Treating Autism with Bumetanide

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By

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FOREWORD

Discoveries are the product of a complex recipe requiring experience, hard work, patience, highly instructive errors that must not be ignored and the capacity of going beyond established dogmas to create novel concepts that in due time will be challenged by next generation. Knowledge evolves by unpredictable steps with most of us contributing small increments and one of our successors having the intuition and the talent to draw from our observations far reaching conclusions that we failed to consider. There is, however, also a chance factor, meetings with colleagues and changing environment impacts our vision and facilitates the emergence of intuition – an ill-defined term that is relevant to step forward advances. When Fleming discovered penicillin, he did not look askance at the bacteria that failed to grow next to a particular fungus. Rather, he was ripe for observing the unexpected, which might lead him down new avenues of research and treatment. Passion rewards the original researchers who study their surroundings with a fresh gaze. Intuition, a quality fashioned from knowledge, experience, and a generous helping of curiosity, can lead the lucky to the top of creativity. At the end of the day, chance smiles to those who are aware and ripe to harvest the fruits!

The icing on the cake is that these discoveries often lead to a questioning of established concepts. For a scientist, that is the Holy Grail. Science is never set in stone, anything at any time can be questioned, and this everlasting process is its fuel for growth. Science does not do well under rigidity and strict programming. Numerous examples illustrate how guided, and sterile research has a low probability of fostering qualitative leaps in knowledge. All the tools that are now part of our daily life – from the theory of relativity and its applications a century later, to X-ray, imaging, discoveries in genetics, stem cells, transplants, antidepressants, GPS, e-mail, radar – are the product of minds that were ahead of their time, of theories that were considered zany before becoming obvious. Along the way, some of those responsible for new discoveries ended up incarcerated. Defeat is orphaned whilst success has many parents. The road is littered with pitfalls. There is the conservatism of those who oppose all questioning. The temple keepers are scientific research's perennial nemesis. Then, there are financial interests, which influence the direction of science depending on the importance attached to the field by the global economy at any given

time. Contrary to common belief, science is not neutral, ethereal nor flavourless; it is the product of women and men who are prey to their egos, to fashions, to power relations, and to dominant forces. Science is becoming exceedingly expensive, increasing researchers' dependency on those who hold the purse strings. This state of affairs is at the root of numerous false discoveries, the lifespan of which is but the length of a sigh. Their discoverers are bestowed with a moment of media exposure, fame, and ephemeral honour. It is the era of Kleenex researchers, like the pop singers who beguile teenagers for a handful of weeks. Yet passion remains indispensable as a fuel of discovery.

This story is the product of an encounter between a biophysics researcher passionate about understanding the mechanisms governing brain maturation and a psychiatrist who seeks to treat autism, one of the most debilitating developmental disorders. The researcher tries to understand the role genes and the environment play in brain formation (the innate and the conditioned) and how neural pathways develop. He does not have a diagnostic or therapeutic goal in mind. Rather, he is guided by curiosity, which acts as both compass and talisman. The psychiatrist wants to enter the bubble within which afflicted children enclose themselves. In the face of this trying disorder, parents often latch onto false hopes. He is guided by permanent suffering, life stories, and the despair of parents who have no handbook to help them understand their inability to communicate with their children. In short, the breadth and boundaries of neuropsychiatry.

This unexpected meeting will create a dialogue between two parallel worlds that usually ignore each other. Researchers are seen as spoiled children who allow themselves the luxury of virtual reality and abstract questions, whereas psychiatrists bring support *hic et nunc*. The present is brought face to face with the promises of a possible future – definitely a difficult dialogue to foster. A common language develops around a passion for understanding and treatment. A presumably crazy idea is born from this meeting: to treat autism with a diuretic. A third person then joins the two, someone just as iconoclastic, an expert in brain imaging persuaded that visualising brain activity can help us identify the afflicted neural networks and their footprint. An ideal approach that allows us to move from animals to humans better than any other method, a bit like a painter who continuously retouches the same canvas.

Indirect and potentially simplistic observations have helped the basic science expert to detect high concentrations of chloride in the nerve cells of immature neurons but also in adult neurons recorded in animal models of a

large number of brain disorders including epilepsies, brain trauma, spinal cord injuries, infract and chronic pain. These observations suggest that insults produce neurons endowed somehow with immature features – a sort of return to the future! A complex situation endowed with numerous philosophical, experimental and even ethical issues. It is, however also endowed with possible novel common therapies to treat for many neurological and psychiatric disorders. This is counterintuitive as these disorders have different aetiologies, clinical manifestations and outcome. Yet, if high levels of neuronal chloride are observed in these disorders, then a diuretic known to reduce neuronal chloride might be used to attenuate them. As we cannot determine chloride levels in neurons in children with autism (this is best performed in vitro in brain slices!), it is instrumental to rely on experimental animals that mimic autism either by administration of a drug or a genetic mutation associated with autism in humans. Usually, basic science that requires years to provide a possible treatment precedes clinical trials. However, the reformulation of a widely-used molecule to treat another disorder that the initial one considerably shortens this latency. Diuretics have been used for decades to treat hypertension and oedemas, by reducing chloride concentrations in the kidneys and their side effects have been extensively documented simplifying the quest for approval from health authorities to perform trials. Consequently, we faced a very unusual situation where the trials were performed well before experimental data in animal models confirmed that neurons have high chloride levels and the diuretic attenuates the clinical and electrographic signatures of the disease. However, the combination of clinical and experimental observations is instrumental as the improvement of a clinical symptom in children by a drug does not imply that the suspected mechanism is the correct one! There are several alternative possibilities that must be taken into consideration (see below).

The idea to treat autism with a simple molecule with diuretic properties annoys geneticists, who are convinced that autism is genetic and that it will only be diagnosed and treated once the hundreds of mutations sometimes associated with it are identified. It does not matter to them that the ‘gene’ for autism cannot be found, nor that the hundreds of mutations concern only a small percentage of those with the disorder, nor that to identify the mutation would not bring us any closer to treating it. ‘We will make it’ they sing in unison and in blind faith. Our era desires this, the certainty that everything, or nearly everything, is genetically determined: intelligence, foolishness, personality, behaviour, religious belief, sexuality, aggression or criminality. Of course, neurological and psychiatric disorders fall under this

spell, a chain of genetic and epigenetic events that lose their mystery, bringing forth healing and improvement. And tomorrow, pigs will fly!

