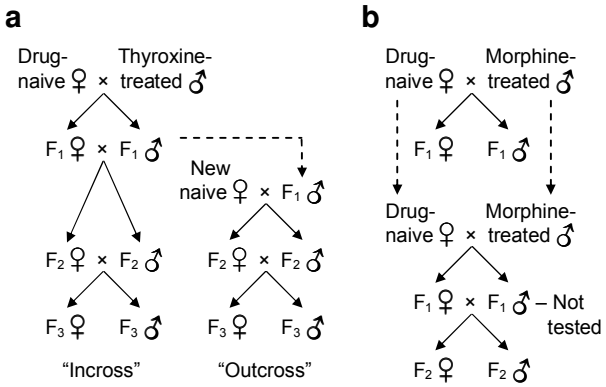

Figure 1 | Breeding paradigms. (**a**) DBA/2J mice, thyroxine study. (**b**) Wistar rats, morphine study. Solid arrows indicate the appearance of progeny, dashed arrows – transition of the same animals.

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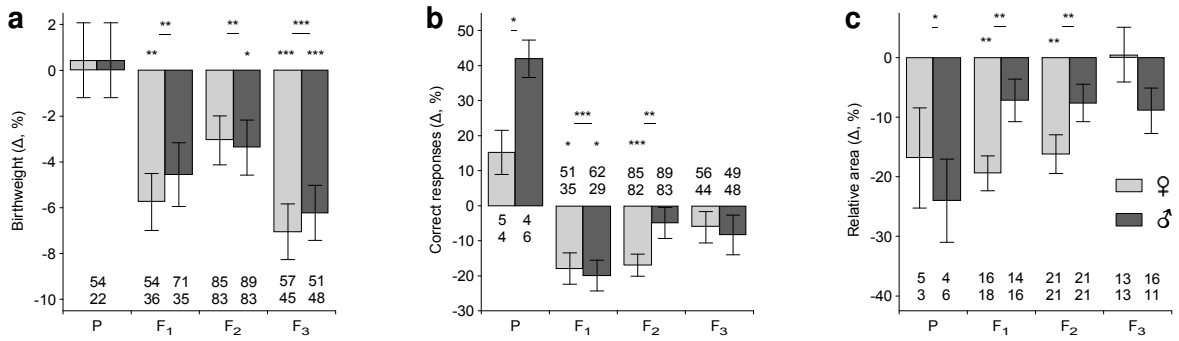

Figure 2 | Phenotype of thyroxine-treated mice and F_1-F_3 descendants. Neonatally thyroxine-treated mice and untreated descendants of thyroxine-treated males. (**a**) Birthweight. (**b**) Two-way avoidance averaged correct responses of 5-day training, 80 trials daily. (**c**) Hippocampal mossy fibers,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

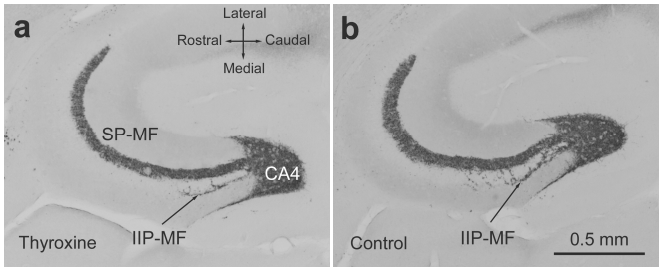

Figure 3 | Hippocampal mossy fiber morphology in the F_3-outcross. Thyroxine study. (**a**) Experimental male mouse. (**b**) Control one. Note the scarce infrapyramidal mossy fiber projection (IIP-MF) in (**a**). Shown samples differ from each other to the greater extent (45%) than mean group values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

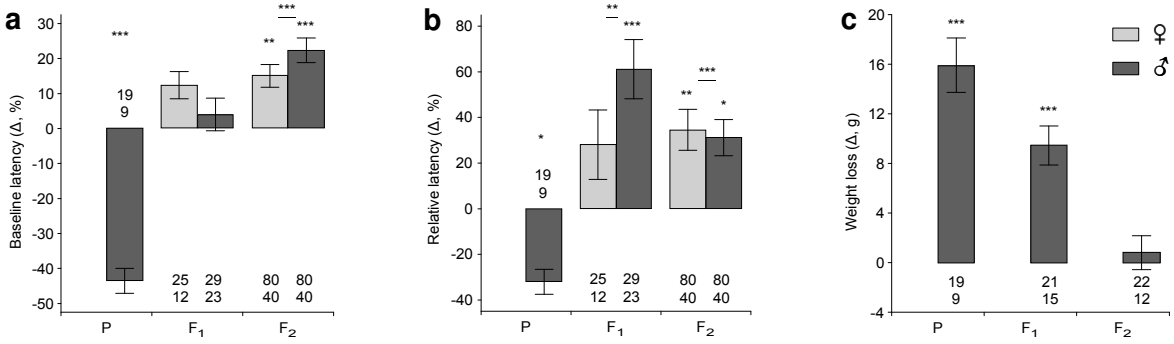

Figure 4 | Phenotype of morphine-treated male rats and F_1-F_2 progeny. (**a**) Pain sensitivity, baseline latency. (**b**) Morphine analgesia, ratio of tail withdrawal latency, measured 30 min after 10 mg/kg morphine administration, to baseline latency. (**c**) Naloxone-precipitated weight loss after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

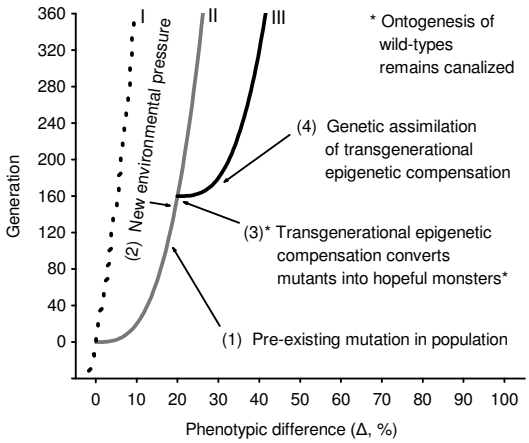

Figure 1 | Transgenerational epigenetic compensation initiates speciation. **I**, **II** and **III** – species or races. Original mutation and its heritable epigenetic compensation are not in the same locus. Speciation demonstrated on hypothetical data.

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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

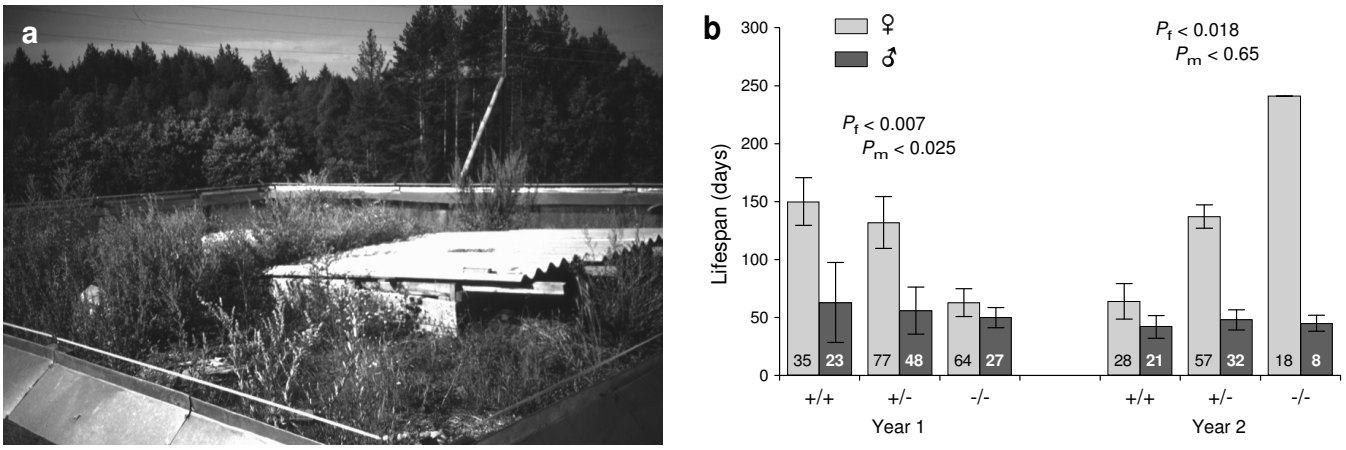

Figure 3 | Lifespan of *Per2*^{Brdm1} mice after release in semi-natural environment. (**a**) Pen 20 × 20 m with two shelters 3 × 2 × 0.7 m each. (**b**) Lifespan (days) after the first release for generations P - F_1 (Year 1) and F_2 - F_4 (Year 2) for all mice that were recorded at least 10 days following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

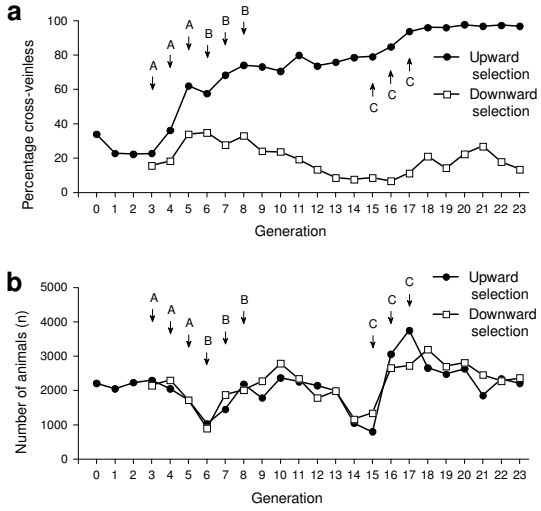

Figure 4 | Transgenerational epigenetic compensation facilitates genetic assimilation. Assimilation of cross-veinless phenotype induced in *Drosophila melanogaster* by heat-shock treatment (40 °C) during 4 hours with onset between 21 and 23 hours after puparium formation. All shown animals (all generations)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

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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

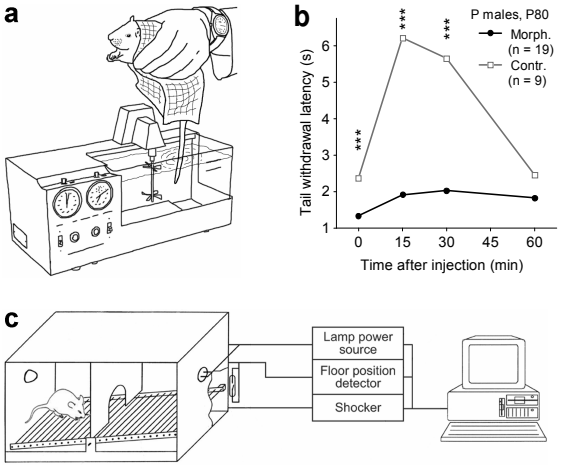

Figure 1 | Tail-withdrawal (**a**) and two-way avoidance (**c**) tests for Wistar rats and DBA/2J mice, respectively. (**b**) Male rats (P) were tested at the age of 80 days in the tail-withdrawal test (56°C), being injected with morphine 10 mg/kg, i.p., after the end of chronic (P42-P79) morphine treatment.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

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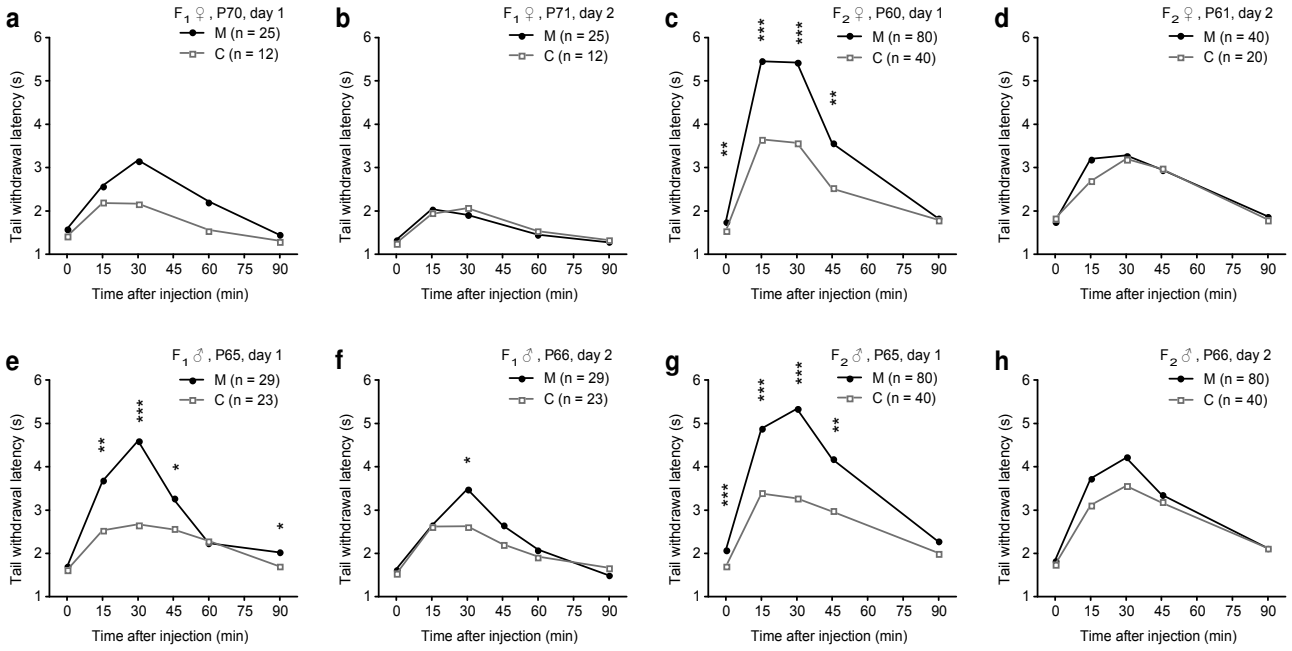

Figure 2 | Tail-withdrawal test in the F_1 & F_2 descendants of morphine-treated male Wistar rats. Each animal was tested twice (days 1 & 2) with the same dose of morphine 10 mg/kg. Morphine was administered i.p. just after the first measurement of tail-withdrawal latency (time "0"). The enhanced analgesic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

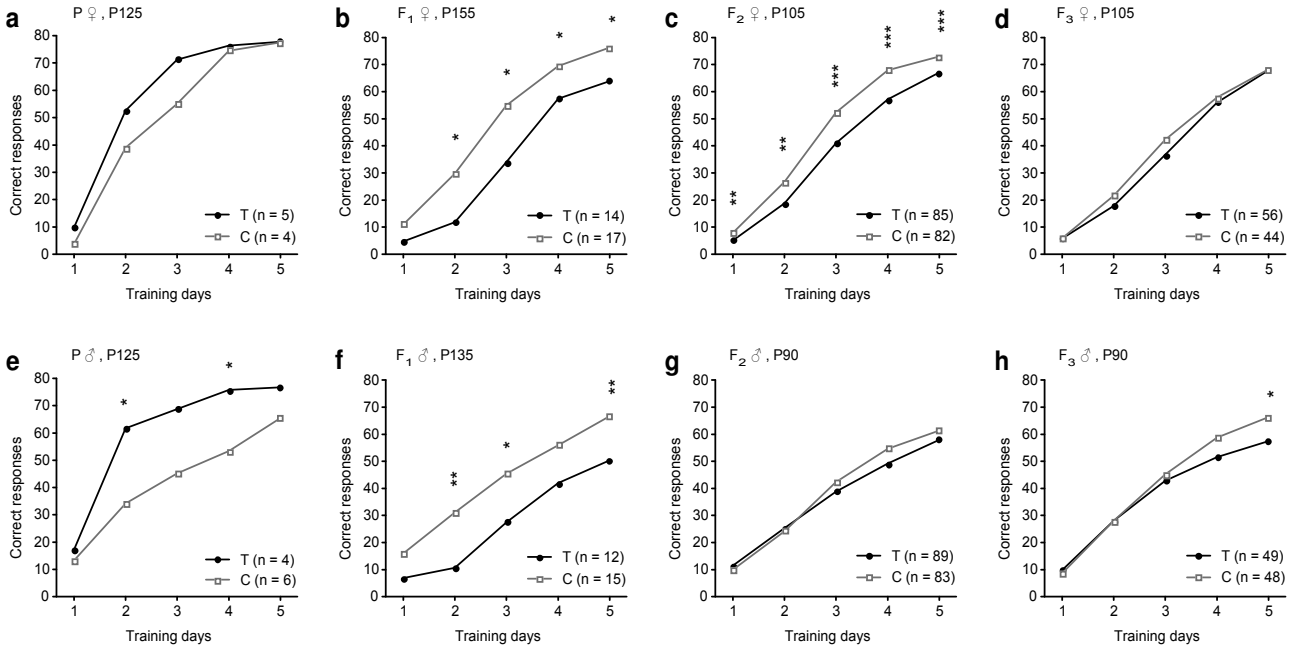

Figure 3 | Two-way avoidance in the thyroxine-treated DBA/2J mice and in the F_1-F_3 progeny of thyroxine-treated males. Note improved performance in the neonatally (P0-P11) thyroxine-treated males (**e**), but decreased performance in their descendants (**b**,**c**,**f**,**h**). Note that the performance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

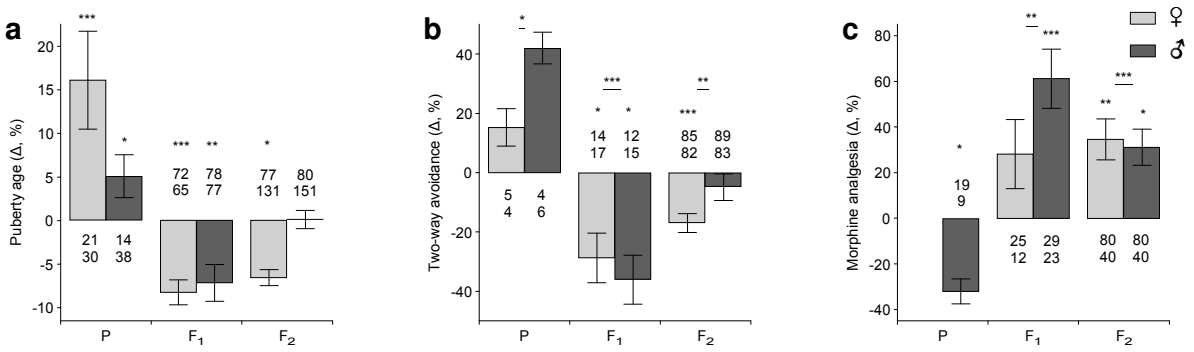

Figure 4 | Phenotypic inversion in the progeny after prenatal (E8-E14), neonatal (P0-P11) and young adult (P42-P79) parental drug treatment. (**a**) Onset of puberty in the experiment of Manikkam and co-authors (2012)[20]: Sprague-Dawley rats (P), both ♀ and ♂, were treated during E8-E14 by i.p. administration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

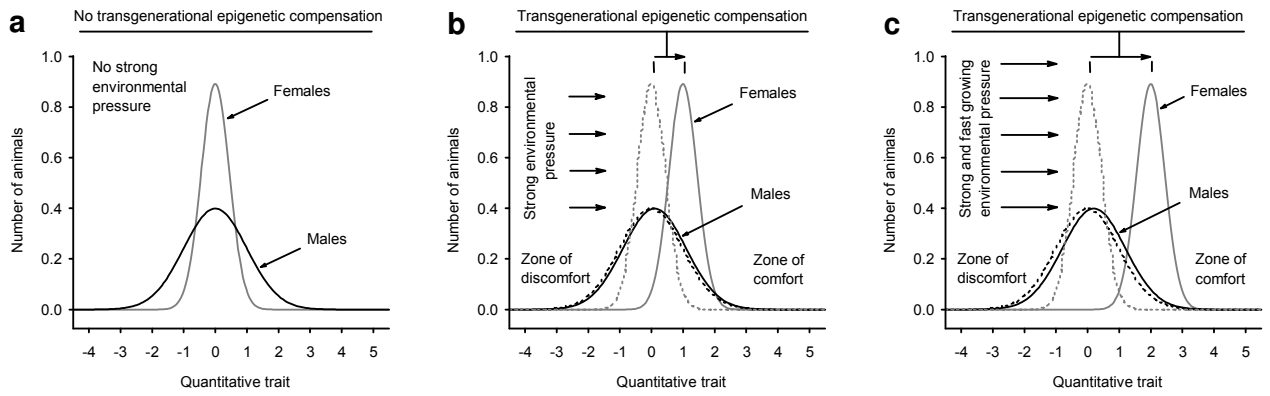

Figure 5 | Distribution of a quantitative trait among females and males in population. Original distribution in males is standard normal distribution. Chosen quantitative trait is functionally linked with given environmental influence (*e.g.*, cold-resistance – low temperatures). Without specific external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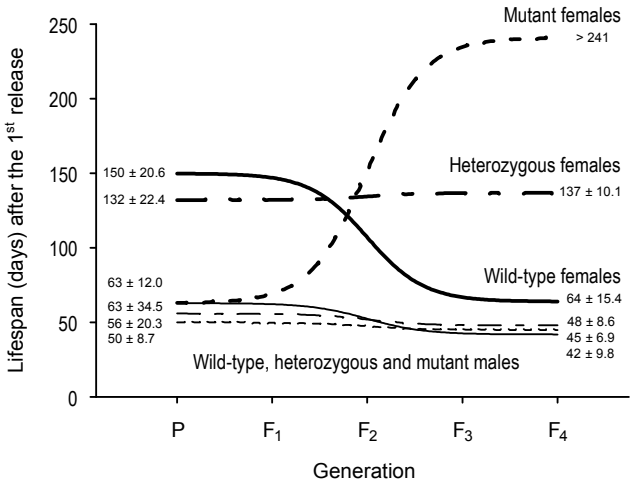

Figure 2 | Lifespan of *Per2*^{Brdm1} mice in pens after the first release[1,11]. All mice were taken out of pens (**Fig. 1**) and released back twice a year. New ones (born during previous half-year) were genotyped. Transponders, bearing individual numbers, were injected into all mice. Antennae, placed 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.

