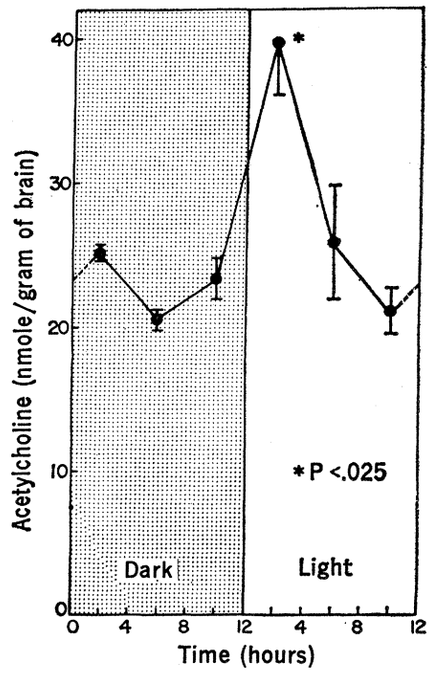


Fig. 2. Acetylcholine concentrations in brain of rats killed by guillotine throughout one 24-hour cycle. Each point represents the mean and standard error of at least three animals. Statistical difference was determined by Student's *t*-test. The *P* value was identical for any point on the curve with respect to the peak at $L_2$.

cally undistinguishable if measured either at $D_6$ ($25.4 \pm 0.2$ nmole/g) ($n = 4$) or at $L_2$ ($26.7 \pm 2.5$ nmole/g) ($n = 4$).

It appears from these results that the regulatory mechanism controlling ACh concentrations in rat brain does not maintain the steady state at a constant level throughout the day. Furthermore, this phenomenon occurs only in grouped animals, while in isolation the steady-state level of ACh is apparently maintained throughout the day.

This pattern was observed only in rats killed by decapitation with a guillotine. When animals were killed by total body immersion in liquid nitrogen, no diurnal pattern was observed. In fact, concentrations of brain ACh were identical at $D_6$ and $L_2$ and both were significantly lower than in rats killed by guillotine at the identical time period. However, freezing the animal in liquid nitrogen before extracting its brain for ACh assay may not reflect concentrations of rat brain ACh in vivo.

We have confirmed elsewhere (*8*) that brain ACh concentrations in rats killed by freezing in liquid nitrogen may reflect physical changes caused by the process of freezing and thawing on tissue compartmentation, leading to increased hydrolysis of the ester by acetylcholinesterase (*9*).

The diurnal pattern for brain ACh does not emerge in a group of rats housed under controlled conditions of light and temperature until a finite time has elapsed. Rats were placed in the controlled environment in groups of eight per cage immediately upon their arrival. Brain ACh concentrations were determined at 2 hours of light and 6 hours of darkness after 6, 13, 18, and 58 days of conditioning. Four animals were used at each time tested. Ratios of ACh concentrations at $L_2$ with respect to $D_6$ were calculated to be 0.94, 0.79, 1.79, and 1.93, respectively. These data indicate that at least 18 days are required before the concentrations of brain ACh are consistently higher at $L_2$ than they are at $D_6$. This probably reflects a synchronization of the diurnal pattern in animals housed under control conditions, as opposed to a random pattern prevalent in a group of rats which have just been delivered from the supplier.

This observation emphasizes the generally accepted belief that, in order to obtain meaningful and consistent results in animal experimentation, the animals have to be preconditioned to the experimental environment for a finite number of days. At least 18 days are required in studies involving the rat brain cholinergic mechanism. The obvious biochemical implications of the observed diurnal variation in concentrations of rat brain ACh provide a tool for gathering insight into the working hypothesis presented in this report. This diurnal rhythmicity could be used to detect physiological mechanisms regulating steady-state concentrations and turnover rate of brain ACh content.

