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Article

Transgenerational epigenetic compensation

Heritable compensation of disturbed functionality

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

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

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

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

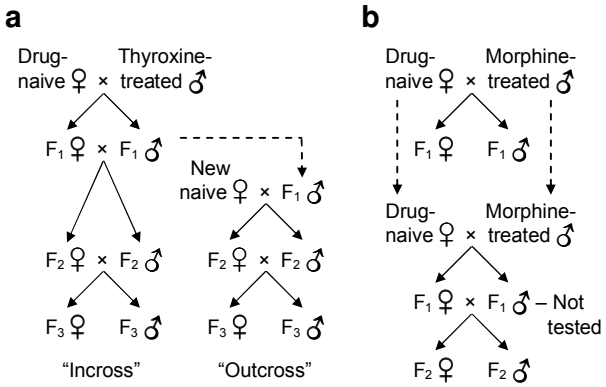

Figure 1 | Breeding paradigms. (**a**) DBA/2J mice, thyroxine study. (**b**) Wistar rats, morphine study. Solid arrows indicate the appearance of progeny, dashed arrows – transition of the same animals.

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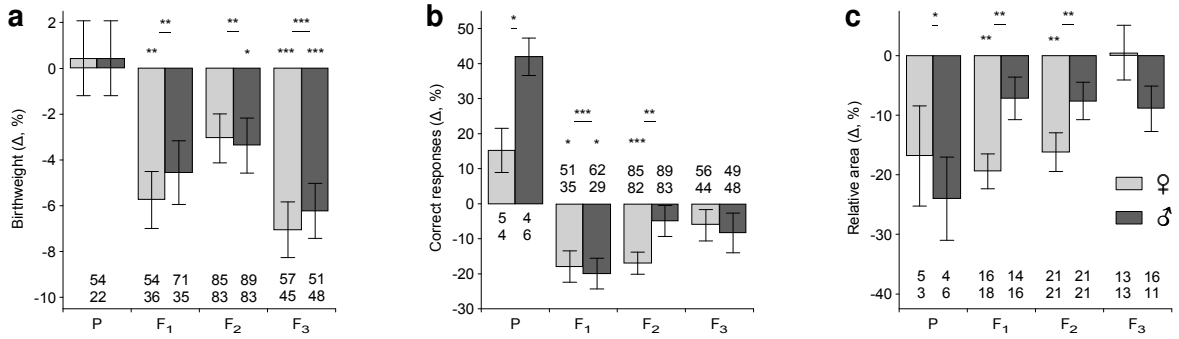

Figure 2 | Phenotype of thyroxine-treated mice and F_1-F_3 descendants. Neonatally thyroxine-treated mice and untreated descendants of thyroxine-treated males. (**a**) Birthweight. (**b**) Two-way avoidance averaged correct responses of 5-day training, 80 trials daily. (**c**) Hippocampal mossy fibers,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

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

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

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

Results

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

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

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

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

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

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

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

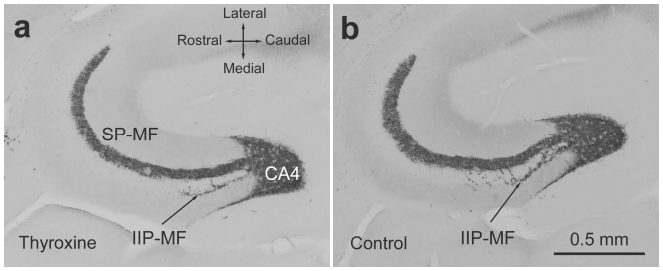

Figure 3 | Hippocampal mossy fiber morphology in the F_3-outcross. Thyroxine study. (**a**) Experimental male mouse. (**b**) Control one. Note the scarce infrapyramidal mossy fiber projection (IIP-MF) in (**a**). Shown samples differ from each other to the greater extent (45%) than mean group values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

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

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

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

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

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

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

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

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

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

Discussion

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

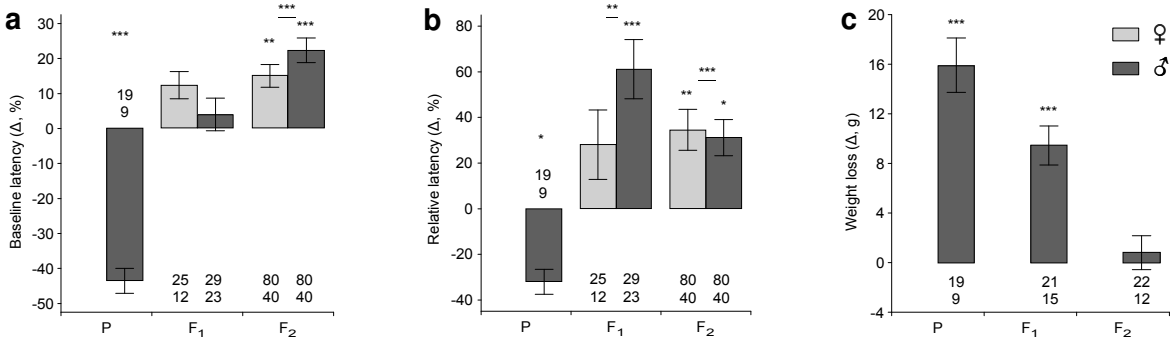

Figure 4 | Phenotype of morphine-treated male rats and F_1-F_2 progeny. (**a**) Pain sensitivity, baseline latency. (**b**) Morphine analgesia, ratio of tail withdrawal latency, measured 30 min after 10 mg/kg morphine administration, to baseline latency. (**c**) Naloxone-precipitated weight loss after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

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

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

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

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

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

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

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

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

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

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

Methods

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

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

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

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

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

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

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

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

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

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

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

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

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

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