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Neural-immune system interactions in *Xenopus*

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1. ABSTRACT

In Xenopus, as in mammals, there is a functionally significant bidirectional communication between the neuroendocrine and immune systems. In this review, we describe the evidence for the neural innervation of Xenopus lymphoid organs, review the effects of neurotransmitter and hormone manipulations on measures of immunity, and discuss the role of hormones on immunological changes during metamorphosis. We also speculate as to the phylogenetic significance of these data, and outline possible areas of future research

2. INTRODUCTION

The existence of a robust functionally significant bidirectional communication between neuroendocrine and immune systems has progressed from a "fringe" hypothesis to a well-established and well-accepted fact (1-4). This evolution of an idea has been fueled by research from many different perspectives and disciplines, resulting in a new discipline known by several names that include psychoneuroimmunology, neuroendocrinimmunology, neuroimmunomodulation, or more simply, neural-immune system interactions. As with many newly established areas

of inquiry, psychoneuroimmunology has "progressed" from phenomenology to detailed mechanistic studies. As might be expected, the overwhelming majority of studies pertaining to neural-immune interactions has been conducted with mammals, chiefly rodents and primates, and it is in these species that our understanding of mechanisms is most developed. While an exhaustive review of the literature of this research area is quite beyond the scope of this paper (5, 6), it is worthwhile to outline six major lines of evidence for bidirectional neural-immune communication, as demonstrated in mammals.

1. Various stimuli (generally lumped under the term "stressors") can elicit behavioral responses ("stress responses") with associated central and peripheral neuroendocrine and autonomic nervous system changes that can alter both laboratory measures of immune responses and the health of the organism following immune challenge (7) or challenge with a pathogen (8). Such stressors can be physical (e.g., footshock, restraint) or psychosocial (e.g., differential housing or handling in animals; bereavement or taking examinations in humans (2, 9-11). It has even been shown that clinically relevant immunopharmacologic effects and immune responses can be conditioned classically (12-14).

2. Lymphoid tissues are innervated by autonomic nerve fibers in patterns that extend beyond just a close association with the vasculature. (6, 15, 16). Using both sucrose-potassium phosphate-glyoxylic acid (SPG) histofluorescence for catecholamines (described in (17)) and immunohistochemistry for tyrosine hydroxylase (TH), the rate-limiting enzyme in catecholamine synthesis, Felten and Olschowka (18) have demonstrated sympathetic innervation of the mouse spleen, with fibers almost exclusively confined to the lymphopoietic white pulp. While the case for direct neural input is most clear for the catecholamines, there is also circumstantial evidence for neuropeptidergic synaptic transmission, implicating vasoactive intestinal peptide (VIP), cholecystokinin (CCK), substance P (SP), and neuropeptide Y (NPY), some of which may be co-localized with monoamines (6, 19-22). One noteworthy neurotransmitter system which does *not* seem to be involved in peripheral neuroimmune transmission is the parasympathetic cholinergic (acetylcholine) system (6).

3. Cells of the immune system (i.e., lymphocytes, monocytes/macrophages, and natural killer cells) express receptors for neurotransmitters, neuropeptides, and hormones (23, 24). While the best known of the hormone-immune interactions involves hormones of the hypothalamo-pituitary-adrenal (HPA) axis and the resulting immunomodulatory effects of elevated levels of glucocorticoids (25), many other pituitary-derived hormones have also been implicated in immune modulation (26-29).

4. Leukocytes respond to altered concentration of the ligands for such receptors, whether pharmacologically (that is, by direct addition of the ligand) or via manipulation of the endogenous system (e.g., sympathetic nervous system ablation) with alterations in function (24, 30-32). Ablation of sympathetic nervous system (SNS) activity has been associated with changes in splenocyte proliferation in response to either polyclonal mitogen stimulation or antigen-specific stimulation (31, 32), as well as alterations in serum immunoglobulin levels from mice immunized after sympathectomy.

5. Leukocytes also produce neuropeptides and hormones, thus allowing for bidirectional communication as well as autocrine regulation (33-35). Such products have been demonstrated to be associated with altered activity and metabolism in the CNS during an immune response (36), including activation of the HPA axis, thereby allowing for a complete loop in communication pathways (37). Some of these CNS changes resulting from an immune response are associated with the well-known "sickness behaviors:" fever (which may be a behavioral response in ectotherms see (38)), loss of appetite, and changes in slow-wave sleep (39).

6. Conversely, neuroendocrine cells can both produce and respond to molecules classically assigned to immune regulation (i.e., cytokines) (40-43). Microglia in the CNS produce several proinflammatory cytokines, and can change their production profiles depending on activation state (44, 45).

3. A CASE FOR COMPARATIVE PSYCHONEUROIMMUNOLOGY

After having arrived at a point where such bidirectional communications can be examined in detail, and whose consequences are at least beginning to be understood, the time is ripe for understanding the phylogenetic history, and hence the evolutionary significance, of such interactions. The extensive "common vocabulary" of information molecules and their receptors used by both the neuroendocrine and immune systems argues for a long history of interaction between the two systems, and is also supportive of a long-term adaptive significance for neural-immune crosstalk. Our understanding of the phylogenesis of such interactions and significance would be enhanced greatly through exploration of the existence of homologues of the mammalian system in other vertebrate (and even invertebrate) species.

A review of comparative studies in psychoneuroimmunology confirms the existence of a long phylogenetic history of interactions between the neuroendocrine and immune systems. These interactions even appear to predate the emergence of the vertebrate adaptive immune system. For example, hemocytes from several molluscan, annelid, and insect species exhibit immunoreactivity for, and functional responses to, a variety of vertebrate neuropeptides (5).

Among vertebrate classes, teleosts, amphibians, reptiles, and birds have all received at least an occasional examination for neural-immune communication. In all classes, such interactions have been demonstrated, although the methods involved and exact nature of the evidence (e.g., anatomical evidence of innervation of organs,

demonstration of receptors on lymphocytes, stress effects on immunity, etc.) vary dramatically (reviewed in (5)**)**. Although there has been a substantial amount of work in the area of comparative neural-immune interactions encompassing many species and all major vertebrate classes, no single nonmammalian species has yet emerged as a model for such studies, and no single nonmammalian species has been characterized by all the approaches used for mammals.