Historically, not very long time ago, the other extreme was filled by the ayatollahs of psychoanalytic sophistry – and this to some extent is still very much the case in France and French speaking countries. Largely disconnected from biological reality and floating in the ether, it seems that the only justification for their approach is to ensure that their caste becomes perennial – to the detriment of the patients. Here, biology, randomised clinical trials and statistics are barbaric terms reserved for use by narrow-minded scientists. But the evidence speaks for itself and only ignorant people will claim that autism is a disorder reflecting a mother's supposed lack of desire for a child. We will, therefore, scrutinise the distant past with a telescope in search of the trigger. Bettelheim has popped by, mixing concentration camps, phallus, the desire to procreate, maternal love, grandiose language and academic discourse. When we try to remind these clinicians that there is a biology of molecules behind all this, a malformation of the brain, and children who do not even master language when the disorder is in its first stages, they still reply that speaking heals and that *we* are the ignorant ones who have forgotten the separation between mind and body. They insist on viewing these molecules and biochemical processes without cure with snobbery. The source is again and always childhood, troubled relationships with the mother, sexuality – the general hullabaloo of psychoanalysis. Speaking is sufficient in itself; it does not require experimental proof. The majesty of speech and beautiful phrasing: French sickness *par excellence*. Descartes is obviously the export product; a prophet hath no honour in his own country. Admittedly, this is now less accepted because science has progressed, yet, at times when fake news become prevalent, other vicious attacks of scientific observations challenging the usefulness of vaccines suggested to cause autism have emerged. This is indeed an endless fight! Elsewhere, pragmatism dominates and, like Saint Thomas we only believe what we see and luckily psychoanalysis became out of fashion in most countries at least as far as autism in children is concerned.

Admittedly, the genetic reductionism that exaggerates the therapeutic benefits it brings has played a decisive role in this abandon and helped the mother to get rid of the guilt that dominated a couple of decades ago. Yet, the discovery of mutations compellingly associated with "simple" monogenic disorders – like Huntington's disease or the Duchesne dystrophy – has not led to suitable treatments! The principal reason for this failure is that, as will be discussed below, the plasticity of brain networks responds to

insults by modifications that disrupt the simplistic link between one gene and the disease. The superficial link one gene – one protein – one disorder ignores the essential roles of development, life conditions and environmental factors that translate the gene to a given outcome in most cases. Homeric struggle leaves little room for a third voice: those who want neither option. The two sides rally against those who seek to understand how neural networks function in the face of adversity. After all, if there has been a communication breakdown, we should expect traces of it in neural networks' electric activity that generates the brain's rhythms.

Is that debate truly devoid of an ideological approach? The situation is further complicated by the fact that autism is not like other disorders. First, it seems to be affecting an increasing number of people – more than 1% in the last epidemiological studies. Second, it combines elements that are particularly sensitive to us: a handicap that originates in the womb and the first few years of childhood, affecting communication and socialising, aspects at the heart of modern society. Since early diagnosis is difficult and cures do not exist, the inability to communicate at an early age creates a vicious cycle that invalidates this crucial stage in life when we adapt to society. Moreover, because autism has a wide range of symptoms, it is difficult to understand its mechanisms and to develop new therapies. Autism has been linked to a plethora of factors, from the mother's or father's age to birth issues (preterm delivery, programmed caesarean, delivery complications, anoxic periods…), and a lot of environmental factors. In brief, a disorder with an early start, a history characterised by variable geometry, cumulating genetic and environmental factors… A nightmare situation for the researcher and the clinician but a boon for swindlers.

Our story will present why a diuretic, which has been prescribed for four decades to treat hypertension and cerebral oedema, can be used to improve the quality of the life of children (and adults) with autism and that of their parents by extension. This conclusion has its roots in a theoretical concept and experimental data. It has been fostered by the wonderful decision of a brain imaging expert to join the team and her demonstration that treatment normalized crucial areas of the brain linked to social and emotional processing. Thus, we escape murky water, whilst in parallel, less constraining data has shown us that high chloride concentrations exist in the model neurons of autistic animals. From there, the fight involves overcoming administrative difficulties and finding the right financing to create a treatment adapted to autistic children. This story highlights the difficult practices of the biomedical world, which are characterised by the medical and scientific fields' conservatism, and the axiomatic slowness and

grotesque bureaucracy that must be conquered to successfully develop a drug.

It is not an exaggeration to state that our efforts to develop a novel treatment based on a diuretic – in spite of the compelling experimental observations and clinical trials, have been met with profound skepticism. Many psychiatrists considered this almost as fake news, papers published on the difficulty to develop novel medicaments and their inefficacy did not even mention our successful trials. From the basic science world, things have been slightly but not significantly better. Somehow, conservatism to novel ideas prevails. To convince, we needed far more basic and clinical data than was required from other approaches more in accord with the dominating modes – we will show that this is what we did.

It goes without saying that our main reason for writing is the parents' experience. It is to them that we dedicate this book. They are the true heroes, who retain trust in science and progress and fuel our passion to overcome all obstacles. Moving on from these testimonies, we attempt to convince the reader that a healthy measure of humility is necessary when we are dealing with such a complex illness. There cannot be a solution as simple as the aspirin used to cure a headache. This is because autism's early origins mean its symptoms cannot be completely erased. But parents are themselves describing the treatment's benefits: 'Our child is more present.' Our book will devote its better part to the testimonies of autistic children and their families.

To contextualise the problem, we will first summarise the mechanisms that govern the brain and its construction and how they can go wrong, giving rise to neurological and psychiatric disorders. Then, we will present the main points on autism, before moving on to the findings from our therapeutic trials and the critical input from brain imaging. The testimonies from autistic children and their parents will allow us to explore the treatment's benefits and its limits.

