An Afternoon with Professor George Bentley

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This year, BSJ had the pleasure of interviewing Professor George Bentley. Professor Bentley's research interests include avian reproductive biology, neuroendocrinology and behavior. As an ornithologist, Professor Bentley has completed extensive studies on song control systems. Endocrine and behavioral responses to stimuli such as vocalizations have been documented for decades, yet the "black box" approach has been applied to any explanation of the brain's involvement. Any external stimulus has to be first monitored by and then responded to by the brain for the stimulus to have a physiological effect. To affect the reproductive axis, these stimuli must influence the gonadotropin-releasing hormone (GnRH) system. Much of Professor Bentley's recent work has been on the recently identified neuropeptide, gonadotropin-inhibitory hormone (GnIH). BSJ had the opportunity to learn more about the role of stress in the GnRH system.

BSJ: How did you get involved in your research in stress regarding birds?

Prof. Bentley: I started my research career looking at what is called "photoperiodism" -- how birds and other mammals respond to changes in day length and how their reproductive system changes. I was interested in how this phenomenon occurs on a basic level -- how birds change from breeding to a nonbreeding status. I ended up working in a lab in Seattle that focused on the role of stress influencing different behaviors within discrete stages of the annual breeding cycle. I am not a stress biologist, historically. When I moved here, because of my interest in regulation of photoperiodism in seasonal breeding, I started working on a neuropeptide called Gonadotropin-Inhibitory Hormone (GnIH), which was discovered in Japan by a friend of mine, Kazu Tsutsui (Kazuyoshi Tsutsui). We started working on GnIH and we knew nothing about how peptides are regulated and their associated regulators. We initially thought this discovery was very exciting because this inhibitory peptide could explain or could be part of the mechanism with which birds and mammals terminate (switch-off) reproduction at the end of the breeding system when their gonads regress. Turns out that was not the case. We started to look at different hormones that might modulate the expression of GnIH. The obvious thing to look at was the HPA (Hypothalamic-pituitary-adrenal) axis. We wanted to see if stress hormones could modulate the GnIH. A graduate student of mine, Becca Calisi (Dr. Rebecca M. Calisi-Rodriguez), started by doing some work on stress at different times of the breeding season in house sparrows. We saw that GnIH was regulated differently at the start of the breeding season as compared to the end of the breeding season. We caught these birds from the wild and simulated a predation event -- we catch them, put them in a cloth bag, and hang up them for an hour. While the birds are sitting there in the bag, we assumed that they perceive that they would be eaten by predators and this elicits a strong stress response and allows us to see how GnIH was regulated. We found that it was regulated more at the start of the breeding season than later in the breeding season. This makes sense, as at the start of the breeding season, breeding can be delayed or advanced depending on what kinds of supplementary cues these birds are receiving. The drive of changing day length can switch on the reproduction system, so the birds do not enter full reproduction until everything is just perfect. Now in birds, we have been historically limited in terms of the tools we have to manipulate at the level of the gene. We first have to clone the gene. Daniela Kaufer and I, we were talking about collaborating and see how GnIH can respond to stress. One of her graduate students, Liz Kirby (Elizabeth Kirby), did a lot of this early work and culminated in a

PNAS paper about stress influencing the mammalian form of GnIH in male rats, inhibiting reproduction.

BSJ: What parts of the brain does stress affect the most?

