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CHAPTER 33

Transgenerational effects of drug and hormonal treatments in mammals: a review of observations and ideas

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Introduction

The consequences of disturbing neuroendocrine systems of young developing animals persist for various lengths of time. Short-term acute effects are the easiest to study. However, changes which outlast the presence of an injected drug or altered hormone level are no less important. A teratogen acting in utero can damage an animal for life. Even more enduring changes carry over from a stressed animal to its offspring. Insults to a female which perturb her progeny are well known. These 'maternal effects' present little problem for the imagination although those resulting from treatments made very early in the mother's life imply some lasting deficits in her reproductive functions. More unexpected are effects which continue to grandoffspring and great-grandoffspring of treated animals.

This article is concerned with disturbances to several neuroendocrine systems of young animals having the following remarkable combination of consequences:

1. Experimentally treating a young animal induces characteristic abnormalities which persist into adulthood.
2. Crossing treated males to normal females gives offspring with altered phenotype.
3. Abnormalities carry over to multiple generations (usually demonstrated in F2 progeny of treated females).
4. Some of the transmitted alterations coincide with known functions of the perturbed neuroendocrine system or with the changes that the treatment induced in the parent animal.

Transgenerational effects of hormone or drug exposure lie at the intersection of genetics and physiology and have different meanings for the two disciplines. Endocrinologists are interested in their physiological significance for the individual neuroendocrine system or for neuroendocrine systems in general. They design studies to describe the transmitted alterations. The geneticist is troubled by their contradiction of the long-standing, basic principles of Mendelian inheritance. Transgenerational effects are suggestive of Lamarckian inheritance or the transmission of acquired characteristics from parent to offspring. The geneticist's overriding concern is whether a study proves the heresy to be real, and if so, what new genetic mechanisms must be acknowledged. Physiological descriptions of the transmitted condition, for him, are secondary. Unfortunately, designing a study toward one of these objectives often weakens it for the other.

In order to include both perspectives we will describe some of the more notable induced carry-over effects, discuss the technical problems for

their genetic verification and review the possible mechanisms which underlie them.

Terminology

Two special notations will be used to avoid certain ambiguities in the literature. Physiologists have counted generations of animals from various starting points. When a pregnant animal is injected with a drug her offspring have been called the 'F0', 'F1' and even 'second' generation. In this paper the animals exposed to the experimental conditions in utero are the starting generation. Their progeny, the grandoffspring of the original pregnant animal, constitute the F1 generation. We will designate them F*1 to emphasize this counting scheme which differs from the usage in some of the original reports. Offspring of the F*1 individuals are F*2. If a male animal is treated and subsequently bred, or if a female is treated *before* mating, it will be described as F0 and its offspring will be of the F*1 generation. The important fact to emphasize is that F*1 animals were not conceived at the time of treatment, although their gametes or gamete progenitors were extant.

Offspring of treated animals have been bred mainly by two regimes. Crossing female descendants of treated animals with normal males for repeated generations will be called *outcrossing* and generate a *maternal line*. In contrast, breeding experimental line males and females together at each generation will be *incrossing*. The main difference between the two regimes is that only one parent can transmit the acquired trait in the former while both can contribute the altered influence in the latter. Also, outcrossing dilutes the original genetic heritage from the treated parent at each generation.

Examples of transgenerational effects

We begin this section of examples with a caveat. Each case reviewed is substantially more complex than its brief description here. The interested reader should consult the original cited articles for further details and interpretations.

Thyroid

Thyroid hormones play key roles in both development and adult metabolism. Injecting a single dose of thyroxine into newborn rats will permanently depress the circulating levels of that hormone and of thyroid stimulating hormone (TSH) (Bakke and Lawrence, 1966; Gellert et al., 1971). Moreover, if neonatally treated female rats are bred their F*1 and F*2 descendants will also have low TSH levels, as well as other alterations.

Removing the thyroid glands of a pregnant rat also alters the control of TSH for multiple outcrossed maternal generations (Bakke et al., 1975; Fujii et al., 1985). Undisturbed levels of TSH are normal in F*1, F*2 and F*3 animals but their response to injections of TSH-releasing hormone or to removal of the thyroid is muted.

In experiments originally intended as controls, Bakke et al. (1975) removed the thyroids of male rats prior to mating with normal females. Unexpectedly, their F*1 progeny had enlarged pituitaries and thyroids, lowered TSH levels (although the drop did not reach statistical significance in their data) and significant changes in various other developmental, physiological and morphological characteristics of one sex or the other.

Thyroxine levels are stabilized in a complex way by feedback loops between the thyroid, pituitary and hypothalamus. These controls have been called the 'thyrostat', as an analogy with a thermostat for maintaining constant temperature. Bakke et al. (1975) propose that the set point of an animal's thyrostat is established during a critical perinatal period on the basis of the prevailing hormonal environment. This setting then persists for the life of the animal. From this model they suggest that their experimental animals transmitted altered thyrostat settings to subsequent generations. Physically, these settings might correspond to the numbers of receptors for TRH on the thyrotroph cells of the pituitary.

Insulin

The etiology of insulin diseases is complex and confused. Offspring of female diabetics are born with enlarged islets of Langerhans and increased incidents of macrosomia and congenital deformities (Jackson, 1960; Neel, 1962). Diabetes also interferes with male gametogenesis (Oksanen, 1975) and there are poorly supported suggestions that it affects offspring of men (Jackson, 1954; but see Rubin, 1958). At least nine groups of workers have treated animals with diabetogenic drugs, such as alloxan, and examined their descendants. All except for Gept (1965) have reported transgenerational effects of one kind or another to persist for the duration of their studies (Bartelheimer and Kloos, 1952; Baranov and Sokoloverova, 1966; Okamoto, 1965; Ohno, 1969; Foglia et al., 1970; Spergel et al., 1971; Aerts and Van Assche, 1977; Milner and Sheffrin, 1982).