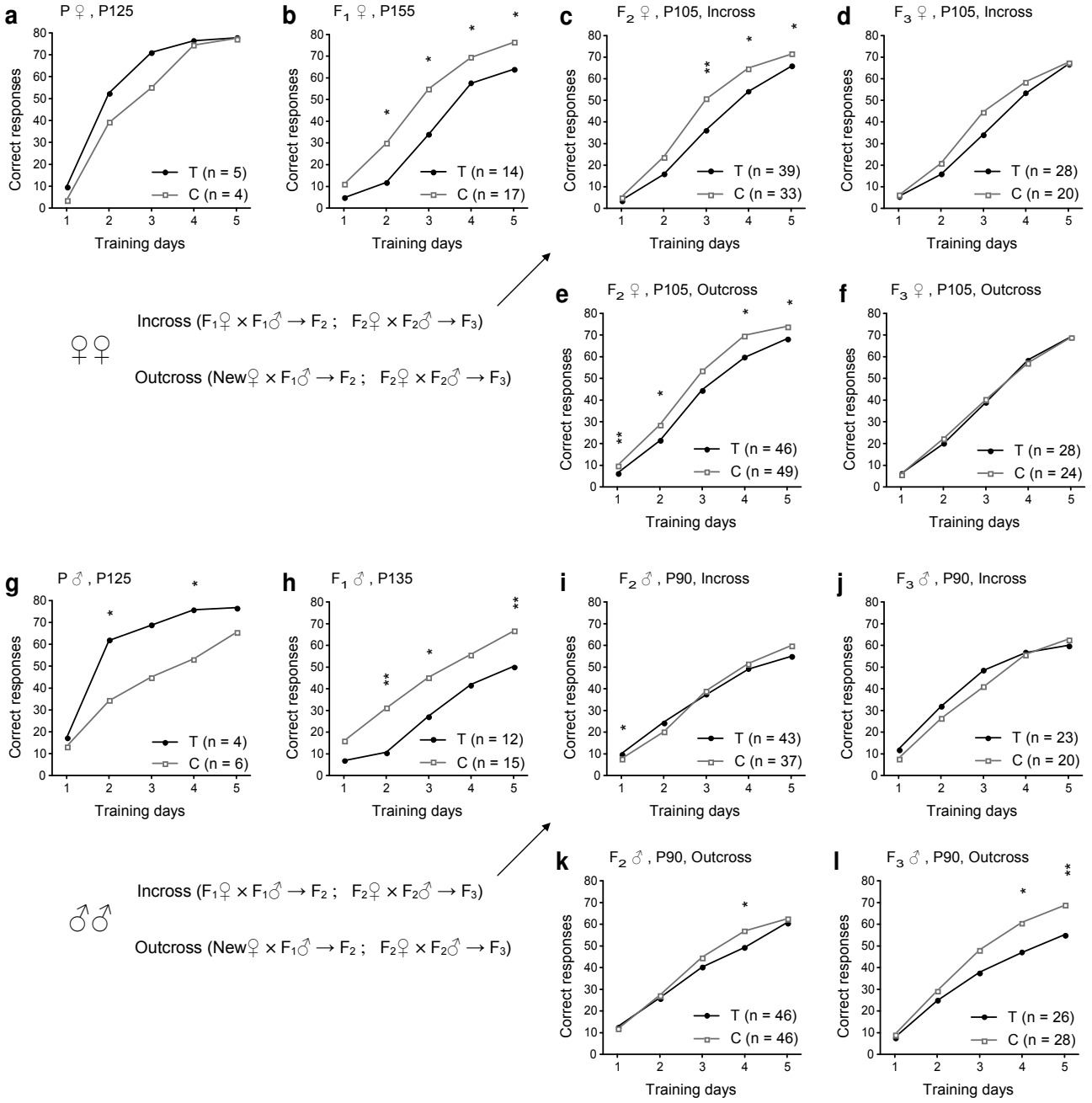

Figure 3 | Two-way avoidance in the thyroxine-treated DBA/2J mice and in the F_1-F_3 progeny of thyroxine-treated males. Note improved performance in the neonatally (P0-P11) thyroxine-treated males (**g**), but decreased performance in their descendants (**b**-**c**,**e**,**h**,**l**). Both Incross and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

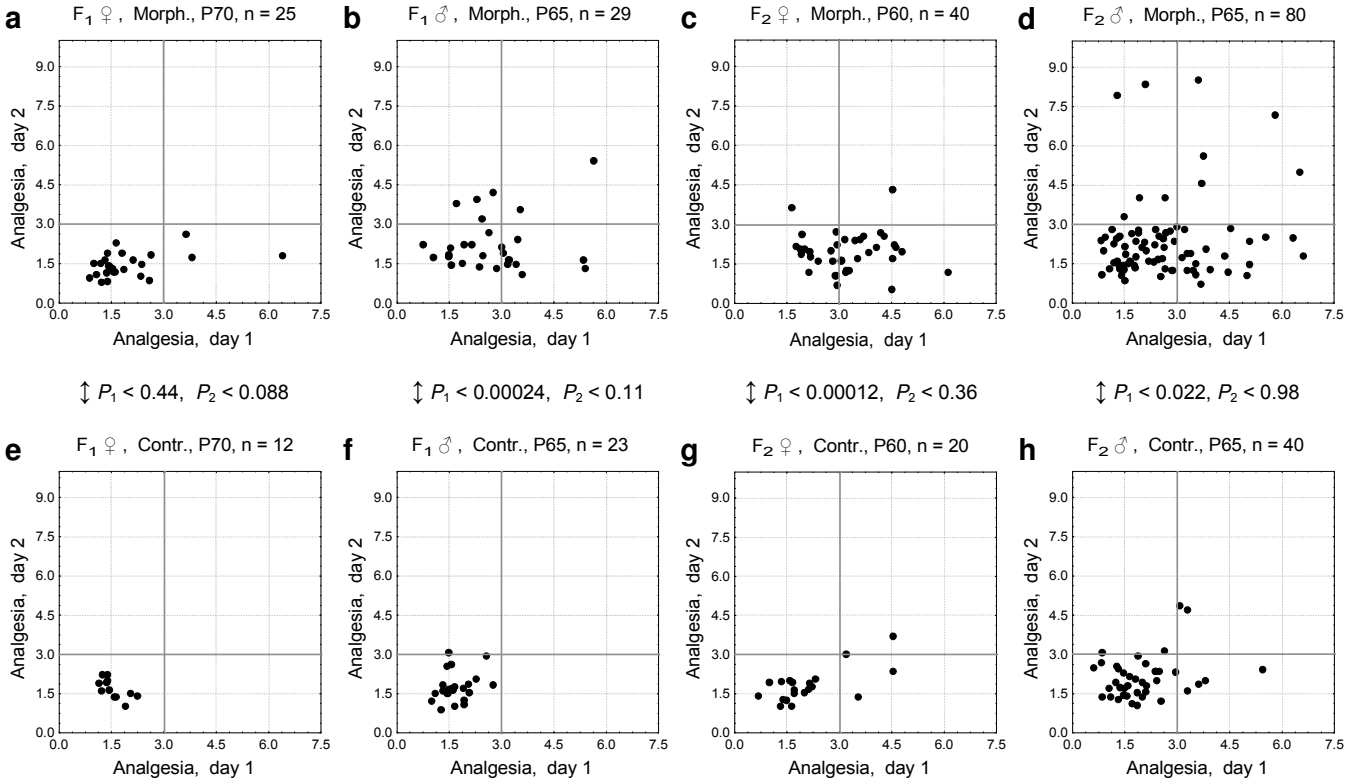

Figure 4 | Tail-withdrawal test in the F_1 & F_2 descendants of morphine-treated male Wistar rats. Each animal was tested twice (days 1 & 2) with the same dose of morphine 10 mg/kg. Morphine was administered i.p. each day after the first measurement of tail-withdrawal latency (baseline latency). Abscissa (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

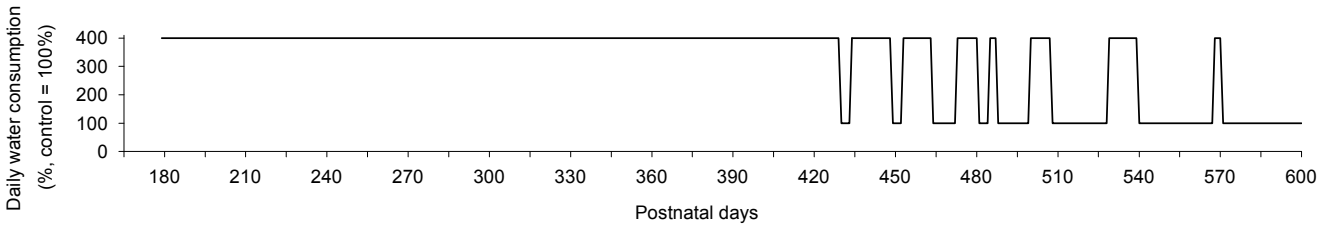

Figure 5 | Water consumption of one female guinea pig, obtained from female with unusually low water consumption (schema). In our heterogeneous outbred stock of guinea pigs (*Cavia porcellus*), one female was obtained that had unusually low (20-25%) water consumption during her adulthood. Contrary to 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

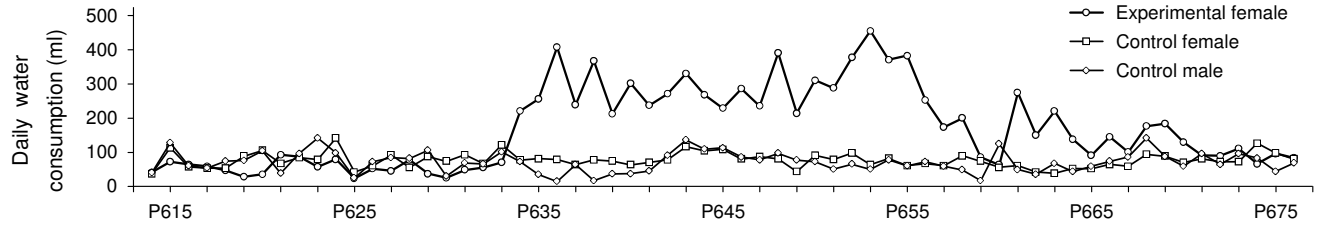

Figure 1 | Randomly expressed increased water consumption in the experimental female guinea pig, obtained from female with low adult water consumption and normal male. Postnatal days P614-P676 are shown. The stochastically increased water consumption in this female is in contradiction with the phenotype 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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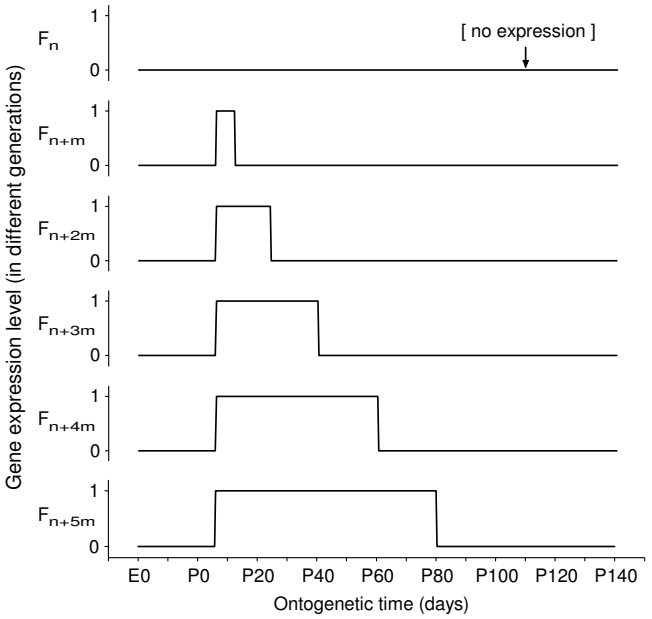

Figure 2 | Expression of one previously dormant genetic locus. Leo S. Berg has described the "precession of characters" in 1922: "… latent characters (factors, *genes*) originally manifested in the young alone… in the course of time and evolution are displayed also in the adult descendants (or supposed 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.

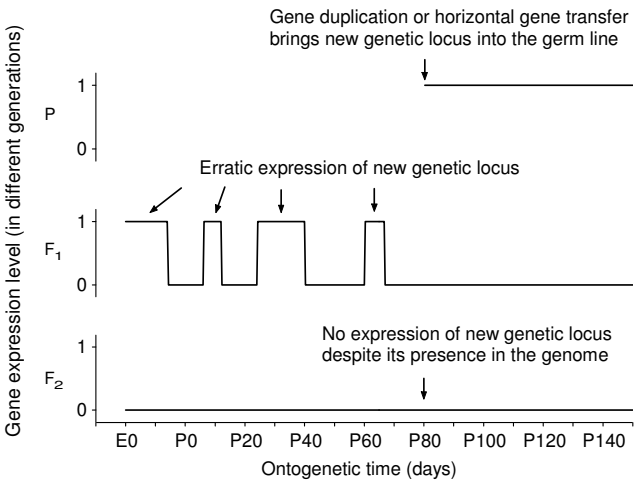

Figure 3 | New genetic locus is submerging into dormancy. In mammals, this process needs at least three shown generations (theoretically, in an idealized situation). In real life, 6-12 generations are required to bring new genetic locus into completely dormant state (many experiments with transgenic 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).

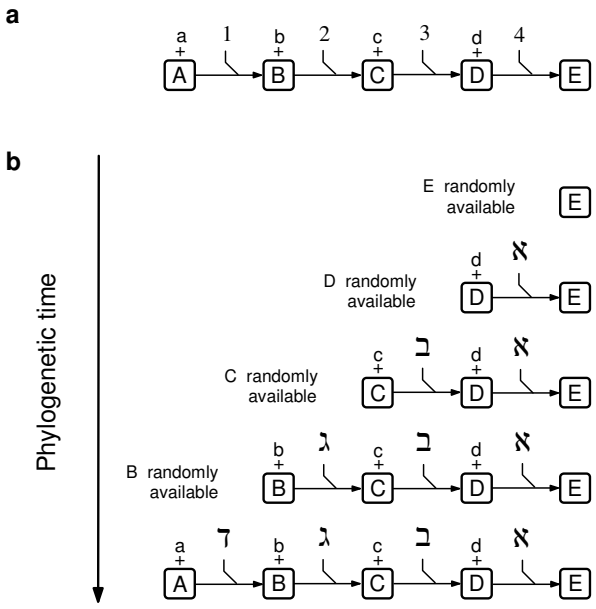

Figure 4 | The evolution of biochemical syntheses by Norman H. Horowitz (1945)[25]. **a**, Chain of biochemical reactions, shown schematically from substrate A to product E, is catalyzed by a set of specific enzymes 1, 2, 3, 4. **b**, In evolution, the order of appearance of specific enzymes is the opposite 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,

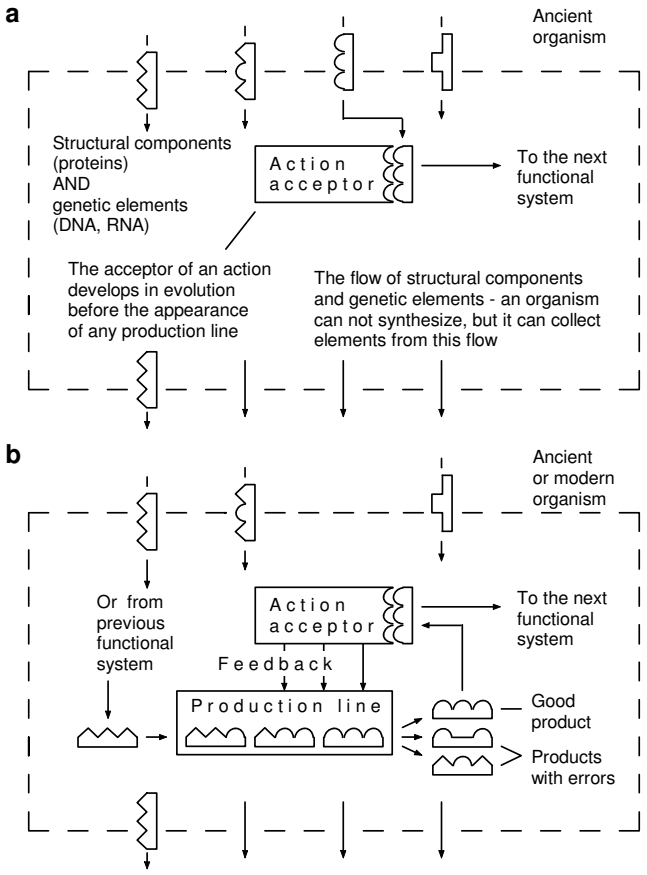

Figure 5 | Action acceptor in evolution. **a**, Early (ancient) organism was an open system not only in terms of energy, but in terms of its structural and genetic components also. It was not able to synthesize, but it was able to collect many components from the environment. The process of collection 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].

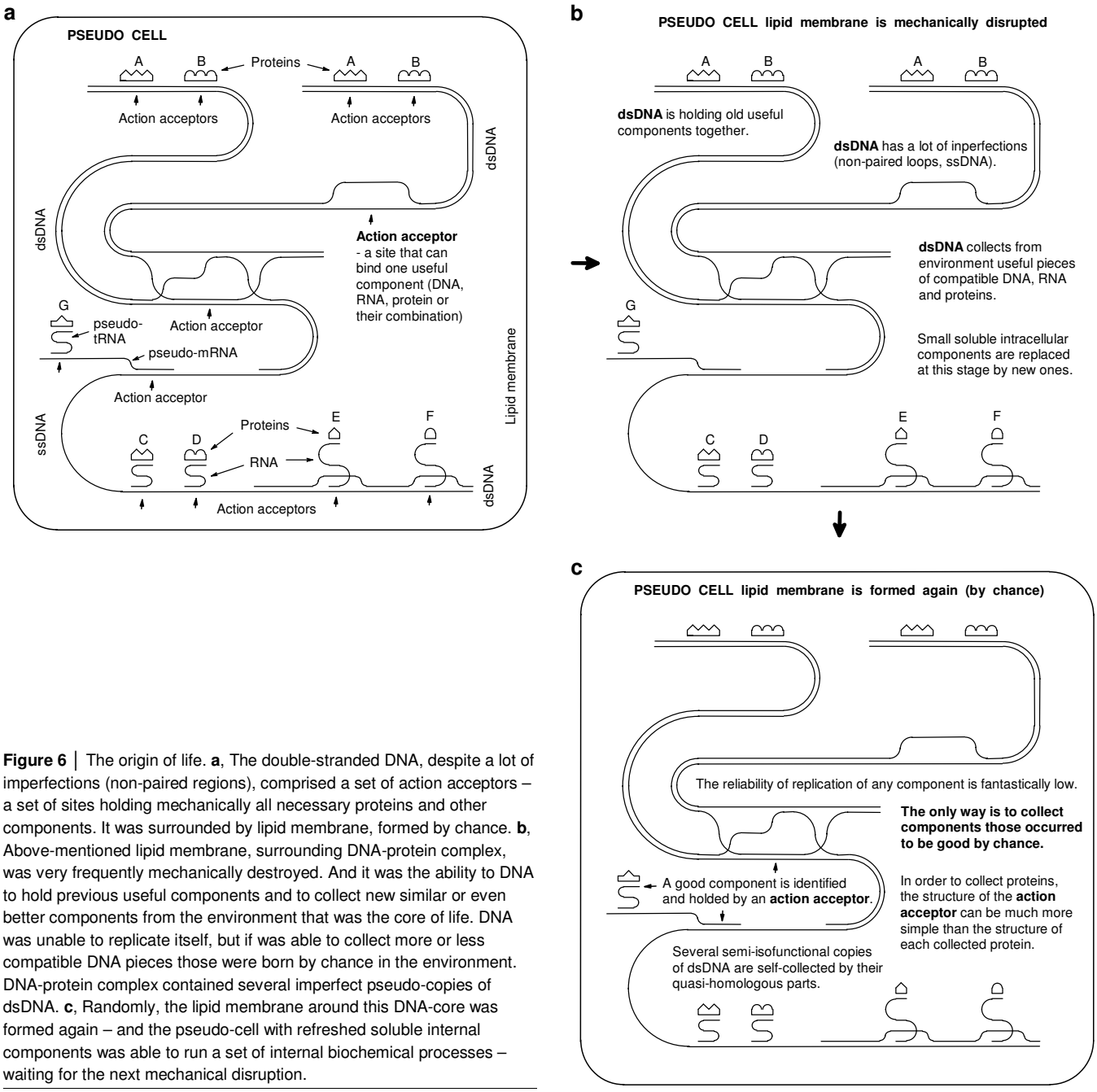

Figure 6 | The origin of life. **a**, The double-stranded DNA, despite a lot of imperfections (non-paired regions), comprised a set of action acceptors – a set of sites holding mechanically all necessary proteins and other components. It was surrounded by lipid membrane, formed by chance. **b**, Above-mentioned 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

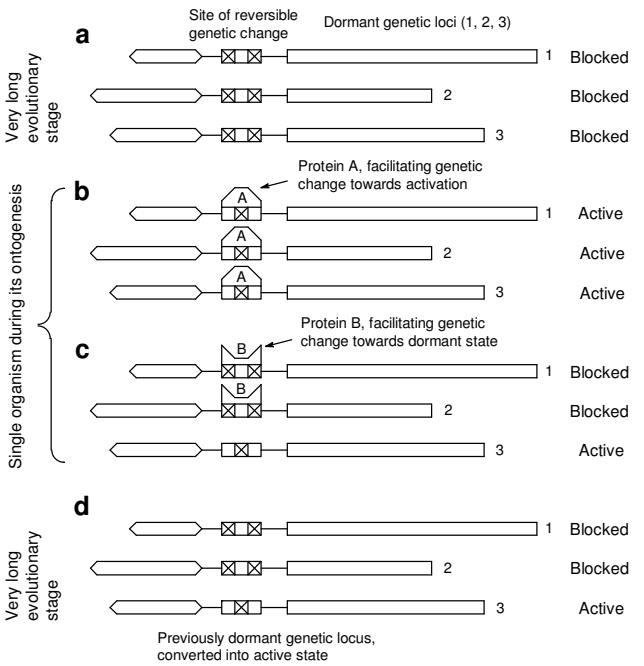

Figure 7 | Activation of previously dormant genetic locus in evolution. **a**, Three dormant genetic loci, each with reversible genetic change in the area of regulatory sites, are shown. **b**, In a deeply stressful situation the specific protein A is expressed, it binds to the site of reversible genetic 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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Article

Hybrid vigour and hybrid dysgenesis

The effect of early in life enrichment of living conditions

Dmitri L. Vyssotski[1,2,3]

The formation of a hybrid phenotype in mammals remains a mystery. In order to resolve this mystery we have chosen inbred mouse strains C57BL/6J and DBA/2J, and their F_1 hybrid B6D2F1, took only females for experiment and housed them during postnatal days P22-P60 either in standard or enriched living conditions, always 4 mice per cage. This adolescent cage enrichment has evoked either induction of hybrid vigour or its significant enhancement, observed in different operant behavioural tasks during the rest of their life (Morris water maze, Go/NoGo sound frequency and sound duration discrimination tasks). This induction or enhancement can be understood only if ontogenesis is an active process, driven by action acceptors – by entities that percept the achievement of positive developmental results, even if the last ones have appeared with a help of random or unexpected factors of stochastic or genetic nature. The same way action acceptors direct evolution on Earth.