It can be argued justifiably that the class Amphibia mirrors a potentially pivotal transition in terms of immune function. The colonization of land is, at least loosely, associated with a substantial reorganization of the immune system, most likely in response to altered immunological challenges and selective pressures. If neural-immune crosstalk is adaptive for immune function, it seems logical to examine amphibians for important phylogenetic transitions in the development of such crosstalk.

The Amphibia are a highly diverse class of vertebrates in terms of their immune system as well as body plan and life history (46-48). Architecture of lymphoid tissues from representatives of the orders Urodela and Apoda resembles that of fish, whereas the organization of lymphoid tissue from the anuran species examined is more similar to that of mammals (49). Functionally, amphibian immune systems exhibit similar diversity. For example, relative to anurans, urodeles (salamanders) have a substantially delayed courses of antibody production and allograft rejection (50)**.**

The typical anuran amphibian undergoes two distinct developmental periods; the first spans the periods of embryonic and larval life, the second, the hormonally driven transition from larval to adult life. From detailed studies with the South African clawed frog, *Xenopus laevis*, we know that this latter period is accompanied by a substantial remodeling of the immune system (reviewed in (51) and by Izutsu in this volume). Each period provides a window of opportunity for examining neural-immune communication in the context of the mammalian standard.

Historically, *Xenopus* has served as the primary model for immunologic studies of the Amphibia. In part, this is because both larval and adult stages are totally aquatic which facilitates experimentation before, during, and after metamorphosis. The popularity of this species for immunological research has led to a substantial array of reagents (monoclonal antibodies, molecular probes, etc.) and cell culture technologies. In turn, this has resulted in the immune system of *Xenopus* being the best characterized of any amphibian species (51, 52). Additionally, several cloned and inbred lines of *Xenopus* now exist (53), allowing for more fine-tuned analyses of immunity along the lines of those used so successfully in mice. It is not surprising, therefore, that, among amphibian species, *Xenopus* is also most thoroughly studied in terms of neural-immune interactions. To date, this work has involved primarily anatomic demonstration of neural innervation of lymphoid organs (54), effects of neurotransmitter and hormone manipulations on measures of immunity (55), and the role of hormones on immunological changes during metamorphosis (56).

4. CURRENT UNDERSTANDING

4.1. Innervation

Initial evidence of the potential for communication between the nervous and immune systems stemmed from observations of innervation of lymphoid organs. Interestingly, by the time studies demonstrating such innervation in mammals appeared in the late 1980s (18, 57), several studies revealing innervation in anuran amphibians had already been published (58, 59). This research not only demonstrated the presence of sympathetic nerve fibers in the spleen of *Bufo marinus* (58), but also direct contacts between nerve endings and lymphoid cells in the jugular body of *Rana pipiens* (59).

In *Xenopus*, the spleen is well-organized into morphologically distinct zones of red pulp and white pulp reminiscent of the pattern seen in mammals (49, 60). The pattern of neural innervation of the spleen is also similar to mammals. *Xenopus* splenic white pulp is innervated with noradrenergic nerve fibers (demonstrated by SPG histofluorescence as well as by TH immunohistochemistry) as well as with neuropeptide Y (NPY)- and substance P (SP)-containing fibers (54). Noradrenergic fibers are seen in association with the central artery, with varicosities extending into the parenchyma of the white pulp, and also in the boundary cell/perifollicular area where secreted neurotransmitter may come into contact with: B-cells and macrophages of the white pulp; nonlymphoid dendritic cells involved in trapping and retention of soluble antigen (60); and possibly the T-cells at the extreme boundary of the white pulp.

It is worth pointing out that this pattern of splenic innervation, while seemingly characteristic of adult anurans, is distinct from NE innervation patterns of representatives from other amphibian orders. Examination of the spleen of several species of salamanders revealed substantial innervation, but in a diffuse pattern of nerve fibers associated with the splenic reticular network (54). A lack of compartment-associated innervation in the salamander spleen is not unexpected, since the salamander spleen is not compartmentalized into lymphoid white pulp and erythropoietic red pulp. To complete the picture (to the degree possible in such a field), the Gymnophiona (apodan amphibians or caecilians) have splenic compartmentation somewhere between that of anurans and urodeles, with some white-pulp aggregations of lymphocytes (60). SPG histofluorescence staining revealed very low levels of innervation that is found both in association with the splenic vasculature and in remote areas of the spleen (54). Taken together, these comparisons suggest that changes in lymphoid organ organization across phylogeny have been paralleled by changes in sympathetic innervation.

A few studies have examined the ontogeny of splenic innervation during the larval and perimetamorphic life of *Xenopus*; these studies were conducted in an attempt to determine whether there are changes associated with the

well-characterized immune system changes that occur during development (51). Interactions between development of innervation and immune system reorganization appear to be complex and dynamic as might be expected in such a rapidly changing system. Splenic innervation is observable as early as Nieuwkoop and Faber (61) larval stage 54 (62), with a drop in splenic NE levels (assessed by HPLC and SPG histofluorescence, (63)) at stage 58, a developmental point at which lymphocyte numbers and their mitogen responsiveness have also been reported to decrease (64). The adult pattern of innervation confined to white pulp then emerges at metamorphic climax (65). The appearance of this splenic innervation appears to be very sensitive to environmental conditions (e.g., animal density, temperature, etc. (62)). However, early larval thymectomy which renders animals T-deficient does *not* alter the development of normal innervation (66, 67). Prevention of overt metamorphosis by sodium perchlorate blockade (68) prevented the development of NA splenic innervation in some, but not all, tadpoles examined (62). Further, chemical removal of sympathetic innervation during larval life prior to the appearance of splenic compartmentation did not alter the subsequent appearance of distinct red and white pulp compartments (62).

A neuropharmacologic avenue for endocrine-immune interactions in the *Xenopus* thymus is also present, as implied by the work of Palmer and colleagues revealing several neuroactive peptides (CGRP, NPY, somatostatin, and SP) in the thymus of this species (see the article in this issue) as well as several other species (69); thus, there is no doubt a long phylogenetic history of thymus-neuroendocrine interactions that warrants further investigation.