I

THE EXTRAORDINARY STORY OF THE BRAIN'S CONSTRUCTION

1. Neurons, synapses, neural networks, and ionic currents

The brain. It is a complex organ, which receives, analyses, and integrates information, memorising some of it and producing a response that takes into account both recent and not-so-recent history. This occurs through the intermediary of neurons and the synapses that interconnect them, creating functional networks composed of millions and sometimes billions of neurons working in concert to generate brain activity. This activity will translate into the responses we have to our environment: our behaviour.

Neurons. These are cells specialised in the reception of information and its transformation first into chemical signals and then into electrical ones. Their activity is propagated via hundreds of millions of synaptic connections and will be translated into behaviour, reaction, and memory… A neuron is a cell with positive and negative ion charges, a bit like a battery that generates electrical current via negative and positive electrolytes. An essential part of its energy is used to preserve ionic gradients between its interior and the exterior.

Neurons have elongations, dendrites, and axon. Myriad types of neurons exist. They are always made up of a core, the cell body, and tree-like elongations. The dendrites and the axon extend from the cell body, constituting respectively the start and end of the neuron. The electrical activity reaches the dendrites and then propagates towards the cell body and the axon to transmit the information to the neuron's target. Information is analysed at every step of the transmission.

The synapses. These are points of contact and of information transmission between two nerve cells. They are also the main location for the integration of our activities, their analysis, memory building, and all the

properties that allow our brain to be such a high-performing and flexible machine. They are the true heart of our integrative capacity. Defective synapses are a sign of numerous brain disorders, autism included. The human brain is made up of thousands of billions of synapses. Comparing the brain's complexity to the processing power of a mobile phone puts this in perceptive. To achieve the brain's complexity, each person would have to carry hundreds of thousands of mobile phones. We understand the organisational difficulty and functioning of this hub, but how does it actually work?

Synaptic transmission. The arrival of an electrical influx into the terminals of a nerve cell will unleash a chemical mediator that will be recognised by the target cell and will generate an ionic current. This current will, in turn, act on the targets of this neuron. The activation of a synapse and the transfer of information to its target lasts a few thousandths of a second, a delay that incorporates the release of the mediator, the activation of target structures and the genesis by the target cell of an electric current that will propagate towards other targets. Synapses are the true heart of our brain's functioning. It is therefore important to understand its functioning and its anomalies. To regulate brain activity and to generate discharge patterns in addition to the associated behaviours, neurons have excitatory and inhibitory synapses that act a bit like a brake and an accelerator. On the one hand, the brain must maintain a constant equilibrium between brake and accelerator and, on the other, it must produce rhythms at specific times that correspond to particular brain activities.

Chemical mediators. We are aware of a number of chemical mediators but two principal ones govern most of the field: glutamate and GABA. They are both synthesised in distinct neural groupings and exercise complementary roles in the behaviour-generation factory. The glutamatergic neurons, which release glutamate, are the most common. The release of glutamate into the synaptic space is recognised by the target neuron's receptors and triggers excitatory currents of positively charged ions called cations. The introduction of cations (sodium, potassium or calcium) will decrease the target cell's rest potential and will excite it. The GABAergic neurons that release GABA are less common (representing between 10% and 20% of neurons). The release of GABA translates into inhibitory currents in the target cell, which are generated by negatively charged ions called anions, of which chloride is a well-known example. The introduction of chloride into the target cell will increase the rest potential – charging the battery, if you will – and the cell will have trouble generating an excitatory response. These synapses are therefore inhibitory because they

tend to reduce the target cells' activity. The GABA synapses are the target of many tranquilising and treatment molecules, such as benzodiazepines (Valium®), barbiturates and other anaesthetics and anxiolytics. They work by increasing the efficiency of GABAergic currents and reducing brain activity enough to produce their calming effects. We therefore understand how neurons must constantly control the equilibrium between excitation and inhibition, knowing that if excitation dominates or if inhibition is depleted, the network will overexcite and, for example, generate epileptic fits.

Brain oscillations. These are electrical activities generated by groups of neurons connected by synapses. The oscillations reflect at once the networks' analyses, the integration of information included in these analyses, and the expression of the corresponding behaviours. Adult brains constantly generate oscillations usually defined by their frequency (cycles per seconds, or Hertz), which range from a few cycles per second to tens of cycles per second and sometimes more, especially in pathological situations. Thus, brain oscillations signal what we do and the behaviour that results from it.

2. The main phases of brain development

The brain's complexity has increased throughout evolution. The main phases in its development are clear and consist of five principal moments.

The formation of a neural tube. During embryogenesis, a tube is formed with cells encircling it. It has dorsal and frontal poles, and dorsal and frontal faces. The general schema has remained the same since our oldest ancestors.

The proliferation. To generate the countless number of nerve cells that will make up the brain, the cells divide following a set rate and schema. A modification in the duration of cell division and/or the period during which the cells divide can change the result dramatically. We indeed think that evolution has partly developed through such variations. Nerve cells are generated in excess and the number will adjust itself during a period of time lasting until puberty. Thus, our cortex can lose many thousands of cells and millions of synapses per minute (as we will see below). Once the final number of cells has been reached, they stop regenerating themselves, save for those located in the central structures (olfactory cells and the neurons of the dentate gyrus). Contrary to the publicity surrounding this subject, which is as mistaken as it is pervasive, our cortical cells do not renew themselves,

and the brain is not a fountain of eternal youth. There are genetic disorders caused by mutations that alter the proliferation, giving rise to an excess of neurons, like autism, or to fewer neurons, like certain forms of early-onset epilepsy and brain malformations.

Neural migration. Our cortex, the most developed structure in *homo sapiens*, simultaneously generates our language and our species' extraordinary integrative capacity. Although not larger than whales and other cetaceans, it has the largest surface and volume relative to our closest cousins, the chimpanzee, and is composed of six neural layers with precise organisation, composition and connections.