The term "hybrid vigour" defines all superior attributes of a hybrid organism in comparison with similar gender representatives of both parental lines[1-2]. The term "hybrid dysgenesis" defines the opposite – all inferior attributes of an organism in comparison with both parental lines (pp. 76-77[3], 156[3]). Hereinafter we use the word "strain" for inbred laboratory animals (*e.g.* C57BL/6J and DBA/2J mice), the word "stock" – for outbred ones (*e.g.* NMRI mice, Wistar rats, albino and multi-coloured guinea pigs), the word "line" is used to describe both inbred and outbred laboratory animals together, as well as all intermediates, in accordance with recommendations of ICLA-72. The term "good stock" is applicable to healthy outbred laboratory animals, those are good breeders and, as a rule, females from such stock can be used as foster mothers.

Hybrid vigour is typically observed if we have two inbred strains as parents; hybrid vigour is typically expressed as increased body weight and increased "strength" (a bit subjective term, but F_1 hybrid mice in fact can survive in semi-natural outdoor conditions, wherein parental inbred strains cannot survive a winter)[4]. Hybrid dysgenesis is typically observed if we have chosen both parents from two good outbred stocks; hybrid dysgenesis is expressed as decreased lifespan together with various health-related issues, appearing during aging and/or detectable early in life. Among such health-related issues there are over-reaction of immune system, allergies, up to various auto-immune diseases (dogs, cats, guinea pigs), problems with digestive system (dogs, cats, guinea pigs), problems with nervous system (guinea pigs; *e.g.* semi-spontaneous seizures, resembling audiogenic ones), problems with reproductive system (cats, *e.g.* Bengal cats – F_1 and F_2 infertility in males). Bengal cats are becoming more and more popular today as pets, and their F_1-F_4 generations can serve as a good illustration of hybrid dysgenesis in mammals, but the same or about the same hybrid dysgenesis is observable in guinea pigs at much low cost.

Two brief conclusions concerning hybrid dysgenesis – one practical and one theoretical: 1) hybrid dysgenesis is evident in species those whole lifespan is practically accessible, and laboratory mice and rats do not belong to this category; 2) hybrid dysgenesis is expressed as problems in regulation in one or several functional systems, these problems can be expressed differently in different subjects of the same cross and sometimes

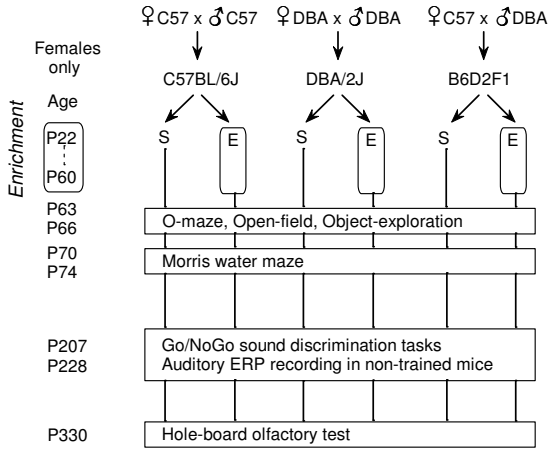

Figure 1 | Breeding paradigms, cage enrichment and behavioural tests. Female mice (strains C57BL/6J, DBA/2J & their F_1 hybrid B6D2F1) were housed during postnatal days P22-P60 either in the cages "Type 2a" (365 × 207 mm) – "Standard" or in the cages "Type 4" (595 × 380 mm) with different toys renewed twice weekly – "Enriched"; always 4 mice per cage.

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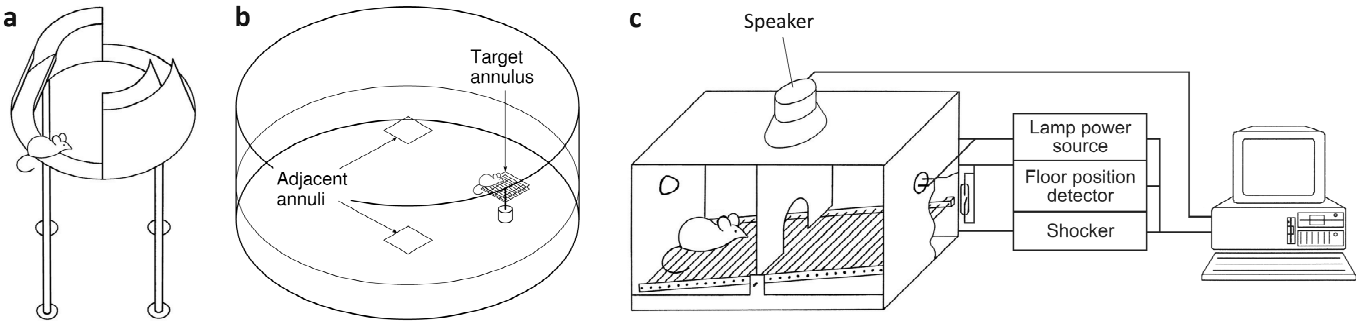

Figure 2 | Equipment for behavioural tests. (**a**) Elevated 0-maze (D = 46 cm, elevation h = 40 cm; 5 min test). (**b**) Morris water maze (d = 150 cm, walls H = 50 cm from the bottom; water level (+ 1 L of milk) h = 15 cm; platform 14 × 14 cm placed 0.5 cm below the surface; annulus – square 16 × 16 cm). The mice performed 16 training trials in 4 days (4 daily, max. duration of each trial 90 s, with an inter-trial interval of 30 s spent on the platform – massed training). On day 5, the mice performed a 60 s probe test without the platform. (**c**) Go/NoGo sound discrimination task (box 270 × 115 × 130 mm with two parts; arch opening 38 × 49 mm) had 40 Go and 40 NoGo daily trials with 7 training days for both sound frequency and duration discrimination tests.

it is practically impossible to discriminate between primary and secondary problems in different affected systems of one animal. Animals of the same cross can demonstrate very different abnormalities and during lifespan of a single individual an abnormality can be sometimes expressed stochastically in all-or-none fashion, *i.e.* it can be unstable in time.

Traditional explanation of hybrid vigour is based on mechanistic interaction of previously dissociated genetic elements, whereas the unstable and destabilized expression of hybrid dysgenesis is pointing out to epigenetic mechanisms[5-8]. If epigenetic interactions have prevailing influence on hybrid phenotype, then its ontogenesis should be sensitive to external influences. In order to test this opportunity we have chosen two inbred mouse strains: C57BL/6J and DBA/2J, and their F_1 hybrid B6D2F1, obtained from cross: ♀C57BL/6J × ♂DBA/2J.

We have selected only females due to practical reasons (the absence of fights) and placed one half of them into enriched living conditions[9] at P22 (one day after weaning) and they were removed from the enrichment at P60, three days before the beginning of behavioural tests (P63) – thus, the whole adolescent period was included into the P22-P60 enrichment period (**Fig. 1**).

Sometimes such cage enrichment is thought as a tool that makes life of a mouse closer to the wild nature. In wild mouse populations (*e.g. Apodemus sulvaticus*), both in the USA (upstate NY) and Russia (Tver region), a lifespan of a mouse is terminated by an interaction with an aerial or terrestrial predator, and the rate of reproduction is determined by food availability, which is always scanty (mammals are horny when they are fed *ad lib*; when they are not fed *ad lib*, they are not so horny). A wild-caught mouse has big head (in comparison with laboratory one), attached to under-developed body, because it needs brain to predict the appearance of a predator, and it has small body due to malnutrition, because any search for food is risky. In a laboratory mouse the lifespan is not determined by an interaction with a predator and the rate of reproduction is not limited by food availability. Thus, we are using cage enrichment only as a tool to reactivate some epigenetic mechanisms.

Elevated 0-maze was the first test that was applied after the end of enrichment period (**Fig. 1**). This test measures the anticipation of an interaction with an aerial and/or terrestrial predator in the particular environment by a mouse (**Fig. 2a**, **Fig. 3a**). Hybrid non-enriched mice have the strongest anticipation of

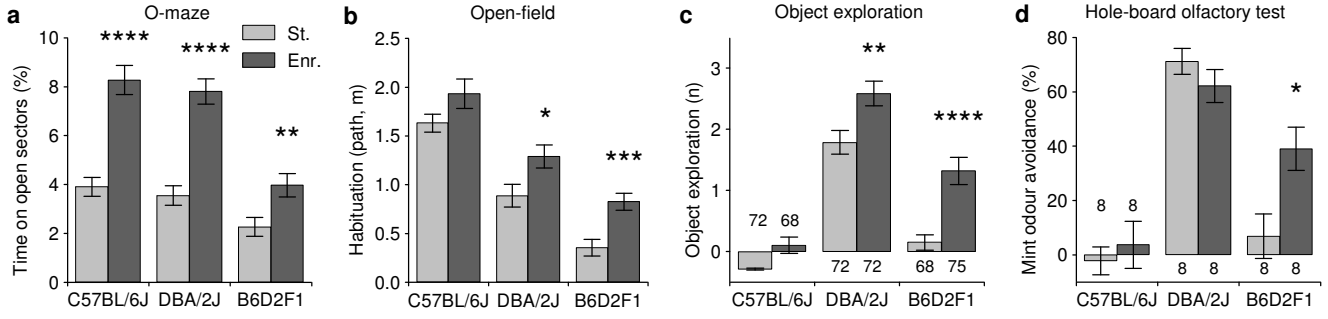

Figure 3 | Exploratory behavioural tests. (**a**) Elevated 0-maze. (**b**) Open-field (arena 50 × 50 cm, wall h = 37 cm; 30 min). "Habituation (path, m)" – the difference in the path travelled between the first and the last 10 min. (**c**) Object exploration (the same arena) – 24 h after the open-field test the animals were tested during 30 min once again, but during the last 15 min a semi-transparent 50 ml Falcon tube (h = 12 cm, d = 4 cm) was placed vertically in the centre of the arena. "Object exploration (n)" – the difference in the number of small movements in the object zone between the last and the first 15 min. (**d**) Hole-board olfactory test (arena 40 × 40 cm, 16 holes d = 2.5 cm, wall h = 32 cm). This test was done after usual hole-board test without odour that consisted of 3 days, one 6-min session daily. During the fourth day under the one half of the floor a dry Mint powder was added. Mice avoid Mint odour. Avoidance (%) was calculated during 6-min session using total exploration time of holes with (O) and without (NO) odour: ((NO – O)/(NO + O)) × 100. Hereinafter: asterisk, $P < 0.05$; double asterisk, $P < 0.01$; triple asterisk, $P < 0.001$; quadruple asterisk, $P < 0.0001$. Mann-Whitney U-test. Mean ± SE.

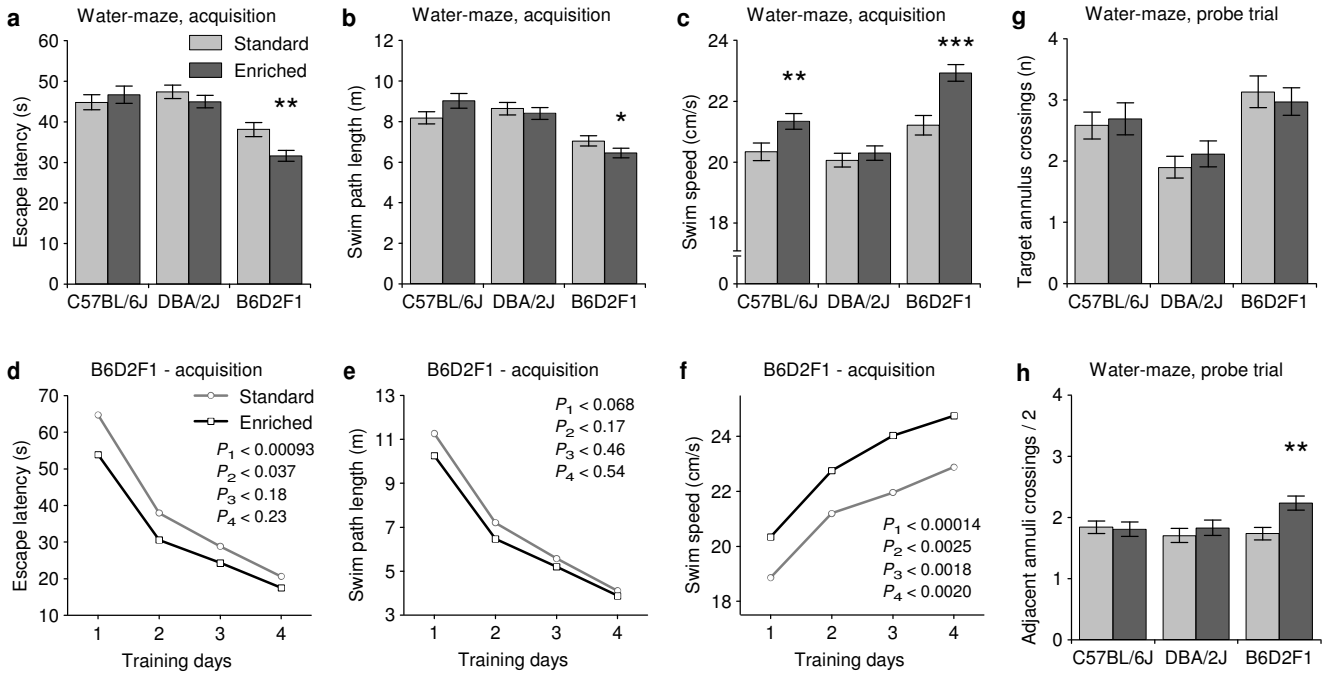

Figure 4 | Morris water maze. (**a**-**c**) Mean values of four training days. (**d**-**f**) Mean values of each training day separately for hybrid B6D2F1 mice. Similar values for inbred C57BL/6J and DBA/2J mice are shown in the **Supplementary Fig. 4**. (**g**-**h**) Probe trial (60 s without platform, day 5). Note that during the probe trial, the hybrid mice have shown the increased number of adjacent annuli crossings – however the platform was never placed here and it is not the memory, but the anticipation of the future – the mice believe that the platform should be here with higher probability than in other places. Mice never had material evidence for such anticipation, but nevertheless their idea leads to better overall performance (**a**) and shorter swim path (**b**).

such a dangerous event (**Fig. 3a**, the shortest bar). The enrichment does decrease the anticipation of an interaction with a predator in both inbred mouse strains, with very high statistical significance (**Fig. 3a**, the two longest bars), but the same enrichment only slightly potentiates such potentially dangerous behaviour as the presence on open sectors in hybrids, and the enriched hybrids finally show the same anticipation of a predator as non-enriched inbred mice (**Fig. 3a**, the most right bar). Thus, if the effect of enrichment is potentially dangerous – it is minimal in hybrids, and it looks like the effect of enrichment is controlled by a prediction from the side of a mouse.

In the next test (Open field) the non-enriched hybrids demonstrate the slowest habituation (**Fig. 3b**), but the effect of enrichment is the most pronounced in these animals. After the introduction of a new object into this open field (**Fig. 3c**), we can see that the enrichment has converted B6D2F1 phenotype from C57BL/6J-type into DBA/2J-type. All three above-mentioned tests were done soon after the end of enrichment period (**Fig. 1**), and here the effects of enrichment could be considered as "temporal", but not "ontogenetic" (they are, in fact, ontogenetic, but we cannot say this on the basis of these three tests).

The olfactory test with Mint odour avoidance was done 9 (nine) months after the end enrichment period. During all these 9 months all animals were housed in standard cages. Nevertheless, the Mint odour avoidance was converted in the hybrid mice from C57-type towards DBA-type (**Fig. 3d**). Statistical significance is not very high here, because we have 8 mice in each group only, contrary to 9 independent batches [each with 8 mice per group] in early tests [0-maze, Open field, Object exploration and Morris water maze], wherein n = 72, 68, 72, 72, 68, 75 (**Fig. 3c**).

The interpretation of all exploratory tests, with some exception of 0-maze, is always controversial, because it is unclear which type of behaviour is "better"; there are no objective means to discriminate between the "superior" and the "inferior". We have chosen two operant behavioural tasks with negative reinforcement – Morris water maze (**Fig. 2b**) and Go/NoGo sound discrimination task (**Fig. 2c**) – those provide clear distinction between "good learners" and "bad learners".

In the Morris water maze all mice have to learn how to find a platform, covered by water made opaque by an addition of milk (**Fig. 2b**), using several trials. The presence in the water, despite it is not very cold, is aversive for a mouse and the mouse would like to find a platform as soon as possible. The escape latency serves as a main indicator of performance (**Fig. 4a**). Classical hybrid vigour is evident without any enrichment (**Fig. 4a**, the light bars), whereas the enrichment has developed the existing hybrid vigour even further, but the positive effect of enrichment was evident only in hybrids, but not in the inbred mouse strains (**Fig. 4a**, the dark bars).

The improvement of performance by means of early in life enrichment was possible only for hybrids. The enriched hybrids had not only shorter escape latency (**Fig. 4a**), but shorter swim path length (**Fig. 4b**). The enriched hybrids had also increased swim speed, observed during all four training days, and it cannot be explained by slightly shorter swim path length due to relatively high statistical significance of the increased swim speed (**Fig. 4c**). The swim speed was also slightly improved by the enrichment in the inbred C57BL/6J mice (**Fig. 4c**), but no other enrichment effects were observed in the Morris water maze in the inbred mice.

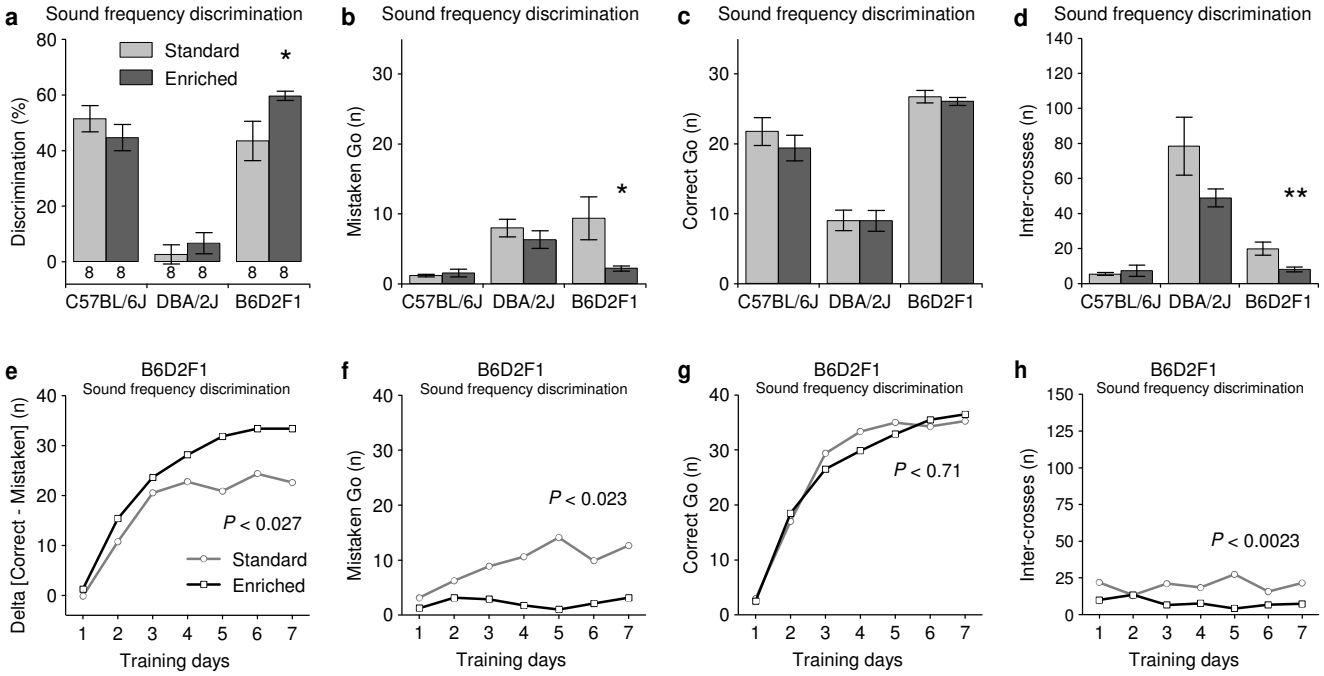

Figure 5 | Go/NoGo sound frequency discrimination task. "Go" signal consisted of two sounds: 50 ms 2.5 kHz and 50 ms 10 kHz, which were separated by 200 ms of silence. "NoGo" signal consisted of two identical 50 ms 5 kHz sounds separated by 200 ms of silence. Each "Go" trial consisted of 5 "Go" signal presentations with inter-signal interval 1 s (onset-to-onset). But if the animal did not move to the opposite compartment, it received additional "Go" signal presentations (maximum 5), paired with negative reinforcement – with electric current, 200 ms, 0.20 mA. Each "NoGo" trial consisted of 5 "NoGo" cue presentations. If the animal was moving to the opposite compartment during these 5 sec, it received negative reinforcement – current 200 ms, once.

