# The Immune-neuro-endocrine Network: the Interface between Environmental and Internal Signals

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Good morning, ladies and gentlemen. I would first like to thank the organizers of this splendid meeting for providing me the opportunity to talk here. I would also like to say how impressed I was with the abstracts I read in the book and that I see how much progress has been made in the field of endocrine disrupters. I would also like very much to thank the organizers of this symposium and the chair persons because they gave me the chance to talk about immune-neuro-endocrine interactions. I also have to say that this is a difficult task because this field is too broad to be summarized in 25 minutes. It is quite a difficult mission, but I will try to do my best. That is why I would like to start with something that you all know. The brain receives information about the external and internal environment using sensory organs and internal receptors and part of this information is conveyed to neuro-endocrine systems. The same occurs with immune system that has specialized cells sense for the intrusion of foreign antigens, the expression of modified self antigens and the integrity of tissues and organs. However, much less is known about the fact that the two systems, the neuro-endocrine system and the immune system are linked together in a common network of interactions. This is the main topic of my talk today.

Immune cells express receptors for hormones, neurotransmitters, neuropeptides and respond to these agents. Conversely, the immune system can send messages to the brain and neuro-endocrine associated structures that recognize and respond to these messengers and altogether compose a network of complex interactions that have immunoregulatory implications and that also can influence the activity of neuro-endocrine systems.

We have been working for many years in this hypothesis that is symbolized in the following scheme. We have here, down, the immune system and up, the central nervous system that includes neuro-endocrine mechanisms which can feedback to the periphery.

We know now if one subjects the immune system to an antigenic stimuli, we do not get only an immune response as we all know, but it also induced a neuro-endocrine response that feeds back on the immune system. This is the "core" of our general working hypothesis. Immune cells release products, for example, cytokines, particularly pro-inflammatory cytokines, and these products act at brain levels or directly affect endocrine glands or peripheral nerves bringing about a neuro-endocrine responses which feeds back into the immune system.

The very first evidence that the immune system and the brain "talk" to each other was based on ~~a~~ very simple experiments. For example, many years ago we decided to make recordings of neurons in the hypothalamic levels to see if one activates the peripheral immune system we can have in some parts of the brain, try to detect changes in the activity of certain types of neurons which would attest for the flow of information from the immune system to the brain. The experiments we did were based on the administration of innocuous antigens, antigens that were not bound to any disease to see if, after we elicit an specific immune response, we observe changes in activity of certain populations of neurons.

This is a very old experiment that we did more than 25 years ago in which we immunized an animal with sheep red blood cells, that elicit a very strong and well characterized immune response, that can be measured by counting the number of specific antibody producing cells in the spleen. In the very same animal we have recorded the extracellular activity of neuronal units in this particular experiment of the ventromedial region of the hypothalamus. As you can see in the slide, when one injects either the control or the antigen on day one there are no changes in the number of antibody producing cells in the spleen and

no changes in the rate of firing of the neurons, the ones that we were recording. However, at the peak of the immune response, we have about a 2-3 fold increase in the rate of firing of neurons in the ventromedial hypothalamus. This was one of the very first evidences that the activation of the immune systems results in a flow of information to the brain, which indicates that the two systems are in very close contact and work together during a host response to an antigenic challenge.

We have studied other neuro-endocrine host responses during immunization, but I will restrict my talk to the hypothalamus pituitary-adrenal axis (HPA axis) because for time reasons I can only provide few examples, based on our own studies. Using the very same antigen as in the experiment described before, sheep red blood cells, to immunize the animals, we have studied the activity of the HPA axis. Nothing happened to the activity of this axis within the first three days, the induction phase, of the immune response to sheep reed blood cells. However, at the peak of appearance of antibody producing cells in the spleen, increased corticosterone blood levels was observed, which last until the immune response extinguishes.

We have experimental evidence showing that the increase in corticoid blood levels detected during the immune response is biologically significant, but I have no time to talk about that. Instead, I would like refer on how the link between two distinct structures (immune and endocrine) of the body is stablished. The immune system is symbolized in the lower part and the endocrine system in the upper part of this slide. It has to be pathways and some messengers derived from the immune system capable to activate the HPA axis.

Our primitive approach at the time that almost nothing was known about the existence of cytokines. When was to try to imitate in *in vitro* what happens in the *in vivo* by means of inducing what is called a mixed lymphocyte response using human peripheral blood leukocytes. We found out that the cell free supernatant from human lymphocytes stimulated to elicit an immune response *in vitro* induced an stimulation of the HPA axis in naïve animals. Thus, this effect is induced by products from activated lymphoid cells indicating for first time how the immune system can be the hypothalamus pituitary axis.