To build this six-storey building, we need scaffolding, an elevator, and automatic guidance control so that the elements can migrate to their assigned location. The neurons will take the lift, transforming into specialised cells called radial glial cells, and head downstairs to their assigned location. The elevator always starts by bringing the cells that reside in the lower floors and then those closer to the surface. Changes in this migration process causes severe developmental problems, such as certain mental disabilities, double cortex syndrome and tuberous sclerosis.

Differentiation. Nerve cells specialise only once they have reached their 'floor'. At which point they acquire cellular, morphological, and biochemical properties, which differentiates them into one of the thousands of cell types present in the brain.

Sprouting, the formation of synapses and neural networks. At this stage, the neurons sprout, emitting axons and dendrites (as we saw earlier) and connecting specifically with the neurons that will be part of the same functional entity. The selectivity is absolute: we can see the extensions coming into contact with a neuron, deciding that it is not the right target and moving on to find the correct one. As soon as the contact is chosen, synapses form and the two neurons will act in concert. The cellular entity is connected by millions of synapses, creating a functional whole responsible for the integration of information and/or the genesis of a particular response. At each stage, a mechanism can be defective, which can give rise to repercussions that are often severe and that can cause neurological and psychological disorders. Thus, either an excessive or insufficient proliferation will take place, which will be translated into an anarchical organisation of part of the brain that can lead to intellectual deficiency and epilepsy. A flawed migration of nerve cells can also create a double cortex. This will generate atypical activity that prevents the brain from functioning

properly and give rise to severe epileptic fits that are resistant to all treatments save that of successfully removing the double cortex surgically.

To summarise, the brain's construction is organised around essential meeting points that must follow a precise timing. Any delay will lead to costly malformations, which cause many severely debilitating conditions. The genetic programme is at the heart of this construction and modulated at every step by information received from the earliest stages of its construction. The brain, when forming, is neither blind nor deaf. It is not on autopilot; it is paying attention to its environment and taking it into account as it forms its neural networks.

3. Brain maturation is controlled by genes and the environment

How is an organ as complex as the brain built? What role do genetics and the environment play in this process?

The brain's volume is between 1,200 and 1,300 m^3, three times that of our close cousins, the chimpanzees. It is the result of a true evolutionary leap in the animal kingdom. Evolution is both qualitative and quantitative. Important regions in the brain are developed preferentially. A good example is the cortical envelope and its associated cortices, which congregate information so that it can be contextualised. It took less than two million years to evolve and this evolution did not occur continuously. Instead, it developed by specialisation, as can be seen by the lack of continuity present between animal species. Thanks to this, *homo sapiens* is a social animal that can gather knowledge and use it collectively to benefit others. Humans are less sensitive to genetic determinism than their close cousins. This is because our intellect is not the result of a hyper-constrained genetic programme but is instead the consequence of complex interactions in which the environment played a crucial role.

Carrying out such a programme is not simple. For example, we need millions of ganglion cells to extend from the retina to the optical centres. This requires green and red lights and stop signs to guide the neurons' axons across the median line towards their goal. If the retinal axons do not reach their target, which happens with some genetic mutations, the visual system will not build itself. Consequently, numerous genetic mutations can make it impossible for this complex programme to develop correctly.

That being said, the genetic programme is not everything. During its construction in the uterus, the embryo listens, perceives and vibrates in response to its surroundings. The construction of its cortices is modulated by the electrical activity generated by its sensory organs, and this before any precise perception is possible. We open our eyes very early on during pregnancy, towards the end of the first trimester, and our retina is active while forming, though sight is not yet possible nor does it correspond to any visual activity. This electrical activity that is observed in a wide range of animal species from frogs to chimpanzees and humans is nevertheless important because, if we block it *in utero*, the visual centres fail to develop properly. They maintain their scheduled organisation, but this organisation lacks context and visual capacity.

How to translate and illustrate this situation? When building a car or a plane, the elements are assembled and, when all is ready, we pump in the petrol and start the engine for the first time. In contrast, the brain works from the earliest phases of its building, as if the airplane or the car already had the motor running from the first phases of construction. A very long breaking-in period follows, which will continue in part for the rest of our lifetime. Today, we know that the immature brain is very active on an electrical level, including during the early stages. Immature cells generate electrical activity that plays a crucial role in the brain's construction. *In utero* the little one is moving, exciting itself and generating an impressive amount of activity, as mothers know well. The studies we have undertaken for three decades on the brains of rodents or primates have allowed us to describe the sequences of maturation of brain activity. These activities are specific to the developing brain and are not observed in the adult one. In other words, the brain speaks a lot as it builds itself, but with a language different to that of the adult's. The data also highlights how important it is that specific drugs are developed for babies, rather than simply administering reduced doses of drugs developed for adults.

The general rule is that ionic currents are slow, which means fast signals like those needed by the adult brain for day-to-day activities (walking, driving, responding to external stimuli…) cannot be generated. It is nevertheless enough for the needs of a developing network. The developing brain is extraordinarily heterogeneous and must coordinate neurons that are at different stages of maturation so they can work together. Remember that the neurons in the human cortex are dividing during roughly a hundred days (approximately between the 40^{th} and the 140^{th} day of gestation). Consequently, they must cohabit and discharge together, despite some already having hundreds of synapses while others have a few or even none.

The slow currents generated by immature neurons are a perfect way of making these very different neurons work together. It is a bit like a gigantic symphony orchestra, one that is many thousands of times larger than that required playing Berlioz's *Symphonie fantastique*. During rehearsals, the members of the orchestra organise themselves by resemblance in small groups, which play pieces that are very different to the symphony. Actually, each group plays a pre-planned baroque minuet in perfect execution. When the time comes, the orchestra comes together to play the symphony without preparation. The quality of the concert will depend on the quality of the execution of the minuet by each little group. If the groups give a poor performance, the overall recital of Berlioz's symphony will be a dud. The embryo's brain harmonises these minuets, which are generated by a giant, heterodox orchestra that is seemingly without any kind of organisation, and translates it into a musical work intelligible to adult ears.