Prof. Bentley: It depends on what type of stress you are talking about, because stress is an overarching term for many different kinds of stress, such as food stress, emotional stress, psychological stress, and predation stress. Yes, stress feeds back to the hypothalamus and then influences the endocrine status of the animal by binding to peptide neurons. It can also influence the hippocampus. It influences the fate of neuronal precursor cells. If you were talking to me thirty years ago, no one would have believed that, because neurogenesis in the adult vertebrate brain was not thought to occur until it was discovered in a songbird study. Songbirds have this network of interconnected brain nuclei called the "song control system" and this allows them to learn vocalizations. The song control system grows and shrinks seasonally in response to changing gonadal steroids and melatonin. In the early 1980s, Fernando Nottebohm from Rockefeller University discovered this shrinkage and growth of particularly HVC, Area X, and RA. It is not just cells changing size. It is new neurons being born and migrating to different brain areas. Since then, there is a debate whether mammals can exhibit neurogenesis in adulthood. Some people pooh-poohed the idea and other people stuck with it. Now, we know that humans and primates can exhibit neurogenesis in adulthood. So, returning to the topic of stress, neurons are born from neuronal precursor cells. With stress, the fate of the neuronal cells will be changed. They will become astrocytes, for example, versus neurons. This process can occur in the hippocampus, which is involved in memory.

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Stress has several effects on the brain.

BSJ: As mentioned earlier, there would be different effects for different types of stress, how do you classify types of stress?

Prof. Bentley: There is also nutritional stress that is thought to influence the size of the song control system. So, if young were fed an impoverished diet, then their song control system will be smaller and they will be less able to learn songs as well. That may be partially mediated by glucocorticoids and nutritional status.

BSJ: In terms of measuring the reproductive ability, what type of stress do you use? How would you classify that?

Prof. Bentley: I would say that is simulated predation stress. To understand this concept, we'll have to delve a bit into acute vs. chronic stress. You can have a single predation event. Imaging a zebra being chased across the plain of Africa by a lion or cheetah, it leads to an elevation in the glucocorticoid to get the energy reserves mobilized. So, acute stress is actually quite adaptive and in some ways can help you survive. Chronic stress can be very detrimental because we haven't really adapted to deal with chronic stress.

BSJ: What initial indicators do you look for when stress is first induced in birds?

Prof. Bentley: Well, one thing we typically do is we take blood samples and we have to get a baseline measure of the bird's stress response. We have aviaries at the field station where we can catch birds and take a blood sample in under three minutes -- that is how long glucocorticoids remain in the blood. First, we take that initial blood sample and then take several subsequent blood samples. However, typically, we can't see the bird in the bag, so I don't know if you're talking about the internal indicators or external indicators?

BSJ: I guess both. I was just curious as to how you measure that

Prof. Bentley: We measure glucocorticoid in the blood. But, if they aren't hunched up, it's very hard to tell if they're exhibiting a stress response. But, if they are, we

really don't want to use those measurements.

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BSJ: You mentioned the HPG axis, Could you elaborate on that? Specifically the tropic effects of GnIH on GnRH (Gonadotropin-releasing hormone)?

Prof. Bentley: On the HPG axis, you have GnRH at the top. The hypothalamus ejects GnRH to the pituitary gland and GnIH is released to the pituitary portal system, which is separated from the rest of the blood supply. So, that neuropeptide can be released in small amounts and not have large effects. If they were released in the peripheral blood supply, then it'll just be diluted. The pituitary releases gonadotropins, LH (Luteinizing hormone) and FSH (Follicle-stimulating hormone). GnIH neurons project to GnRH neurons, as the GnRH is receptive to the GnIH. Moreover, we know, not from our work, but work done in some other labs, Yale in particular, that GnIH can inhibit the firing of GnRH neurons and inhibit their activity. In most species, GnIH projects to the anterior pituitary gland and can be released in the portal system that is receptive to the GnIH expressed in the pituitary gland, thereby directly inhibit the synthesis and release of LH and FSH. GnIH is also made in the gonads, so that the gonads can make it even though it's called a "neuropeptide". The receptor of GnIH is also found in the gonads. We have data to support that GnIH can inhibit gonadal steroid release. And not just in birds!

BSJ: How would you measure the levels of GnIH and GnRH since they are very localized, especially in the median eminence?