Later, it became evident that there is not one factor, but at least 12 different cytokines, which can induce the same effect. It is an extremely highly redundant effect. And when one looks at the med-line for cytokines or Interleukines and the HPA axis, one gets more than 1000 papers published on this aspect. But the first cytokine that we explored was IL-1 (interleukin-1) and so far it is still the most powerful one capable to elicit this whole response.

In the slide is shown the effect of injecting a low, sub-pyrogenic dose of IL-1. We see that both the ACTH and the corticosterone levels are increased and we also know CRH producing neurons in the hypothalamus are activated. Thus, the whole HPA axis is activated by IL-1. As I mentioned there are more than 12 factors produced by immune cells capable to exert a comparable effect.

For example, as can be seen in the two next slides, IL-6 and TNF stimulate the HPA axis but one needs about 20 times higher dose than IL-1 to have a comparable effect. So basically it has the same effect, but IL-1 is still more potent.

The mentioned data allowed us to delineate the first immune neuro-endocrine circuit. There are now several others circuits involving the sympathetic nervous system and also several hypothalamus-pituitary endocrine axes but I will circumscribe to this one.

I am still using the scheme that you see in the slide. Is a very old slide. We know that when one stimulates the immune system, not only IL-1 is released but also, depending on the type of the immune response, other cytokines of a family that we call generically, glucocorticoid increasing factors are also released. These cytokines act either at hypothalamic pituitary or adrenal levels to increase glucocorticoid levels. The increased release of this hormone establishes a feedback mechanism since the production of most cytokines are inhibited by increased glucocorticoid levels. Thus, cytokines induce activation of the

HPA axis and this activation feeds back on the immune system and dumps the production of several cytokines. The cytokine-mediated activation of the HPA axis serve also to impede the expansion of lymphoid mass and activity which might favour the appearance of autoimmune and lymphoproliferative diseases in susceptible subjects. I shall provide only two examples about the relevance of this circuit.

If one injects a very low dose of LPS, a gram-negative endotoxin, which does not induce fever and does not kill any of the animals. We have an increase corticosterone blood levels occurs. This increase is so important that when it is blocked 100% of these animals will die. So it is a question of life or death.

This effect is not caused of any direct effect of the endotoxin, but based on the release of cytokines such as Interleukin-1, indeed, if one blocks IL-1 receptors, as you can see in the slide, with the IL-1 receptor antagonist, the effect of the endotoxin on the HPA axis is abrogated to a large extent. There are many examples related to the operation of this circuit after the stimulation of Toll like receptors by bacterial products, illustrating the relevance of the cytokine-HPA axis circuit for natural immunity. However, also during the specific immune response this circuit is relevant for death or life.

Just one example, if one induces what is called an experimental autoimune encephalomyelitis (EAE), by giving for example as an antigen, myelin basic protein to Lewis rats, these animals develop a paralytic attack that occurs 12-14 days after the immunization.

If we measured the corticosterone serum blood levels in these animals that develop an specific immune response to myelin basic protein, we see an increase in the levels of this hormone. We know that if we adrenalectomize these animals will all die. Otherwise, all these animals will survived. This protective effect is again based on the cytokine-HPA axis circuit because if one blocks IL-1 receptors in animals with EAE, we see the increase in corticosterone levels during the disease is significantly reduced.

So, these two examples are indicative of how relevant are the interactions between the immune and neuro-endocrine systems, but it is also telling you how much relevant is for the host if these interactions are disrupted by different factors including environmental factors. If we consider that coupled immune-neuroendocrine responses contribute to the control of several infectious, autoimmune and neoplastic diseases, anything that causes disruption of these responses would represent profound medical problems.

I have referred until now to what we call a long loop circuit, but at this stage, we have realized that the same cytokines that from the periphery mediate neuroendocrine responses are also produced in the brain. So, why and what are they produced for? Cytokines such as IL-1, IL-6, TNF and interferons are produced by brain cells, particularly by astrocytes, microglial cells and also by some neurons.

We have also seen that if one injects LPS in the periphery of an animal, even very low doses that are known not to disrupt the blood brain barrier and will not penetrate into the brain, we see that this endotoxin can induce the gene expression of several cytokines in the hypothalamus, brain stem and the Hippocampus. Very low cytokine expression happened in the cortex, cerebellum and the thalamus. Thus, activation of peripheral immune cells results in production of cytokines in the brain or at least gene expression of cytokines, for example for IL-1, IL-6, TNF and γ-interferon, indicating that these cytokines can be expressed in the brain when immune cell stimulation occurs at peripheral levels.