In the first long-term study, Okamoto (1965) chemically destroyed the beta cells of male and female rats, guinea pigs and rabbits one month before mating. The F*1 progeny developed fewer beta cells per islet. Pituitaries and adrenals also were histologically affected. Some of the offspring in turn were injected with alloxan and the deficiency was exaggerated further in the next generation. The progressive cell decrease from this experimental regime eventually led to overt

TABLE 1

Condition of beta cells and incidence of diabetes in descendents of serially diabetized Wistar rats

	Pancreatic beta cells			Number of animals	
	No. of cases	Beta cells per islet	Cell size (μm)	Examined	With diabetes
Normal controls					
	33	66.3	110.3	30	0
Both parents with alloxan diabetes					
F1	13	48.3	107.0	28	0
F2	4	33.4	100.4	5	0
F3	3	24.6	94.9	5	0
F4	1	20.8	91.8	8	0[a]
F5	1	17.9	89.1	14	12 severe[b] 2 mild[c]
Fathers with alloxan diabetes crossed with normal females					
F1	25	58.5	107.7	22	0
F2	4	53.2	101.9	16	0
F3	4	44.7	94.2	7	0
F4	4	37.6	91.9	9	0
F5	4	29.4	80.7	11	0
F6	4	20.8	85.2	17	0[a]
F7	4	16.6	79.3	20	17 severe[b] 3 mild[c]

From the data of Okamoto (1965).
[a] Sometimes slight and intermittent.
[b] Severe or persistent.
[c] Intermediate or transient.

diabetes without drug treatment (see Table 1). Diabetes erupted among F*5 animals when both mothers and fathers were injected at each generation and in the F*7 generation if only males were treated. Ohno (1969) confirmed these findings in a smaller parallel study and Foglia et al. (1970) found that surgically removing the pancreases of six generations of female rats produced glucose intolerance in the seventh generation.

Spergel et al. (1971, 1975) and Goldner and Spergel (1972) used a simpler paradigm in a study involving one thousand rats. They injected subdiabetogenic doses of alloxan into one generation of preweanling rats and mated the animals among themselves and to normal controls. The progeny from treated males mated with normal females statistically were less glucose-tolerant than normal, as were those from exposed females crossed with normal males. The F*1 animals were pooled and incrossed for six more generations without further treatment. Glucose tolerance progressively deteriorated. By the fifth generation some rats were overtly diabetic and the others were unusually sensitive to administered alloxan.

Morphine

Morphine stimulates endorphin receptors involved in a diversity of neural, hormonal, immunological and developmental functions. Injecting narcotics into neonatal rats slows development and leads to permanent defects in nervous system structures, behavior and responses to hormonal stresses (Smith et al., 1977; Glick et al., 1977; Sonderegger and Zimmermann, 1978; Sonderegger et al., 1979a)

These same types of abnormality also appear among the F*1 offspring (Smith and Joffe, 1973; Joffe et al., 1978; Friedler, 1974, 1978; Friedler and Cochin, 1972; Friedler and Wheeling, 1979; Sonderegger et al., 1979b; Zimmermann and Sonderegger, 1980). Fig. 1 compares two 7-day-old offspring of a morphine-treated male inbred mouse with an age-matched control from our laboratory. The two F*1 pups weighed half as much as normal and obviously were behind in their development. Such carryover effects are highly sporadic, however, and vary markedly in degree from one litter or individual to another.

Zimmermann and Sonderegger (1980) found that most progeny of neonatally narcotized female rats grew abnormally slowly. The rats eventually caught up with controls in size but retained a higher tolerance to pain, a characteristic that mimics direct exposure to morphine. A few F*1 progeny had permanent extreme deficiencies. They were smaller, lacked pain sensation and maintained their hind limbs tonically in full extension. Two such females successfully conceived but delivered their F*2 pups stillborn over a two-day-period.

Parathyroid

The parathyroid is a key regulator of calcium ion levels in the blood, acting on kidneys, bone and other organs. Fujii (1978) removed the glands from pregnant rats on day 5 of gestation and incrossed the resulting offspring. Blood calcium levels were significantly depressed for at least four generations and responded substantially less

Fig. 1 Delayed growth of the F1 offspring of a male mouse treated neonatally with morphine. The two pups on the left were sired by an inbred C57BL/6 male mouse which had been injected twice daily for 21 days after birth with increasing amounts of morphine to a final dosage of 8 mg/kg body weight. The father of the control on the right was injected with saline. The two male parents were bred with normal C57BL/6 females on the same day and the pups were photographed 7 days after birth.

when the parathyroids were removed from these animals. Also, F*2 rats were killed by smaller quantities of intravenous calcium chloride than controls ($LD_{50} \approx 26$ mg/kg vs. ≈ 33 mg/kg for normals; Fujii et al., 1980). By the seventh incrossed generation, blood calcium levels had reverted to normal but gonadal alterations remained (Fujii and Yamamoto, 1983). The ovaries senesced late in aged F*7–9 females and testosterone levels declined less with age in the males.

Few effects of parathyroid stress have been shown to pass through the male line. F*3 males of Fujii's incrossed parathyroidectomized line sired apparently normal progeny with control females (Fujii and Morita, 1978). Unfortunately, the reciprocal *outcross* of F*3 females to control males was not reported for comparison. Also, Fujii and Morita (1978) saw no transgenerational consequences of removing the parathyroids from adult male rats, possibly because the animals become aspermic within three months. However, grafting extra parathyroid glands onto adult male rats did affect the male F*1 offspring (Sakamoto and Fujii, 1980). Testes matured early in F*1 males, with testosterone levels elevated 2–4 fold at three weeks of age. Concomitantly, testosterone-dependent somatic organs were smaller, indicating a decrease in tissue sensitivity to the hormone.