Prof. Bentley: It's very hard to do in birds. We can measure the transcription of the genes with qPCR or PCR. Through in-situ hybridization, we can check for the expression in birds. We can do immunohistochemistry and label the neurons in the brain with antibodies specific to those neuropeptides. We can also cut out the hypothalamus, extract the neuropeptides, and then perform an ELISA assay to measure the quantities of the neuropeptide in question. That doesn't tell us about the release to the pituitary gland, though! This is where animal models like sheep come in very handy because you can cannulate this portal system, using live X-ray, taking quite large blood samples of the portal system over a long period of time. Then, you can see the pulsatile release of the neuropeptides once you do the assay. We've collaborated with some people on that; we don't have sheep here. I've thought about it, it would be a bit useful for that. But, we know people who have a long history of studying sheep and can help us measure these neuropeptides.

BSJ: So, in terms of what you mentioned initially about what your research focus has been: the differences in the GnIH levels in the spring and the fall/non-breeding seasons. Would there be a relationship of stress in there whereby they would not produce as much GnIH if exposed to stress and impact reproductive activity?

Prof. Bentley: Yes, we've done some studies looking at that, what we call a type of "social stress". A graduate student of mine looked at a type of social stress. In this experimental pyridine, we limited the number of nest sites available for the pairs of starlings. She found that early in the breeding season, the birds that had successfully competed under these natural stresses had lower GnIH content in the brain than the birds, which had not been successful in nesting on the same day.

BSJ: So this would be during the spring?

Prof. Bentley: Yes, during early spring!

BSJ: So, you would say that since they've been exposed to those stressful conditions… Everyone would be equally exposed to the stress, right?

Prof. Bentley: Yes! But, some are dealing with it a different way. Some are successfully competing. There might be different factors coming into play here to modulate the GnIH. It might not be a stress effect, it might be a perception of "Oh, I've successfully competed for the nest spot, and now I can go into the next stage of my breeding cycle!" and therefore, to put it simply, GnIH decreases. So, maybe not a stress response per say, but you can see social competition as having been "stress-induced".

BSJ: How does GnIH affect LH and FSH? Are these hormones involved in a negative feedback pathway?

Prof. Bentley: This gets quite complicated the more that people work on it! There are many factors to consider, such as the species or the length of daytime. In general, GnIH inhibits the synthesis of LH and FSH. LH and FSH are glycoprotein hormones and these glycoproteins have an alpha-subunit that is identical in LH, FSH, and TSH

(thyroid-stimulating hormone). All have the same common alpha-subunit, but they have a different beta-subunit, which confers their differential biological activity. We know that in vitro and in vivo can inhibit synthesis of both, the common alpha-subunit and the beta-subunits of the gonadotropins. So, presumably, that's acting via binding to its receptor, a G-protein covered receptor. In terms of the simple steroid negative feedback pathway, I know of no evidence that testosterone is involved in negative feedback to the GnIH system. Lance Kriegsfeld has done some work looking at the role of estrogen in terms of its action on GnIH and negative feedback. He's interested in ovulation and he studies female mammals, specifically hamsters, prior to ovulation. Estrogen, of course, is involved in negative feedback. But just before ovulation, there is a switch to positive feedback and Lance has some data indicating that GnIH is responsive to estrogen and it may be involved in this switch between negative to positive feedback but he's still working on that.

BSJ: So would you refer to GnIH as a "stress hormone"?

Prof. Bentley: I would not call it "the stress hormone", only because I think it's doing many other things, as well responding to many different kinds of input. They can influence sexual behavior, regulate feeding behavior, and of course, stress response. They respond to melatonin, which is secreted at night. They probably do much more, but keep in mind GnIH was just discovered 13 years ago, so there is a lot more to be discovered. I think it's a mediator of stress, well a mediator of a perception of a stressor, whether it is psychological or physiological, and then integrating that stress signal into the appropriate endocrine response.

BSJ: So if you have low levels of LH and FSH would that directly correspond to an ability to successfully reproduce.

Prof. Bentley: Depending on how low, yes I believe so.

BSJ: From our understanding GnIH and RFRP3 are orthologs. What are the structural and functional differences between mammalians and birds?