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**References and Notes**

1. P. Albrecht, M. B. Visscher, J. J. Bittner, F. Halberg, *Proc. Soc. Exp. Biol. Med.* **92**, 703 (1956); W. B. Quay, *Gen. Comp. Endocrinol.* **3**, 473 (1963); *Proc. Soc. Exp. Biol. Med.* **115**, 710 (1964); R. J. Wurtman and J. Axelrod, *Life Sci.* **5**, 665 (1966); D. J. Reis, M. Weinbren, A. Corvelli, *J. Pharmacol. Exp. Ther.* **164**, 135 (1968); R. J. Wurtman, C. Chou, C. M. Rose, *Science* **158**, 660 (1967); L. E. Scheving, W. H. Harrison, P. Gordon, J. E. Pauly, *Amer. J. Physiol.* **214**, 166 (1968); A. H. Friedman and C. A. Walker, *J. Physiol.* **202**, 133 (1969).
2. R. J. Wurtman and J. Axelrod, *Biochem. Pharmacol.* **12**, 1439 (1963).
3. S. H. Snyder, J. Axelrod, J. E. Fischer, R. J. Wurtman, *J. Pharmacol. Exp. Ther.* **147**, 371 (1965).
4. A. H. Friedman and C. A. Walker, *Fed. Proc.* **28**, 251 (1969).
5. D. J. Jenden, I. Hanin, S. I. Lamb, *Anal. Chem.* **40**, 125 (1968); I. Hanin and D. J. Jenden, *Biochem. Pharmacol.* **18**, 837 (1969).
6. A preliminary account of this work has been presented in abstract form [I. Hanin, R. Massarelli, E. Costa, *Physiologist* **12**, 246 (1969)].
7. C.-G. Hammar, I. Hanin, B. Holmstedt, R. J. Kitz, D. J. Jenden, B. Karlén, *Nature* **220**, 915 (1968).
8. I. Hanin, R. Massarelli, E. Costa, in *The Effect of Drugs on Cholinergic Mechanisms in the CNS* (Skokloster, Sweden, in press).
9. J. Crossland, *J. Physiol.* **114**, 318 (1951); K. A. C. Elliott and N. Henderson, *Amer. J. Physiol.* **165**, 365 (1951).
10. We thank Miss S. Lofstrandh for technical assistance.

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## Brain Enhancement in Tadpoles: Increased DNA Concentration after Somatotrophin or Prolactin

Abstract. *Frog tadpoles, injected with prolactin or somatotrophin during early stages when the brain cell population is rapidly increasing, exhibited marked increases over sham-injected controls in body weight, brain weight, and brain DNA, throughout subsequent development. Animals treated with somatotrophin attained the increase in brain DNA during the infection period. Prolactin had little effect during this period, but brain DNA accumulation continued at an accelerated rate over the next 15 days, when the rate of DNA increment normally declined. Patterns of incorporation of tritiated thymidine confirmed that both hormones increased DNA accumulation, suggesting increased cell proliferation rather than decreased cell death.*

Mammalian prolactin and mammalian somatotrophin both promote general body growth in anuran tadpoles (*1*). The actions of the two hormones on the growth and development of the brain, however, are only vaguely understood. Early experiments, in which crude pituitary extract was adminis-

tered during embryonic development, produced heavier brains in frogs and in newborn rats (*2*). More recently, purified somatotrophin was shown to produce similar, but smaller, effects in rats and chicks (*3*, *4*). In some of these studies, the increase in brain weight appeared to be the result of an increase in the brain cell population, as determined by counting the cells in histologic sections (*2*) or by colorimetric estimation of total brain DNA (*3*). In contrast, histologic studies (*4*), in which rats were treated prenatally with somatotrophin and allowed to grow to adulthood, indicated an increase in cellular size (rather than numbers of cells) as the end result of treatment with somatotrophin. Research into the neural effects of prolactin has not been reported.

Using *Rana pipiens* tadpoles, we have repeated and extended the experimental study of the neurogenetic effects of somatotrophin and have conducted a parallel study with prolactin. Our primary goal in expanding the earlier experimental designs was to monitor changes in the brain, not only in the resulting mature animal, but also during the period of injections and at frequent intervals thereafter, through metamorphosis.