Suppression of insect diapause by LSD

A well known hormonally regulated process of insects is pupal diapause, the delayed emergence of the adult from the pupa. The butterfly *Pieris brassicae* produces several broods a year. The last of the season overwinter as resting pupae in response to the short day lengths of autumn. Injecting lysergic acid (LSD) into the caterpillars counteracts this photoperiodic signal and its effects linger for multiple generations (Vuillaume and Berkaloff, 1974). Table 2 records that 78% of *P. brassicae* reared under short-day laboratory conditions diapaused. Twenty μg of LSD dropped this incidence to 18%. It also affected the three subsequent generations. Exposure of even one male or female grandparent to LSD significantly decreased the likelihood that an F*2 pupa would diapause. Injecting LSD into both parents, as caterpillars, is lethal to offspring. These F*1 progeny die either at the end of the pupal stage or as malformed imagos.

LSD appears to induce two superimposed changes in offspring. Besides suppressing their diapause it makes them more resistant. F*1 caterpillars tolerate twice the normal lethal dose of LSD.

Carryover effects on immunity and development

In addition to hormone dysfunctions, perinatal disturbances of the immune system and organogenesis reportedly can also affect descendants of males for multiple generations. However, these claimed carryover effects are controversial.

Early in this century biologists induced carryover changes in morphology of various organisms, especially unicellular forms. A notable case in

TABLE 2

Effects of LSD on *P. brassicae*

Progeny generation	% of pupae which diapause[a]		LD_{50}
	Not treated	+20 μg LSD[b]	
Parental	78	18	35 μg
First			
A ♂(*) × ♀(n)	99	100	60 μg
B ♂(*) × ♀(*)	25	97	
C ♂(*) × ♀(n)	79	48	
Second			5–10 μg
♂ from A × ♀(n)	45		
♀ from B × ♂(n)	57		
Third			5–10 μg

Data abstracted from Vuillaume and Berkaloff (1974).
[a] Each percentage figure represents 60–378 individuals.
[b] Injected into 5th instar larva.
*, parent injected with LSD.
n, normal parent.

mammals involved defects from injecting anti-lens antiserum into pregnant rabbits (Guyer and Smith, 1918; 1920). Some offspring exposed in utero were born with malformed eyes and they transmitted eye defects in the sporadic manner expected for a recessive genetic trait. The abnormalities grew more and more pronounced over the course of five incrossed generations, rather than dying out (resembling the case of Spergel's rats treated with alloxan; see above). Several physiologists tried to repeat these experiments. However, they were unable to prepare antisera that were teratogenic and therefore could not follow the inheritance of induced eye defects. For a perceptive evaluation of these and other early studies, the reader is referred to Detlefsen (1925).

Inheritance of immunity has been studied more recently. It is well accepted that an immunized mother can transmit antibody molecules or lymphocytes to her progeny through the placenta or milk supply (Auerback and Clark, 1975). More unexpectedly, it was found that inoculating *male* neonatal mice with antigens also modifies the immune response of offspring (Gorczynski and Steele, 1980, 1981; Gorczynski et al., 1983; Steele, 1984). Cellular as well as soluble antigens and both immunizing and tolerizing schedules were effective. Up to half of the F*1 and F*2 descendants of tolerized mice were tolerant or partially tolerant to that antigen. Several other investigators have observed other, less spectacular male carryover effects (Guttman and Aust, 1963; Mullbacher et al., 1983) but attempts directly to replicate Gorczynski and Steele's results so far have failed (Brent et al., 1981, 1982; Smith, 1981; Nisbett-Brown and Wegmann, 1981; McLaren et al., 1981). The discrepancies have been analysed in detail by Steele (1981b and Steele et al., 1984) and his critics (Lewin, 1981; Brent, 1981). It seems likely to us that the immune system can transmit alterations across generations although not necessarily by the frankly Lamarckian mechanism proposed by Steele (1981a). See, for example, the complex findings of Gorczynski et al. (1983).

Other more restricted transgenerational effects

Effects of a variety of other parental treatments carry over to a narrower range of offspring. Some have been documented only among the first generation from treated males (see listing in Soyka and Joffe, 1980; also Adams et al., 1982). Others cross multiple generations but only through female lines (see examples in Caspari, 1948; Jinks, 1964; Svetlof and Korsakova, 1972; Denenberg and Rosenberg, 1967; Wehmer et al., 1970; Barnett, 1973; Ratner and Tchuraev, 1978; Zamenhof and Marthens, 1978; Beach et al., 1982) or through asexual cycles (see Goldschmidt, 1938; Csaba and Lantos, 1977). A third type of effect progressively intensifies from generation to generation while the animals are maintained in the inducing environment (Lints and Lints, 1965; Koloss, 1966; Mampell, 1968; Kahn, 1982; Wallace et al., 1983). Some of these cases eventually may turn out to include the full range of hereditary properties of the examples reviewed above (especially those of Mampell, 1968, and Fried and Charlebois, 1979). Others may be inherently simpler.

Mampell (1966) has advised that transgenerational effects may be far more prevalent than generally believed. We concur. Physiologists and endocrinologists can expect to discover substantially more examples if they examine the offspring of their experimental animals. We likewise agree with Barnett (1973) that cumulative carryover effects 'should be looked for, and recorded when found, even when they are extremely inconvenient to the experimenter and cannot be explained.' The same is true for observed absences of carryover effects, despite the difficulty of reporting 'negative' findings.

Difficulties for verifying induced transgenerational effects

Carryover effects as described above are complex and present a number of difficulties for experi-

mental verification. The most important are the following.

