Neuronal tunes

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*European Journal of Neuroscience, Trends in Neurosciences, The Journal of Neuroscience*: the work resulting from the collaboration of Professor Jacques Balthazart's team from the University of Liège (GIGA-Neurosciences) and the group of researchers led by Professor Annemie Van der Linden (Bio-Imaging Lab at the University of Antwerp), was in the spotlight three times in one year. It relates to the strange plasticity of the songbird’s brain. This peculiar phenomenon is helping neurobiologists in their quest to elucidate the neural bases of complex behaviours.

When we hear sparrows, chaffinches or canaries singing, we have no idea of the strange way their brain behaves all year round. Throughout the seasons, the size of the cerebral nuclei that control the vocalisations of passerines (1) changes, sometimes significantly. Examined from the angle of neurobiology, the study of this spectacular phenomenon is favourable to the development of models that allow us to better determine how a complex behaviour (song in this case) is based on the "dynamics" of the living, involving close relations between a neuroanatomical substrate (neural structures) and neuroendocrine and neurochemical mechanisms.

The presence of an intense periodical neurogenesis within certain song nuclei in several species of passerines has not escaped the researchers. Understanding the laws that govern the emergence of new neurons in these regions of the brain, falls under the scope of fundamental research, while also marking a way that could one day lead to man and form the outlines of new therapeutic pathways for the treatment of degenerative diseases of the central nervous system.

Learning to sing

It was Peter Marler, an English ethologist working at the University of Cambridge some fifty years ago, who sparked the sustained interest scientists have had in songbirds ever since. First of all, he confirmed what ornithologists had sensed: passerines learn their vocalisations from the environment in which they live. We know today that psittacines (parrots, budgerigars, cockatoos) and certain hummingbirds share the same
characteristic, but that other birds have vocalisations that can be qualified as innate. Thus, a cockerel placed in total isolation from birth, will nevertheless develop its crow.

Marler's essential contribution was to demonstrate the song-learning mechanism in passerines. Let us take the case of the chaffinch, in which he took particular interest. "In the beginning (from September to December), the animal is happy to listen to its fellow birds and store their songs in its memory", explains Professor Jacques Balthazart of GIGA-Neurosciences (previously known as the Centre de Recherche en Neurobiologie Cellulaire et Moléculaire or CNCM). "Then, in January and February, it practises, producing rather disorganised vocalisations - or subsong - which it compares with the examples it has memorised. In short, it proceeds by trial and error. This phase is followed by the third step: crystallisation, which takes place in March, during which the bird selects the vocalisations from those it has emitted that correspond to the songs inscribed in its memory. The chaffinch produces a stable song, the adult song." In some species, like the zebra finch, an Australian passerine, there is nevertheless a partial overlap between the auditory phase and the active production phase. As for the starling, it learns new vocalisations all its life.

(1) Passerines are a category of bird to which three-fifths of all the living species belong.

A two-sided mirror

When he was working at Rockefeller University in New York, Marler had two extremely brilliant students: Fernando Nottebohm and Mark Konishi. For a long time, scientists had been trying to understand the neuroanatomical bases on which the control of complex behaviours was based. The song system offered a particular advantage, insofar as bird vocalisations are emitted by a specific unique organ: the syrinx. Nottebohm's idea was that it must be possible to map the areas of the brain connected to the muscles activating the syrinx, by studying degeneration resulting from the severing of the axonal endings.
In the beginning, he pinpointed an intermediary motor nucleus situated in the brain stem, the nucleus of nerve 12 (nXIIts), before identifying several other interconnected nuclei in the brain: those that control song. "The latter define a caudal motor pathway and a more rostral pathway", explains Jacques Balthazart. "The first one is composed of the HVC (formerly known as the "High Vocal Center"), which unilaterally projects onto another nucleus, the RA (Robustus archistriatalis). In turn, the latter projects onto the nucleus of nerve 12, which innervates the syrinx. Besides the motor pathway, there is also a far more complex frontal pathway, where the HVC projects over an area called Area X. The neuronal connections take the following route: from Area X to a nucleus called DLM, from here to another nucleus called IMAN, and from IMAN to the RA."