Each tadpole received a total of six intraperitoneal injections of either 5 $\mu$g of prolactin (P-group), 25 $\mu$g of somatotrophin (S-group), or 0.05 ml of Ringer solution (C-group) beginning in the middle of larval stage III (*5*) and continuing, on alternate days, to stage VII (*6*). These doses produce extensive effects on body growth without inhibiting metamorphosis. We removed the intact brains from exsanguinated animals at larval stages II and III (untreated), V, VII, X, XIII, XVI, XX, and after metamorphosis (XXV). After the brains were weighed and frozen, they were homogenized; the DNA was then extracted and measured in triplicate by the diphenylamine method (*7*).

To examine the total synthesis of brain DNA during the interstage periods III to VI, VII to X, and X to XIII, six animals from each group were given daily injections of [$^3$H]thymidine (specific activity, 20.2 c/mmole; 1.5 $\mu$c/g body weight) during each of the periods under study. Portions of DNA extracted from the brain were counted directly in Bray's solution (*8*) in a liquid scintillation system. As an additional counting control, we extracted DNA from unlabeled brain tissue that had been killed by freezing and thawing and that had subsequently been injected with [$^3$H]thymidine; radioactivity in this DNA was insignificant, confirming that unincorporated precursor was separated from the DNA during the extraction.

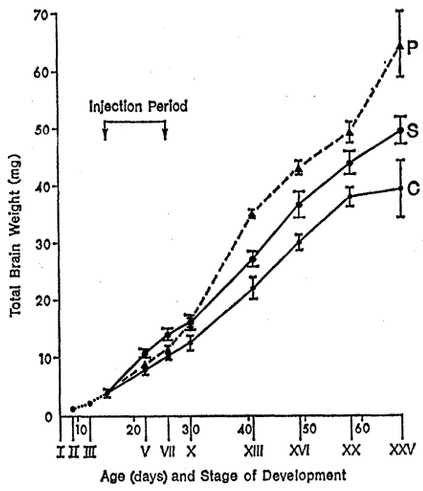


Fig. 1. Wet weight of whole brains removed, at different stages of development, from frog tadpoles after receiving injections of prolactin (P), somatotrophin (S), or saline (C). Each point is the mean value derived from 11 to 16 animals. The vertical bars show two standard deviations about the mean.

Neither hormone altered the time at which successive stages were reached, and none of the groups exhibited physical or morphological abnormalities of any kind. Similarly, animals injected with [$^3$H]thymidine developed at the same rate and to the same sizes as their similarly treated, but unlabeled, counterparts.

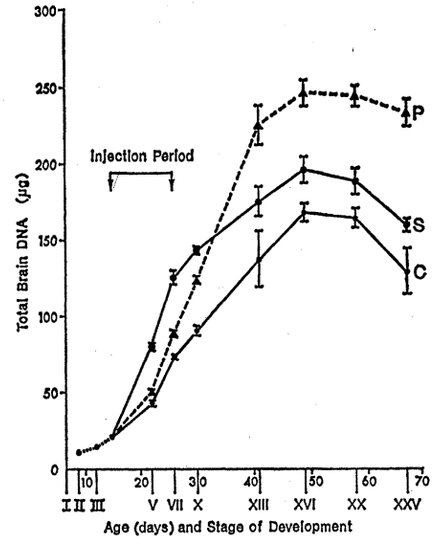


Fig. 2. Total brain DNA of frog tadpoles at different stages of development after receiving injections of prolactin (P), somatotrophin (S), or saline (C). Each point is the mean value derived from 11 to 16 animals. The vertical bars show two standard deviations about the mean.