This effect is controlled by noradrenergic (NA) neurons. Because if one destroys these neurons in the brain using a neurotoxin called 6-hydroxy-dopamine the IL-1 gene expression in the hypothalamus, it is blunted remaining close to basal levels of expression and in parallel the corticosterone levels are significantly decreased when compared with that of animals with intact NA neurons in the brain.

While studying cytokine gene expression in the brain we got another surprise that was that when one injects intraperitoneally IL-1 into an animal, also IL-1 gene expression occurs in the brain. Thus, IL-1 in the periphery triggers the induction of its own gene in the hypothalamus. To explore the meaning of that we have blocked the receptors for IL-1 in the central nervous system and evaluate the effects of peripheral administration of IL-1 and LPS on the HPA axis and glucose blood levels.

Coming back to the HPA axis, we see in the slide that when one blocks the IL-1 receptors in the brain, nothing happened to the induction phase of the stimulation of the HPA axis both by LPS and IL-1 injections. However, a decrease in corticosterone blood levels is observed during the maintenance phase of the response to these substances. This decrease is paralleled by the expression of the IL-1 gene in the brain. Thus, endogenous brain-born IL-1 mediates the maintenance of the response of the HPA axis.

This same happened, next slide please, with another effect that we have noticed after giving IL-1, that is that glucose levels goes down for a prolonged time when one gives IL-1 at peripheral levels. This effect can be blocked either by over-expression in the brain the IL-1receptor antagonist using an adenovirus as a carrier or by using the IL-1 receptor antagonist protein. You can see in the slide that blockade of IL-1 receptors in the brain interfere with the peripheral hypoglycemia that we see in these animals.

We have also asked the question of whether an increase in neuronal activities also results in the production of cytokines in the brain. For that, we have used a procedure to induce a prolonged increase in neuronal activity that was to elicited a long term potentiation (LTP) of neuronal activity in the hippocampus. This is a phenomenon that is very much studied because it is related to some forms of learning and memory. However, we have used LTP as a tool for our research because this process is based on a very short neuronal stimulation at pre synaptic levels to trigger a long lasting neuronal stimulation that can be maintained for hours, and days and even weeks when is triggered *in vivo.* Using this procedure we wanted to see if such prolonged increase in neuronal activity could result in a local induction of cytokines.

In this slide it is shown what is LTP. The neuronal activity at basal levels were first recorded. Then, within one minute, three trends of tetanic stimulation is applied that induces an rapid and long lasting increase in synaptic efficacy. This can be measured in different ways, for example, as the population spike amplitude. I will show the results obtained *in vivo*, but we have comparable results in hippocampal slices.

We can see in the slide that eight hours after triggering LTP there is in the ipsilateral part of the hippocampus an increase expression of IL-1 messenger RNA when compared with the contralateral side, an effect that is not noticed sham operated animals, that have also electrodes implanted chronically for two or three weeks in the brain. When LTP lasts only three hours, five hours later we see no increase in the IL-1 gene the expression. More importantly: if one blocks NMDA, glutamate receptors which are the trigger of LPT we also block the induction of the IL-1 gene.

Obviously we wanted to see what is the relevance of IL-1 expression in the hippocampus and for that we have to block the expression of IL-1 receptors because we are measuring the gene and not the protein. When one injects the natural IL-1 receptor antagonists (IL-1ra) into the lateral ventricle we see that nothing happens if this injection is given before tetanic stimulation or at the time of tetanic stimulation. However when the blocker it is given 2 hours later, that is the time in which the gene for IL-1 is fully expressed, we see that the levels of activity potentiated neurons is reduced to baseline levels. Thus, IL-1, endogenous IL-1, contributes to the maintenance of LTP in these animals. Next slide please.

We did the very same and I am not going into details with regards to IL-6. The gene for this cytokine is also expressed the ipsilateral part of the hypothalamus of animals undergoing LTP. No animals of the control groups show the increase in gene expression of the cytokine. Thus, IL-6 is also increased during LTP. However, we know that not all pro-inflammatory cytokines are expressed during this process, because TNF gene expression is not increased during LTP. In contrast other members of IL-1 family like IL-1 receptor antagonists and IL-18 are expressed during LTP.

And this is what happens to LTP (slide) if one inject into the brain a neutralizing antibody against IL-6 with regard to LTP. Animals injected with this antibody are showing a clear increase in LTP duration

so IL-6 is doing just the opposite of IL-1 indicating that both cytokines are involved in the "fine tuning" of the maintenance of LTP.