4.2. Transmitter Regulation of Immunity

As with mammals, there is pharmacologic evidence that cells of the amphibian immune system express receptors for neuroendocrine transmitters/hormones. Radioligand studies have demonstrated the presence of specific, saturable beta adrenergic receptors (beta-AR) on splenocytes and activated peritoneal leukocytes from several species of amphibian; *Xenopus*, however, was not examined (70). The Plytycz lab had previously demonstrated the presence of muscarinic-type cholinergic receptors on anuran peritoneal leukocytes (71), but in light of the considerable literature indicating a *lack* of direct cholinergic effect on immunity in mammals (6), the functional significance (if any) of such receptors is unclear. Given that the status of cholinergic innervation of mammalian spleen was a matter of debate for some time, further investigation of cholinergic effects on amphibian lymphocytes is needed.

Hodgson *et al.* (72, 73) reported adrenergic receptor sensitivity of *Xenopus* antigen-binding splenocytes, as well as splenocytes from several other amphibian species. Alpha-AR stimulation decreased, whereas beta-AR stimulation increased the number of antigen-binding lymphocytes (thought to be T-cells by these investigators). *In vitro* phytohemagglutinin (PHA)-stimulated T-cell mitogenesis was reduced when splenocytes were co-cultured with a beta-AR agonist (10^{-10}-10^{-12}M isoproterenol).

The same group (74, 75) reported different effects of *in vivo* NE administration on *in vitro* antibody responses in *Xenopus*; these differences appear to be based on the thymus dependency of the response and the timing of immunization relative to NE administration. Chronic NE administration (by means of an implanted pellet) before antigen priming with a T-dependent antigen led to increased antibody productions, whereas NE exposure after priming had no demonstrable effect. Responses to T-independent antigens were studied using an acute injection of NE rather than a pellet. A reduced splenic anti-TNP antibody response was seen in splenocytes from frogs that had received an injection of NE at the time of immunization with TNP-LPS. Given that the route, timing, and duration of NE administration was varied in addition to the antigen type, we feel that no general conclusions can be put forward from these studies.

The same investigators also demonstrated effects of *in vitro* pharmacologic manipulation on antibody production. A low dose of NE (10^{-12}M) or the beta-AR agonist, isoproteranol (10^{-12}-10^{-15}M) enhanced the production of anti-TNP antibody by splenocytes from animals primed *in vitro* with TNP-LPS, whereas treatment with the alpha-2 agonist clonidine (10^{-9}-10^{-15}M) resulted in a reduced response, an effect that was blocked by the alpha-antagonist, yohimbine.

In vitro stimulation of beta-AR (isoproterenol (ISO, a ß2-agonist) at 10^{-8}-10^{-10}M) was also reported to reduce PHA-stimulated T-cell mitogenesis (74). We have only been able to replicate this finding when we used much higher (i.e., supraphysiologic) concentrations of ISO (Figure 1). Given that the authors did not detail their culture methodology, we remain unable to provide any explanation for the disparity between the two reports.

Adrenergic effects on *in vitro* apoptosis of *Xenopus* splenocytes have also been reported (76). This effect was modulatory rather than direct: no AR agonist induced apoptosis over the 4-20 hour culture time; both alpha and beta AR stimulation altered the apoptosis of lymphocytes cultured with a calcium ionophore; alpha-AR stimulation (clonidine, and alpha-2 agonist) enhanced ionophore-induced apoptosis in 4-hour (but not 20-hour) cultures; and beta2-AR stimulation (ISO) diminished apoptosis in 4-hour cultures but increased apoptosis in 20-hour cultures. Whatever the underlying pathway(s), there appears to be some specificity for the agent used to induce apoptosis, as results differed when apoptosis was induced through culture with the synthetic glucocorticoid, dexamethasone (DEX); clonidine had no effect, and ISO enhanced the DEX-induced cell death at 4, but not 20 hours of culture.

One avenue for exploration of functional *in vivo* interactions between nervous and immune systems that has been used extensively in mammals has been the ablation of the SNS using the selective neurotoxin 6-hydroxydopamine (6-OHDA) to destroy catecholamine-containing nerve

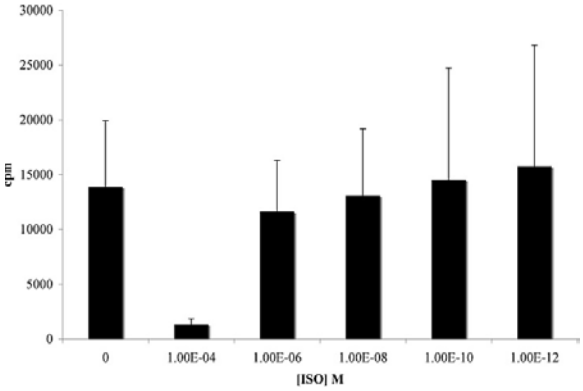

Figure 1. ß-adrenergic receptor stimulation affects PHA-stimulated splenocyte mitogenesis only at supraphysiologic levels of the ligand. Splenocytes from adult *Xenopus laevis* were removed and cultured per a standard protocol (see (55), here using 1µg/ml PHA), with the exception that the indicated amount of isoproterenol was added at initiation of culture. At 48 hours in culture, all cultures were pulsed with 1µCi ^{3}H-thymidine. Cells were harvested 24 hours later and counted for incorporation of radioactive label. Treatments were not significantly different except at an isoproterenol concentration of 10^{-4} M, which is substantially higher than a physiologically relevant concentration.

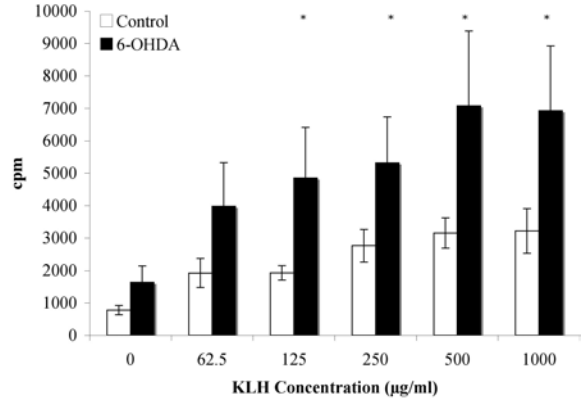

Figure 2. Sympathectomy enhances the antigen-stimulated proliferative response of splenocytes from KLH-immunized frogs. Frogs were injected with 6-OHDA or vehicle and 2 days later, all frogs were immunized with KLH. Spleens from were harvested at days 7, 21, and 28 days post-immunization (N = 3-4 frogs/group/day). Splenocytes were stimulated with KLH in a 3-day ^{3}H-TdR incorporation assay. Since no effects of experiment or day after injection were seen, data were collapsed across these variables. Results are expressed as mean cpm ± SE. Asterisks indicate significant differences (Fisher's PLSD simple effects, $p < 0.05$) from the corresponding vehicle-treated animals.