1. Sporadic occurrence of altered progeny among the offspring of stressed animals. Some parental treatments affect only a portion of offspring. There are several possible reasons. The alteration might have variable expressivity in offspring, as seems to be the case for DDM induced by morphine. A few F*1 individuals exceed a threshold between a minor and major degree of affliction. Alternatively, an experimental treatment might induce an alteration in only some germ line cells of an animal. A low or variable incidence of affected progeny does not in itself cast doubt on the reality of a carryover effect but it can create serious problems for demonstrating and characterizing them. Any adequate study of a sporadic carryover effect should precisely describe the incidence of affected offspring, attempt to identify the factors which influence that incidence and present an experimental protocol that maximizes it.

2. Quantitative alterations. All variations are 'quantitative' in a purist sense, but some are large enough that affected and unaffected individuals can be distinguished unambiguously. Unfortunately, some carryover effects are not that great. They can be demonstrated by comparing distribution curves of measurements for progeny from experimental and control crosses but some or many individual animals cannot be unmistakably identified as affected or normal (e.g. Steele, 1984). Statistical analysis of distributions within a population is a valid analytic technique. However, geneticists simply will not accept statistical tests on the tails of distribution curves, or modest shifts in means as proof for biological phenomena as radical as 'Lamarckian inheritance' (Brent, 1981). Too many alternative possible explanations are no less believable. A foremost goal of a methodology must be to dichotomize cleanly affected and non-affected animals. This is especially important for studies which continue into subsequent generations.

3. Variations among strains. Phenotypic traits that are subject to modification by conditions experienced by parents or earlier ancestors can be expected to vary from one strain or substrain of animals to another. This makes it difficult to ensure that different laboratories (especially at different times) are working with equivalent animals. In fact, laboratories trying to replicate others' studies often obtain significantly different results. The differences observed among the more than half-dozen studies on progeny of various animals with chemical diabetes are a good example. Presumably, variation among strains has a genetic basis as is the case in plants. Some varieties of flax are susceptible to experimental induction of hereditary alterations called genotrophs while others are not (Cullis, 1977). Plant breeders have found it possible and informative to characterize the basis for this difference through crosses of 'plastic' and 'nonplastic' varieties by standard genetic procedures. It is not necessary for an experimental procedure to be effective for all strains or species, but a treatment must give reproducible results in some animals available to other laboratories.

4. Unintentional selection. Selection changes the characteristics of animals from one generation to another. It turns out to be very difficult to eliminate the *possibility* of this factor intruding in multigenerational experiments. Prenatal death, variation in litter size, failure of some pairs of animals to mate or conceive and even choosing which animals to breed for the next generation all can cause artifacts if they are biased toward the character under study. Such bias is distinctly possible for a neuroendocrine alteration. Cryptic selection is particularly capable of increasing or decreasing the intensity of carryover effects during the course of a multigenerational study (Guyer and Smith, 1920; Korec, 1981; Spergel et al., 1975).

It is important to realize that natural selection cannot be eliminated from an experiment. The best that one can do is estimate its total possible

effect from litter size variation, amounts of perinatal death and so forth and compare the 'worst case' magnitude with experimental findings. Protocols for untreated control lines should be designed very carefully to indicate the possible effects of selection.

A very different level of selection distorts the literature. Scientists naturally publish data which is statistically significant and ignore negative findings. If twenty people examine the progeny of experimentally stressed male animals, one is expected to find them to differ from controls by a 'statistically significant' amount ($P < 0.05$). If only this 'significant' observation is reported the literature will become skewed.

Examining twenty traits in one study presents the same problem. Obviously, investigators must examine various inducing regimes, look at a variety of phenotypic measures, and even test subsets of data for significance: transmission from females only, progeny of the treated males, total numbers of animals vs. numbers of litters in which defects show up and so forth. Unfortunately it is usually impossible to tell from an article how many possible parameters the statistically verified ones correspond to. We suggest that investigators use separate standards to evaluate the confidence of two aspects of their findings: (A) how solidly the observations show that a treatment did induce changes in succeeding generations; the usual significance level of 0.05 is far too loose here: and (B) what syndrome of changes is carried over. Conventional measures of significance are appropriate for this purpose.

5. Inadequately defined genetic background of the experimental animals. Most transgenerational studies of drug and hormonal treatments have been conducted on inadequately characterized strains of animals. Because geneticists are skeptical of purported Lamarckian inheritance, this field has been left mainly to physiologists. These latter scientists naturally design studies around the logic of their field instead of genetic paradigms. For example, Goldner and Spergel (1972) deliberately chose outbred animals for their studies of the transmission of latent diabetes because 'A non-inbred strain of rat... more closely resembles the genetic state of the human population than does the inbred animal, brother-sister mated for more than twenty generations. Any genetic information gleaned from such an inbred strain could be applied only with difficulty to the understanding of human diabetes mellitus.' More extreme problems arise for a carryover process which operates only in genetically obscure species (such as insect diapause). Ultimately, inheritance must be related to contemporary genetics. That paradigm is to relate genetic phenomena to the base sequence of a DNA molecule. Geneticists will fully accept induced carryover effects only when the DNA responsible for them is isolated. This goal is becoming more and more realistic. However, it is feasible only in genetically standard organisms.

6. Subjective measures. Neuroendocrines have sophisticated functions. Some can be observed only with complex assay systems or technical expertise. In particular, judgement is required to conduct and interpret some behavioral tests. Such tests are valid, and indeed underlie whole fields of research. Yet geneticists will reject demonstrations of the inheritance of acquired characters by assay methods that have a possible subjective component or seem more complex than necessary. They demand that at least one transmitted characteristic be completely objective, such as body weight, a simple count or a measurement that anyone can make.