It is interesting to note that simple combinatorial model of hybrid vigour is not working for the results of Morris water maze: if some genetic elements were randomly fixed in the C57BL/6J genome, and some others – in the DBA/2J genome, and if they were combined together in the B6D2F1 hybrid like a key-lock interaction, then we should expect to see already full hybrid vigour in the non-enriched hybrids, and the enrichment should be able to do nothing for its further improvement.

The second mystery is that we can see specifically the improvement, but not the degradation of performance in all hybrids (both enriched and non-enriched). The fixation of genes in an inbred strain is basically a stochastic (random) process, with negligible effect of natural and artificial selection. And it is statistically impossible that two randomly selected groups of genes being combined together in hybrids will produce superior functional system without a help of any purposive activity (at least, with the same probability the effect will be negative as well as positive). These two arguments lead us to the assumption that the development of hybrid vigour, as well as ontogenesis in general, is an active and purposive process.

During the probe trial the platform was removed from the tank for the whole 60 seconds of testing and all mice were searching for it without any positive result. No effect of enrichment was observed here (**Fig. 4g-h**), except one curious observation: the number of adjacent annuli crossings was significantly higher for enriched hybrids than for all other mice (**Fig. 4h**). Usually water maze is classified as a test for spatial memory. However the enriched hybrid mice have demonstrated here not "better memory" (the platform was never placed into the adjacent annuli for any given mouse), but "better anticipation" of the future.

The fact that the individual behaviour of an animal is driven by an anticipated future has been recognized by Peter K. Anokhin many years ago (before the World War II), on the basis of his experiments with dogs. The term "action acceptor" was introduced by Peter K. Anokhin in 1955[10-11] to describe the entity that senses the appearance of the anticipated result (typically – positive result – the animal is in search for this result). An action acceptor plays similar role in ontogenesis, including early ontogenesis: if a group of cells is in search for some result that could be, for example, some mechanical tension of cell layers in early ontogenesis, as soon as this result is achieved/sensed by a sufficient number of cells, the rest of the cells and/or the cells that have achieved the above-mentioned result are switching their efforts to search for the next anticipated ontogenetic result.

Action acceptors, as well as other components of phenotype, can be partially genetically determined, partially learned or induced by local or external environment of the organism or environment of given cell group. The most important thing is that not only ontogenesis, controlled by a sequence of action acceptors, becomes more robust to external and internal disturbances (to so-called "developmental noise"), but the results of ontogenesis can be improved by unexpected events[12]; the ontogenesis can utilize or it can extract unexpected benefits from random/stochastic developmental deviations and from the appearance of new unexpected entities in the genome of this organism. Exactly the same new/unexpected genetic entities are present in the hybrid genome. The functionality of the ontogenesis of Metazoa is based on the action acceptors to the extent that without developmental noise (variability in the

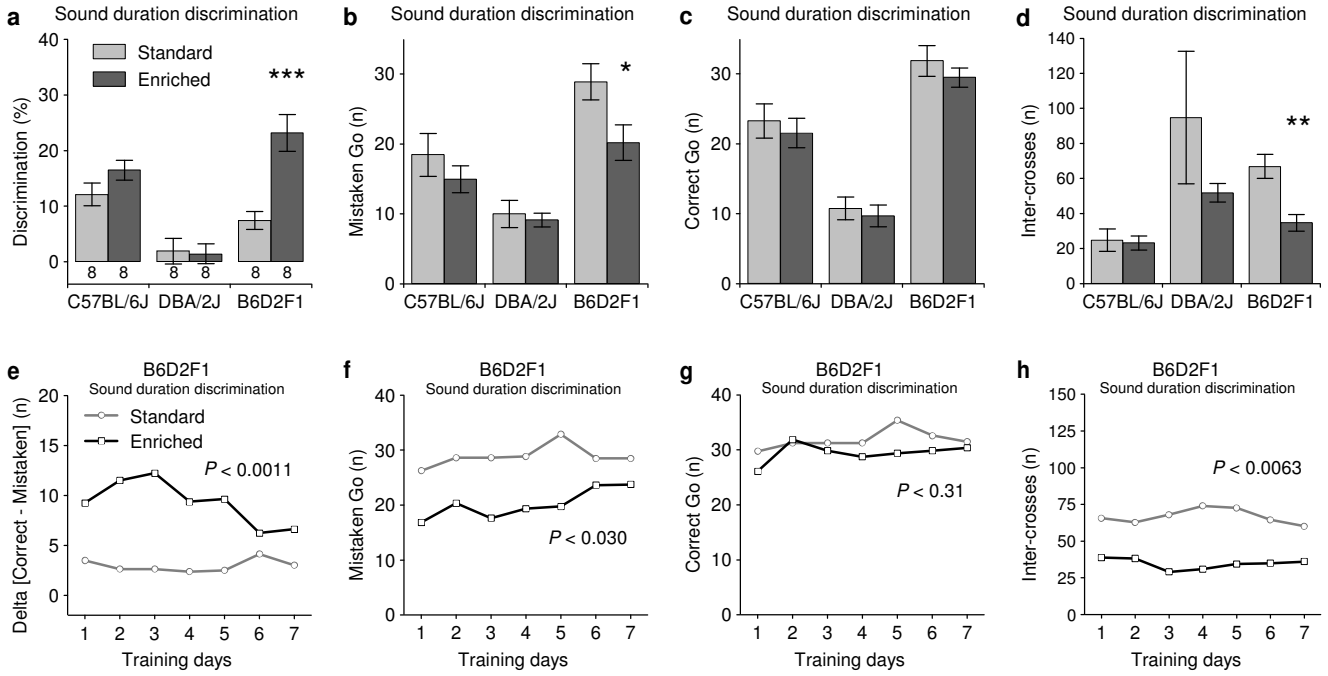

Figure 6 | Go/NoGo sound duration discrimination task. After Go/NoGo sound **frequency** discrimination task (**Fig. 5**), wherein animals were trained during 7 days (40 "Go" and 40 "NoGo" trails daily) to discriminate pairs of sound 5-5 kHz and 2.5-10 kHz, and 7 days of task-free period, the same animals were trained in Go/NoGo sound **duration** discrimination task, also during 7 days (40 "Go" and 40 "NoGo" trails daily). "NoGo" signal was taken from the sound frequency discrimination task. "Go" signal consisted of two sounds: 50 ms 5 kHz and 150 ms 5 kHz, separated by 200 ms of silence. An animal should be able to discriminate the duration of the second sounds – 150 ms in "Go" and 50 ms in "NoGo". This is a very difficult task for all mice.

individual behaviour of the cells that is not genetically fixed) the ontogenesis as a process becomes impossible (Supplementary Fig. 9[13]). And, in principle, the same anticipated result can be achieved by different ways, of course. That is why we have remarkable individual variability in human brain functional morphology (fields, *etc.*).

Go/NoGo sound discrimination tasks, as well as all other Shuttle-box-based tests, were always criticized for being non-ecological for a mouse. During this task mouse learns to go from one compartment to another one during presentation of one sequence of sounds and it learns to stay in the same compartment during presentation of another sequence of sounds, whereas during the absence of any sound sequence presentation the mouse can change compartments freely. Despite the absence of any analogues of this task in the wild nature, the enriched hybrids show superior performance with respect to all other mice in both sound frequency (**Fig. 5a**) and sound duration (**Fig. 6a**) discrimination. In both tasks the enriched hybrids have significantly decreased number of mistaken Go in comparison with non-enriched hybrids (**Fig. 5b,f**, **Fig. 6b,f**) It seems that only the early in life enrichment makes hybrid vigour evident in this Go/NoGo sound discrimination task (*i.e.* no hybrid vigour without enrichment; **Fig. 5a**, **Fig. 6a**), and this test was done 5 (five) months after the end of 38-day enrichment period (**Fig. 1**). How on Earth a random combination of genetic factors plus adolescent enrichment entails superior performance in absolutely

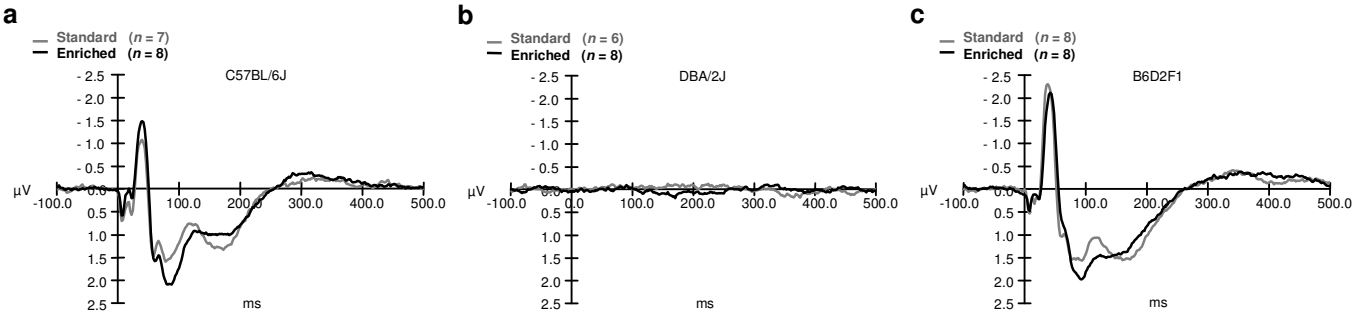

Figure 7 | Auditory evoked potentials. The record was done from the surface of primary auditory cortex in Standard and Enriched C57BL/6J, DBA/2J and B6D2F1 mice. These mice were never trained in Go/NoGo sound discrimination paradigm. This is a grand-average of four paradigms, wherein the stimuli had duration either 50 or 150 ms and consisted of accords either 3 + 6 kHz or 4 + 8 kHz with inter-stimulus interval (onset-to-onset) 500 ms. Note that the enrichment did not change the amplitude of N1 (25 - 50 ms) and produced non-significant similar alterations in P2 (50 - 200 ms) in C57 and F1.

non-ecological task (**Fig. 6a**)? It remains a mystery, unless there are action acceptors which can consolidate functional systems from unexpectedly available components. Sometimes functional systems are thought to be some systems with feedback loops (after cybernetics), wherein the current process is manipulated from the side of the action acceptor in order to achieve the positive result, detectable by the above-mentioned action acceptor. However the described above function of an action acceptor is deeply secondary: feedback can be weak, feedback can be strong, feedback can be absent at all and the positive result can be achieved randomly, but as soon as it is achieved the system is switching to the search for the next ontogenetic result – that is the main function of an action acceptor.

Note that the enriched hybrids have decreased number of inter-crosses in comparison with non-enriched ones (**Fig. 5d,h**, **Fig. 6d,h**), *i.e.* they have decreased spontaneous locomotor activity, whereas in Morris water maze they always have increased swim speed in comparison with all other animals (**Fig. 4s,f**), *i.e.* they have enhanced locomotion. These observations cannot be explained together, unless we are dealing with purposive behaviour in both cases.

If ontogenesis is under significant control of action acceptors those are at least partially heritable and are at least partially genetically fixed, the same action acceptors must be active on the evolutionary time-scale, the same action acceptors are directing evolution. If from a randomly available pool of genetic components some can be activated to serve as a reminder about action acceptor, or to serve as its part, or to comprise the action acceptor as a whole, then evolution becomes internally purposive (as well as ontogenesis currently is) and Darwinian natural selection occurs to be a process of minor importance.

Any action acceptor contains in itself the part that is an anticipated future, and this part is not material at the particular time point of the existence of this action acceptor (**Supplementary Fig. 1**). Here we are at the border of the contemporary natural sciences, at the border between vulgar materialism and religious idealism, and further discussion can be placed only in the **Supplementary Information**.

Methods

Freshly weaned females (C57BL/6J, DBA/2J & B6D2F1) were ordered from Taconic M&B A/S, Ry, Denmark. Received mice had the following body weights: C57BL/6J: 9.71 ± 1.65 g; DBA/2J: 9.33 ± 2.16 g; B6D2F1: 9.96 ± 1.76 g (mean ± SD), corresponding well to P21-P22. Upon arrival (on Tuesday), animals were weighed and ear-marked and assigned in groups of 4 of the same genotype to either standard or enriched housing. Mice were housed under standard and enriched conditions during postnatal days P22-P60 in temperature (21±1°C) and humidity (50±5%) controlled conventional colony rooms under reversed 12-12 h light-dark cycle (lights on at 19:00 h) with water and standard rodent pellets ad libitum. Standard housed mice were kept in "Eurostandard Type II L" cages (365 × 207 × 140 mm; polycarbonate, transparent; "L" means "long"; these cages are also known as "Type 2a") with sawdust as bedding. Enriched housed mice were kept in "Eurostandard Type IV" cages (595 × 380 × 200 mm; polycarbonate, transparent; known also as "Type 4") with sawdust as bedding and a "Mouse House" (Tecniplast, Indulab, Gams, Switzerland) as shelter. In addition, twice a week (Tuesdays and Fridays), one enrichment item (autoclaved) was added to the enriched cages. Enrichments added on Tuesdays (when also new cages with fresh sawdust were provided to all mice) remained in the cage for one week until the next cage change (they were so-called "soft enrichments").

Enrichments added on Fridays remained in the cage until the end of the housing period ("hard enrichments"). Soft enrichments included a soft paper tissue (wk 1), a coarse paper tissue (wk 2), a handful of straw (wk 3), a handful of shredded paper in stripes (wk 4), a handful of pieces of bark (wk 5), and a handful of rodent pellets that were hidden in the sawdust (wk 6). Hard enrichments included a wooden tunnel (25 cm long, inner diameter: 4 cm) with several holes (wk 1), a trapeze (12 cm long, diameter: 1 cm) hung from the cage lid (wk 2), three wooden branches (ca. 30 cm long, wk 3), a cardboard roll (15 cm long, diameter: 4 cm, wk 4), and a cardboard house "Shepherd shack" (Shepherd Speciality Papers, Indulab, Gams, Switzerland, wk 5). Thus, enrichment was a combination of more space, additional resources, increased environmental complexity, and novelty (novel items and environmental change). On the last Friday (wk 6), mice from enriched cages (Type 4) were placed in standard cages (Type 2a) until testing started on the following Monday.

Behavioural testing and other procedures are described in **Supplementary Methods**.

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Additional information

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Article

Action acceptor in evolution

The anticipated future in biological evolution

Mit'ka Vyssotski[1-4]

The desired result of any process is represented in an organism by an action acceptor, consisting of two parts: the part which is absent yet and the part that is represented by material reminders of the said action acceptor. The absent part belongs to ideation space. There is no physical time in the ideation space: all entities are given simultaneously. The entities comprise, in general, a computably non-enumerable set – one entity can contain in itself one or more the same or different entities (no conservation laws). This feature provides the possibility to resolve contradictions that are unsolvable in 3D physical space. Evolution proceeds through the interaction of ideation space with 3D physical space by means of action acceptors. The development of each action acceptor precedes the appearance of its positive result by several generations and consists of selection of its material reminders through the self-election of organisms for differential reproduction.

In eukaryotic organisms, the appearance of material reminders of an action acceptor is possible from the pool of dormant genetic loci (or "dormant genes", named so by J.W. Harms in 1929[1,2]) – through the involvement of epigenetic inheritance. The self-selective reproduction, namely reproduction of organisms with better developed action acceptors, can proceed despite possible identical phenotype of all above-mentioned organisms. In other words, even with identical phenotypes (the achieved developmental results), biological evolution can proceed through the differential reproduction, wherein organisms with better developed action acceptors (with higher quantity and quality of the material reminders of the said action acceptors) reproduce better (even when the achieved results of these action acceptors are absent in the ontogenesis of these organisms). Contrary to the differential reproduction, the differential mortality is relatively rare in nature and its evolutionary results are rather limited – in nature the mortality is mainly a statistically random and genotype-independent process, as it was discussed by Nikolai Ya. Danilevski in 1885[3-5].

The existence of an anticipated future is so important for all organisms that it can be used as a definition of life: if an organism has its own anticipated future – it is alive, otherwise it is not alive. Not only animals, but plants, fungi, bacteria and viruses all have their own action acceptors and anticipated future. The interaction between the ideation space and the space of vulgar materialism (our 3D physical space) is important up to the level when it can be used as a definition of consciousness: the consciousness exists only when there is an interaction between ideation space and 3D physical one. There is an unavoidable link between life and consciousness, and evolution of life can be considered as evolution of consciousness, as it was declared by Pierre Teilhard de Chardin in 1955[6].

The statement that the differential reproduction is important for evolution is not new *per se*, however the idea that it provides possibilities for selection of material reminders of action acceptors and, thus, provides possibilities for evolution of the said action acceptors, was not discussed previously. The above-mentioned evolution of action acceptors always goes several generations ahead of the subsequent appearance of the corresponding morphological or other easily detectable phenotypic traits. This in advance development of an action acceptor serves as a basis for the "law of precession of characters" or "nomogenesis", defined as "evolution determined

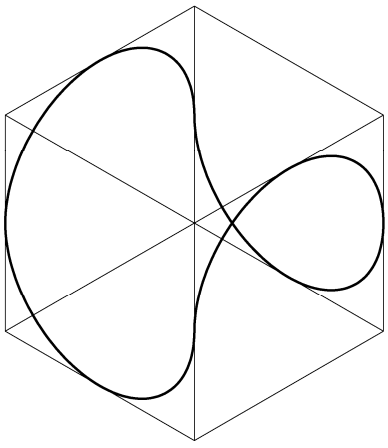

Figure 1 | Problem. Heavy chain or liquid moves without a friction in a narrow tube with speed V_1. The tube is looped in four half-circles around a cube in 3D. What will be the speed V_2 of the said heavy chain or liquid if this contour will be transformed from the said 3D structure into a simple circle in 2D? The total length of tube remains the same. No friction losses.

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by law" by Leo S. Berg (1922)[7]. 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. Evolution is determined by law. The law is determined by action acceptors.

The term "action acceptor" was introduced by Peter K. Anokhin in 1955[8], however the necessity of this entity was recognized by him much earlier and can be traced back to 1932[9]. At that time typical examples for illustration were taken from the behaviour of dogs. Any activity of organisms can be used as a source of examples for illustration of the role of action acceptors: any foraging behaviour, any predator-prey interaction, any reproductive behaviour, etc. We will start with complex human behaviour, but not with reproductive one. Any reference to reproductive behaviour will block further discussion at the beginning (it is impossible to discuss human reproductive behaviour with humans – neither in folkloristic terms, nor in scientific or medical ones) – the role of anticipated future is self-obvious in human love, especially in human females, but the subject is so emotionally super-charged (a reference to Sigismund Schlomo Freud could be placed here), that we will use other examples to be safe. In addition to the overwhelming complexity of this issue we can refer to the observation of Bernard Shaw[10]: he has mentioned that the said entity [human love] is much rare in human population than humans would like to believe. Bernard Shaw wrote on the 6th of August of 1889 with respect to "Tristan und Isolde" of Wagner (pp. 275-276)[10]: "Tristan and Isolda comes off better than Parsifal by just so much as the impulse to play it is more genuine and the power to understand it more common. To enjoy Parsifal, either as a listener or an executant, one must be either a fanatic or a philosopher. To enjoy Tristan it is only necessary to have had one serious love affair; and though the number of persons possessing this qualification is popularly exaggerated, yet there are enough to keep the work alive and vigorous".

We have chosen the following two examples of human behaviour to illustrate the properties of an action acceptor and the features of ideation space.

Cube

The first example consists of the process of solving some problem in the field of classical mechanics (**Fig. 1**). In the process of solving this problem we would like to find an answer and solution – and the answer and solution could not be taken from our memory or other sources (very difficult situation). However the action acceptor – the desired solution and answer – are directing our behaviour, despite they are not known to us yet. Note that this or similar problem could be equally solved in 1725, 1825, 1925 or 2025. And the solution and the answer remain the same. There is no physical time in the ideation space – everything is given in the ideation space simultaneously – as it was mentioned by Mikhail M. Bakhtin[11] with respect to novels of Fyodor M. Dostoevsky, and we can derive the same idea from the contemporary service (*Mahzor Lev Shalem*[12], *Siddur Lev*

Figure 2 | G. Pruefer large-bore clarinet (s/n 4987, circa 1910, USA). It has articulated C#/G#, Selmer large-bore barrel, "Denman 3+" glass mouthpiece, narrow metal ligature and Vandoren Traditional 1.0 reed.

Shalem[13], *Haggadah Shel Pesach*[14], etc). However, some local physical time can always be introduced, if necessary. Another example of the local time introduction can be found in a human song, when a singer first explains a tragic story in a calm voice and then, several seconds later, when a topic and wordings are switched back to everyday life, the expression of emotions goes up to extremely high level, atypical for the everyday life tasks.

Clarinet

The second example is about playing a clarinet (**Fig. 2**). When we are playing this instrument we would like to have nice and clear sound. All human actions, position of human lips, direction of air flow inside mouthpiece are optimized for this purpose. Reed, mouthpiece, ligature and barrel (**Fig. 3**) are all selected (and sometimes from very large spectrum of these items) in order to have the desired sound. This situation is typical for any interaction of material items with ideation space: sound quality depends on material elements and laws of physics; however these material items have been chosen themselves by a human to provide the anticipated sound and this choice was dictated by the ideation space. Some musicians have hundreds of mouthpieces, dozens of barrels and several clarinets. Sometimes even the whole main instrument is chosen by a musician with a large bore, in order to sound like a cello in its lower register – I mean here the old British clarinets that were praised by Bernard Shaw, namely on the 1st of August of 1894 (pp. 116-122)[15]. One such sample was played by John Denman (famous British clarinettist; the glass mouthpiece from the **Fig. 3** was developed by John Denman for large bore clarinets; the shown sample is called "Denman 3+"). Today all mass-produced mouthpieces are for narrow-bore clarinets or, like "Vandoren M30D", – for mid-bore clarinets like Noblet metal clarinets with "raised diamond", "non-raised diamond" or "raised non-diamond". "G. Pruefer Silver Throat Deluxe", compatible with "Vandoren B45 Dot", also should be considered only as a "mid-bore clarinet", in view of large-bore British clarinets, praised by Bernard Shaw.