terminals. Administration of 6-OHDA leads to an immediate release of NE from sympathetic nerve terminals, followed by a profound, but transient, drop in NE levels until the terminals regenerate and normal levels reoccur (77)**.** The immunological consequences of depletion of this sympathetic neurotransmitter are complex in that it has been associated with an enhancement of both polyclonal and antigen-driven *in vitro* proliferation of splenocytes from mice on the one hand (31, 32) and unaltered (78) or reduced (30, 79) responses to thymus-dependent (T-dependent) antigens on the other. Not only do these diverse real effects point out considerable interstrain variability of sympathectomy (SyX) on immune function (79) but they highlight the importance of other variables that include the nature and concentration antigen and the timing of SyX relative to antigen administration (80, 81).

Chemical sympathectomy is immunomodulatory in amphibians as well as in mammals, and has been used in a few experiments to examine the effects of sympathetic innervation on immune function in larval and adult *Xenopus*. Splenocytes from adult *Xenopus* that had been chemically SyX two days prior to sacrifice, reproducibly exhibit an increased proliferative response to the mitogens lipopolysaccharide (LPS), concanavalin A (Con A), and PHA (55). Increased *in vitro* proliferation has also been seen using keyhole limpet hemocyanin (KLH) to stimulate splenocytes from frogs that had been first sympathectomized and subsequently immunized with KLH (Figure 2).

Sympathectomy was reported to: reduce anti-hapten antibody responses to TNP-SRBC; enhance primary anti-hapten response when frogs were primed with TNP coupled to a T-independent carrier (74, 75); and have no effect on primary serum anti-KLH IgM in frogs immunized 2 days after SyX (Figure 3). These data indicate a complex relationship between SNS activity and the immune response that depends on the nature of the antigen. In recent studies from the Kinney lab, SyX frogs that had been immunized with KLH received a secondary challenge with KLH following a repeated 6-OHDA injection (on days 47 and 49). Serum anti-KLH IgM (Figure 3) and IgY (Figure 4) levels were elevated when compared to the appropriate controls. This may be the first examination, in any species, of the impact of SyX on secondary antibody responses.

In terms of cell-mediated immunity in mammals, investigations on the effects of 6-OHDA denervation on delayed-type hypersensitivity (DTH) responses (82) revealed that SyX of C3H/HeJ and BALB/c mice was associated with reduced ear swelling induced by antigen challenge of sensitized animals, an effect which was, at least in part, a result of reduced antigen-specific T-cell response. SyX mice exhibited reduced antigen-specific interleukin-2 (IL-2) production and cytotoxic T-lymphocyte activity by cells from draining lymph nodes (LN) harvested five days after contact sensitization.

In amphibians, a commonly used *in vivo* measure of cell mediated immunity is rejection of skin allografts (83). Chemical SyX two days before transplantation and repeated weekly during the course of the experiment to maintain denervation, had no effect on skin allograft rejection in *Xenopus* regardless of whether the host-donor disparity involved MHC plus minor histocompatibility (H) locus antigens or just minor H-antigens (Table 1). Further,

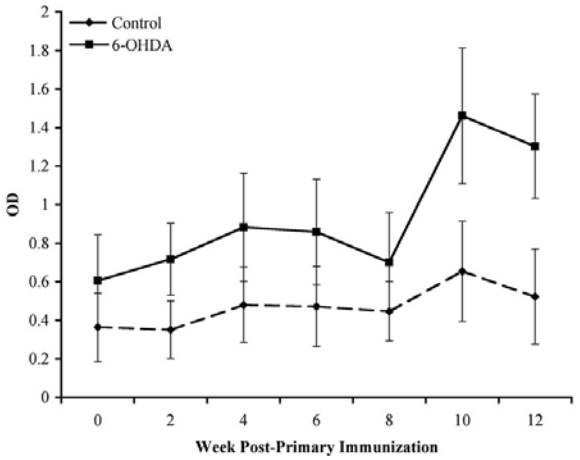

Figure 3. Denervation is associated with enhanced secondary anti-KLH IgM antibody production. Ten frogs were each given 5.5mg of 6-OHDA in 0.1 ml of a 0.1% ascorbate/H_2O solution by i.p. injection. A control group of ten frogs was given 0.1 ml of a 0.1% ascorbate/H_2O solution. Two days after the injection, we obtained blood samples from all frogs (pre-bleed, time 0), and then injected them all with 55mg of KLH. Blood samples were obtained 2, 4, and 6 weeks following primary injection. In week 8 following treatment, frogs were again injected with 6-OHDA 2 days before a secondary injection of KLH. Blood samples were obtained at weeks 8, 10, and 12 (relative to initial injection). Serum samples were diluted 1:75 and assayed by ELISA using 10A9 antibody. Results are average optical densities of 10 frogs per group. A repeated measures ANOVA revealed a highly significant main effect of treatment ($p<0.0001$) as well as time ($p<0.0001$) and a significant time by treatment interaction effect ($p<0.0001$).

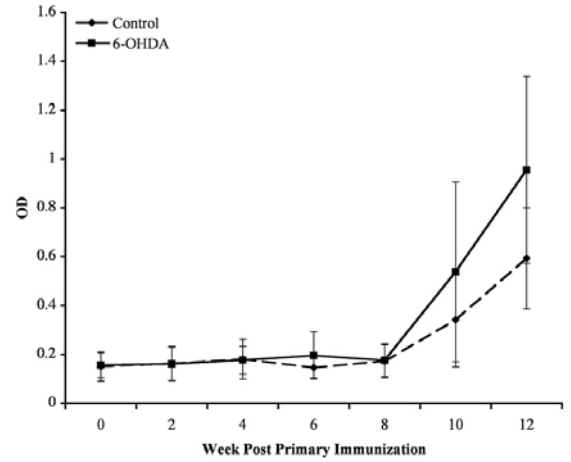

Figure 4. Denervation is associated with enhanced secondary anti-KLH IgY antibody production. Animals used were the same as those described for Figure 3. Serum samples were thawed, diluted 1:75 and assayed by ELISA using 11D5 antibody. Results are average optical densities of 10 frogs per group. A repeated measures ANOVA revealed no significant treatment effect ($p= 0.19$), but a significant time effect ($p<0.0001$), as well as a highly significant time by treatment interaction ($p<0.001$).

there was no effect of SyX on the second-set rejection of grafts that differed from the cloned recipients only by minor H loci.