What purpose do immature minuets serve? Another analogy will help us answer this. During the construction of a building or a bridge, we proceed first by choosing an architect and a programme of works, armed with a plan and many details – this is the genetic programme. Then the work is delegated and construction site meetings ensure that it all develops as planned. The programme is checked constantly throughout the process. Thus, in the case of the Channel tunnel, building works were undertaken from both ends, with each part meeting in the middle down to the last millimetre, guided by a GPS. We think that electrical activity plays the role of the GPS and, at the same time, this activity modifies itself progressively, allowing the process to be followed – a kind of construction site meeting. This activity acts like a checkpoint, running continuous quality control, similarly to compliance checks in industrial production chains (see discussion in (Ben-Ari & Spitzer, 2010)). The studies that we have conducted over many decades on rodents, primates, and on prematurely born babies for certain parts confirm this sequence, its maturation, and its electric signals.

This system seeks to inform the neurons' targets as to the location and maturation stage in which the neuron finds itself. For the visual system, each ganglionic neuron in the retina, of which there are a million, lengthen towards our visual centres and send this information to their targets. A visual system builds itself from this information. It is therefore a sophisticated GPS, which can indicate space, time, location, and age all at the same time. A second function of the system is the taking into account of external conditions and adaption to these. It will do this by changing the programme if need be. Nothing is immutable; continuous control is exercised, which

takes external conditions into account. Complex genetic biochemical pathways fine-tune and modify the programme's execution at every point in time. Thus, we can identify at the DNA level the regulatory genes that control the turning on or off of thousands of other genes, enabling this true strike force to totally amend the execution of a programme. These genes are turned on by other genes or by external events, the role of which is to cause a chain of events when the time comes, as well as the chance to correct or amend any given part of the programme. We will see that these genes are also often the targets of mutations that cause severe disorders like Rett syndrome, the symptoms of which are also shared by autism.

To conclude, one of the major discoveries in the study of brain maturation is the electrical language of immature neurons that differs from adult ones. The developing brain is not a little adult brain. It has its own ionic currents, discharge patterns and electrical properties. The goal of these activities is different from that of the adult brain. It does not codify information precisely and swiftly like those that enable quick responses to stimuli from the outside world or, for the blessed ones among us, that allow the playing of Bach's violin sonatas. The brain's construction is therefore built from a genetic programme, whilst also maintaining flexibility by allowing the environment to influence its execution and adaptation to conditions. The developing brain receives a lot of information generated by the periphery. The data are analysed in real time and provides the elements for the programme's functioning and adaptation. In the next chapter, we will look at an example of a maturation sequence to illustrate how the brain develops; this has been extensively investigated and used as a model for developmental sequences.

4. A maturation sequence: immature neurons are richer in chloride than adult ones

It all started when one of us, Yehezkel Ben-Ari, was named director of an INSERM unit (the French National Institute for Health and Medical Research) at the Port-Royal Maternity Hospital in 1986, taking over from Professor Alexandre Minkowski. In order to immerse ourselves at the heart of the maternity hospital, we decided to study the maturation of brain activity and the various pathologies that can afflict the brain. The first recordings we took allowed us to discover utterly unexpected properties, which were clearly difficult to place within our contemporary frameworks. The GABAergic currents clearly had properties that were totally different from adult ones. These currents were supposed to inhibit neural activity but

instead excited it because of the high intracellular chloride concentrations present in immature neurons (Ben-Ari, 2014; Ben-Ari, Cherubini, Corradetti, & Gaiarsa, 1989; Ben-Ari, Gaiarsa, Tyzio, & Khazipov, 2007). The activation of a receptor causes chloride to exit rather than enter, like in adult neurons, such that the GABA then causes the rest potential to depolarise rather than hyperpolarise, often resulting in neural excitation. This observation, which has been confirmed in very many species and brain structures, seems to have been maintained throughout evolution and constitutes a major developmental property of the brain.

When chloride transporters mature

A chloride importer with a mouthful of a name, NKCC1 is among the important regulators of intracellular chloride concentrations. It allows two chloride molecules to enter in exchange for one sodium molecule (Na+) and one potassium molecule (K+). Another one also exists, a chloride exporter called KCC2 that returns the process to equilibrium. It allows two chloride molecules to exit for two potassium molecules. This mechanism ensures that the flux of chloride is controlled because it must retain a specific stability to ensure the proper functioning of neural networks. The transporter that lets the chloride in starts working before the exit transporter does, resulting in an accumulation of chloride in the nerve cell (Rivera et al., 1999). We can study the functioning and the role of these transporters thanks to molecules that prevent the selective means by which one transporter or another is blocked. Thus, a molecule like bumetanide selectively blocks NKCC1, lowering the intracellular chloride concentration and reinforcing the inhibition of young neurons. This molecule, which we will explore at length later, is a diuretic that acts at the kidney level, where NKCC1 controls diuresis. This state of affairs is not unique, indeed it is present in the brain and in peripheral organs, where a good number of proteins and transporters exercise the same functions. Intestinal motility, for example, is controlled via neuron receptors that work in this way.

What is the point of having neurons and GABA synapses that excite in the first place?

The excitation of GABA receptors in immature neurons that have high chloride concentrations is linked to an increase in intracellular calcium. This calcium, which actually exists in small concentrations when free, plays a central role as trophic factor acting on growth, the burgeoning and the

formation of synapses, or cellular plasticity. Essentially, GABA first excites, taking on a trophic role, then, when the density of the glutamate synapses is high enough, the transporter that drains chloride becomes operational, changing the GABA's action polarity (Ben-Ari, 2014; Ben-Ari et al., 2007). Nature does things well. The alternative mechanism with a precise minute by minute control of the excitatory and inhibitory drives is far too rigid implying a genetic automated control of every one of the hundreds of molecules involved in synaptic operation.

What are the clinical implications of this sequence?