7. Nonspecificity of effects. Some transgenerational changes have no obvious relationship to the disturbed neuroendocrine system. Their ad hoc character makes these persisting changes more difficult to believe and less interesting than specific changes. It is primarily their similarity to the short-term disturbances that induced them that distinguishes carryover effects from ordinary mutation. The preceding paragraph describes the

reasons for tracing changes in generalized characteristics, such as body weight, because they are objective and unambiguous. However, it is necessary *also* to demonstrate specific changes in progeny if they occur. This is important even if it requires specialized analytic procedures that may be complicated and not wholly precise. One generally useful and objective indicator of specificity is an altered sensitivity of progeny to the drug or treatment used to induce the change in the parent. Exposing animals to LSD, alloxan, morphine and tolerizing antigens makes their descendants more sensitive to those particular agents.

Mechanisms

Induced carryover effects have been compared to Lamarckian inheritance of acquired characters. The essential difference is that Lamarckism presumes that acquired modifications in phenotype automatically carry over to progeny as an innate, primary feature of inheritance. In contrast, the carryover effects of stress to neuroendocrine systems must be ascribed to special mechanisms that individual neuroendocrine systems have happened to evolve (Campbell, 1982). Without those evolved specializations, insults to an animal would be confined strictly to that individual. Whether these mechanisms evolved in order that experiential information can be usefully transmitted to progeny (as suggested for glucose regulation by Goldner and Spergel, 1972) or whether transmission is merely a byproduct of elaboration that a system evolved for other functions is an open question.

The artificially induced transgenerational changes in studies reviewed above are obviously maladaptive, but this may be misleading. Presumably the adaptive purpose for a carryover mechanism would be to pass along fine adjustments of a thyrostat, a glucosestat, calcium regulation, tendency to diapause, tolerance to an antigenic specificity or developmental rate. It is easy to believe that tuning any of these properties in an animal of one generation according to the slight imbalances experienced by its parents could be useful. However to demonstrate carryover effects, experimentalists must abuse these systems to degrees far beyond any functional meaning. Nudging the thyrostat setting of mice by amounts that could be adaptive would be experimentally unnoticeable. Thus, the gyrations induced by addicting baby mice to morphine or extirpating the thyroid are irrelevant to the issue of adaptiveness of carryover mechanisms.

An adaptive genetic explanation for induced carryover effects must answer two separate questions. One concerns the type of change made in the structure of the gene. The other is how a neuroendocrine imbalance triggers that genetic change to take place. Thirty years ago both problems seemed intractable. In the words of Waddington (1957), 'It seems to be the opinion of nearly all recent authors, with the exception of Lysenko and his followers in Russia, that the lack of conclusive evidence for such effects and the difficulty of envisaging even in theory a mechanism by which they might operate, justify one in completely rejecting this theory.' Fortunately, the enormous progress in genetics over the past 30 years has overturned this situation.

Before the DNA revolution, genes were considered to be abstract units of sacred information, transcendental to the physical world except for rare, spontaneous, random and blind mutations. We now have a more realistic material view (Campbell, 1982, 1983). Genes are biological molecules and, like all components under the control of cells, are substrates for enzymes. Cells have enzymes to catalyse many deliberate sorts of alterations in gene structure, including inversions, transpositions, duplications, base pair substitutions, and corrections of one gene according to the sequence of another. Each of these types of alteration is known to produce transgenerational changes in variable characteristics of lower organisms or plants. It would fall well within precedents set by other species for any of them to underlie carryover effects in a neuroendocrine

system of mammals. Identifying the type of DNA alteration responsible for a particular carryover effect, the gene involved and (eventually) the enzymatic pathway that catalyses that change, amounts to a conventional, if ambitious, program in molecular genetics.

The more challenging puzzle is how a neuroendocrine imbalance induces a germ cell to change its genome. The Weismann barrier between the soma and germ-line is formidable and certainly most experiences of the soma have no effect whatsoever on heredity (Weismann, 1893). It is this barrier and not the presumed inviolability of the structure of gene molecules which makes carryover effects in animals surprising. In fact, text books on cell biology describe without reserve examples of induced carryover effects in organisms which do not separate somatic and germ lines, such as bacteria, single-celled protista and plants (e.g. Alberts et al., 1983). We also accept labile gene changes which occur 'spontaneously' in metazoans because they do not imply a communication between the soma and germ-line (Belyaev et al., 1981a; but see Belyaev et al., 1981b).

Various investigators have suggested that drugs and hormone imbalances exert effects on progeny by acting on germinal cells. According to Guyer and Smith (1920) 'Ever since the discovery of the existence of such special internal secretions as hormones and chalones doubtless every biologist has thought of the possibility and many have expressed the idea that such substances might be concerned in some way in transmitting the results of somatic modifications to the germ, although, to our knowledge, no one has yet supplied a plausible explanation of how somatogenic [traits] are converted into blastogenic modifications by such means.'

Our increased understanding of hormones make this speculative activity of neuroendocrines increasingly plausible. It is becoming apparent that most neuroendocrines have multiple target tissues and functions. Some are broadcast throughout the bloodstream as endocrines while at the same time secreted regionally to regulate adjacent cells as paracrines and even produced ultralocally by cells to stimulate themselves as autocrines. Also, many are used simultaneously to regulate and coordinate somatic physiology (as hormones), the nervous system (as neurotransmitters and neuromodulators), the immune system (as immune regulators) and development (as trophic and tropic agents). We have suggested (Campbell, 1982) that it is more meaningful to call these chemical messengers 'cybernins'; conveyers of information, rather than 'hormones', 'neuroendocrines', 'neuroimmunoendocrines', 'hormone-like growth factors' and other like terms which emphasize individual physiological roles. Cybernins carry out their functions in an integrated fashion. A major emphasis in contemporary endocrinological research is to demonstrate that a cybernin known to regulate one aspect of physiology also functions in other areas and that these roles are interwoven. Any cell potentially can tap into a cybernin circuit merely by expressing on its surface receptors for that messenger molecule. Some cybernins are indeed perceived by many different cell types. We have included in this review examples of carryover effects from the immune system and from development, despite their more speculative status, in order to emphasize the scope of the cybernin system.