In other words, there are two ways the information from the HVC can end up in the RA, directly or indirectly. The work of Nottebohm led to a complete description of the neuroanatomical substrates of the control of song. Hence, it attracted the attention of numerous neurobiologists because it offered them a perfectly defined frame of reference to carry out their studies on the neuroendocrine and neurochemical mechanisms governing complex behaviour.

What do we know about the role of the various nuclei that constitute the song system? Firstly, various experiments have revealed that the lesion of a nucleus in the motor pathway makes the bird completely silent. Secondly, it was discovered that the destruction of the rostral pathway in the adult had no immediate effect on song. However, in the case of a lesion in Area X or the IMAN nucleus, the stability of the vocalisations produced is somewhat altered after a few months: the rhythm slows down or accelerates and the quality of
the harmonics diminishes. On the other hand, if any link in the rostral chain of a young bird is damaged, it will never learn to sing.

This means that the rostral network is involved in learning and the caudal network (the motor pathway), in production. "It is clearer now why two paths lead from the HVC to the RA", says professor Balthazart. "They allow birds to establish a comparison between their vocal production and the elements stored in their auditory memory. This explains the shifts encountered after several months in adult birds where Area X or the IMAN nucleus were damaged."

In the same way, a bird made deaf continues to sing perfectly for a long time, but since it is incapable of "recalibrating" its vocalisations in relation to those it has memorised, it finally falls prey to a decline in the rhythm and harmonics of its vocal production.

**Neurogenesis**

Nottebohm and his colleagues revealed major differences in the size of the nuclei in males and females. Furthermore, researchers realised that these differences varied in degree according to the species. In zebra finches, for instance, the HVC nucleus in the male is five times more developed than in the female, while the ratio is two and a half in the canary. In certain tropical species, on the other hand, there is no dimorphism in the song nuclei. But what else do we know? In the zebra finch, the female does not sing, while the female canary sings a little and, in the other above-mentioned tropical species, they sing as much as the males. Consequently, this has led to theories linking the size of the nuclei to the quantity of song produced by the animal or the number of songs it has encoded. Nottebohm has also shown that in the canary, the size of HVC correlates with the diversity of the repertoire. There was only one step involved in concluding that this nucleus was linked to a library where songs were stored, that was big enough to hold a high number of "books". This conclusion was reached at the beginning of the 1980s, but it is currently partially being called into question. "One thing is certain: the vocal activity of songbirds is closely linked to the production of steroid hormones, which influence the size of the nuclei", points out Professor Balthazart. He goes on to explain that "testosterone fulfils a major role at this level. Furthermore, when it is administered to a female canary, for instance, she starts to sing abundantly."

The size of the song control nuclei is not constant; it fluctuates according to the season. In concrete terms, the bird's nuclei are very big in spring, decrease in the summer when the volume of the testicles diminishes, reach a minimum size in the autumn, then increase progressively once again to reach their maximum size at the end of winter and the following spring.
The question is then: how do these variations occur, which we believe to coincide with those of the secretion of testosterone by the testicles and those of the repertoire produced? We now know that the mechanisms governing the variations in volume of HVC and RA - the most extensively studied nuclei - are not the same. In RA, we observe modifications in the size of the neurons, their distribution and their dendritic connections. In spring, for instance, they are bigger, make more connections and are more spread out. Hence, a more voluminous nucleus. In HVC, the mechanism in force for RA is partly present, but it is not the only one. We also observe the incorporation of new neurons in the nucleus. "In other words, neurogenesis is involved here", says Jacques Balthazart.

When Nottebohm made this discovery, this phenomenon was something of a revelation, because it went against an old dogma of neurobiology: the brain of warm-blooded adult vertebrates (homeotherms) does not produce new neurons. So what was then discovered? In short, the existence of an extremely active neurogenesis in the entire telencephalon of songbirds. This discovery led researchers to reconsider the problem in a more general manner. As a consequence, the presence of significant neurogenesis in at least two regions of mammals' brains has been demonstrated.