If GABA excites immature neurons while inhibiting adult ones, a chemical that would improve GABA's effectiveness would have an opposite effect on the brain of the mother than on the brain of the foetus. Valium® and other benzodiazepines thus have anxiolytic, soporific or anaesthetic effects in adults by increasing the inhibitive effect, while the opposite is true for the immature brain. We can easily imagine what the implications of such an observation are, especially given that many molecules are often given to pregnant women to treat insomnia and depression. The same goes for cannabis, which acts on the GABAergic synapses and can cause undesirable effects on the development of the embryo brain. Other circumstances are just as delicate. With a pregnant woman who is epileptic, for example, we seek to prevent fits during pregnancy that may cause an abortion by administering molecules that exert aberrant actions. Thus, there are antiepileptic agents that act on GABA signalling and that have been found to increase the incidence of autism in newborns, having a deleterious effect on the foetus brain development. Experimental observations provide compelling observations that blocking the high chloride levels *in utero* for instance by maternal administration of bumetanide during gestation can be toxic leading to offspring with autism (Wang & Kriegstein, 2011). These observations also underline another important aspect of the development of new medicine: a pharmacopoeia specifically dedicated to pregnant women and new born is much needed. Indeed, until recently, the molecules used to treat new born were the same as those used for adults. There was no obligation to test these molecules on young animals. Legislators have since changed tack and it is now obligatory to test molecules developed for newborns on young animals. We will see other examples later on where different treatments are necessary for developing brains.

5. Childbirth: a special highly vulnerable moment

Childbirth is a complex process during which hundreds of molecules and signalling pathways act in concert. It is in fact the most complex biological event in mammals. The goal of all these adjustments, which happen only at birth, is that of adapting to a new environment. There is, for example, the preparation of the lungs for a shift from an aquatic to an oxygenated environment, involving the reduction of water levels in the lungs but also major shifts of metabolic, cardiovascular, immune system and microbiota during this period. Birth is a very stressful event (Lagercrantz & Slotkin, 1986) with levels of stress molecules in the baby higher than anything that we shall experience in our lives. These levels are in fact much higher than the ones observed in the mother giving birth. This stress is indispensable for some of the events that must take place rapidly notably the first breath. This probably underlines the observation that following C-section delivery, there is less stress molecules but also some initial difficulty to breath. During evolution, the challenge was to both provide a system –stress molecules – that will enable the abrupt and rapid shift but also to protect brain neurons from this excessive stress. Indeed, neurons are not at ease in presence of very high levels of stress molecules being associated with enhanced excitability and even seizures produced notably by an accumulation of chloride in neurons and a shift of GABA actions from inhibition to excitation. How is it possible to reconcile these two opposite requirements?

Among the molecules involved, oxytocin appears to play a particularly important role. This hormone, which already operate to trigger uterine contractions and childbirth, also plays a key role in the mother-child relationship after birth and during breastfeeding in humans and animals. In experimental conditions when this hormone is prevented from operating, the mother is indifferent to her offspring. Indeed, there exists a close relationship between oxytocin concentrations and the sociability of certain animal species. We reasoned that perhaps it also, plays a role in the alterations of GABA polarity during birth.

During a focused study conducted on rodents recording neurons from the embryonic to the adult stage, we discovered a completely unexpected result when examining the change curve of intracellular chloride concentration (Tyzio et al., 2006). It must be admitted that we were confounded, as it did not initially make sense! That is the surprise of discovery. The curve describing the fall in chloride concentrations had an abrupt inflexion a few hours before and after birth. It was as if birth was announced by a dip in intracellular chloride concentrations in the newborn's

brain. Knowing that oxytocin triggers birth, it was of course necessary to check the hypothesis that oxytocin was at the origin of this fall in chloride concentrations. The key experiment consisted in injecting a molecule that specifically blocks oxytocin's action. We were able to see that administering this molecule stops the fall in chloride in the neurons of newborn rats. In other words, the same molecule that prompts labour acts also on central neurons of the newborn, causing a diminution in chloride. Oxytocin released by the mother, or by the future baby in some species, provokes birth and at the same time acts on the newborn's brain. That is some multitasking of Swiss Army Knife proportions! This fall in chloride, the readers will have understood, means a reduction in GABA's excitatory effects in favour of increased inhibition. This translates into a reduction in central neural activity and a greater resistance to external aggressions, such as reduced oxygen levels, that are a major cause of neurological disorders.

The brutal fall in chloride during birth is hugely important. Indeed, one of the major causes of brain injuries, birth handicaps, and motor disabilities are temporary absences of oxygen called hypoxic-ischaemic episodes. They are generated by a number of factors, such as the umbilical cord contracting or poor irrigation of the brain during labour. They are even more dangerous if there are high levels of electrical or metabolic activity. These hypoxic-ischaemic episodes will translate into brain injuries that often cause neonatal epilepsy that can persist throughout life. On the other hand, oxytocin has a neuro-protective effect on the newborn's brain by inhibiting brain activity during birth (Tyzio et al., 2006). And that is not all: it also has analgesic effects, relieving pain. The initial observations were made by Dr Lagercrantz's group in Sweden, which showed that vaginal delivery babies feel less pain than C–section delivery ones (Bergqvist, Katz-Salamon, Hertegard, Anand, & Lagercrantz, 2009). The baby endures a painful process during birth and this observation suggested that oxytocin might reduce that pain. We confirmed that this is the case in rodents. The concentration of intracellular chloride in pain cells is high and reduced by the hormone while its antagonist worsens the sensation of pain in animals right after birth (Mazzuca et al., 2011). So, the hormone that triggers labour and delivery also protects the child's brain and reduces pain during childbirth. We cannot be anything but awed by Mother Nature's genius that, here as elsewhere, she uses one mechanism to solve many problems. It is not impossible that we will become increasingly interested in molecules of this type in the future when seeking to prevent dangerous effects from occurring during childbirth (Ben-Ari, 2015). Therefore, during evolution, the possible deleterious actions on neurons of stress needed for major biological functions during birth including respiration was compensated by

the same hormone that triggers birth. The fact that the various actions of oxytocin are mediated by an action on chloride levels and the polarity of GABA actions reinforces the importance of this parameter that stands at the core of so many developmental and pathological issues.