An intriguing extension of the cybernin concept is that these same molecules also regulate the genetic system. By this perspective cybernins would signal germ-line cells in the same general way that they do other targets. They would bind to specific cell surface receptors (presumably expressed from the same gene as in many or all target cells) and initiate the same pattern of intracellular and genetic activities as in other cells (Campbell and Zimmermann, 1982).

It is suggestive that most treatments which induce transgenerational effects also produce long-lasting somatic changes in the treated animal. One injection of thyroxine or insulin into a neonate alters TSH or glucose regulation for the

life of the animal (Bakke et al., 1975; Csaba, 1980). An obvious possible basis for this persistance is that signals from hormone receptors induce specific changes in DNA structure which are stable through chromosomal replication and cellular division.

We have proposed the term *automodulation* for the case in which cell surface receptors regulate the enzymatic alteration of their own genes (Campbell, 1982; Campbell and Zimmermann, 1982; Fig. 2). The definitive example in mammals occurs as part of the immune response. B lymphocytes of the immune system synthesize immunoglobulin molecules and plant them on their outer membranes as cell surface receptors for antigens. When these receptors are stimulated they send signals to the cell nucleus to induce specific enzymatic alterations in the structure of the immunoglobulin genes. These change the receptor's subsequent responses to stimuli.

Automodulation is a plausible mechanism for permanently setting levels of hormones during critical periods in development, such as for the thyrostat. Moreover it would allow changes to cross generations with no extra physiological machinery beyond that evolved to mediate the somatic functions. Inheritance of thyrostat settings would only require the genes activated at the critical perinatal period in the thyrotroph cell also to be expressed at that time in germ-line cells.

There is no direct evidence for gene automodulation in germ-line cells. However, the physiology of these cells is poorly known. The most obvious prediction of the model is that germinal cells have receptors for various neuroendocrines. Interestingly, receptors have been detected on *Xenopus* or mouse eggs for acetylcholine, adrenaline, 5-hydroxytryptamine and dopamine (Kusano et al., 1977), progesterone and insulin (El-Etr et al., 1979, 1980; but see Wallace and Misulovan, 1980), and purines (Lotan et al., 1982). Germinal cells of males are harder to study than large oocytes but circumstantial evidence suggests that they too carry receptors for cybernins. For example spermatogenic cells in mouse testes synthesize endorphins. If these cybernins have autocrine functions, as Kilpatrick and Millette (1986) suggest, then male germ-line cells would have morphine receptors. Also, deficiencies in a variety of hormones arrest sperm production (including, by the way, insulin and parathyroid hormone, Oksanen, 1975; Fujii and Morita, 1978). It would be interesting to test egg cells systematically for receptors for the whole range of hormones, cell growth factors, neuromodulators etc. that have been discovered in mammals.

Automodulation in germinal cells would directly explain why injecting drugs into animals changes the sensitivity of their offspring to those

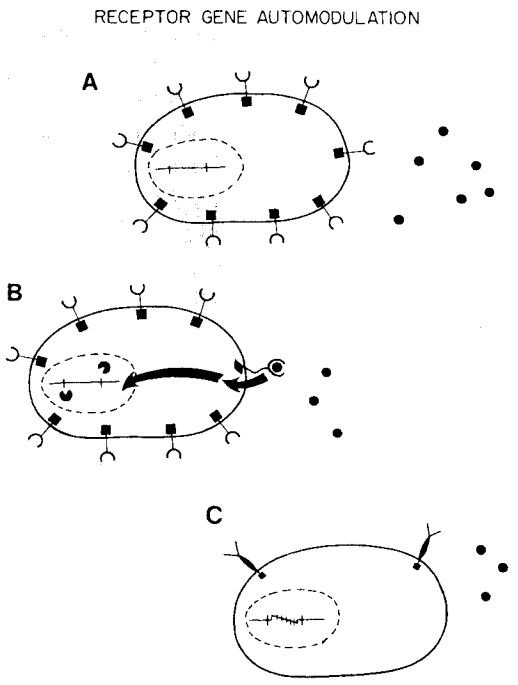

Fig. 2 Model for automodulation of a hormone receptor gene. A. A cell is shown with hormone receptors in its membrane and the receptor gene in the nucleus. B. Upon binding the hormone an activated receptor signals a set of specific nuclear enzymes to rearrange the structure of the receptor gene. C. The modified gene changes the cell's responsiveness to the hormone. Automodulation might affect the number of receptors on the cell membrane, their binding properties or the signal that they generate when activated.