Body weight in both experimental groups was markedly increased over control tadpoles at every stage, reaching increments of 25 to 100 percent in the later larval stages. Animals receiving prolactin were always heaviest and were consistently 1 to 2 g heavier than S-group animals from mid-larval stages through metamorphosis. Both hormones also increased brain weight, although the two experimental groups exhibited small but consistent differences in the timing of brain growth (Fig. 1). During the injection period, the brains of S-group tadpoles were consistently larger than those of their P-group counterparts. The latter overtook the S-group tadpoles, however, and continued to enjoy an advantage in brain weight from larval stage X through metamorphosis.

Brain DNA (Fig. 2) was rapidly accumulated in control animals between mid-stage III and stage XVI, although the maximum rate of DNA increase was attained before stage VII. Prolactin had little effect on the amount of DNA accumulated or on the extent to which [$^3$H]thymidine was incorporated into brain DNA (Table 1) during the period of hormone administration, stages III to VII. However, during the subsequent 15 days, in mid-larval stages, the tadpoles of P-group failed to show any decline whatever in the rate of DNA accumulation in the brain. Thus, DNA content rose 55 percent more rapidly in the P-group brains than in control brains from stages VII to XIII. Cumulative [$^3$H]thymidine-labeling during these stages (VII to X and X to XIII) confirmed a continuous, augmented rate of DNA accumulation in the brain, and suggested that the additional DNA was mainly accumulated by increased synthesis, rather than by decreased cell death. The end result of prolactin treatment was a 50 to 80 percent enrichment of brain DNA in the postmetamorphic frog.

In contrast to prolactin, somatotrophin enhanced brain DNA accumulation by 90 percent during the injection period (Fig. 2), producing an increment of 50 $\mu$g of DNA per brain over the stage VII controls. This increment

Table 1. Radioactivity in brain DNA extracted from frog tadpoles after receiving daily injections of [$^3$H]thymidine during the periods shown. Each range of values is derived from three portions of the total DNA extracted from six pooled brains. Measurements of DNA were done on other portions. All tadpoles received six injections of either prolactin (P), somatotrophin (S), or saline (C) on alternate days, beginning at mid-stage III.

| Group | Radioactivity (count/min) | |
|---|---|---|
| | Per milligram of DNA | In whole brain |
| | *Mid-stage III to stage VI* | |
| P | 40,200 ± 1800 | 2816 ± 126 |
| S | 56,190 ± 510 | 5900 ± 54 |
| C | 33,600 ± 3400 | 2016 ± 196 |
| | *Stage VII to stage X* | |
| P | 32,360 ± 2780 | 3883 ± 333 |
| S | 16,056 ± 1656 | 2240 ± 232 |
| C | 17,380 ± 40 | 1564 ± 4 |
| | *Stage X to stage XIII* | |
| P | 33,617 ± 1133 | 7059 ± 238 |
| S | 12,092 ± 483 | 2060 ± 82 |
| C | 21,310 ± 2710 | 2660 ± 337 |

waned during subsequent development, and was ultimately reduced to a net increase of 15 to 25 percent in the postmetamorphic frogs of the S-group. The labeling experiment (Table 1) showed comparable changes in the accumulation of newly synthesized DNA. Only during the injection period did the S-group animals exceed the controls in uptake of [$^3$H]thymidine into brain DNA.

There are reasons, however, for exercising caution in interpreting the significance of the increased radioactivity in the DNA of the experimental groups. First, increased [$^3$H]thymidine incorporation suggests an enhancement of cellular proliferation, but it does not exclude the possibility that the normal number of polyploid neurons is increased. Moreover, our evidence that both hormones primarily increase DNA synthesis—temporal differences notwithstanding—does not preclude the possibility that cell death is also affected. In particular, neuronal death is extensive in normal animals during the metamorphic stages XX to XXV (*9*); and during this period, P-group tadpoles suffer a mean decrease in brain DNA that is only 25 percent as great as the DNA losses in the other two groups (Fig. 2). This observation suggests either that a diminution of cell death may also occur after prolactin treatment, or that enhanced cell proliferation and normal cell death occur concurrently in P-group animals during metamorphosis.