Now coming back to the immune system, (slide) look what happens when a very low dose of LPS is injected intraperitoneally. LTP, a phenomena that as I mentioned is related to many types of behaviors and to memory formation, is inhibited when LPS given in the periphery.

So let me now summarize. (Slide) Cytokines acting at brain levels play an essential role in the communication between the immune system and neuro-endocrine mechanisms. However, under basal condition, these mediators act as immune regulators in the periphery and neuro modulators in brain. Cytokines produced at peripheral levels indirectly, either following humoral or neuronal pathways or acting at the level of vascular structures in the brain, initiated neuro-endocrine responses and trigger cytokine production in different structures of the brain.

Cytokines produced in the brain prolong neuro-endocrine responses and by this way contribute to immuno-regulation. Both, peripherally immune signal, and signals induced during prolonged neuronal stimulation result in cytokine production in the brain. These two kinds of signals acting on cytokine producing brain cells allows, in our view, the integration of central levels of immune and neuronal influences and would provide an explanation on how immune neuro-endocrine interactions are integrated at brain levels. The coordinated response to neuro / sensorial stimuli and immune stimuli derived from the external and internal micro environment constitute an adaptive mechanism during disease. However, these coordinated adaptive responses can be disrupted by external agents.

I would like to leave you all with this message. Cytokines, predominantly proinflammatory are produced the interface, as seen in the scheme are produced in the interface between the external and internal environment and these cytokines can mediate neuro-endocrine responses that when are prolonged can act as disrupters of the endocrine system. I propose to this audience to study cytokines as potentials mediators for endocrine disrupters. I believe that such studies can bring us in the future, to a better understanding of the mechanisms of the disruption of endocrine functions during the course of exposure to environment pollutants, for example. Thank you very much for your attention.

## Q&A

Nohara: Thank you very much Prof. Besedovsky. So may I ask a Question?

Besedovsky: Yes.

Nohara: I want to make sure, if the cytokines which are produced by the immune cells, can pass the brain blood barrier?

Besedovsky: It is a very open question and indeed most people say they do not cross the blood brain barrier, but there are indirect ways for interpreting the effects of cytokines on the brain and that is why we came to the scheme showed in my last slide. For example, there are several factors that can be induced by cytokines in the endothelium interface of brain vessels, for example, prostaglandins and NO, from which, they can activate brain cells indirectly. Other people, and there is also evidence in favour to that, suggest that there are neural afferences, specially vagal nerve afferences that can be stimulated by cytokines and transmit information to the brain. But basically we need to know then what happens once cytokine signals arrive to the brain, independently of whether the pathway used is direct or indirect. What prolongs the effect of these mediators is their capacity of inducting their own production in the brain. That was the issue I addressed in the second part of my talk.

Nohara: But the reason I ask this question is if the cytokine can pass through the blood brain barrier, the cytokines may induce the same kind of network of cytokines which is seen in the immune system, also in the brain?

Besedovsky: In my view, they do not cross the brain blood barrier. They exert their effects on the brain indirectly following different pathways and especially because peripheral signals can trigger the production of low levels of brain born cytokines that act locally there.

Nohara: But still we can see a similar network in the brain as in the immune system.

Besedovsky: Exactly.

Nohara: O.k. Thank you.

Q: Prof. Besedovsky, you said very strongly that you thought cytokines may be the mediators of endocrine disrupters.

Besedovsky: It may be, but little was done to study this possibility.

Q: It may be, you said it strongly but then you said it may be, that is true. But do you have any, could you elaborate on that a little more? Are there any examples, where you think that this may be so?

Besedovsky: No, that is my surprise. That is why I looked in the literature, and I really that found very little, if any, information about that. That was the reason of the proposal I wanted to bring to this audience. The reason is very clear, as you know, in the skin is produced more IL-1 than any lymphoid organs of the body. And the skin and mucosa are our main interface with the environment. At these interfaces it is triggered the production of a lot of these cytokines. Thus, these cytokines could really serve as mediators which might be adaptive to a certain level, but when they are produced because of a chronic exposure of an irritant or toxic, either chemical or other nature they can interfere with cell metabolism. This could lead to an abnormal, long lasting release of cytokines would end in disrupting many of the neuro-endocrines systems that are known to be affected by cytokines. This is the message that I would like to leave to this audience: the possible role of pro-inflammatory and other cytokines on endocrine disruptions should be seriously considered.

Nohara: Thank you very much, Prof. Besedovsky.