Prof. Bentley: It's interesting, in birds, there is one gene that encodes three mature peptides -- there's a precursor polypeptide that's encoded and then cleaved into three mature peptides. We've only been working on GnIH. We don't know much about the other peptides that are called GnIH-related peptide 1 and related peptide 2. We know from in vitro binding studies that they can bind in a similar way to the GnIH receptor, so it may just be that the precursor is cleaved differently under different circumstances, but it ends in the same result. Alternatively, it may be that there is another receptor that we haven't discovered yet and this differential cleavage is a precursor in different brain areas to perform different processes. Now, in mammals, typically, there is a precursor polypeptide, but only two mature peptides: RFRP-1 and RFRP-3. In humans, it seems that there may be three mature peptides, but we've only managed to isolate two; however, based on the genome, it seems that there ought to be another one in there. And functional differences? Well, there are some functional similarities. One interesting difference is that, in hamsters - I don't remember which species, RFRP-3 is administered to these hamsters on long days, so when they should be breeding you can inhibit the HPG-axis, you can inhibit LH release. But, if it is administered to the same species on short days, when the reproductive system is regressed, you can increase LH release. We have not looked at that in birds yet to see whether there is a differential response to the administration of GnIH under different day lengths, but there are many similarities in RFRP-3 and GnIH. Both influence sexual behavior, feeding behavior and the response to stress.

BSJ: What does it mean for humans to have more than two to have three?

Prof. Bentley: Evolutionarily or functionally?

BSJ: Both maybe? Would you say there is greater variety between how those would function?

Prof. Bentley: Possibly, if there is a third mature peptide it looks like it's probably an RSamide, instead of an RFamide. I don't know if I want to speculate too much on that because we haven't isolated it.

BSJ: Is it an ability to react to different types?

Prof. Bentley: Maybe. Several of these neuropeptides haven't been looked at in great detail across all vertebrates; the most studied organisms are rats and mice. So, there are neuropeptides that we know of that were probably present early on in the evolution of vertebrates like GnRH2 more basal vertebrates have more forms of GnRH than rats and mice. Typically rats have one form, fish have three or four forms. Humans have expressed GnRH2, as well as GnRH1, whereas rats don't. You have to think about how we evolved and what type of accidents that occurred along the way for us to lose one or keep one.

BSJ: Do high levels of stress affect the production of RFRP3 in mammals like stress triggers the production of GnIH in avians?

Prof. Bentley: Yes. In male rats, that's certainly the case. This was the experiment I was talking about that Daniela Kaufer's graduate student Liz Kirby performed. Daniela now is actually doing some cool work. We're collaborating on this with another graduate student, Anna Geraghty. She's looking at stress and RFRP3 in female rats. People typically don't study female model organisms. In birds, its because it is hard to get them to breed in captivity if they're a wild bird. You get this nice testicular response in the lab; in the wild, you don't get that with the ovary unless they are in the correct conditions, which we have at the field station. With rats, people are worried about the data being muddled by the stage of the estrous cycle that the female is in at the time. Therefore, they just find it much easier to work on males. And that's a little shortsighted, I think, because you're missing half of the population of your studied organism. So, Anna very bravely wanted to look at the effects of stress on GnIH in female rats and how that might influence pregnancy success and eventual total reproductive success. And, doing some very cool manipulations, she's found that yes, stress increases RFRP3, yes it influences reproductive success. Using a very cool viral vector technique, she can knock out RFRP3 and then block those stress effects. Those virus-treated animals with RFRP3 knocked out showed the same reproductive success as the controls.

BSJ: Are GnIH and RFRP3 conserved evolutionarily across species or observed only in birds or mammals?

Prof. Bentley: They are pretty well conserved across species. When I first started working on GnIH, I was submitting around proposals and getting comments back along the lines of, "Yeah this is very interesting, but it may only be in birds. We don't know if this substance even exists in mammals, let alone humans." When I came here, I made it one of my missions to see if GnIH was conserved across vertebrates. Obviously, you can't study every vertebrate. But I wanted to look in rodents, fish, nonhuman primates and humans. We ended up isolating GnIH or RFRP3 from the monkey brain and the human brain. We've also been looking at GnIH and RFRP3 in carnivores because nobody had been doing any work on carnivores.