Metamorphosis in *Xenopus* is associated with an ease of tolerance induction of MHC identical but minor H-locus incompatible grafts transplanted during this period (see chapter by Izutsu in this volume). It is thought that such allotolerance is a reflection of the tolerance that must develop to new self-antigens that emerge during this transition from immunocompetent larva to immunocompetent adult. Chemical SyX also had no discernible effect on the development of tolerance of perimetamorphic animals to skin allografts from adult donors differing at only minor H loci (84). Given that tolerance induction during metamorphosis is critical for a successful transition, it is perhaps not surprising that there is no effect of the sympathetic nervous system on the process. Much of the research to date suggests that the SNS often has an immunomodulatory role that could be too subtle to have an effect on such a crucial process.

Additional experiments in other anuran species (*Rana esculenta* and *R. temporaria*), using pharmacologic administration of neurotransmitters confirm the lack of influence of such neurotransmitters on allograft survival (71). In contrast, rejection of xenogenic skin grafts transplanted to *R. esculenta* was accelerated by repeated injections of propranolol and in some cases, retarded by atropine (a cholinergic agonist). It should be noted, however, that xenograft rejection in frogs is generally thought to involve innate and antibody-mediated immunity, rather than T-cells (85, 86).

4.3. Endocrine Effects on Immunity

When discussing neuroendocrine influences on immunity, we generally refer to two "arms" or routes by which information flows from the CNS to the immune system- the autonomic nervous system (discussed above) and the endocrine system. Most of the hormones studied in mammals are pituitary-derived, and some studies, most notably those from the lab of Louise Rollins-Smith, have examined the role of pituitary hormones in immunity in *Xenopus*, particularly during metamorphosis.

4.3.1. Hypophyseal axis hormones

Endocrine-immune interactions have a long history of study, with the immunosuppressive effects of glucocorticoids underpinning one of the classic hallmarks of a generalized stress response (87). The impact of these steroids on immunity (sometimes only seen with pharmacologic rather than physiological concentrations), remains an active area of research, and is now seen to be more complex than a simple immunosuppressive effect (88).

Glucocorticoids effect similar changes in the lymphoid system of amphibians as they do in mammals (e.g., thymic involution, loss of cortical lymphocytes, redistribution of lymphocytes (89)). Some of these effects may depend on the season when the hormone is administered experimentally (90), and the effect may vary

Table 1. Sympathectomy does not affect allograft rejection

Histocompatibility barrier	Median survival time (in days) (95% confidence interval)	
MHC + minor H	Ascorbate control	6-OHDA
	16.0 (14.0-18.3)	17.4 (15.6-19.4)
	N = 6	N = 7[1]
Minor H only		
minor first-set	28.3 (23.1-34.7)	25.8 (17.6-37.9)
	N = 5	N = 6[2]
minor second-set	12.0 (10.9-13.2)	13.0 (11.1-15.2)
	N = 4	N = 3

[1]One animal did not reject during the experimental period, which lasted for 30 days postgrafting. The nomographic method used to evaluate these experiments (175) corrects for such non-responding individuals. [2]One animal did not reject before it died, at 40 days postgrafting. Procedure: Adult outbred *Xenopus laevis* were purchased from Xenopus I, Ann Arbor MI, and allowed to acclimate to laboratory conditions for at least two weeks prior to any treatment. Laboratory-bred *X. laevis* x *X. gilli* hybrid LG-15 clones were anesthetized and injected with either ascorbate vehicle or 75 µg/g BW of 6-OHDA in ascorbate vehicle. Two days later, animals were transplanted, on their dorsal surface, with a 2 mm x 2 mm piece of ventral skin from either an outbred adult frog differing by major plus minor histocompatibility (H) antigens (N = 8 vehicle injected; N = 8 6-OHDA-injected) or from an MHC identical LG-6 frog which differed from the LG-15 host only by minor H antigens (N = 7 ascorbate vehicle injected; N = 8 6-OHDA-injected). Animals received weekly injections of 6-OHDA to prevent regeneration of sympathetic nerve fibers; control animals received injections of vehicle at these times. Animals were grafted according to the published technique of Chardonnens and Du Pasquier (176). Briefly, ventral skin was removed from anesthetized donors, placed in APBS, and cut into 2 mm x 2 mm pieces with a scalpel. A 2 mm transverse incision was made in the recipient's dorsal skin posterior to the eyes and the graft was inserted beneath the skin such that equal parts of the graft were located anterior and posterior to the incision. Two days later, animals were again anaesthetized and the skin overlying the graft was removed to allow observation of the graft. Grafts were scored daily by recording vascular changes and the percentage of viable donor pigment cells remaining on the graft. Rejection was considered complete when all xanthophores were destroyed. Following rejection of their minor H-disparate first-set grafts, LG-15 animals received second-set grafts of LG-6 skin to evaluate the effects of SyX on immunological memory. Due to mortality and technical loss of grafts, only five ascorbate-injected animals and three 6-OHDA-injected animals received such repeat grafts. In addition to the aforementioned study, an experiment was conducted in which LG-15 frogs were injected with either ascorbate (N = 21) or 6-OHDA (N = 15) within one week after they reached metamorphic climax (i.e., stage 66 of Nieuwkoop and Faber (61)); two days later, they were grafted with LG-6 grafts. Frogs grafted with minor-H antigen disparate grafts at this developmental stage invariably become tolerant of their grafts (124). To maintain denervation, frogs received weekly injections of 6-OHDA; controls received weekly vehicle injections.

by lymphoid organ (91). In *Xenopus*, corticosterone treatment *in vitro* inhibits PHA-induced proliferation of splenocytes (92), and high (pharmacologic) doses of hydrocortisone *in vivo* suppresses antibody response (91).