We have confirmed that purified somatotrophin increased DNA in the developing brain, although our results are not in complete agreement with the hypothesis advanced by previous researchers (*3*). They inferred, from observation of a net DNA change and some histologic estimations, that somatotrophin extends the period of rapid neuronal proliferation. In our experiments, neither prolactin nor somatotrophin, administered during early larval stages, lengthened the period over which brain DNA is normally accumulated (to stage XVI). Rather, somatotrophin induced a very rapid rise in the rate of brain DNA accumulation during the period of its administration; but shortly after the termination of the injections, the rate of rise in DNA content declined to below that of the control group. In contrast, the effects of prolactin on the accumulation of DNA in the brain became manifest only after the injection period: an extraordinarily rapid rate of increase of DNA was maintained through the mid-larval stages, and considerably less brain DNA was lost during metamorphosis than in normal tadpoles.

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**References and Notes**

1. C. S. Nicoll, H. A. Bern, R. C. Strohman, *Amer. Zool.* **5**, 738 (1965); A. Enemar, B. Essvik, R. Klang, *Gen. Comp. Endocrinol.* **11**, 328 (1968); P. S. Brown and B. E. Frye, *ibid.* **13**, 139 (1969).
2. S. Zamenof, *Growth* **5**, 129 (1941); *Physiol. Zool.* **15**, 281 (1942).
3. ———, J. Mosley, E. Schuller, *Science* **152**, 1396 (1966); S. Zamenof, E. Van Marthens, H. Bursztyn, in *Proceedings of an International Symposium on Hormones in Development*, M. Hamburgh and E. J. W. Barrington, Eds. (National Foundation, New York, in press).
4. B. G. Clendinnen and J. T. Eayres, *J. Endocrinol.* **22**, 183 (1961).
5. A. C. Tayler and J. J. Kollros, *Anat. Rec.* **94**, 7 (1946).
6. Prolactin (NIH-P-S-8, ovine) and somatotrophin (NIH-GH-S8, ovine) were gifts of the Endocrinology Study Section of the National Institutes of Health.
7. E. Howard, *J. Neurochem.* **12**, 181 (1965); modified after K. Burton [*Biochem. J.* **62**, 315 (1956)].
8. G. A. Bray, *Anal. Biochem.* **1**, 279 (1960).
9. For references and discussion, see M. Jacobson, *Developmental Neurobiology*, (Holt, Rinehart & Winston, New York, 1970).
10. Supported by NSF grant GB 8315.

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## Mammalian Evolution: Is It Due to Social Subordination?

The basis of J. J. Christian's (*1*) evolutionary hypothesis is that in certain vertebrate species the available stands of optimum habitat are allocated to socially dominant individuals. Subordinate, chiefly young, animals are compelled to pioneer new kinds of habitat where they may become prospective founders of new evolutionary lineages while the original population is preserving the status quo. Though most of Christian's examples are small rodents, he extends the hypothesis to animals in general and to Darwin's finches in particular.

In spatially shifting but temporally stable habitats (for example, successional communities) the dominant-subordinate social system indeed facilitates the evolutionary status quo as subordinate pioneers are destined to discover newly available stands of the habitat and, as Christian rightly states, become dominant settlers there. Many polymorphic dispersionary systems exist in the animal world (*2*) and some—for example, solitary and gregarious locusts and alate or apterous aphids—function on a phenotypic basis, as Christian claims the cyclically expansive small rodents do (*3*). However, contrary to what Christian claims, I believe this mechanism does not easily lead to evolutionary differentiation. Colonies on secondary or marginal habitats have poor reproductive success, and they owe their prolonged existence to steady reinforcement by surplus individuals from optimal habitats (*4*). Such overwhelming gene flow slows down or prevents local adaptation on whatever basis this could happen (*5*). Christian's hypothesis does not explain how subordinate and surplus mammalian emigrants isolate themselves from the parent population. Yet, without isolation—in the cited cases geographic isolation is the most likely mode—they could hardly have evolved into different species, let alone into different adaptive types.

Colonization by barrier crossing is an essential phenomenon in island or archipelago situations where geographic