We were approached a while ago by a private foundation interested in creating a novel technique for sterilizing cats and dogs. They looked at how GnIH worked and thought, "Well, maybe you could apply that to create this single injection sterilization technique for cats and dogs". Its established by a billionaire, who basically is very upset that there are millions of cats and dogs euthanized every year in this country and around the world. We are in the process of isolating GnIH from cats. We've cloned it from cats, and we think we can manipulate GnIH in cats, hopefully to sterilize these animals. So yes, quite a long answer, it does appear to be conserved from fish to humans.

BSJ: So just to bring it a little bit back home, do you see your research being applied to the effects of stress on humans and as Berkeley students, we have many different types of stresses upon us. How would it affect humans?

Prof. Bentley: Well, we need to do the studies, but one would predict that you probably have elevated GnIH because of chronic stress and it may well influence the libido or fertility. But, we simply don't know really in humans, yet, what the effects of chronic stress are on reproduction via GnIH. What we do know is that chronic stress in humans can affect fertility and libido, as well. But, bear in mind that there are other factors that affect GnIH. Melatonin can increase GnIH release, staying up late at night messes with your melatonin rhythm. Similarly, you might be influencing GnIH in that way, too.

BSJ: With birds, would you be able to test acute stress versus chronic stress? What are the ways you would do that?

Prof. Bentley: We're starting to look at chronic stress in birds now. With acute stress, we typically use this very well defined paradigm - you go and catch the birds, put them in a bag for 30 minutes to an hour. Many labs have used this technique to measure different aspects of the stress response in birds. Chronic stress, again, it can come in different forms. The stress could be a worry about being eaten; it could be being forced to live in a poor habitat as compared to a good habitat. Now, we're able to house birds in a nice outdoor environment, which we call a "semi-natural environment". At the same time, we have birds indoors in an environment, where we know they won't breed. We know from data from many different animals that have been housed in a non-natural environment that such conditions can elevate the baseline glucocorticoid level. I think the chronic elevation of that baseline glucocorticoid level could be classed as a chronic stressor, but the effects might be subtler than a potent chronic stressor. Some labs go up to the birds' aviaries every day and spray them with water, or blow wind at them, or make startling noises. Those would be really strong chronic stressors.

BSJ: Since "stress" can be used in many contexts, do you have a term that you prefer to use in lieu?

Prof. Bentley: I would have to think about that. I don't have one. Even if I coined one, I think other stress biologists would disagree with me. They would have their own word that they want to use. I think stress is a whole spectrum of physiological responses to a whole spectrum of different cues. If I picked a word, I might be thinking about a specific part of the spectrum, but someone else might be thinking about the next.

BSJ: Where do you see your current research heading in the future?

Prof. Bentley: I am currently collaborating with Daniela Kaufer on this study, where we are trying to develop a non-sterilization technique on cats and dogs. I think that would be fantastic; this would be one very applied aspect of our research. It really could change the world, really, if we did it. It's just the mission of the foundation that's funding us is to develop this single injection sterilization technique that could be used without any surgical expertise and could be used in developing countries, for example. If you travel around the world, in some countries, there are dogs running everywhere. In this country, you tend not to see it so much, but there are millions of animals euthanized every year and we wouldn't need to do that. That's something I'm very excited about working on. In terms of other aspects of GnIH biology, I'm interested in going the other way. So, rather than sterilizing animals, I'm interested in finding out how GnIH is modulated by captivity, and I think I mentioned to you that it is really hard to get wild species to breed in wild captivity, especially birds. We think GnIH might be something we can manipulate to enable captive breeding of endangered species. I'm starting a collaboration with some people from the Smithsonian, on the East coast, to look at that as well. So two very different endpoints—sterilization and reproduction—but using the same mediator.

BSJ: We would like to thank you for your time.