Prolactin (PRL) may be "immunopermissive" for lymphocyte proliferation since in initial experiments, incubation of *Xenopus* cell cultures with anti-chicken PRL antibodies suppressed proliferation of larval and adult splenocytes that had been stimulated with LPS, PHA, or Con A (Rollins-Smith and Cohen, unpublished). Similarly, preliminary studies with lymphocytes from the salamander, *Ambystoma mexicanum*, also revealed that anti-PRL antibodies inhibited PHA-induced mitogenesis in a dose-dependent fashion (Cohen, unpublished). Moreover, addition of PRL to culture of mitogen-stimulated axolotl lymphocytes was significantly enhancing. If these effects are confirmed, such a permissive role is consistent with PRL effects in mammals, and suggests a long phylogenetic history of this function (93).

4.3.2. Seasonal Effects on Immunity

Vertebrates of all classes are known to exhibit regular endocrine changes in association with reproductive and other seasonal cycles. There is now a substantial literature on seasonal variation in immunity in a variety of ectothermic species, much of which has been related to endocrine changes (for review, see (5)). Overall, there appears to be a winter-associated period of immunocompromise in anurans, which may have profound effects on survival of populations in terms of both exposure to pathogens during winter months and immunological memory across seasons (94, 95). Amphibian lymphoid organs exhibit seasonal morphological changes (96, 97). The thymus of at least one frog species (*Rana temporaria*) undergoes cyclical changes with maximal development occurring during the summer and involution in the winter (98-100); these changes are not due solely to changes in the ambient temperature (98, 101). Although there are some indications of functional differences (102, 103), relatively little has been done to examine the effects of the normal winter hibernation period (or its laboratory equivalent) on the anuran immune system. Given issues associated with climate change and amphibian "extinctions" (see below), there is a pressing need to expand our understanding of seasonal effects on immunity; *Xenopus* as the laboratory model is currently underutilized in this regard.

4.3.3. Metamorphosis and Immunity

Metamorphosis is driven by changes in circulating levels of many endocrine hormones, most notably triiodothyronine (T3) and thyroxine (T4) (104-106). Thyroxine appears to drive the terminal maturation of larval erythrocytes (51, 107) and the expansion of a separate adult erythrocyte population (108), but does not have any consistent effects on lymphocyte viability or proliferation (92).

Alteration of thyroid hormone levels alters the progression of metamorphosis and allows a dissection of the development of adult-type immunity in relation to normal metamorphosis. Such experiments, then, reveal the

very essence of neuroendocrine-immune interactions. One thyroid-manipulation strategy is to administer sodium perchlorate, a goitrogen that blocks iodine uptake by the thyroid follicle cells (68) thereby preventing overt metamorphosis. Under such a blockade, some features of the immune system change in a manner comparable to normal (i.e., unblocked) control animals. These features include: expression of MHC class I antigens (64, 109); immigration and expansion of T-cell precursors in the thymus (110); and development of high titer IgY antibody production (111). These changes occurring during blockade of thyroid function are assumed to be thyroid-independent. Other morphologic and functional features of immunity do not change in perchlorate-blocked tadpoles, and thus are (directly or indirectly) thyroid dependent. For example, in blocked larvae: the thymus does not assume an adult-type morphology (112); splenic compartmentation and innervation by the SNS (described above) is dramatically delayed (62); and lymphocytes in the thymus and spleen do not achieve the expanded cell numbers characteristic of postmetamorphic adults (113, 114). Functionally, blocked larvae retain: larval-type tolerogenic responses to skin allografts (Cohen and Crosby, unpublished), larval patterns of antibody response to a specific hapten (111); and a lack of adult-type MHC Class II^+ T cells in the periphery (113).

In addition to thyroid hormones, PRL, growth hormone, and corticosteroid levels increase during metamorphosis (104, 115-118). All of these hormones have been shown to be immunomodulatory in mammals (93, 119, 120), and some are also known to be immunomodulatory in amphibians (56, 67, 109).

As mentioned above, corticosteroid hormones (CH) can be immunosuppressive in adult frogs. In general, this also appears to be true for larvae: concentrations of corticosterone as low as 1-10 nM induce apoptosis of larval thymocytes and splenocytes (109, 121), a sensitivity that is comparable to that of adult lymphocytes (92, 109, 121). This sensitivity, coupled with the rise in hormone levels during metamorphosis, may be a major cause of the extensive die-off of larval splenocytes at this time (67), and thus critical in the turnover of larval cells believed to be necessary for post-metamorphic self-tolerance**.** Additionally, both corticosterone and aldosterone (concentrations of which also rise during metamorphosis) inhibit PHA-induced proliferation of both adult and larval lymphocytes, an effect that can be blocked with the CH-receptor antagonist, RU486 (67, 92, 109, 121, 122).

The effects of hypophysectomy (HypX) of tadpoles on their immune function during metamorphosis appear less profound than might be expected. Such HypX tadpoles continue to grow, can generally reject MHC-disparate grafts, and have no apparent delay in repopulation of an implanted thymus with host thymocytes (123-126). HypX tadpoles maintain the low larval pattern of MCH class I and II antigens, even in skin at 1 year of age (127) and, like control tadpoles, do not reject minor H-locus disparate grafts or grafts expressing organ-specific antigens (124, 125). HypX tadpoles also exhibit decreased lymphocyte numbers in both thymus and spleen (relative to age-matched intact controls) as well as inhibited proliferation of cells in the developing thymus (110, 128).

4.4. Heat shock proteins and neural-immune system interactions

Several years ago, the Cohen-Robert lab at the University of Rochester asked whether the hsp 70 and hap 90 families of heat shock proteins (hsps) play any role in the *Xenopus* defense system. Although hsps are typically induced by heat, cold, and glucose deprivation (hence their designation as "stress proteins"), our interest in them at that time focused on their possible ancestral roles in immunity. Immunological properties of gp96 and hsp70 have been conserved during vertebrate evolution. Molecular, biochemical, and serological characterization confirms extensive structural conservation of gp96 homologues in all vertebrates. Hsps limit protein aggregation, facilitate protein folding, and act as molecular chaperones in all species studied (129, 130).

In frogs and well as mammals, hsps can have profound affects on both innate (danger signal) and adaptive immunity (52, 131-133). A wide range of endogenous peptides associate *in vivo* with hsps released from necrotic cells (134). They potentially represent the antigenic repertoire of a particular cell (i.e., tumor, viral, and minor H-antigens). Immunization of *Xenopus* with homologous gp96 stimulates MHC-restricted thymus-dependent immunity against minor H-antigens *in vivo* (135). Further, soluble complexes of peptides and either gp96 or hsp70 purified from autologous murine (136) or frog (52, 132) tumors specifically immunize recipients against tumor challenges. Such immunity is abrogated by depletion of $CD8^+$ T-cells or macrophages during priming (136), and of $CD8^+$ or $CD4^+$ T-cells during the effector phase. Gp96 alone (i.e., without peptide) has been demonstrated to enhance cytokine production and non-specific killing (132), as part of a non-specific immune response.