TABLE 3

Suggested mechanisms for induced carryover effects

Suggested mechanism	Ref.
A. Effects transmitted by males and through multiple generations	
Automodulation	1
Direct genetic transformation: uptake of DNA from soma by germ cells	2
Transfer of genetic material from affected somatic tissue to germ cells by a vector	3
Mutation	4
'Paramutation' as a presumed heritable 'restraint on gene expression without altering structural DNA'	5
Combination of two mechanisms: one in males, the other for multiple female generations	–
Contrived data or artifact	6, 7
B. Male transmission through a single generation	
Damage to the sperm by a drug	8
Change in component of sperm besides DNA	9
Transmission of a drug through semen	10
Change in a component of semen other than sperm e.g. hormone, antibody, or lymphocyte	8
Methylation of DNA at specific sites	–
Altered coital activity of male	8
C Transmission through multiple female generations	
Self-perpetuating alteration in:	
a cellular organelle	11, 12
the uterus or placenta or milk supply	13
the immune system (auto-antibodies)	14
a neuroendocrine system	15
behavior	16
Changes in the numbers of copies of:	
extrachromosomal genetic elements	17
extranuclear intracellular particles	18
genes in multigene families	19, 20
Altered heritable pattern of gene regulation	21
Integration of an episome which can be in either a cytoplasmic or chromosomal state	22
D. Progressive change while animals are kept under inducing conditions	
Artifact from improved rearing conditions instituted over the course of the study	23
Progressive loss of virulence of an endemic pathogen or change in a symbiont organism	24, 25
Progressive depletion of an essential dietary nutrient	26
Extra replication of heterochromatin	27
Selection	28

1. Campbell and Zimmermann, 1982.
2. Kanazawa and Imai, 1974.
3. Steele, 1981.
4. Detlefsen, 1925.
5. Spergel et al., 1971.
6. Koestler, 1971.
7. Huxley and Carr-Saunders, 1923.
8. Joffe, 1979.
9. McLaren, 1979.
10. Lutwak-Mann et al., 1967.
11. Jinks, 1964.
12. Caspari, 1948.
13. Zamenhof et al., 1972.
14. Goldsmith et al., 1973.
15. Aerts and Van Assche, 1979.
16. Denenberg and Rosenberg, 1967.
17. Bucheton and Picard, 1978.
18. Mampell, 1968.
19. Cullis, 1977.
20. Ritossa, 1976.
21. Bussey and Fields, 1974.
22. Barigozzi et al., 1962.
23. Agar et al., 1954.
24. Barnett, 1961.
25. Mampell, 1965.
26. Wallace et al., 1983.
27. Beardmore et al., 1975.
28. Waddington, 1952.

compounds. As another detail, Kusano et al. (1977) have called attention to the substantial variation in the presence and abundance of receptors for particular neuroendocrines among batches of *Xenopus* eggs. Perhaps germinal cells vary in which receptor systems are active, or which are active at a particular time. A stochastic element in receptor expression would agree with the sporadic carryover effects seen among the progeny of animals exposed to certain cybernin stimuli.

Gene automodulation is only one of several proposed mechanisms for induced carryover effects. Kanazawa and Imai (1974) have suggested that DNA is transferred from soma to germinal cells in a process resembling transformation in bacteria. Steele (1981a) believes that a retrovirus vector carries genetic information to the testes from stimulated antibody-producing cells. He has elaborated his model for the immune system but it could be further developed to apply to endocrine organs. Such direct flows of nucleic acid from the body to the germ-line would correspond to a true Lamarckian process.

Another class of explanations for male transmission depends on components other than DNA of sperm (such as a patch of membrane possibly with cybernin receptors or a 'cytoplasmic' structure). As a pertinent example, Sastry et al. (1982) have detected enkephalin (as immunoreactivity) in mature spermatozoa. Extracellular agencies are also possible. Gorczynski et al. (1983) showed that immunologically tolerant male mice seem to transmit some factor to female mates inducing them in turn to transmit hyporesponsiveness maternally to their progeny. This factor might be lymphocytes or antibody molecules in both sexes.

Table 3 catalogues a number of suggestions for how drug influences might be transmitted through one sex or the other. These ideas are germane because several of them might be superimposed to produce the full range of carryover effects seen in the examples described above. For example, hyperparathyroidism might change calcium metabolism through multiple generations in female lines by one mechanism and testosterone levels in descendants of males by another. A particular possibility that must be kept in mind is that a drug which induces an automodulation process might also alter uterine or placental function as an entirely independent secondary action. A second subsidiary mechanism could explain why F*1 animals sometimes differ from those of the F*2 generation (as in the cases of thyroxine and LSD exposure described above). Because transgenerational effects of physiological manipulations are outside conventional orthodoxy it is generally felt that they must be rare. This may be a wrong perception. The alternative is that there are many possible ways for events in one generation to affect the next, ranging from trivial to sophisticated. As a complex multifunctional trait adaptively evolves it tends to accumulate associations with other genes that may affect progeny. As physiology becomes more integrated many traits may interact with ones that affect offspring.

Nonspecific carryover effects

A final question with a major bearing on mechanism is the degree of specificity of the transgenerational effects induced by stress. Presumably the carryover effects described here are specific. Diabetogeneic drugs induce glucose intolerance and thyroidectomy disrupts thyroxine balance in progeny. However, the consequences of these treatments may overlap more than the literature suggests. Blood glucose levels have not been measured in progeny of hypothyroid animals nor have studies of induced diabetes included response times of F*1 animals placed on a hot plate. Endocrinologists naturally concentrate their attention on traits in progeny related to the cybernin that they are studying.

Various investigators have found that the most obvious carryover effect of neonatal morphine exposure is a delay in postnatal maturation. Retarded postnatal weight gain also carries over from thyroidectomy, protein deprivation and certain subdiabetic regimes. There are two plausi-

ble reasons for these overlaps. One is that various stresses trigger a common response mechanism. Organisms do have generalized stress-response systems. One that extends taxonomically from bacteria to higher animals was defined originally as heat shock responses but now is known to be stimulated by a variety of stresses (Hammond et al., 1982). Animals, plants and bacteria all have been found to respond to certain general stresses by enzymatically changing DNA sequences in their chromosomes (Echols, 1981; McClintock, 1984; Strand and McDonald, 1985).

The existence of a common carryover mechanism for a variety of neuroendocrine stresses is an attractive idea. It probably cannot account for all of the effects discussed above. However, from a medical point of view induction of a DDM syndrome by a variety of neuroendocrine imbalances could be substantially more important than more restricted carryover responses.