Figure 3 | Clarinet parts. Box with reeds, barrel, mouthpiece, ligature and single reed. This reed has strength 1.0 and it is called "very soft". The ligature with one screw is prepared from the upper part of standard metal ligature (with two screws). Mouthpiece "Denman 3+" is made from heavy glass ("crystal glass") and it is compatible only with large-bore clarinets.

Clarinet, as it was known to Bernard Shaw at the end of 19th century, has nothing in common with modern instruments. During the 19th century the leather pads were invented. They provided good sealing without previously annoying leaks. At that moment clarinet was played only by musicians who were able to play nicely even with significant pad leakage. Humans were providing compensation by air flow, optimizing its direction, position of lips, etc. And as soon as all leaks were eliminated, the sound of British clarinets was widely praised. A lot of people wanted to buy and play this instrument after the end of WW1.

And they were unable to do so. Clarinet was a large-bore one, designed to provide outrageously beautiful sound in its lower register, whereas all upper registers were intended to be played... by experienced musicians. Now we have a market-driven economy and narrow-bore clarinets, called "professional", but optimized for lazy kids from wealthy families (the main customers for the most expensive instruments today).

Saxophones have avoided this fate only because they all have exactly one octave between registers and random and unintended jumps between them produce less terrible impression.

Clarinet has one octave plus 7/12 of octave (octave + perfect fifth) between registers. Exactly for this reason the term "articulated C#/G#" means that the same key is C# for the first (the lowest) register, but G# for the next one (called "clarion"). And a random and unintended jump between them makes any lazy kid ridiculously funny. This is the reason for degradation (in terms of sound quality, but not in terms of gross income) of the whole clarinet industry in a market-driven economy.

Anticipated sound

However, where the desired sound can be found, where it is "stored"? Contrary to usual sound, which can be recorded by a microphone and stored in a digital or analogue form, the desired sound cannot be investigated by means of physics and chemistry, and, thus, it is not material in accordance with definition of vulgar materialism, introduced by Ludwig Büchner[16] in his book "*Force and Matter*" (1855) many years ago, even before the first publication of "*The Origin of Species…*" (1859) by Charles Darwin[17]. The desired sound can be found in the ideation space. Similar ideas were discussed by Henri Bergson[18] in his book "*Matter and Memory*" (1896), because memory is also an entity from the ideation space. The term "ideation space" (but not the "space of ideas") was not used at that time (introduced much later, at the end of 20th century, by Boris L. Zlotin[19]). However it was Bergson who has correctly pointed out to the main feature of the ideation space: ideation space may contain computably non-enumerable sets of objects. One object can contain in itself one or more similar or different objects and their number is unknown in principle. Many known mathematical theorems are not applicable to such space. The number of dimensions in this space is also computably non-enumerable and it cannot be claimed to be 3D, 4D or any other. Contrary to the ideation space, the material 3D physical space contains usually only computably enumerable sets (number of neurons, number of genes, number of synapses, etc). And some known technical difficulties of calculation, which can be present sometimes, do not make those sets "computably non-enumerable" – they are still fully computably enumerable sets, and the vast majority of theorems of the contemporary mathematics is fully applicable to them.

The anticipated future in simple organisms

When we are speaking about "anticipated future" humans have a tendency to imagine *Homo sapience*, or at least some mammal with higher brain functions, but not a mould or a virus. However it was Nikolai Ya. Danilevski who has pointed out in 1885 that the main question of evolutionary theory is not to explain how a monkey has developed from a frog, but how a frog has developed from a mould (and Darwinism can nothing to do in this respect). When we have a virus with its specific receptor for interaction with particular mammalian cell, we do know that this virus is prepared for interaction with this mammalian cell, even if the cell is absent in the vicinity of this virus during given period of time. It means that this virus has its own anticipated future and this anticipated future includes in itself the anticipated interaction with mammalian cell, even if this cell can never be found (both by virus and by humans – we can consider even this extreme example). It means that the anticipated future determines to some extent the behaviour of this virus and it determines to some extent the evolution of this virus. For the virus the anticipated mammalian cell is exactly an imaginary entity (until the real interaction with it in 3D physical world will happen, at least). When we have a regulatory site of dsDNA for binding of specific transcriptional factor (a protein), we may know that this site is prepared for binding of particular protein, even if this protein is absent or cannot be found in this cell and its nucleus during given period of investigation. For a regulatory site of dsDNA the anticipated transcriptional factor comprises its own anticipated future (which will or will not be materialized with some realistic or non-realistic probability). Anyway we should not think about an anticipated future as about something super-intellectual, because even a plasmid has its own anticipated future (because it has sites for binding of proteins and for interaction with cellular machinery).

Reminders

However if there is one computably non-enumerable set in the ideation space and one, presumably corresponding, computably enumerable set in the 3D physical space, there could not be an unambiguous point-to-point projection from the computably non-enumerable set to the computably enumerable one and *vice versa*. The material entities that could be found in brain or genome can have only status of a "reminder" or, even better, a "material reminder" for computably non-enumerable entities from the ideation space.

The importance of "reminder", however with slightly different meaning, more like a procedure than an element, was extensively discussed and investigated with respect to learning and memory at the end of 20th century by Konstantin V. Anokhin[20,21], a grandson of Peter K. Anokhin. Look at the **Fig. 5e**, where we have a short episode of clarinet record – the transition from the first note to the next one from the **Fig. 4b**. Each note, as we can see from the **Fig. 5a**, has its own "the beginning", "the middle" [of the stream], and "the end", and all these three parts do have their own duration and can be played differently, whereas on paper (**Fig. 4b**) each note is characterized only by some frequency of sinusoidal wave and by its duration. In the record we do have very complex signal, which is also sort of periodical, but it is as far from sinusoidal wave as one can imagine – and it

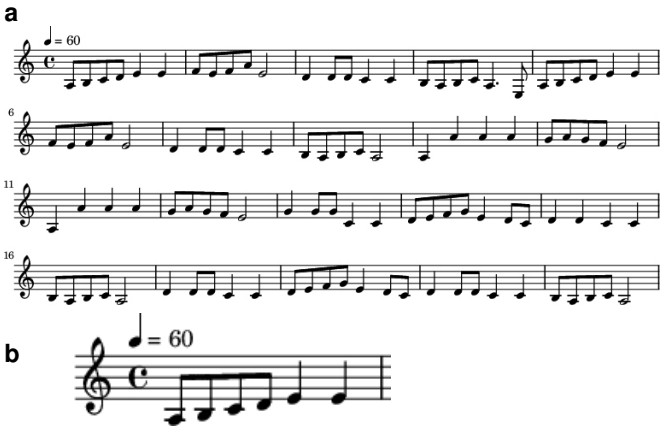

Figure 4 | Musical pieces for clarinet. The lowest note in (**a**) is the lowest one that can be played by this instrument. References to audio files:

http://www.evolocus.com/evolocus/v1/evolocus-01-037-sa1.mp3 – MP3 file, 16-bit, 48 kHz sampling rate – small file, good for listening (3.2 MB) – clarinet sound of the piece (**a**) (recorded by mid-side technique).

http://www.evolocus.com/evolocus/v1/evolocus-01-037-sa2.wav – WAV file, 24-bit, 192 kHz – large file, the same record; the next **Fig. 5** was prepared using "Mid" (the 1st) channel of this file (112.9 MB).

is only a simple transition from one note to another one played by a rather primitive instrument (**Fig. 2**), which cannot play chords, for example, contrary to a piano. However, the phase of the sound wave is preserved here during this transition, as we can see from the **Fig. 5e**; and humans can feel this preservation of the phase as pleasant. This record was done by Warm Audio WA-87 condenser microphone. The signal passed through Neve Portico 5012 preamplifier and after very mild compression by Neve Portico 5043 compressor, placed after preamplifier, it was digitized by LynxTWO-A audio card at 192 kHz with 24-bit resolution (further details can be found in the **Methods** section).

Any notes, printed on paper, are only reminders for music, but they do not contain music themselves. The anticipated sound belongs to a computably non-enumerable set, whereas the already recorded sound contains only computably enumerable entities. A single note can be played by a plurality of ways and these ways comprise a computably non-enumerable set. However it will be an erroneous simplification if we will say that a playing human has some "anticipated sound" and that this player is just "pushing" the "real sound" (that can be recorded by a microphone) towards the anticipated one. The above-mentioned process does exist, of course. However in addition to it, the production of sound using a clarinet or cello involves a lot of procedural memory. And this memory is only to some extent under the direct feed-back-loop control in real time, because it was mainly acquired in advance, during previous self-training. It means that the performance is always slightly out of direct control of a human consciousness, the consciousness of a player. That is why it is so interesting to play music. When we are playing, it is seems to us that the music is played, just a little bit, by somebody else. And this "somebody" can sometimes play even better than we can think about ourselves!

If our performance would be based solely on declarative memory (this is the memory which we are trying to use solving problem about speed during the conversion of 3D movement into

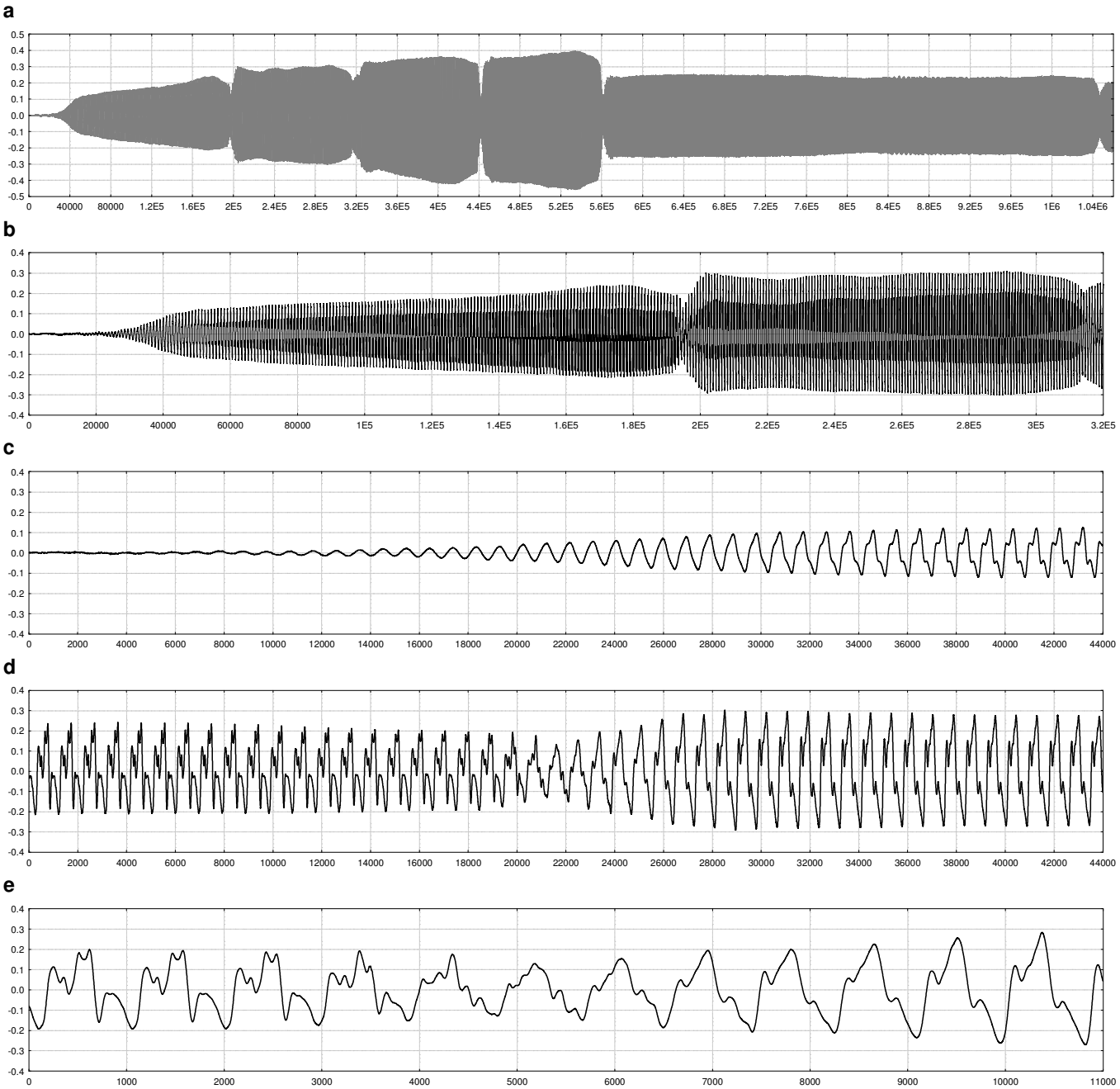

Figure 5 | Clarinet sound records. Abscissa – counts of ADC (192 kHz); ordinate – linear amplitude (24-bit original resolution). (**a**) The first measure (six notes, **Fig. 4b**) of the **Fig. 4a**; it contains slightly more than one million counts of the analogue-digital converter (ADC). (**b**) The first two notes. (**c**) The beginning of the first note. (**d**) The transition from the first note to the second one. (**e**) The same as (**d**), but shown with higher temporal resolution.

2D, **Fig. 1**), we will be able to hear nothing new, nothing unexpected in our own music. However due to the procedural memory (it is called sometimes, especially in the field of neuro-linguistic programming (NLP)[22,23], a "kinesthetic memory") we can hear sound as it would be played by some "other", by another human being. And this is a miracle! And there is one more complexity on the top of this: when we are playing music, it represents not our spirit, but a soul of another person, the soul of a character of this musical piece. So, it is not only feels like music of another human being, but it is officially and *de facto* a representation of another soul. A human "soul" is always a representation of human spirit in a consciousness of another person (or G-d, as it was mentioned by Mikhail M. Bakhtin[24]). All published articles of Bakhtin are strictly secular; however he was definitely familiar with religious thought-style [*Denkstile*, after Ludwik Fleck[25]], contrary to many contemporary scientists.

As soon as we have a computably non-enumerable set, we automatically do not have in this set any conservation laws, such as physical laws of conservation of energy, conservation of impulse, conservation of the moment of impulse, conservation of

charge, etc. We can speak, for example, about "psychic energy", if we would like to do so, but there is no "law of conservation of psychic energy", because the psychic energy, being mostly an entity from the ideation space, can appear from nothing and it can disappear without a trace in the middle of nowhere.

Contradictions

Biological evolution rather often proceeds through differentiation, when something (one entity) is splitting into something different (two slightly or significantly different entities). In many cases the differentiation is driven by a contradiction. However, in a living organism something remains always unknown or hidden from us. It is easier to understand the role of contradictions in differentiation using evolution of technical systems. Technical systems are basically known for humans, because they were constructed, but not discovered. A contradiction between positive effects and cost factors is among the most popular ones. A contradiction between performance and efficiency is not rare either. When we are trying to improve something, something else is going to be disrupted, degraded or just will not be good anymore. Let's consider two examples.

Performance cores and efficiency cores in processors

In the evolution of processors (CPUs) the first central processing unit (CPU) had only one core. Then, processors with two and four cores were introduced. In a stationary system, with mild limitations of power usage, all cores can be perfectly identical. However in a mobile system, like in a phone, which should not be in a completely "off" state, the requirement of lower power consumption and high performance are in contradiction with each other: it means that when we would like to increase performance, we will increase power consumption, and when we would like to increase efficiency, we will disrupt performance. Some optimization is always possible, even with a single core or with a plurality of identical cores, which will have slightly better performance and slightly lower power consumption. However, the above-mentioned contradiction was in fact resolved, when the first processor with two categories of cores was introduced. The most remarkable example had two "performance" cores, optimized for the best performance, and two "efficiency" cores, optimized for the best efficiency. It was so called "A10" "Fusion" processor, developed for "iPhone 7" by "Apple" company at the beginning of 21st century. It was using either two "performance" cores or two "efficiency" cores, but "performance" and "efficiency" cores were never active simultaneously. Later processors, like Intel processors for desktop and laptop computers, were able to use "performance" and "efficiency" cores simultaneously, but we will not discuss their further evolution here. In order to avoid a simplistic conclusion, the second example is always necessary.

Honda VTEC engine in cars

At the end of 20th century the first mass-produced combustion engine with VTEC ("Variable Valve Timing & Lift Electronic Control") was developed by Honda, and we will use some version from the year 1989 as an example (**Figs. 6-8**). A combustion engine, in addition to crankshaft with pistons,

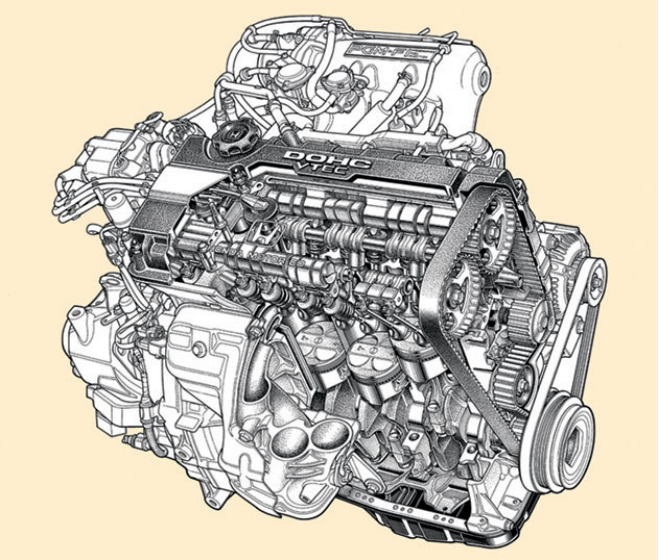

Figure 6 | VTEC engine. VTEC (Variable Valve Timing & Lift Electronic Control) engine was developed by Honda and introduced in 1989 in mass production. Note 3 cams (1 central & 2 peripheral) for each two valves.

always has at least one camshaft which opens and closes intake and exhaust valves by means of corresponding cams. A form (a profile) of a cam can be optimized for given speed of crankshaft and camshaft rotation, taken into account air flow and its properties; it can be optimized for the best performance – the highest possible torque. However when the speed of crankshaft and camshaft rotation goes up several times (let's say from 2000 RPM to 6000 RPM), we need to open valves wider and for a longer period of time, in order to have the highest possible torque at the highest possible rotation speed. It is obvious that one single cam, one single profile, cannot be optimal for both high and low speed of rotation simultaneously.

This contradiction cannot be solved with single cam profile. That is why it was invented a camshaft with two different cams (with two different profiles) for a given valve, and a special mechanism, manipulated by oil pressure, was developed in order to engage one or another cam to handle this valve. At the level of the whole engine it was one set of cams of camshaft to be in use at low rotation speed and another set of cams of the same camshaft to be in use at high rotation speed. The first VTEC engines were optimized by Honda for the highest performance (highest torque) for both high and low rotation speed, and were very good for cars with manual transmission (I still remember "Rover 623si Sport" with Honda engine (2.3L non-turbo) that was able to reach 220 km/h between Zürich and Bern and 230 km/h between Zürich and Rome, at some parts of the way). Later, taken into account an automatic transmission, it was possible to optimize cams for low rotation speed for high economy, whereas cams for high rotation speed – for high performance, and several other steps were possible, but we will not discuss further evolution.

If we look at any technical system or living organism – it contains only computably enumerable set of elements. In our previous example it is either old engine with one single cam per valve or new Honda VTEC engine with two different cams per

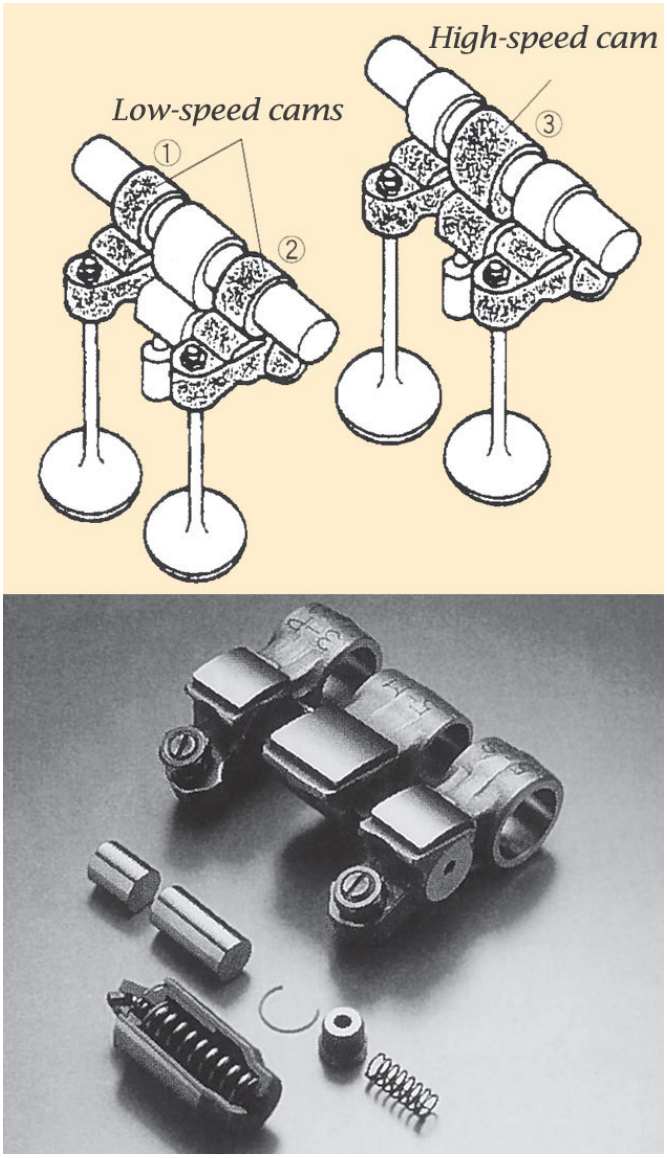

Figure 7 | VTEC schema & photograph. Two cylindrical pins are moving (↔) by application of different pressure of engine oil by a control system.