It is not unreasonable to assume that increased expression of such ancient and important molecules as hsp70 and gp96 might significantly influence immunity. Of relevance to our discussion of neural-immune system interactions in *Xenopus* are the recent and exciting observations from the Fleshner laboratory that, in rats, hsp72 could be induced by physical stressors (intense exercise, inescapable electric tail shock (137)); this upregulation had consequences in terms of several parameters of innate immunity. More recently, Fleshner *et al.* (138) reported a similar elevation of central and peripheral hsp72 following exposure of a rat to a cat predator (psychosocial stressor); this response could be blocked or attenuated by adrenalectomy). To the best of our knowledge, no one has studied whether hsp-peptide complexes play any role in the mediation of those changes in innate and adaptive immunity that are causally associated with either physical and/or psychosocial stressors. *Xenopus* could serve as a worthy model system for such studies

5. SPECULATIONS, APPLICATIONS, AND FUTURE DIRECTIONS

In *Xenopus* as in mammals, it is important that studies progress from their current state of phenomenology to those that are more mechanistic. In mammals, such a progression is exemplified by the work of Virginia Sanders and her colleagues. Following up on her classic denervation studies (139-141), Sanders began studying how ligation of $beta_2$AR on B and T cells by catecholamines (23) might bring about modulated immune responses. She demonstrated that cells secreting Th1-type cytokines, but not cells secreting Th2-type cytokines, express the functional $beta_2$AR that binds to catecholamine agonists and antagonists (23, 142). In response to $beta_2$AR stimulation, the levels of Th1 cytokines produced are modulated (e.g., decreased IL-2 production by freshly isolated murine splenic CD4+ T-cells (23, 24, 143)). Clearly, anything that differentially regulates cytokine responses of the Th1 and Th2 variety has a potentially important impact on the ensuing immune response.

Sanders also investigated the function of $beta_2$AR expressed on B cells and found that NE stimulation is necessary for the maintenance of an optimal primary and secondary Th2 cytokine-dependent antibody response *in vivo* (144). Depletion of NE *in vivo* decreases levels of antigen specific IgG1 production cell proliferation and germinal center formation. More recently, this group has reported that signaling through the $beta_2$AR receptor increases expression of the critically important co-stimulatory molecule, CD86 or B7-2 (145, 146) both *in vivo* and *in vitro*. Stimulation of the B cell receptor, CD86, and the $beta_2$-AR in concert modulates the level of IgG1 and IgE produced on a per B cell basis (147). Additional cell signaling studies have revealed insights into the mechanisms by which CD86 expression is regulated (148, 149). Thus, from simple ablation studies, we now have a progressive, step-by-step understanding of at least some of the pathways by which the sympathetic nervous system can influence immunity. Sadly, this systematic and thorough research is not yet typical of the field; many phenomena in the discipline are still awaiting similar characterization in conventional murine and human systems; for comparative studies, the field is wide open.

Approaching the study of neural-immune interactions from a comparative perspective allows for a variety of other questions to be addressed. For example, amphibians are at an interesting phylogenetic position in terms of immunity given their position at the tetrapod transition from aquatic to terrestrial lifestyle. It is reasonable to posit that leaving the water for land resulted in differences in the pathogenic spectrum and other immune challenges to which the first terrestrial tetrapods were exposed. If this is the case, then the Amphibia offer a prime model for examining the extent to which the neuroendocrine and immune systems have coevolved.

"Psychoneuroimmunology" is a compound word reflecting the complex interactions that define this field. In terms of evaluating the state of this interdisciplinary science as it has been applied to the Amphibia, clearly the "neuro" component is the best studied. As reviewed above, much of the available information concerns the effects of neuroendocrine manipulation on measures of immunity. The "psycho" (i..e., behavioral) component of this field offers significant potential for research. For example, studying hormonal (particularly corticosteroid) manipulation in an ethologically relevant fashion (e.g. physical or psychosocial stressors in nature) is one neglected area. Another is the applicability of the immunocompetence-handicap hypothesis (ICH) to amphibians. The ICH was originally developed to explain the maintenance of the often flamboyant sexual traits of male birds (150). Briefly, it suggests that traits such as bright plumage are promoted by testosterone and preferred by females as an indicator of male quality. The testosterone-dependence of the trait maintains a form of honesty in the signal if the higher levels of testosterone needed for optimal trait expression is also immunosuppressive- only a *very* high-quality male can afford to use an immunosuppressive hormone to exaggerate an attractive trait (one of the senior author's students has termed this more colloquially the "swimming with dumbbells" hypothesis- demonstrating one's fitness by performing under an additional handicap). While still open to verification, this hypothesis has been tested and found viable for both birds (150-152) and reptiles (153, 154); it has only been addressed in a very limited fashion with amphibians (155). Thus, it would be interesting to further examine whether the behavioral mate-attracting traits of male amphibians (e.g., calling) impose any sort of immune handicap.

Much of what has been published in psychoneuroimmunology has been derived from various laboratory measures of immunity. Whether these sometime subtle changes that result from some neuroendocrine perturbation have significant bearing on the health of the organism is often questionable. Thus, another substantive topic in need of further investigation in amphibians is the effects of neuroendocrine changes (whether pharmacologic, developmental, seasonal, or via environmental challenges) on infection and disease outcomes. Even as we progress toward a complete mechanistic understanding of the field, we need to keep in mind that the true measure of the relevance of a behavioral manipulation is how it affects the survival and normal function of an intact behaving animal in a real-world setting.

At present, we have very limited understanding of the true adaptive value of neural-immune crosstalk. The following section presents an area that may provide a framework for understanding exactly this issue.