**Complex relations**

How can we define the type of link uniting a morphological element, the change in size of some of the brain's nuclei, and a learnt behavioural expression, the production of songs? Such is the key question that numerous laboratories concerned with research on songbirds are studying.

In the 1980s, Fernando Nottebohm suggested the existence of a correlation between the number of different songs produced by the canary and the volume of the HVC. Hence, the idea already evoked that this nucleus
was a sort of library and its size, the reflection of the complexity of the animal's repertoire. Subsequent studies showed that this conclusion was not always valid. According to Jacques Balthazart, we now tend to believe that the size of HVC does not necessarily correlate with the diversity of the bird's repertoire, but rather with the quantity of vocalisations it makes.

"Perhaps there is an inversion in the causal link", he insists. Indeed, it was initially thought that the volume of HVC influenced the variety in the repertoire, while we now think that the size of the nucleus is the consequence of a more abundant vocal activity, as if the bird was "exercising" its brain by singing a lot."

In these conditions, it is justifiable to question the role of the new neurons that establish themselves in the HVC. It has been revealed that they all project onto the RA, and never onto Area X. And yet, the HVC and RA constitute the motor pathway, while X belongs to the frontal pathway involved in learning the songs. The decrease in the size of HVC in the summer and part of the autumn is the result of an accelerated neuronal death that affects the exchange of information between the two components of the motor pathway. If such a phenomenon affected the frontal pathway, the song memory would perhaps deteriorate and diminish. "Is that the reason why it is preserved?" Jacques Balthazart wonders. "Undoubtedly. But this is a finalist interpretation that is not currently based on any scientific certainty."

On the other hand, we know that seasonal variations in the nuclei and neurogenesis coincide with those in the size of the testicles and, subsequently, the production of testosterone. The volume of the male gonads is itself controlled by the photoperiod which, incidentally, also has a direct effect on the size of the brain, regardless of the action of the testosterone.

In fact, this is unbelievably complex and the web is very difficult to untangle. Why? Because there are several types of closely interlinked correlations involving the photoperiod, the level of testosterone, the number of songs emitted, the size of the HVC nuclei and, up to a certain point, the vocal repertoire.

**In vivo exploration**

Up until recently, all the studies relating to songbirds' brains relied on classic anatomical techniques that could only provide single post-mortem measurements. To overcome this stumbling block, neurobiologists were led to make comparisons between animal cohorts. For instance, a series of birds was killed in the spring and another in the autumn in order to dissect their brains and thus learn about the evolution in the size of certain nuclei, such as the HVC or Area X. "For several years now, medical imaging techniques, such as nuclear magnetic resonance, allow us to visualise various structures in the nervous system in man and to study how it functions in vivo", our interviewee explains. "For a long time, the application of these methods to fundamental research on animal models was limited by the low resolution of the images obtained and by various technical problems mainly associated with the time it takes to take these images - the subjects have to stay still."

The appearance of magnetic resonance imaging systems (MRI) with a high magnetic field (7 Tesla and above) has overcome the first obstacle, making it possible to obtain images of the brain of a living animal (anaesthetised) with an anatomical resolution of 50 to 70 microns. Furthermore, this new technology avoids sacrificing numerous animals because a single group of subjects can be studied repeatedly and they do not need to be killed to observe the structure of their brain.

At the end of the 1990s, the Bio-Imaging Lab research group, led by Annemie Van der Linden at the University of Antwerp, decided to adapt these techniques to the study of animal models and focused a major part of its work on the study of seasonal nervous plasticity in songbirds. These concerns matched those of the Professor Balthazart's team, which had already been studying the behaviour and nervous anatomy of these birds for a long time. They soon began working together. In June 2009, the magazine *Trends in Neurosciences* published
a review paper (2) dedicated to imaging work carried out in conjunction by the two laboratories. These studies require both a structural approach (MRI) and a functional approach (MRIf).