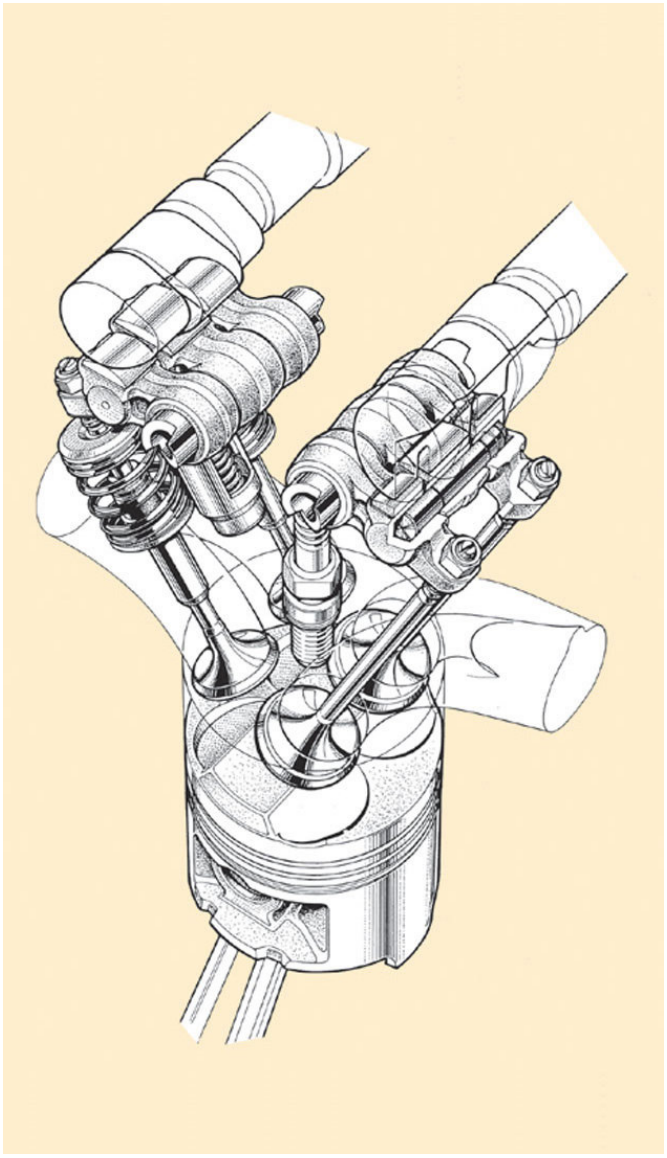

Figure 8 | VTEC drawing. One piston is shown with four valves (2 intake and 2 exhaust valves) and two camshafts (intake & exhaust camshafts).

valve – it is always a computably enumerable set in 3D physical world. There is no splitting or emergence of two different cams from one cam and no splitting or morphing of one processor core into two different cores in our material world. Cams of different types and cores of different types are manufactured as different pieces from the beginning of their technological process. However the above-mentioned splitting, emergence, division or morphing was done in the ideation space, where it is technically possible, due to a computably non-enumerable set. The solution was found in the ideation space, where the entities are not computably enumerable. Only afterwards the same solution was materialized in computably enumerable set in 3D physical world.

Ideal functional system

Contradictions are always present in evolution of any technical system and in evolution of any functional system of a living organism. And if those contradictions would be handled only by means of optimization, but not by means of resolution (which is possible sometimes only in a computably non-enumerable set), the results of evolution would not be so impressive, as we can see now, both in the field of technical progress and in biological evolution. The similarities between technical progress and biological evolution exist due to common driving factor: a drive towards ideal technical system or ideal functional system of an organism. The ideal system is a non-realistic system that contains only positive effects/results, whereas all negative effects or cost factors are equal to zero. Ideal system has no weight, it cannot be broken, it does not require any energy, and it does not require any time for its ontogenetic development, however it produces the desired positive result/effect where and when it is necessary. And because an ideal system typically cannot be found anywhere (it is an idealization *per se*), any real system can evolve further through not only its own optimization, but through

resolution of contradictions between its positive effects and cost factors, those always can be found. And in order to resolve contradictions, we have to go to the ideation space. Ideation space is in use by all organisms, which do have their own anticipated future and remembered past, *i.e.* by animals, plants, fungi, and by all unicellular organisms and viruses.

Action acceptor and hopeful monsters

An action acceptor is necessary for two purposes: to resolve a contradiction and to materialize in ontogenesis the result, the solution of the contradiction that is already resolved. The role of action acceptor in resolution of contradiction is similar to its role in solution of any problem with atypical appearance, like problem from the **Fig. 1**. Thus, it is a rare event and a field of "hopeful monsters" of Richard Goldschmidt[26,27], who has entitled his book as "*The Material Basis of Evolution*", keeping in his mind that there is also "a non-material basis of evolution", and it is even more important than the material one (Goldschmidt has received traditional education; that is why we are sure in our speculations). Now we do know that biological evolution always goes through the interaction of the ideation space with the space of vulgar materialism. And our current task is to show how exactly it proceeds in very different species, just from the beginning of life, literally.

Differentiation in ontogenesis

Ontogenesis of animals, plants and fungi, and *Metazoa* in general, was always known as a rather stable and canalized process, where the final functionally (useful phenotype) can be achieved despite unexpected mutations, sometimes mechanical damage or chemical poisoning, detectable or experimentally introduced during ontogenesis. When a single group of cells is going to be differentiated into two different ones, one of them is typically can be named "new", whereas another one remains mainly "old". In order to be a successful differentiation, it is sufficient that at one time point all "old" cells, without an exception, were trying to convert into "new" ones, but only the successfully converted cells will send a feedback to the rest of old cells that will stop their further conversion. See Supplementary Fig. 9[28] & its discussion[28]. In early experiments with morphogenesis such feedback was shown to be a diffusible substance (in some cases) and the term "embryonic inductor" (and in the earliest publications – "evocator") was associated with this process. Thus, the "old" cells, those have received this feedback, being morphologically the same as at the beginning of this process of differentiation, are not in the same state.

In old times humans were saying that the "embryonic inductor" has changed the state of these "old" cells, directing them to some other route of differentiation, not towards already achieved and quantitatively sufficient population of "new" cells. We would like to say about the same process that each cell at the beginning of this process of differentiation has an action acceptor, the activated action acceptor at the beginning of this differentiation, and as soon as the desired result is achieved, the cell produces a feedback towards the rest of similar cells, and this feedback "says" to them that this result is already achieved to some minimal extent. Taken into account statistical dispersion, both temporal and spatial, among any group of cells, we can see why ontogenesis can be so well canalized. Nothing can be done really "simultaneously" in early ontogenesis, and the discussed variability at the cellular level, being completely random, is not a disrupting factor, but an absolutely necessary factor of ontogenesis.

In order to have an evolutionary new group of cells it is sufficient to have an activated new action acceptor during ontogenesis of one old group of cells and it is absolutely necessary to have a feedback and a reception of this feedback by the old group of cells, the reception that will stop their further conversion/movements towards the new group of cells, because it is absolutely necessary to suppress such conversion. And whether this suppression will be specific or barely specific, or even general, depends on evolutionary stage of this new cell group. If the cell group is really evolutionary new, may be the only general mechanisms of suppression are readily available (like immune system can suppress cancer cells in adult healthy mammals), whereas the specific feedback is always preferable, if available, from the standpoint of the efficiency of ontogenesis (like in a classically known case of "embryonic inductor", which is a feedback from the morphologically achieved result, as we have discussed earlier).

Early evolution without replication

At the earliest stages of evolution, when not only all multi-cellular organisms (*Metazoa*) were absent, but any cell itself it was impossible to find, and replication of any heritable material (known today as RNA and DNA) was possible with so many errors that any propagation of any lineage was technically impossible, the action acceptors played an important role in collecting of more or less acceptable variants of replication or pseudo-replication (pseudo-replication – because it was often impossible to say, which lineage has produced given molecule or a "piece" of expected molecule – it was just available or "found" in the environment). We do not know "how" these molecules or semi-molecules were collected, but we know that the only way to have a self-propagated process, keeping very low reliability of replication, was to use simple and "short" (in a molecular sense) sequences to collect (and "select") from the environment the sequences that occurred to be by chance more or less good-replicated or seemingly "good-replicated", because at these early stages they could be just randomly born in the environment due to unknown and may be irreproducible sequence of events ("unknown" not only to us, but unknown to any living entity during these early stages of evolution) – it could be just in a vast majority of cases the result of some statistically random processes. But they were selected in a non-random way by simple (at that time) action acceptors. Those action acceptors had very simple task: just "keep and hold" what has been found useful or seemingly useful by them. We all know that DNA was discovered as a structure due to its ability to "crystallize" or to form long-range order in a semi-crystal (see "*The Double Helix*" by Jim Watson)[29] – and the same ability may be was used many millenniums ago to collect and to "keep together" more or less similar pieces of DNA, when replication in modern sense (as a "covariant reduplication") was technically impossible (no DNA-polymerase, no RNA-polymerase – nothing useful for this purpose was available)! The old human idea about "self-replicated" molecules at the earliest stages of evolution is false:

such molecules were never available. The action acceptors were self-collected and self-selected as a whole and "in pieces" and the first "reparation" was done to fill out the holes between available and collected pieces. It is self-obvious that the mechanisms of reparation were introduced in evolution many-many billions of generations before the invention of the first very primitive mechanisms of replication. The replication has evolved on the basis of reparation, but not *vice versa*.

And cells also, at the beginning were randomly organized foam particles (bubbles). They were randomly formed and randomly destroyed, wherein the genetic material, more robust mechanically in general, was holding necessary components together, when the foam or envelope was temporarily destroyed. Then, instead of random and semi-stochastic disruption, the sell "division" was introduced as an anticipated facilitation of stochastic disruption into the two semi-equal pieces. See Fig. 6[28] & discussion[28]. And the nucleus was introduced even later, when chromosomes were so long, that they were difficult to distribute during cellular division or "anticipated disruption" as we have said earlier – it is the same process. And with the introduction of nucleus, or may be a little bit later, the dormant genes were introduced and widely spread in all eukaryotes and especially in all *Metazoa*, as it was discussed buy Wilhelm Jürgen Harms (1885-1956), known also as J.W. Harms, before the Second World War. The existence of *Metazoa per se* was impossible without dormant genes.

Action acceptor and its propagation in population

Dormant genes were immediately recognized as an important resource for both material reminders of further action acceptors and for building of solutions for formulated problems in the form of action acceptors that have been developed earlier. In all eukaryotic organisms dormant genetic loci should be considered as a primary source of genetic novelty, in addition to traditionally considered mutations. In the **Fig. 9** it is shown the appearance and propagation in population of some action acceptor and its corresponding solution, the solution of this local evolutionary problem. An action acceptor always consists of some material part (that we describe as "material reminders" of this action acceptor) and another part, which belongs to the anticipated future and it is not material yet (it cannot be investigated by means of physics and chemistry). Thus, we can describe the propagation in population of a new action acceptor as a propagation of material reminders of said action acceptor.

Said material reminders can be registered by scientific means, but they do not bring to the organism any positive effect. They are propagating in population only because their carriers do have subjective drive for better reproduction than others. When a component of the desired morphological trait randomly appears in population (as a classic mutation or as a dormant gene that is brought out of dormancy by epigenetic means), said action acceptor detects this event and makes particular organism more happy than others, and by such subjective way it becomes self-elected for further reproduction.

Let's consider for the sake of simplicity only one material reminder in one locus. Let's assume that there is one organism or very small group of organisms which has one new mutation or one previously dormant gene that has been taken out of dormancy by epigenetic mechanisms. Note, that because is it just

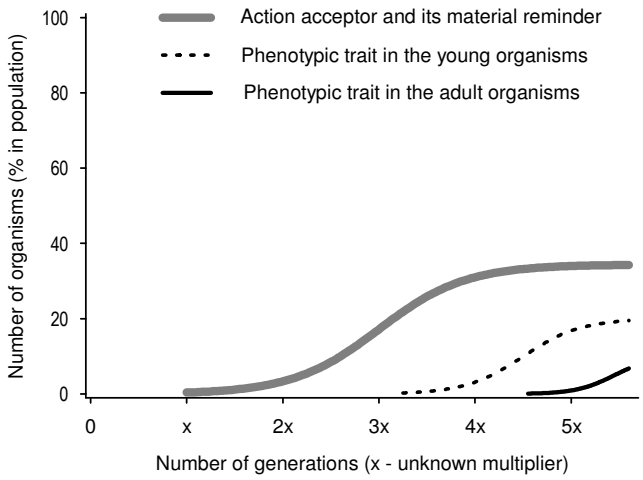

Figure 9 | The propagation of an action acceptor and, then, its desired phenotypic trait in population (schema). The appearance of an action acceptor in the form of its material reminder(s) always precedes the development of any complex morphological trait in population.

a reminder of action acceptor, it does not have any effect on detectable phenotype – it is technically invisible for an external observer. The phenotypic trait, corresponding to this action acceptor, may appear many-many generations later. And at this time point it will be visible in phenotype. Let's consider multi-cellular organisms. First, this phenotypic trait will appear only in very young organisms, those are too young for reproduction, and then this phenotypic trait will disappear in ontogenesis and will not be detectable in adult individuals, those are good for reproduction. And only many generations later this phenotypic trait will be present in both very young and adult organisms, *i.e.* it will be found in both non-reproducing and reproducing ontogenetic stages of these organisms.

It seems to humans that they do know how a phenotypic trait can propagate in population (their opinion is erroneous, but we will discuss this later). However, how a material reminder of an action acceptor can propagate in population? How an entity that has not any impact on phenotype can propagate in population? Humans usually are saying that such entity can propagate only "randomly" or "by chance", see, for example, book of Eugene V. Koonin "*The Logic of Chance: The Nature and Origin of Biological Evolution*", published in 2011[30]. The random propagation is an important process. However this propagation can be not only random, because the abilities of an organism are more diverse and stronger than the abilities of an external observer. We shall explain it right now.

Natural selection

"Natural selection" can be considered in at least two different ways: as a trademark of Darwinism or Darwinian thought-style, expressed in such books as "*The Origin…*"[17] and "*Autobiography…*"[31], and as a combination of two independent words, namely "natural" and "selection". If we consider these two words literally, we see that any event that was observed or could be observed in nature can be called "natural" and we see that there are many processes of selection in living organism(s),

including, but not limited to, processes of selection in immune system, processes of neuronal target selection in early ontogenesis in nervous system, the survival of the fittest in population genetics, selection of germ cells during reproduction, selection of neuronal groups during behavioural episodes (see Gerald Edelman)[32,33], and may be there are many more even undiscovered yet processes of selection in living nature. To call all these processes "natural selection" is counter-productive (it is a sort of idolatry), and we will use the term "natural selection" only as a trademark of views, explicitly expressed by Charles Darwin in his published materials, including all his books.

Then, even the full title of Darwin's the most famous book comprises an erroneous statement (in its second part): "*On the Origin of Species by Means of Natural Selection or the Preservation of Favoured Races in the Struggle for Life*"[17]. There is the preservation of favoured races and there is the struggle for life, but they are irrelevant with respect to each other and the last one has no direct relation to biological evolution.

"*Autobiography…*"[31] of Charles Darwin is a compact book about historical events, including explanation of the role of Thomas Malthus population theory (p. 40)[31]. Humans were impressed by excessive reproduction and limited resources for survival, including food, that along leads to survival of some portion of all organisms. Many organisms should be born and many ones should be found dead during different periods of their lifespan. Humans were glad to see how an inanimate external change, let say change in climate, *e.g.* towards low temperature, leads to evolution of living organisms, in our example – to survival of the most cold-resistant creatures. And, in addition, such natural process did not require any participation of Creator or G-d and, thus, it was fully compatible with all prerequisites of vulgar materialism – it was considered as a complete defeat of all vitalists and religious fundamentalists. However, aside from the last two categories, there were naturalists who were not completely agreed with common joy.

Geographical landscape

Nikolai Ya. Danilevski has demonstrated in 1885 in his book "*Darwinism: A Critical Study*" that Darwinian evolution is fake. *I.e.* it is a fake explanation of biological evolution. We will take only three examples. In Darwinism the attention is traditionally focused on selection by means of differential mortality. What is the main reason of death among organisms in nature? Believe it or not, but it is interaction with a predator or other relevant consumer. The lifespan of a mouse is determined by an interaction with an aerial or terrestrial predator. And the same can be said about the vast majority of animals. The end of an individual is consumption by a predator. Plants, like grasses, are consumed, for example, by goats and donkeys. However the consumption by a predator in real time depends more on geographical landscape and relative position of a prey and predator in this landscape than on the prey phenotype and genotype. And positions of prey and predator are typically statistically random and, thus, just due to this reason they are genotype-independent. The death of a prey depends more on the random location of the prey in geographical landscape with respect to the random position of its predator, than on genotype and phenotype of this prey. This is the first example.

The second example will be about the change of climate, when the temperature becomes colder and colder, and it is a favourite example of Darwinism, applicable to both animals and plants. What will be with plants if the temperature becomes colder and colder? Some of them will be killed by frost, of course, but which ones? Observations in places like Siberia have shown that the plants that a frozen to death can be found only in places where the level of snow is relatively low, whereas in the nearest areas with a lot of snow all plants can be found intact. It means that the survival of a plant depends mainly on its local position in geographical landscape that determines the level of snow during winter. The position of any given plant in local geographical landscape is typically independent of its genotype. The death is random.

The survival of such primitive mammal as hedgehog in Russia during winter provides similar example. It is the third example. If the winter is mild (warm), the population of hedgehogs rises up, if the winter is cold – the quantity of hedgehogs goes down. Someone can speculate that the survival of hedgehogs depends on individual cold-resistance. Hedgehogs are hibernating (sleeping) during winter. And the survival of each individual hedgehog depends on place (warm or cold) where it was sleeping (hibernating). Someone can say that the winter position of a hedgehog is determined by its own behaviour and that a clever hedgehog can choose potentially better (warmer) place for its hibernation. That is correct, but it is not a "cold-resistance". And the ability of a hedgehog to predict the temperature in a given place can be realized only with some probability, because there are always too many statistically random factors that determine the temperature in given place. And the death appears once again genotype-independent. And once again the geographical landscape was found to be more important for individual survival than individual phenotype or genotype.

Misleading correlations

A lot of classically known examples that were told to "confirm" natural selection in evolution are based on really existing strong correlations which are providing an illusion that natural selection is really working as a factor of evolution. For example, when predators are consuming unhealthy and relatively weak prey, humans are commonly saying that this is an example of natural selection. However it is another way around, because when an animal is obviously unhealthy or weak, it is already out of the process of reproduction and its death or consumption by a predator is irrelevant to evolution. Here the strong inversed correlation between the [low] probability in participation in reproduction and the [high] probability to be consumed by a predator provides a false impression that the high probability to be consumed by a predator is a reason for the exclusion of given genotype from further evolution.

Another popular group of classically known "confirmations" of Darwinian evolution consists of examples of effects of infectious diseases and/or small parasites on population. Here humans prefer to talk about survival of the most tolerant individuals with respect to given disease or parasite, whereas the increased mortality among the rest serves as an "obvious confirmation" of natural selection. Here again we have strong inversed correlation between the physical health, important for

participation in reproduction, and the probability to be found killed by given infectious disease. This correlation could be very strong. However once again, the probability to participate in reproduction and the probability to be killed by disease, being strongly and inversely correlated, provide an illusion that the probability to be killed by a disease is the most important factor of evolution during this evolutionary episode. Despite the existence of said strong correlation, the provided human explanation is a fake one, because, in fact, not the mortality-related probability, but the reproduction-related probability determines the course of evolution.

Erroneous additional presuppositions

There are at least three silently introduced presuppositions that are not often discussed in evolutionary literature, but they are always in use in statistical calculations. The first presupposition is: all organisms do have the highest possible reproduction rate – the maximum possible speed of reproduction (sometimes humans are adding the following remark here: the speed, limited by food availability). The second presupposition: all organisms would like to reproduce with maximum speed (humans do not provide any remarks here, because for them this statement seems self-evident). The third presupposition: all organisms with sexual reproduction have statistically random mating (here we have an interesting paradox: humans are happy to speculate about sexual selection – for them it is an example of natural selection, but as soon as we are looking at any related statistical procedure – we can see only random mating, always introduced "for the sake of simplicity", because it is seemingly the only one doable statistical idea). All above-mentioned presuppositions are false.

Instead of them, the following three statements are correct.

1. Different organisms in population have always different rate of reproduction, even if we suppose (erroneously) that they all have the same motivation for reproduction.
2. Different organisms in population always have different motivation for reproduction, even those that have physically identical "maximum possible reproductive rate".
3. All mating events in nature in any population are not statistically random, and statistically random mating events can be organized only in laboratory (under artificial conditions).

Why all these statements were if not completely rejected, but silently ignored, during at least the early days of Darwinism? The answer is clear, if we look at the elements that were highly praised in Darwinism. In Darwinism all creatures were considered as passive objects of selection, conducted by inanimate or practically inanimate natural conditions. Even in the case of sexual selection a partner or potential partner of an animal was considered as a passive object of selection, conducted by the said animal. That was the basis that provided full compatibility with materialistic thought-style, depicted many years ago as "vulgar materialism" (Ludwig Büchner). At that time it thought to be very important.

If we are starting to think about selection at the level of reproduction, about self-selection for reproduction, or even about self-election for reproduction, we will arrive and occur very soon at the position when we will discuss "animal wish", "plant anticipated future", "fungus hope", and we will be one step from the acceptance of ideation space as a reality – as some real entity or a set of real items (but not as a "material entity" or "material items", because they are not such), however as an entity that is real for all living organisms. And from the acceptance of ideation space (first – as a reality, then – as a driving force of evolution) there is literally one single step towards religious thought-style. The above-mentioned sequence of events would be a complete defeat of materialistic thought-style and an indisputable victory of the thought-style, whose name the vast majority of contemporary scientists are afraid to pronounce in public.

Differential reproduction

Nevertheless, despite somebody is afraid of something, the evolution proceeds through the differential reproduction, but not through differential mortality, because the cost factors of differential reproduction are significantly lower than the cost factors of differential mortality. If we do have a differential mortality, we should have an excess of newborn animals, in order to eliminate some of them during different stages of their ontogenesis to obtain the desired evolutionary shift in the frequencies of alleles, keeping at the same time the size of population at the more or less stationary level.