5.1. The amphibian decline problem: applied comparative psychoneuroimmunology.

Ecology would not seem to be a prime area for input from psychoneuroimmunological research. There are, however, several areas of ecology that can be advanced by a better understanding of neural-immune interactions. One such area is the ICH mentioned above. Another is the worldwide decrease of populations and species of frogs and salamanders (156). This decline is beginning to receive

considerable attention owing not only to concerns of conservation biologists, but also because these disappearing amphibians have been suggested to be the "canaries in the coal mine" for consequences of anthropogenic environmental changes including global warming. Several not mutually exclusive hypotheses have been advanced to explain the declines. These include the destruction of habitat where amphibians breed and grow, and the introduction of invasive exotic taxa that displace native species. At face value, these two explanations would not appear to involve psychoneuroimmunological considerations. However, they do involve what might be considered environmental stressors (including UV radiation, pollution, and/or global climate change) and costs of inflammatory responses have been demonstrated to be lower for at least one (albeit non-amphibian) exotic invader than a non-invasive congener, leading to speculation about the role of immunity in invasion (157, 158).

At least two pathogens, a Chytrid fungus (*Batrachochytrium dendrobatidis*) and iridoviruses (ranavirus, *Ambystoma tigrinum* virus, frog virus 3) are causally involved (159) in these population declines and extinctions. Although it is reasonable to propose that exogenously altered immunity contributes to the action of these (and other) infectious agents, this has not yet been tested directly in the lab or in the field. With respect to the fungus, this is not surprising since our knowledge of amphibian adaptive immunity to chytrids is basically nonexistent. We do know, however, that naturally produced antimicrobial skin peptides of frogs are capable of inhibiting growth of zoospores and mature fungal cells (160). Amphibian skin gland secretion of these peptides is regulated by the SNS (i.e., the catecholamines epinephrine and norepinephrine; (161-163). Chlorotriazine herbicides and similar agents used as pesticides decrease the intracellular content and release of dopamine and NE from PC12 cells, a cell line with neuronal characteristics, (164), and inhibit NE synthesis by suppressing the activity of dopamine-beta-hydroxylase (165). This leads to the intriguing suggestion that introduction of such compounds into the environment has impaired immune function in amphibian populations via altered SNS activity, resulting in deaths due to infection.

Our knowledge of anti-iridoviral immunity, at least in *Xenopus*, is further along than our understanding of anti-fungal immunity. Thanks to the work of Jacques Robert and colleagues at the University of Rochester, we now know that in addition to antimicrobial peptides (166) and NK cells (167) as components of its innate immune system, *Xenopus* uses CD8 T-cells, NK/T cells, and antibody to deal with laboratory-introduced infections with the iridoviruses, frog virus 3 (FV3; (168)). We also know that: x-irradiation increases mortality associated with FV3 infection (169); larval and metamorphosing frogs are much more susceptible infection with FV3 than are adults (169); and, as previously mentioned, metamorphosing frogs have a naturally down-regulated immune system, presumably to avoid immune responses to adult-specific antigens that are emerging during metamorphosis (51). Finally, we know that in amphibians, glucocorticoids are highly immunomodulatory, especially during metamorphosis (121). It is interesting to speculate that environmental stressors increase glucocorticoids in populations of wild amphibians. Although we've not directly tested this possibility in *Xenopus*, we do know that elevated resting levels of corticosteroids have been detected in toads (*Buffo terrestris*) collected from areas polluted by coal ash, and in mudpuppies (*Necturus maculosus*) exposed to organochlorine where maximal concentrations approximate those associated with the natural glucocorticoid-associated immunosuppression in *Xenopus* that occurs during metamorphosis (92, 170, 171). Furthermore, immunotoxicological studies have revealed the immunosuppressive capacity of various pesticides (e.g., atrazine, metribuzine, endosulfan, lindane, aldicarb, DDT, malathione, and dieldrin) singly and in combination in *Xenopus* and *Rana* (172, 173). Obviously, it is a big jump to conclude from these few studies that immunomodulatory pollutants and other man-made additions to the environment are contributing to the decline of amphibians by suppressing their capacities to fully respond to viruses and fungi, but at the very least, the aforementioned scattered observations delineate a raft of focused experiments that could exploit *Xenopus* as an ideal model system.

Clearly, psychoneuroimmune mechanisms are not the only explanation for our example of amphibian decline. Nevertheless there is considerable potential for a better understanding of the problem through the great explanatory power of a meta-disciplinary approach. Any or all of the observed environmental changes could be immunosuppressive owing to their action on the neuroendocrine system that in turn, negatively signals the immune system and increases susceptibility to fungal and/or viral pathogenicity, eventuating in a population decline (56). Given that as yet no single "smoking gun" causing the decline has been unequivocally identified, it seems reasonable to propose that there is an interaction of two or more of the above "stressors" (along with other as yet unidentified ones), via neuroendocrine pathways, to suppress amphibian immunity thereby increasing pathogenicity (174).

In conclusion, comparative studies of the mechanisms of neural-immune crosstalk in a variety of nonmammalian species will not only provide insight into their evolution, but may help in understanding the adaptive significance of the having of these two major homeostatic systems coordinate their efforts.

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Abbreviations: 6-OHDA: 6-hydroxydopamine; AR: adrenergic receptor; CCK: cholecystokinin; CGRP: calcitonin gene-related peptide; CH: corticosteroid hormones; CNS: Central nervous system; Con A: Concanavalin A; DEX: dexamethasone; DTH: delayed-type hypersensitivity; FV3: frog virus 3; H: histocompatibility; HPA: hypothalamo-pituitary-adrenal; HPLC: High-performance liquid chromatography; Hsps: heat shock proteins; HypX: hypophysectomy; ICH: immunocompetence-handicap hypothesis; IgE: immunoglobulin E; IgG; immunoglobulin G; IgM: immunoglobulin M; IgY: immunoglobulin Y; IL-2: interleukin-2; KLH: keyhole limpet hemocyanin; LN: lymph node; LPS: lipopolysaccharide; MHC: major histocompatibility complex; NA: noradrenergic; NE: norepinephrine; NK: natural killer; NPY: Neuropeptide Y; PHA: phytohemagglutinin; PRL: prolactin; SNS: Sympathetic nervous system; SP: Substance P; SPG: Sucrose-potassium phosphate-glyoxylic acid; SRBC: sheep red blood cells; SyX: sympathectomy; T3: triiodothyronine; T4: thyroxine; TH: Tyrosine hydroxylase; TNP: trinitrophenol; VIP: Vasoactive intestinal peptide

Key Words: Amphibian, Psychoneuroimmunology, Endocrine, Neural-Immune Interactions, Comparative, Sympathetic Nervous System, Stress

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