Whether C-sections increase the incidence of autism has long been debated with contradictory epidemiological observations. This is quite understandable as the heterogeneity of C-sections renders difficult to conclude. C-sections can be performed because of urgency – complications and obvious evidence that the fetus suffers for one reason or another. However, the incidence of seizures has increased worldwide with over 50% in some countries (Brazil, Egypt), around 20 in others (Europe) and slightly higher in the USA. This is the result of both legal aspects with less parents suing the hospital if complications have occurred after C-section than vaginal delivery – but also "fashion" – with well-known actors having done it leading to the "why not me" mind-set' – and career – women wanting to do it rapidly and to go back to work! Earlier studies have suggested that there is no link between C-section and autism in offspring. A more recent study relying on a very large number of papers and studies suggests in contrast that there is a small but significant increase in the incidence of autism after C-section delivery. However, epidemiological investigations aren't sufficient to conclude this because of the heterogeneity of cases and the difficulty to differentiate between C-sections made in emergency and others.

We therefore decided to test the hypothesis in mice, where the conditions are well controlled and homogeneous. Interestingly, this important issue has seldom been investigated earlier with only two laboratories having adapted a surgical intervention to perform a C-section in rodents. We made several attempts to develop the technique and after a long period we succeeded to have pups elevated by a foster mother after a C-section delivery. We then used all our techniques to determine if the brain and neurons are altered in pups born by vaginal delivery and by age matched C-sections. We used both term C-sections – fetuses being removed at term by C-sections – and "preterm" C-section – where fetuses are removed 24 hours earlier. We could not remove the fetuses earlier as the lungs are not mature, but it bears stressing that the duration of gestation is 19-21 days and hence 1 day difference is a significant period (corresponding roughly to two weeks in a human pregnancy). From the electrophysiological aspect, we found no difference between term, preterm C-sections and vaginal delivery, suggesting that delivery does not impact the alterations produced by oxytocin and the abrupt shift of chloride (see above). This was also the case

with a variety of behavioral features that were not altered, with the exception of calls made by the pups when separated from the mother early on, but these effects were short lasting. Also, reconstructing neurons revealed that they were slightly smaller following C-section delivery, but this difference was abolished 1 day later as if there was a delay that was corrected within 1 day. Collectively, this study suggests that C-section at least in rodents and in the short time frame possible does not impact the process, and that the brain is not affected by the lack of vaginal delivery and associated events (Fernandez et al., 2018).

Therefore, the GABA developmental shift during birth is an important signal with oxytocin playing a number of important roles as an anti-stress molecule endowed also with a neuro-protective action on metabolic insults during this vulnerable period.

6. High intracellular chloride concentrations in epilepsy and other pathological disorders: back to the future

Many brain disorders have been associated with an excess excitatory drive mediated by an imbalance between excitation and inhibition. This can be due to an increase of the former and/or a decrease of the latter. Is it possible that alterations of the GABA shift and chloride levels also operate in pathological conditions as they do during birth? Epilepsies was obviously the first type of pathology to investigate in this regard and the initial measures of the effects of pathological conditions on intracellular chloride came from studies dedicated to epilepsies. One of the most frequent brain disorders, epilepsy constitutes a large family of disorders associated with intense highly synchronized aberrant electrical activities of brain networks associated with a large panel of clinical symptoms. Studying epilepsy has helped to better understand the normal functioning of the brain and its pathological disorders. During an epileptic fit, all or some of the brain's neurons discharge their electrical signals in a synchronised manner, as opposed to the large-scale discharge patterns observed in physiological disorders. With epilepsy, the equilibrium between excitation and inhibition is disturbed and the regulation of intracellular chloride undergoes a tough ordeal. Inevitably, chloride levels were expected to increase and this indeed was found to be the case at least in many animal models of epileptic disorders. Perhaps more astonishingly, similar increases of neuronal chloride was then observed in a large panel of brain disorders extending from brain trauma, spinal cord injuries, ischemic insults, Parkinson disease,

chronic pain, Post-Traumatic Stress Disorders (PTSD), developmental disorders and even certain types of carcinogenic tumours (Ben-Ari, 2017).

These observations have important clinical implications. Let's look at an example: a good number of antiepileptic molecules act by improving the efficiency of GABA currents. Since they allow chloride to enter and end up controlling the amplitude of the currents generated by the GABAergic synapses, they end up facilitating cerebral inhibition. Many of these molecules are known to the non-scientific public, such as barbiturates and benzodiazepines (of which Valium® is well-known). Beyond their antiepileptic effect, they also act as anaesthetic, anxiolytic or even analgesic agents. Nevertheless, if GABA excites neurons notably because of high neuronal levels of chloride, these agents will have different or even opposite effects to those they would have in healthy tissue. Thus, as we have seen, Valium® will inhibit neurons in pregnant women, while exciting those in the embryo. If GABA excites neurons rather than inhibiting them, however, these same products can logically have paradoxical pro-epileptic effects and worsen fits!

Many clinical and experimental observations corroborate these paradoxical effects. Furthermore, they show that the paradoxical effects are more important than the fits that happen before the phenobarbital is administered, suggesting that the recurrence of fits produces an increasingly acute accumulation of chloride. This is because chloride concentrations become more difficult to regulate when there is a recurrence of fits. When experiments are conducted, the phenobarbital reduces the number of early fits but worsens those that occur later on. Therefore, the effectiveness of antiepileptic medication is determined by the *timing* of when it is administered. New therapeutic solutions might therefore be possible if chloride concentrations were to be reduced back to their original levels, thereby restoring GABA's hyperpolarising and inhibitory action.