And such excess of newborn animals is not required at all in order to receive the same evolutionary shift in allele frequencies in the case of differential reproduction. If we imagine two populations: one of them is evolving solely by means of differential reproduction and another one is evolving solely by means of differential mortality, which one will be evolving faster or in more efficient way? Of course, the one which is not spending resources for production and further long-lasting support of additional animals (those will perish anyway). The answer is self-evident to the extent that allows us to assume that the population evolving solely by means of selective mortality could never be found during the whole history of life (both documented and undocumented). Such model of evolution is an example of profanation and obscurantism.

In folkloristic terms we can say that the organisms may be self-elected for reproduction not because they are “strong”, but because they are “happy”. And this “happiness” may include in itself a lot: memory about individual ontogenesis, current state of action acceptors, action acceptors’ state during different periods of ontogenesis, the state of development of some barely developed action acceptors, the anticipated future of all kinds. Some of these entities can be investigated by means of physics and chemistry, and some others are not accessible for materialistic investigation, being mainly in the ideation space, where only their material reminders can be investigated objectively, – but all of them are important for organism's self-election for reproduction or for self-exclusion from reproduction.

Opportunities

The self-election of organisms for reproduction provides opportunities that were not evident previously.

1. Organisms with brains automatically become capable of faster and more efficient evolution. Brain is an instrument of evolution, as it was proposed in the hypothesis of evolutionary brain by Boris L. Zlotin & Alla V. Zusman in the article “A natural brain for intelligent design”, published in 2005[19].

2. Contrary to selection by a predator, who does not know much about its prey, about its early stages of ontogenesis, about its general health (the predator sees in the prey only a piece of food), the self-elected organism (not only an animal, but also plant and fungus) may know a lot about its own health, including its own health during early stages of ontogenesis. Such self selection for reproduction may be done using information about all previous ontogenetic stages.

3. Each organism, among both males and females, has different germ cells or groups of germ sells available for reproduction during different periods of ontogenesis. And by selecting this or that period of time in its own life for reproduction, an organism can select this or that group of germ cells. The result of this process may be very similar to the one that was proposed by August Weismann in his theory of germinal selection, first published in German in 1896, in the article "On germinal selection as a source of definite variation"[34].

4. The self-election for reproduction helps us to understand the role of sexual dimorphism in evolution, explained by Vigen A. Geodakian in 1965 in his article "The role of sexes in transmission and transformation of genetic information"[35]. The self-election for reproduction and self-exclusion from reproduction are always stronger in males than in females, due to the higher variability (dispersion) among males. However, because this process is not necessarily linked with the increased mortality among males, there could be no negative impact on population size, counting both males and females. The participation in reproduction in males is more sensitive to an external environmental factor than the participation in reproduction in females. Previously, when our attention and attention of Vigen A. Geodakian[35,36] was focused on differential mortality (in accordance with classical Darwinism), it was difficult to understand benefits, provided by an opportunity of a male to impregnate many females, because typically we do not have a dramatic increase in mortality among males in comparison with females: the observed increase is rather mild in the vast majority of cases. However when we do not have a natural selection, but a self-election for reproduction as a main mechanism, the benefits of sexual dimorphism can be utilized completely. We also can understand, why hermaphroditic organisms (like earth warms and tomatoes) are less successful than bi-sexual organisms in evolution: for a hermaphrodite, in order to have the same evolutionary rate as in the population with males and females, the distribution function of participation in reproduction as a male and the distribution function of participation in reproduction as a female should be completely independent (*e.g.* some hermaphroditic organism could be healthy enough to participate in reproduction as a female, but not healthy enough to participate in reproduction as a male; and these capabilities should be completely independent, whereas in the real life they are always correlated to some extent due to simple physiological reasons – because it is the same hermaphroditic specimen).

5. Any disruptive factor, like an extremely low temperature, always acts in population first of all through the differential reproduction and only afterwards and only in some the most severe cases – through the differential mortality also. Even in the cases when we can see significant mortality (a lot of dead animals or plants), like in situations with infectious diseases or spread of parasites, the evolution goes through the differential reproduction. Here the inversed correlation between reproduction and mortality leads to illusion that the increased mortality is a factor of evolution, whereas it is a fake explanation, an erroneous conclusion, because the decreased reproduction always goes ahead of the increased mortality, and mortality *per se* is irrelevant for evolution (in the vast majority of cases). It does not matter how many organisms were found dead, because all of them (and may be many others also) have stopped their own participation in reproduction in advance. The above-mentioned fake "confirmation" of natural selection exists and remains popular since the beginning of Darwinism, and the above-mentioned inversed correlation masks this error very well (during more than 150 years).

6. In any population with sexual reproduction, *i.e.* with male and female organisms (and even with hermaphrodite organisms, like earth warms or tomatoes) there is no random (in statistical sense) mating of individuals. We may say that organisms are mating "at will", but not "randomly". In mammals, females from large litters prefer to mate with males from large litters, whereas females from small litters prefer to mate with males from small litters. This regularity is evident even in humans (replace the term "litter" by the word "kids", of course). It produces effect of "false bottleneck", where using contemporary statistical methods we see that some population has passed throw very **narrow bottleneck**[37], but it was not so. The bottleneck in population size could be completely absent, but the observed canalization and unification of genome was achieved by means of non-random breeding. In some studies the obtained bottleneck occurred to be so narrow that we do know from independent naturalistic observations that such population must be extinct now. This contradiction between the obtained very narrow bottleneck[37] and the observation that populations of such small size do not survive in evolution is an indicator of an error in the model: this is not a "miracle", but we do have an erroneous model of evolution of control population without any bottle-neck (with erroneous assumption of random breeding).

7. The self-election for reproduction may take into account not only current adult phenotype, but the episodes of appearance and later disappearance in early ontogenesis of those traits that will be typical for adult individuals only many-many generations later in evolution. Thus, these traits may propagate in population and we will see with respect to some morphological traits "the law of precession of characters", described by Leo S. Berg in his book "*Nomogenesis or Evolution Determined by Law*" (1922)[7]. This is not a full explanation of the precession of characters, but it is a demonstration of the possibility of such precession.

8. Dormant genetic loci (or "dormant genes") that were brought out of dormancy by an extreme stress or specific drug treatment (neonatal L-thyroxine treatment, adolescent morphine treatment, *etc.* – *i.e.* treatments with known transgenerational effects)[28,38-43], being brought out of dormancy typically do not demonstrate constant expression, but their expression is jumping in time during lifespan of a single individual from quasi-zero level to some relatively high one and back (multiple times) in a semi-random fashion, demonstrating so-called "destabilized" or "unstable" heredity (these terms were introduced by Trofim D. Lysenko before the WW2)[44]. If one such gene is not expressed during given time interval, it cannot be selected by means of differential mortality, but it can be selected by means of differential reproduction, due to the existence of memory, the

memory that is active during individual lifespan (any memory – not only in the sense of animal higher brain functions).

9. The selection and propagation in population of reminders of action acceptors is possible by the self-election of organisms for reproduction. It means that an action acceptor itself for some desired evolutionary result (let say morphological trait) can be developing in evolution during many-many generations before the first appearance of the said desired result at any stage of ontogenesis. In folkloristic terms, we can say that the development of a "question" may be spread in population during many generations before the beginning of the development of the "answer" to this question. For a given action acceptor it will be done by means of development of the material reminders for this action acceptor, the material reminders that will be accumulated during many successive generations. Thus, the "formulation of a problem" will be always developing in evolution before the beginning of the search for the "solution" of the said problem.

10. Evolution of any trait is a complex process, driven by its anticipated future and distributed among many individuals of the same species during a multitude of generations. Due to this reason many closely related species avoid breeding with each other under natural conditions. Simultaneously, sometimes these species can be bred with each other in captivity without visible problems. Narrow nationalism, understood as reproduction exclusively inside given nationality, comprises the basis of human biological evolution exactly due to the above-mentioned reason.

Lamarckism

The described above schema of evolution depicts more efficient process than any known form of Lamarckism (the inheritance of acquired characters; see book written by Leonid I. Blacher in 1971: "*The Problem of the Inheritance of Acquired Characters: A History of a priori and Empirical Methods Used to Find a Solution*")[45]. I have to admit that I was unable to predict that the defeat of Darwinism will be so shameful. It is replaced not by some form of Lamarckism, as it was proposed by proponents of transgenerational epigenetic inheritance (Eva Jablonka and Marion J. Lamb, book "*Epigenetic Inheritance and Evolution: The Lamarckian Dimension*", 1995)[46], but by evolution of action acceptors, wherein not an external entity eliminates bad creatures, but an internal action acceptor gives command for reproduction when the desired result is achieved.

Epigenetic inheritance

However, the transgenerational epigenetic inheritance[38-43,47] itself can be very useful for evolution of action acceptors as well as for evolution of detectable phenotypic results, obtained by the said action acceptors in the course of evolution. On the other hand, it seems that living organisms do have sufficient mechanisms to organize and to promote the inheritance of acquired characters, if necessary, but it was not done yet, just because some other, more efficient, mechanism of evolution has been found.

The solution of evolutionary question

I know that the described above solution of evolutionary question will not be accepted by English-speaking community.

And there are solid reasons for that.

If we look at any organism (even at unicellular one, but let's imagine a *Metazoon*) we will see a lot of parts and a lot of functional systems. We can see a lot of various parts and a lot of various functional systems during different periods of ontogenesis of that organism. What if somebody will choose some part from some functional system from some period of ontogenesis and improve it? Will choose randomly and improve. Will it be good or bad? Let's imagine that some part is really improved and it is not a joke. Will it be good for this organism and population? Let's temporarily ignore that any improvement and any change in general will have some unexpected consequences those can even disrupt something occasionally. Let's say that we do have the positive effect of this improvement only. Will it be important? The answer is, unfortunately, strictly negative: the improvement of a randomly chosen part or a randomly chosen functional system during a randomly chosen period of ontogenesis is useless.

All periods of ontogenesis have different vulnerabilities, different sensitivities to external disruptive factors and different probabilities of interaction with predators. Even during a randomly chosen period of ontogenesis not all functional systems and not all their parts are equally important. There are a few parts, and sometimes it is possible to localize even a piece of some part, that limits total efficiency or total positive effect of the organism during given episode of ontogenesis. Sometimes the selected part could be optimized further, and the further total improvement could be achieved due to optimization. However there are more interesting cases, where there is a contradiction when an attempt to improve one important feature will lead to degradation of another also important feature, and where their strait-forward optimization is not productive anymore.

The resolution of such contradiction can provide further way of evolution, but we would like to focus our attention upon the previous step in this story: the selection of a period of ontogenesis, the selection of a functional system, the selection of a part of said functional system and sometimes the selection of a piece of said part of the said functional system for further improvement. This choice could not be random. Sometimes humans are saying that the correct formulation of a problem contains 50% of its solution. It is usually said with respect to development of various technical systems. Technical systems are also objects of evolution, but the evolution that is known to humans. The entity or a part of entity that is in the focus of further improvement is not random, but its choice is extremely important.

The formation of an action acceptor, even without any knowledge of possible solution, may take several generations, *i.e.* it could be distributed between several consecutive generations, because it is not an easy task *per se*. An action acceptor for any problem can be formed in evolution slowly, by means of step-by-step collection of material reminders of this action acceptor, and this process can be distributed among several generations. Some material reminders can be distributed in different loci and in order to collect them together and in order to promote and to propagate them in population, the mechanisms of self-election and non-random mating can be used. As soon as an action acceptor is established in population, *i.e.* as soon as it is developed to some extent in some group of individuals in population, it is possible to assume that if some solution or some

even week element of solution will be found, it will not be missed. This element of solution may have no phenotypic effect, or it may have no useful phenotypic effect – for example, it can be so weak, that it will be of no practical importance. However, nevertheless, it will be immediately detected and discovered by the action acceptor. And solely on the basis of this discovery, made by said action acceptor, the said element, which could be very weak at this time point, will propagate further in population through self-election of this organism for reproduction and non-random mating of this organism with similar one(s), if the last one(s) could be found in vicinity. A creature, whose action acceptor has discovered an element of the desired solution, will become the happiest creature, may be not in the whole world, but locally. This happiest creature will be the most suitable for further reproduction.

And what if this creature, let say it belongs to population with sexual reproduction and with males and females, will meet a creature of opposite gender that made similar discovery? It is a rare event, but it is still statistically possible. We will have a happy female creature, whose internal action acceptor has discovered the desired element of solution, and will we have a male happy creature, whose internal action acceptor has discovered slightly similar or very different element of the desired solution. What will we have if these two happy creatures will meet each other? We will have a variety of younger happy creatures. Is it a miracle? Yes and no simultaneously. It is a miracle, because this process is driven by an interaction of the ideation space with the space of vulgar materialism – this process is not a direct consequence of the events in the space of vulgar materialism along. And it is not a miracle, because here we do not have any events that are incompatible with known laws of physics and chemistry.

Here we have an automatic explanation of the law of precession of characters. Some traits are appearing in the young along, and then they disappear very briefly in their ontogenesis. Then, in further generations, these traits can be found not only in the young organisms, but in the slightly older organisms as well, however, nevertheless, they are disappearing before the adult age. And only afterwards, may be many generations later, these traits can be found in the young organisms and the existence of these traits is prolonged into the adult age also. Thus, finally for this evolutionary episode, these new traits will be common for both young and adult creatures of this species. This is a typical picture, produced by an action acceptor, which detects this new trait and promotes it in population by means of the self-election of the carrier organism for reproduction and may be also by means of the further non-random breeding of the said carrier organism. But the self-election for reproduction is enough to obtain this result (**Fig. 10**), even without further facilitation of its evolutionary development by means of non-random mating.

For a material reminder of an action acceptor or for a component of a morphological trait, in order to be selected (by means of differential reproduction), the corresponding genetic change should be dominant. It means that this genetic change is not a suppression of some previously active gene, but it is a new expression of genetic material that was previously silent (non-expressed). For a *Metazoon* (a multi-cellular organism) the typical situation consists of activation of some previously dormant gene or genetic locus. It can be done by genetic or epigenetic means. And we do know from the experiments with

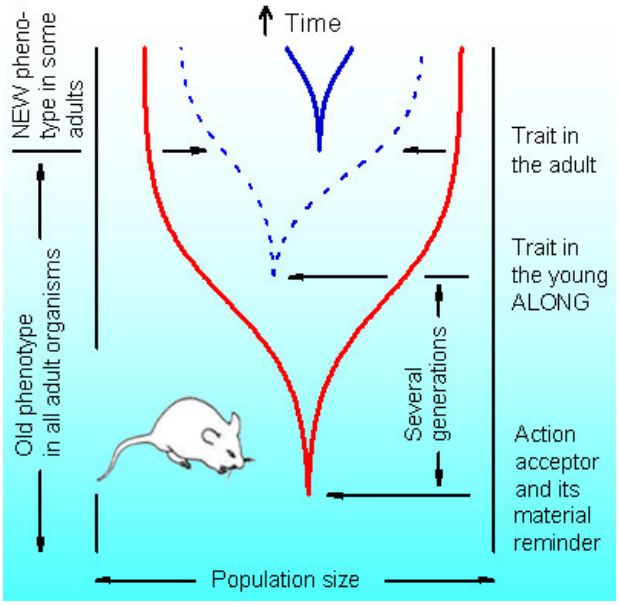

Figure 10 | The precession of characters in evolution. Novel activation of any locus has always its own negative or unexpected effect(s). Many can be compensated separately, though. That is why a previously dormant (and now dominant) gene first of all is activated only during relatively short period of ontogenesis in the young organisms. Later in evolution its expression extends to later periods of ontogenesis, including adult ones.

guinea pigs (Fig. 1[28], Supplementary Figs. 2[28], 3[28]; Fig. 5[43], Supplementary Fig. 3[43]) that such activation is not stable in time during lifespan of a single organism: it some cases it semi-randomly appears and disappears. This flexibility provides opportunities for further optimization in evolutionary time, visible sometimes as the law of precession of characters.

Theory of Inventive Problem Solving

With respect to evolution of technical systems, in the course of human inventive activity, in the previous century, mainly between 1956 and 1985, the Theory of Inventive Problem Solving (known as "TRIZ"[48,49] – it is a transliteration from the Russian abbreviation "ТРИЗ") was developed and introduced through seminars by Genrich S. Altshuller in the USSR, with its main instrument, that is the instrument for thought, known as the Algorithm of Inventive Problem Solving (known as "ARIZ"[50] – this is also a transliteration from the corresponding Russian abbreviation). ARIZ is a very interesting tool. First of all, it is not an algorithm in terms of contemporary applied mathematics, because this algorithm is developed for humans who are capable to work with computably non-enumerable sets, and these computably non-enumerable sets are completely out of the scope of contemporary applied mathematics. We can say that ARIZ contains instructions for humans or human thought. When we are trying to improve some important property of a technical system, we can do it often only at the cost of disruption or diminishing of its some other also very important property. For example, we can make some engine more powerful, but at the cost of its bigger size and weight and at the cost of increased fuel consumption, and this increased fuel consumption will be present through the

whole spectrum of regimes, unfortunately. This is an example of technical contradiction. From the practical standpoint it is often possible to improve situation by means of careful optimization: we can get a not extremely powerful engine, but it will be not extremely heavy as well and with reasonable fuel consumption. Such optimized solution may be practically important, but it is not interesting, because it does not resolve the above-mentioned contradiction. If we take all components of given engine as a computably enumerable set of part, this contradiction probably never can be resolved – the situation will be sort of as in the contemporary applied mathematics: we have a contradiction with given number of components and with given number of components it cannot be resolved. One piece of material cannot be heavy and light simultaneously, for example. However if we imagine that the same engine is comprised from computably non-enumerable components and that each given component may contain in itself several different or identical parts, the situation will be very different, at least in our imagination. But it is enough for us to have it in our imagination only, because we can find the focus of this contradiction, or the most important element, involved into this contradiction, we can split this element into two or more at will and we can resolve this contradiction, because we are working in our imagination with a computably non-enumerable set. At the beginning of this article we have an example with Honda VTEC engine. In this engine we have a camshaft with two different sets of cams, or we can say that each old cam was split into two new cams: one of this cams is in use when the engine has maximum power and maximum rotation speed, whereas another cam is in use when the same engine has low power and low rotation speed. This is an example of a contradiction resolution. If we would not be able to split each cam into two, we will be unable to resolve this contradiction, at least by the above-mentioned way.

There are a lot of peculiarities in TRIZ. One of them is a recommendation to replace special terms by simple words that could be even folkloristic ones. Special technical or scientific terms bring with them an additional psychological inertia, which we would like to avoid during the process of solution of a problem. In this example the term "cam" and "camshaft" would be recommended to replace by something simple, let's say "opener" to indicate just some part of its function. There is also a term "x-element" in TRIZ – just some change, may be not even a new material element in the system, but it should be defined by a human what this element should do. When we are working with unknown yet elements and/or with computably non-enumerable sets, the restrictions provided by our language are more important than in our usual life.

Omissions

One known way to point at unknown yet entity or at the entity or entities from a computably non-enumerable set is to use an omission in human language. Some languages, like Hebrew and Russian, are very comfortable with omissions. In other languages, especially in English, each omission is assumed to be an error that should be corrected as soon as possible. Omissions in general are not allowed in English language. There are some tricks that can be used to introduce an omission in the text using English language, but it is not a trivial exercise and sometimes the desired result cannot be achieved at all. I will illustrate this statement by two known examples.

Example 1. "The Sun Also Rises". This is a title of a book. This phrase indicates that there is an entity, or may be several entities, or may be a computably non-enumerable set of entities, and each of them "rises as well as the sun". The entity or entities is or are unnamed and unknown. This sentence is formally correct. We do not see any error in English language here. However for a native English speaker it is so unusual and confusing – it is looking like some error that should be avoided, if possible. Thus, when this book was published in London, the title was changed to "Fiesta". However, the omission in the original title was introduced by the author intentionally, as well as in the "For Whom the Bell Tolls".

Example 2. "Who brings life to the dead". "Who" is not a question for us – so, we will proceed to the end of this sentence. In English language the word combination "the dead" means only one: "partially or completely decomposed bodies of the dead humans".

This nonsense is a result of translation into English. Originally it is a sentence with omission. It may be written by the following way, the way that is completely prohibited by the rules of English language: "Who brings life to [the] dead [omission]". In this sentence the article "the" is completely removed. And the "dead" entity or entities is or are unnamed and unknown. They belong to a computably non-enumerable set. And we do know that a computably non-enumerable entity cannot be substituted by any set of computably enumerable entities. A computably non-enumerable set cannot be projected into a computably-enumerable one. That is why any word, like "matter", "inanimate matter", "the dead", or any list of specific words, will be an error. Any perceptible entity here will be a complete nonsense. The presence of a specific entity is a requirement of English language. And we need an omission here to depict an entity from a computably non-enumerable set. Thus, this sentence cannot be translated into English without significant deviation in its intrinsic meaning. But why the "dead bodies" are incorrect? First of all, because these bodies would not be found dead, if some one would like to see them alive. Second, we see here a process that is continuous in a non-stop manner, the process occurring with indisputable regularity. This process is given to us not as a rare miracle, but as something that is observable every day and may be even every second.

If we look from the position of our material world at the ideation space, we will see what we can see now. If we look at our material world from the position in the ideation space, we will see what we should see, perhaps. If we are saying about something that it is "alive", it means that we are speaking about an interaction between the space of vulgar materialism and the ideation space. However the side, from which this something is looking, is not specified.

I do know that humans prefer simplistic statements and not like the ones, mentioned above.

Ok, in a few words we can say: those organisms are good breeders that would like to be good breeders, *i.e.* would like to reproduce; those organisms would like to reproduce that do have better state of their own action acceptors, *i.e.* those that are more “happy”.

Some humans will ask: "But where is Natural Selection?" My answer is simple: "In the middle of nowhere!" Natural selection is a fake explanation of biological evolution (and it was shown in 1885 – remark for those who are interested in). Thus, the evolutionary question had knowingly wrong answer during 139 years. Similar to the previously mentioned problem with heavy chain or liquid, moving without a friction in a narrow tube around a cube (**Fig. 1**), wherein some humans are saying that the speed remains the same due to the law of conservation of energy, because the influence from the side of a tube is always tangential with respect to the moving liquid. This is an erroneous answer and a fake explanation as well.