**Unsuspected plasticity**

Structural imaging, first of all. When MRI was first used, the spatial resolution was 50-70 microns and the contrast of these images somewhat limited. In these conditions, the technique revealed axon fascicules, but not those of the song nuclei. How could the latter be visualised? The solution was to resort to the MRI of manganese, an element that is injected directed into the brain, in the HVC. For reasons that cannot be explained here, the RA and Area X, the two projection sites, accumulate the manganese in turn and become visible several hours later during the MRI examination. Hence, the researchers from Liège and Antwerp were able to confirm, through repeated sequential measurements in the same animal, the presence of major variations in the volume of the key brain structures involved in controlling song in passerines such as the canary, starling or zebra finch. For instance, the bench scientists showed - as had already been shown through anatomical techniques - that injecting testosterone into birds caused the song nuclei to increase in size. This time, however, it was by studying the same animal and not animal cohorts that this cerebral plasticity was revealed.

"The MRI analyses have particularly allowed us to fine tune our understanding of the hormonal and social mechanisms that control these variations in volume of the nerve structures", Jacques Balthazart explains. "The possibility of obtaining successive images of the brain of the same subject before and after an experimental manipulation is indeed a major advantage. Moreover, it allows us to greatly reduce the number of experimental animals that have to be sacrificed."

Besides the numerous experiments that use MRI coupled with an increase in contrast through the injection of manganese, the researchers are also carrying out work based on another technique: Diffusion Tensor Imaging (DTI). Based on the polarisation of vibrations of the water in the electromagnetic field generated by the machine, this technique was essentially used to visualise the fascicules of myelinated fibres (axons), through mathematical analysis (computer reconstruction), the aim being to study the seasonal variations in the connections within the animal’s brain. This research was the subject of a publication in the European Journal of Neuroscience (3) in 2008. It demonstrated the existence of an unsuspected seasonal plasticity in the nerve connections linking the song control centres. What was even more astonishing was that this plasticity turned out to also affect many other fascicules of fibres linking the different parts of the brain to each other or to the sense organs, such as the eyes. Thus, the optic chiasm turned out to be smaller in the summer than in the spring. "Do songbirds need a better visual system during the reproductive period than outside this period?" our interviewee wonders. "In any case, the mechanisms behind the seasonal plasticity of the fascicules of fibres must now be analysed, as well as the functional significance of the phenomenon."

**Left or right?**

For technical reasons, it was not possible to relate the studies in magnetic resonance imaging (MRI) to the production of song since they were carried out on anaesthetised birds. The research was focused on hearing. We have known for the past twenty years that the hearing system in birds remains highly active when the subject is anaesthetised. Two major pathways were explored, as recently related in *The Journal of Neuroscience* (4). The first one tried to determine whether there was a difference between the regions of the brain through which the bird recognises its own song (*Bird Own Song* - BOS) and those it uses when it hears a fellow bird singing - conspecific song. As for the second approach, its purpose was to compare the areas of the brain allotted to the recognition of conspecific song and that of heterospecific song (another species) respectively.
The **Bold** MRIf (Bold meaning Blood Oxygen Level Dependent Signal) takes into account the neuronal activity of anatomically limited regions of the brain by measuring the variations in blood flow and the level of the haemoglobin's oxygenation. By using this technique, research at the universities of Antwerp and Liège were able to isolate three areas in the brain, in the zebra finch, that react more intensely when it hears its own song than when it hears a conspecific song: MLd - an auditory nucleus situated in the **mesencephalon**, the HVC and Area X. At the same time, the MLd, Area X and the L Field - a primary acoustic structure - were more activated by conspecific song than by heterospecific song. "Amazingly, these distinctions can already be observed in the ascending auditory pathways in the mesencephalon, when it was thought that they only functioned in the "upper echelon", in the telencephalon", explains Jacques Balthazart.

Another surprise: the distinctions in the mesencephalon are lateralised. The recognition of bird own song compared with conspecific song takes place in the right hemisphere whereas that of conspecific song compared with heterospecific song takes place in the left hemisphere. "These experiments raise numerous questions concerning the evolution and the anatomical bases of cognitive abilities, as well as their lateralisation, in vertebrates distant from man", concludes Professor Balthazart.