It is from these indirect observations that our hypothesis of reducing chloride concentrations with a diuretic was born. The diuretic would increase the efficiency of antiepileptic molecules acting on GABA. Indeed, NKCC1, the co-transporter we looked at previously, is one of the main importers of chloride into the kidneys. It controls the removal of water *by* transporting chloride. However, this very same importer of chloride is also present in the brain and is expressed during embryogenesis, being, as we have seen, one of the factors responsible for high chloride concentrations in immature neurons. We quickly came to the conclusion that a diuretic decreases chloride concentrations in epileptic rodents' neurons and thus

improves the efficiency of GABAergic inhibition. Admittedly, similar changes were observed in some but not all models of epilepsies. In addition, things became more complex when we and others discovered that recurrent seizures acutely increase neuronal chloride suggesting that seizures might beget more seizures by this mechanism, hence the difficulty to generalize the putative efficacy of reducing chloride to all types of epilepsies. An extra complication came from our observations that recurrent seizures generated in a given brain structure can propagate to distal regions leading there to the formation of an epileptic focus. An additional blow came from a small clinical trial on two-days-old babies in Europe who suffered from severe neonatal anoxic seizures. The trial had to be stopped because side effects on the auditory system. This might however have been anticipated considering the susceptibility of the developing hearing system to a mixture of antibiotics and diuretics. Two lessons from these studies may be gleaned: first, the diuretic might raise concerns when applied early in life in particular in conditions where antibiotics are required, and second recurrent seizures have an impact on chloride regulation and the effectiveness of the diuretic might depend on the number of seizures that occur prior to the administration. In addition, using this drug during pregnancy might raise severe concerns.

Following that, and as is often the case in science, we and many other laboratories found that the changes in intracellular chloride concentrations did not only concern epilepsy: other pathologies were also characterised by an accumulation of chloride in neurons. These pathologies include brain injuries, as well as bone marrow injuries, brain trauma or chronic bone pain, and are linked to an increase in intracellular chloride and GABA's excitatory effects. There seems to be a kind of general response to aggressions or brain injuries: a deficient chloride regulation system, a reorganisation of the circuits and the expression of currents and signals usually characteristic of a brain in formation. Why such a variety of insults lead to some common alterations remains a mystery. This is also corroborated by the observation that drugs to develop a given disorder are also often found to be efficient for other ones, raising the possibility that common mechanisms might operate perhaps in relation with at least some of the clinical manifestations in relation with general neuronal excitability that these drugs reduce. These observations are what led us to propose treating autism with a diuretic. Gaining compelling experimental data took a few years but astonishingly they were preceded rather than as usual followed by clinical trials.

II

WHAT IS AUTISM AND RELATED DISORDERS?

Autism is a neurodevelopmental condition affecting one child in 59 and more than 2% of boys, according to the latest figures from the US Centre for Disease Control (Baio et al., 2018). It is a developmental disorder that impacts three principal areas: social relations, communication, and behaviour. We can define autism simply as the absence of a social instinct that characterises people who have a 'normal' development, generally described as 'neurotypical'. Autism was first described by two doctors who did not know each other but who depicted the well-known triad of autism with a single year separating them. Kanner wrote about it in English in 1943, whilst Asperger published his observations in German in 1944 but was only recognised for his contribution in the 1980s when translated in English by Lorna Wing. The cases he described related to children who had normal language acquisition, and behaved like "little professor", and could be very gifted. Incidentally, she regretted later this publicity that was undue because Asperger appeared to have been a convinced Nazi, in favour of forced sterilisation of unfit persons and who sent many children to death, being not suitable for the 'glorious' society planned by the Fuhrer (Czech, 2018). On the other hand, it is possible that Asperger wanted to avoid labelling these children as 'mentally retarded', which would have had them sent to concentration camps. In any case, 'high-functioning autism' can be used as an alternative term for Asperger syndrome, although many high-functioning autistic individuals do like to refer to themselves as "Aspies", and there is some important value in that diagnosis for those affected (Attwood, 2007), and even if obviously there is no way to defend whatever Hans Asperger did during WWII, he still seems to have first described this syndrome.

It was only in the 1980s that DSM-III, the psychiatrists' bible, finally recognised autism as a category in itself rather than a psychosis (Pichot, 1986). Later, the DSM-IV defined categories within ASD comprising of Autistic Disorder, Pervasive Developmental Disorders, Not Otherwise Specified (PDD NOS), Asperger's Disorder, Rett Disorder and Childhood Disintegrative Disorders. This was then revised in the 2013 DSM-5 which define one broad category "Autism Spectrum Disorder", that then needs to

be specified in terms of the presence or absence of intellectual impairment, language impairment, the presence of a known medical or genetic condition or environmental factor, and the association with another neurodevelopmental, mental or behavioural disorder, and Asperger syndrome is no longer a separate category in the DSM-5.

What is most striking about autism is its incredible variety of clinical forms and the fact that we use the same term to qualify people who have pronounced intellectual disability, people who cannot speak, those who display self-mutilating behaviour, and others who subscribe to the triad but who have a normal if not above-average level of intelligence. That is why we prefer to speak of an autism spectrum, or of the autisms (Coleman & Gillberg, 2012), rather than simply of 'autism'.

When it comes to genetics, it has been clearly established that individuals are five to ten times more likely to be autistic when they have a sibling who is autistic. It has also been shown that there is a 70 to 90% concordance between identical twins. Some families are more prone than others to exhibit autistic traits, suggesting that heredity plays an important role. Ever since the pioneering study by a group of French scientists that identified a mutation in a Swedish family, hundreds of mutations that correlate with an autistic syndrome have been identified. Each mutation may only be relevant to a handful of individuals on the planet, others may affect between 0.1 and 1% of people, with the total number of mutations estimated to be relevant to roughly 15 to 20% of autistic people. But there remains a huge majority of afflicted individuals who are without a mutation. Furthermore, the same mutation in two brothers may produce two totally different clinical situations, with the first exhibiting severe autism, including a lack of speech, and the second being diagnosed with high-functioning Asperger's. Technology has allowed the search for the loophole in the huge genetic alphabet to be undertaken speedily and with costs divided by a thousand. From this starting point, geneticists have now postulated that all those with autism have the same mutation or even that there is a master gene that triggers the disorder and that it is simply a question of time as to when we will locate it. The issue is that, in science, it is not possible to extrapolate therapy from a single principle. To say that autism is a 'genetic' disorder is an abuse of language, unless we decide that the weak percentage of autistic people who have a mutation represent the majority. Recent studies however provide a vivid illustration with the progressively emerging observations that genes are controlled by epigenetic factors that provide a link between genes and environment. In essence, the idea is that genes are not *Deus ex Machina* switches that trigger on their