"But why so deep disrespect anyway?" – Someone may ask: "Is it possible to express similar ideas softly?" During previous century we knew many bright and honest naturalists, thinkers and scientists, those were trying to solve problems in the fields of evolution, ontogenesis, behaviour, neuroscience and neuroevolution. And all of them **were honestly thinking** that any acceptable solution in each of these areas should be compatible with principles of natural selection. The life of each of them was wasted for nothing (not completely, sometimes, but mainly for nothing). That's the price for idolatry. No solution exists for the problems in the above-mentioned fields under the assumption of Darwinian evolution. And despite the entity has earned deep and reasonable respect, it must be liquidated.

***P.S.*:** Here we see not only the end of Darwinism as an evolutionary thought-style, but we see the end of vulgar materialism as an exclusive thought-style in natural sciences.

Methods

Equipment for sound recording consisted of NZXT Phantom PHAN-001WT full-tower case (white) with CPU AMD FX-4100, CPU cooler Scythe Kotetsu SCKTT-1000, motherboard Asus M5A97 R2.0 with AMI BIOS 2603 dated 2015-06-26, RAM 16GB (4 × 4GB 1R×8 PC3-14900E Elpida ECC unbuffered EBJ40EG8BFWB-JS-F), video card Matrox Millennium P690 PCIe ×16 MGI P69-MDDE128F (128 MB), Intel SSD 520 Series 180GB + Western Digital WDC WD20EZAZ-00GGJB0 2TB HDD, power supply PC Power & Cooling Silencer Mk-II 750W (with its 135 mm fan facing up), OS Windows 7 Home Premium 64-bit with Service Pack 1 and Convenience Rollup (April 12, 2016).

Four channel PCI audio card LynxTWO-A 24 bit/192kHz was installed in the lowest PCI slot (near power supply); LynxTWO-A has AK5394A (ADC) and CS4396 (DAC), *i.e.* A-D converters are AKM AK5394A with a 123dB dynamic range, while on the output side the D-A converters are model CS4396 from Crystal Semiconductor (Cirrus Logic), with a dynamic range of up to 120dB.

Digital Audio Labs CDX-01 CardDeluxe 24 bit/96kHz was installed as a secondary sound card in PCI slot above LynxTWO-A to provide output compatibility with unbalanced equipment (it has two balanced inputs and two outputs, but they can be used also in unbalanced mode – directly and safely, contrary to LynxTWO-A); CardDeluxe has AK4528 (ADC) and AK4393 (DAC).

Monitor EIZO ColorEdge CE210W (21.1" 1680 × 1050 VA panel; the monitor features a high 1000:1 contrast ratio, a wide 178°/178° viewing angle and a fast 8 ms response time); Compaq PS/2 keyboard P/N 286220-003 RT2156TW, HP PS/2 optical mouse P/N 5188-6230 Rev. B.

Condenser microphone Warm Audio WA-87 (silver) and ribbon microphone Avantone CR-14 with Cloud Lifter CL-1 (CL-1 only for CR-14); all XLR microphone cables were "Mogami Gold Studio", total cable length between each microphone and preamplifier was 18 ft (5.48 m; cables 15 ft + 3 ft); dual channel pre-amplifier Neve Portico 5012; dual channel compressor Neve Portico 5043.

The recording was done using "Mid-Side" technique, wherein Warm Audio WA-87 condenser microphone was used for front ("Mid") recording (Ch 1) and Avantone CR-14 ribbon microphone was placed perpendicular to WA-87 for "Side" recording (Ch 2).

All graphics in the article are based on WA-87 data ("Mid" channel, Ch 1), but Supplementary Audio files contain both "Mid" and "Side" records (Ch 1 and Ch 2, respectfully). Clarinet was placed at approximately 1 meter from the combination of WA-87 and CR-14, wherein CR-14 was placed exactly above WA-87 with 5-10 mm distance between them.

The following settings were used. Warm Audio WA-87 (Ch 1): -10dB – off, filter – off, frontal recording – middle position of the switch.

Avantone CR-14 (Ch 2): the name "Avantone" on the MIC – towards the left hand – side recording. This microphone was working with Cloud Lifter CL-1 and CL-1 was placed between this microphone and preamplifier (Neve Portico 5012). Phantom power +48V was applied from Portico 5012 to both WA-87 and CL-1 (*i.e.* – to both channels; Cloud Lifter CL-1 does not transmit +48V phantom power to the connected microphone, but CL-1 needs at least +15V phantom power for its own operation).

Neve Portico 5012 preamplifier: Ch.1: +48V – ON, Phase Invert – off, MIC GAIN = 54, TRIM = -2, MUTE – off, TO A BUSS – off, HPF – off, but Hz handle – horizontally to the left (it should not be active). "SILK" – off;

Ch.2: +48V – ON, Phase Invert – off, MIC GAIN = 36, TRIM = 0, MUTE – off, TO B BUSS – off, HPF – ON, 120Hz – handle opposite to 20Hz.

Neve Portico 5043 compressor (the following settings provide insight into the term "very mild compression"): Ch.1: IN – ON, Threshold = +2 dB, RATIO = 2:1, ATTACK = 70 ms, FB- ON, LINK – ON, RELEASE = 100 ms, BUSS INPUT – off, METERS SELECT – ON (Ch.B), GAIN = 4 dB,

Ch.2: IN – ON, Threshold = 0 dB, RATIO = 2:1, ATTACK = 65 ms, FB – ON, LINK – ON, RELEASE = 100 ms, BUSS INPUT – off, GAIN = 6 dB.

Data were recorded by the program "Sonic Foundry Sound Forge" (Version 5.0b, Build 162, © 1997-2001 Sonic Foundry, Inc.) at 192 kHz and 24-bit and stored in PCA format (Sonic Foundry Perfect Clarity Audio).

PCA data were imported into the program "Sound Forge Pro Suite" (Version 14.0, Build 33, © 2020 MAGIX Software GmbH), wherein this format (PCA) is called "Sony Perfect Clarity Audio", and saved as FLAC Audio (*.flac) format.

FLAC Audio data were used by the program "Audacity 2.3.3" to convert data into TXT tab delimited format.

TXT tab delimited data were imported into the program "Statistica 8.0" (StatSoft, Inc. (2008), STATISTICA (data analysis software system), Version 8.0, Modules Version 8.0.725.0), wherein all data graphics were prepared.

Figure 1 has been drawn in AutoCAD 2008 [B.51.0 (UNICODE)] 32-bit (Autodesk, Inc.). AutoCAD machine was IBM IntelliStation E Pro Type 6846-31U (originally with PIII-933 Coppermine), upgraded many years ago with motherboard DFI CA64-TC Rev. C (VIA VT82C694T + VIA VT82C686B) with Award BIOS 6.00PG dated 2002-03-26, CPU PIII-S 1.4 GHz Tualatin (SL6BY), RAM 2GB (2 × 512MB PC133 Silicon Technology ECC Registered Buffered SL72R4K64M8H-A75AV [Micron] + 1024MB PC133 Corsair ECC Registered Buffered CM744S1024-133 [Samsung], Memory Parity/ECC Check disabled in the BIOS, because this BIOS option is intended to be used with ECC unbuffered only), video card 3DLabs WildCat VP560 AGP 4x [Rev. D, BIOS Version 3.04, driver 3.01-0852] (64 MB), HDD Hitachi Deskstar HDS722516VLAT20 160GB connected to motherboard *via* Adaptec ATA RAID 1200A PCI card, OS Windows 2000 Professional (5.00.2195) with Service Pack 4.

Monitor IBM ThinkVision L191p Type 9419-HB7 (19" 1280 × 1024 IPS panel; the monitor features a high 1000:1 contrast ratio, a wide 178°/178° viewing angle and a slow 20 ms response time); Logitech Deluxe Plus PS/2 Keyboard Black Y-SW45 P/N 867373-0403, HP PS/2 optical mouse 800 dpi P/N 672651-001 Rev. 0A.

Photographs of clarinet were taken by Vera Vyssotski with a help of Nikon D7200 with lens Nikon DX VR AF-P NIKKOR 18-55mm 1:3.5-5.6G. Pictures and photograph of Honda VTEC engine (1989) are property of Honda (Japan).

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Additional information

Supplementary Audio accompanies this paper at
http://www.evolocus.com/evolocus/v1/evolocus-01-037-sa1.mp3
http://www.evolocus.com/evolocus/v1/evolocus-01-037-sa2.wav

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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

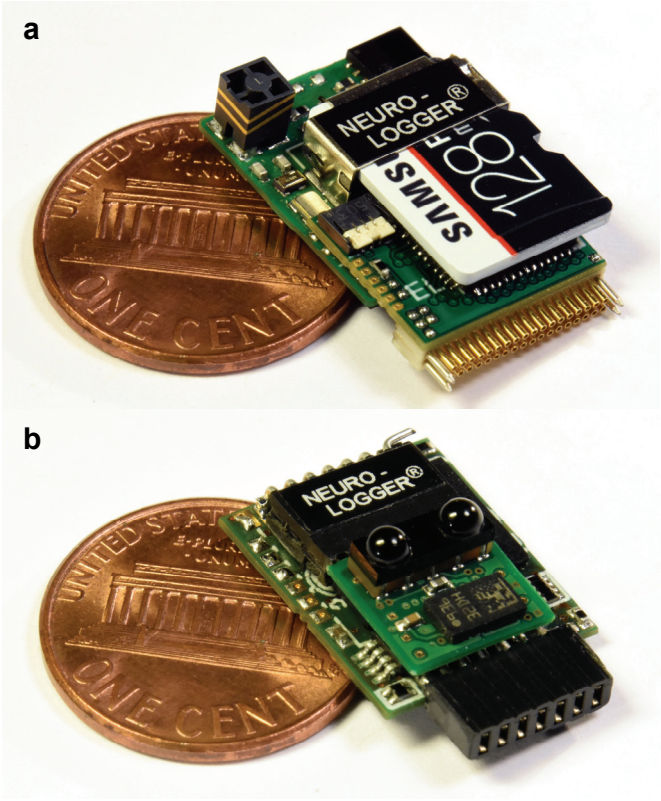

Figure 1 | Neurologger 3 (**a**) and Neurologger 2A/2B (**b**). Shown Neurologger 3 (**a**) has 32 channels and 128 GB Micro SDXC card (Samsung). Both Neurologger 3 and Neurologger 2A/2B have infrared (IR) sensor to receive information from external equipment, sent by infrared (IR) emitter (*e.g.* processed 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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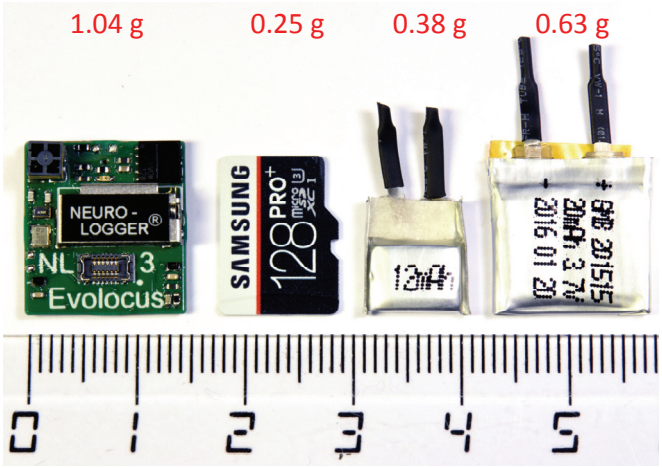

Figure 2 | Neurologger 3, an example of its memory (Samsung 128 GB Micro SDXC) and two examples of its battery. Batteries of different capacities can be used depending on desired record duration. Two examples of batteries of different weight/capacity are shown. The logger needs only one battery. Note 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.

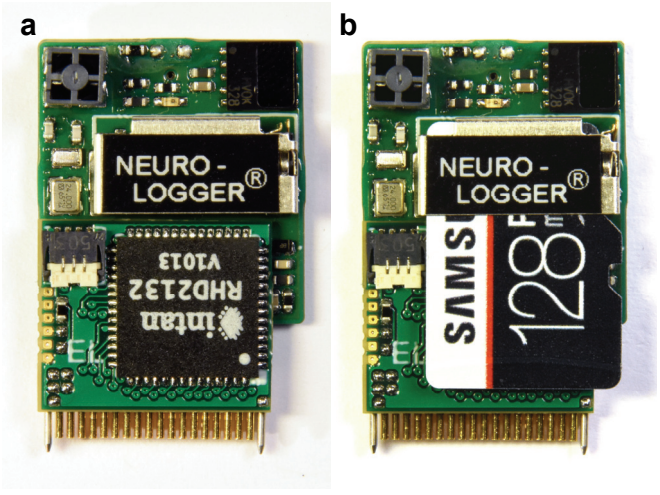

Figure 3 | Neurologger 3 with its headstage and memory. To record neuronal activity the neuronal headstage should be attached to the processor module how it is shown in this picture (**a**). The 32-channel headstage with Hirose connector is placed at the bottom side (invisible). Then, the memory card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

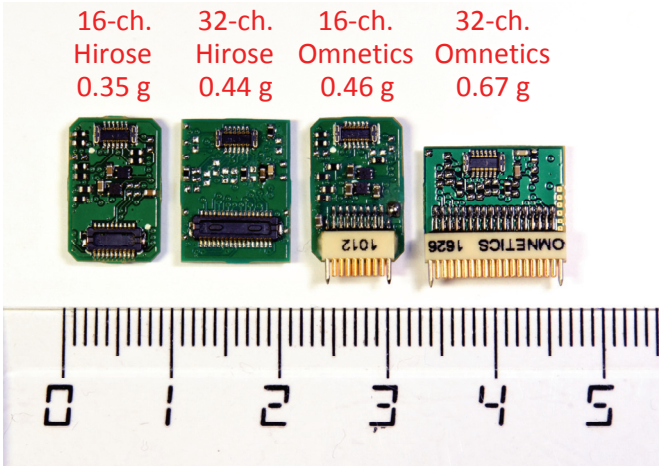

Figure 4 | Four neuronal headstages, available for Neurologger 3. From the left to the right: 16-channel Hirose, 32-channel Hirose, 16-channel Omnetics and 32-channel Omnetics. Both Omnetics and Hirose types are pin compatible with Neuronexus silicon probes. Please see Neuronexus Internet side for the 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.

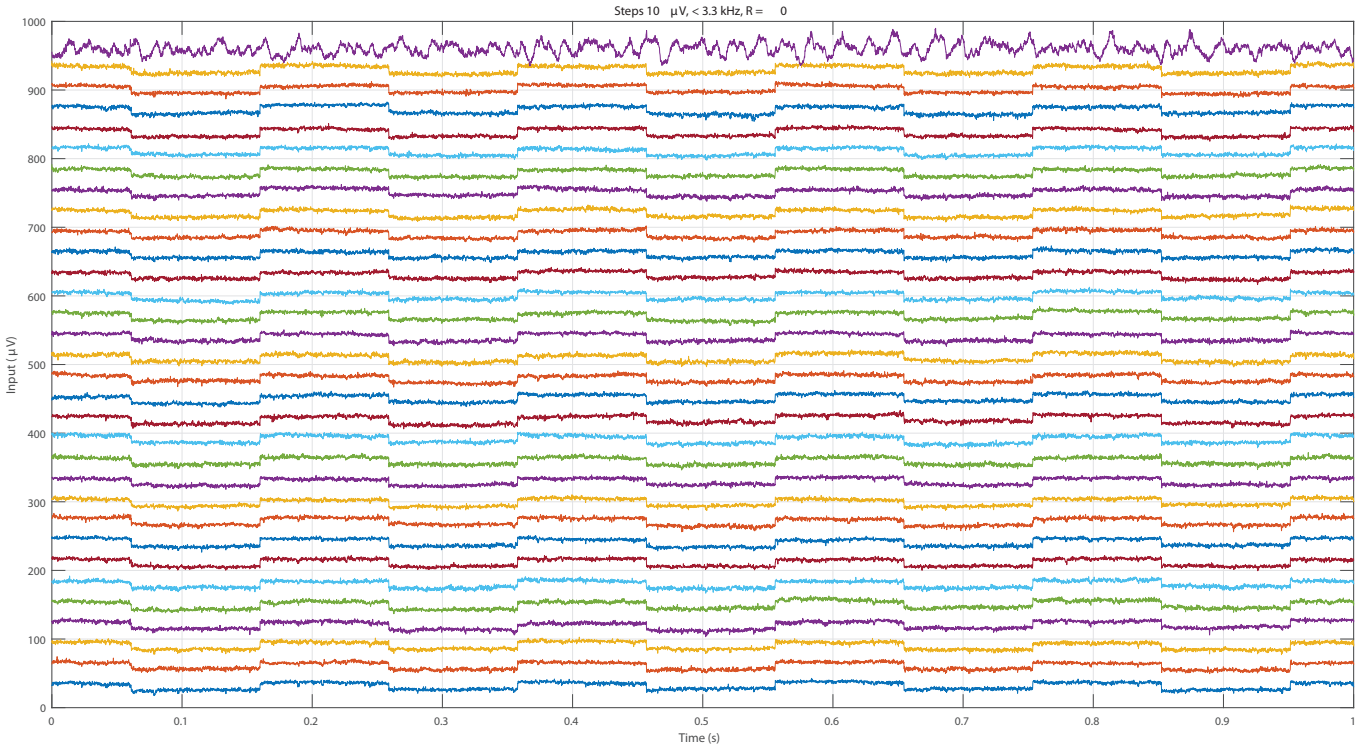

Figure 5 | Noise and electromagnetic disturbances in the Neurologger 3. Neurologger 3 has extremely low noise and electromagnetic disturbances in its frequency range *F* = 1-3300 Hz. The original signal recorded in the frequency band 1-7500 Hz was additionally low-pass filtered at 3300 Hz frequency. 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.

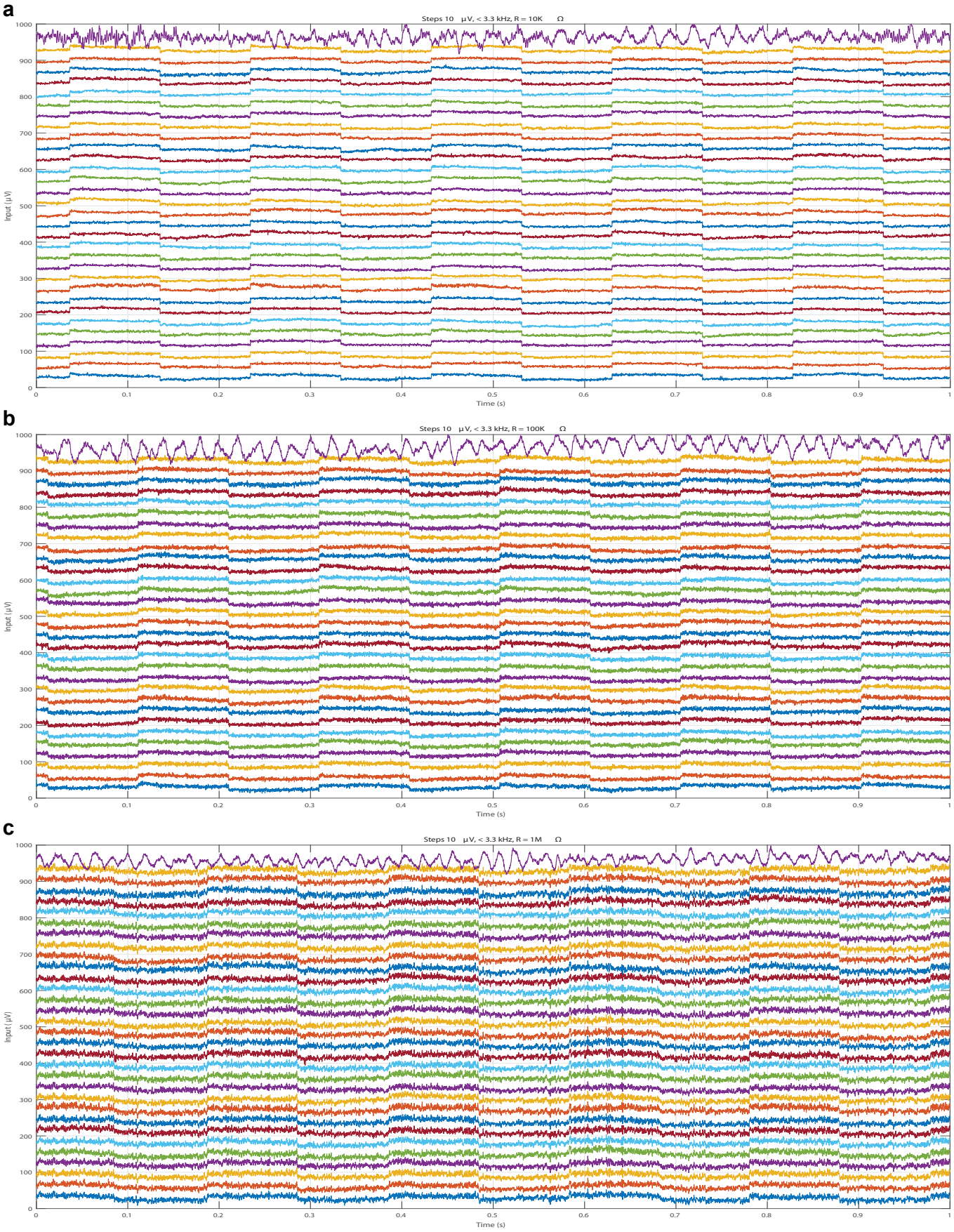

Figure 6 | Noise and electromagnetic disturbances in the Neurologger 3 when a sequence of 10 µV peak-to-peak rectangular pulses was given to the input of the logger through 10 KΩ (**a**), 100 KΩ (**b**) or 1 MΩ (**c**) resistors. All other conditions are identical to the conditions of the record in the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

Supplementary Information accompanies this advertising feature at http://www.evolocus.com/evolocus/v1/evolocus-01-I-s.pdf

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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