A second possible reason for stresses on different hormone systems to induce similar effects on progeny is that cybernin circuits are highly interrelated to one another. They modulate and regulate the sensitivity of cells to each other. Many, if not most, cybernins also act on other cybernin-producing cells. Instead of representing independent communication channels between pairs of tissues cybernins are locked into a network which, at least indirectly, links most together. Alterations in one cybernin component will cascade through the network producing secondary and tertiary effects on an ever-widening array of characters. For example, alloxan-induced diabetes disturbs LHRH and thereby gonadotrophins which in turn alter sex steroids (Cusan et al., 1980). Drug interventions at different points in the network might ramify to a common cybernin system that has evolved the machinery to affect offspring generations. In the progeny a limited number of transmitted changes might again expand to a range of phenotypic properties.

Immunologists have realized the fundamental roles of network properties in the operation of the immune system. The connectivity among cybernins gives it a network capacity as well. However, the cybernins are richer in diversity and function than immune molecules and their potential range of network properties is commensurately larger. The complexities reported for transgenerational effects probably can be understood only by a network level of integration of development, immunity and neuroendocrine regulation with inheritance.

Summary

Hormonal stresses at critical periods during development can lead to long-lasting, even permanent, abnormalities in adult hormone levels. Evidently the steady-state values of certain endocrine control circuits are set in part on the basis of hormone levels about the time of birth instead of being rigidly programmed genetically. This developmental flexibility might allow adapted hormone levels of a mother to be impressed upon her fetal offspring as an epigenetic 'maternal effect'. Two features of the carryover effects in several endocrine systems suggest even more elaborate underlying mechanisms, possibly even involving induced changes in gene structure. These disturbances can persist for multiple generations and be passed on by male as well as female parents.

This article reviews the most notable carryover effects reported in the literature, discusses the difficulties of interpreting their descriptions, and catalogues the rather extensive set of mechanisms that have been proposed to explain transgenerational effects of one sort or another.

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Discussion

W. Lichtensteiger: As far as the thyroid axis and its regulation are concerned, there has been a characteristic deficit in humans, i.e. cretinism. This condition disappeared completely after preventive administration of iodine. This rather appears to speak against a genetic fixation of the setpoint sensitivity.

J.H. Campbell: This is an interesting observation. Insufficient dietary iodine in various parts of the world is extreme enough to cause cretinism, and more generally goiter, but obviously has not induced a permanent continuation of this pathology. You're probably right to add a caveat about interpreting this observation in humans. It would be interesting to know if, indeed, there are residual functional or histological abnormalities in the thyroids of people today with a long suspected family history of iodine insufficiency. Of course, even if this were the case it would be hard to draw any definite conclusions. We really need controlled studies on experimental animals. Even so, your point is important: past widespread iodine deficiency has not imperiled the human gene pool to any detected degree.

M. Mirmiran: Does showing that small mouse offspring produce small offspring in the following generations allow you to conclude that any receptor set point is determined genetically?

J.H. Campbell: If mice made small by neonatal exposure to morphine, or with an induced thyroid imbalance, generate offspring with related defects there must be a mechanism for this. A small size might be transmitted through the female line due to a small placenta or inadequate milk supply but it is trickier to devise an epigenetic mechanism for inheritance through the male line. I doubt that small mice produce small sperm and therefore cause the F*1 progeny to grow more slowly after birth. Bakke et al. (1974, 1976) have characterized the altered phenotype of the F*1 progeny from thyroidectomized male mice. The changes are multifold, as one might expect for an alteration in a hormone system with diverse roles in development and adult metabolism. With respect to the effects of thyroid balance a change in the set point of the thyrostat would explain the changes.

M. Mirmiran (Comment): I can understand your sympathy for genes. However, your conclusion that a carry-over effect occurs for receptor set point is largely speculative with no data. In this regard, I am afraid you neglected the dynamic interactions between neurotransmitters and receptors during development, and our belief that the set point develops largely as a function of neurotransmitter availability on the synaptic level.

V.R. Sara: A receptor is a protein translated from a gene. One way to regulate the threshold of sensitivity would therefore be via changes in the promotor regions of the gene. Any such structural change would provide a means for genetic transmission of a 'hormonostat'. The techniques are now available to map the structure of the gene suspected of being altered in the germ cells.

J.H. Campbell: Yes, the basic prerequisite techniques are available. Moreover, they have been used to prove that various chemicals and drugs can induce specific heritable

alterations in relevant genes of simple organisms. However, the amount of work needed to pinpoint changes in a complex gene of mammals should not be underestimated. You make a good point when you state that sequences regulating a receptor gene might be changed instead of the structural gene itself.

G.J. Boer (addition to V.R. Sara): Before we go into DNA technology, however, we should be sure about the trans-generational characteristics of the effect.

J.H. Campbell: Absolutely.

G.J. Boer: If, indeed, DNA changes are underlying the effect you described, what do you think should be our attitude towards experimentally approaching this.

J.H. Campbell: The obvious approach would be firstly to demonstrate conclusively that a persisting, heritable alteration can be induced reproducibly by a simple experimental manipulation on the male parent. This has not been done as yet. Secondly, it is necessary to characterize the syndrome of changes in affected offspring precisely enough to pinpoint fairly definitively the gene that is likely to have been altered. Only thereafter can one try to isolate, clone and sequence the DNA of that gene in order to prove that a change in structure was induced. Each of these steps is substantial and requires its own sort of work and expertise. To me it seems likely that if certain genes have evolved machinery to modify their structure in response to environmental conditions, the first example to be discovered will probably be in a system that is mainstream in molecular biology. This should perhaps be considered by anyone interested in approaching this problem.

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