Abstract—The developing brain is talkative but its language is not that of the adult. Most if not all voltage and transmitter-gated ionic currents follow a developmental sequence and network-driven patterns differ in immature and adult brains. This is best illustrated in studies engaged almost three decades ago in which we observed elevated intracellular chloride (Cl$^-$), levels and excitatory GABA early during development and a perinatal excitatory/inhibitory shift. This sequence is observed in a wide range of brain structures and animal species suggesting that it has been conserved throughout evolution. It is mediated primarily by a developmentally regulated expression of the NKCC1 and KCC2 chloride importer and exporter respectively. The GABAergic depolarization acts in synergy with N-methyl-D-aspartate (NMDA) receptor-mediated and voltage-gated calcium currents to enhance intracellular calcium exerting trophic effects on neuritic growth, migration and synapse formation. These sequences can be deviated in utero by genetic or environmental insults leading to a persistence of immature features in the adult brain. This "neuroarcheology" concept paves the way to novel therapeutic perspectives based on the use of drugs that block immature but not adult currents. This is illustrated notably with the return to immature high levels of chloride and excitatory actions of GABA observed in many pathological conditions. This is due to the fact that in the immature brain a down regulation of KCC2 and an up regulation of NKCC1 are seen. Here, I present a personal history of how an unexpected observation led to novel concepts in developmental neurobiology and putative treatments of autism and other developmental disorders. Being a personal account, this review is neither exhaustive nor provides an update of this topic with all the studies that have contributed to this evolution. We all rely on previous inventors to allow science to advance. Here, I present a personal summary of this topic primarily to illustrate why we often fail to comprehend the implications of our own observations. They remind us – and policy deciders – why Science cannot be programed, requiring time, and risky investigations that raise interesting questions before being translated from bench to bed. Discoveries are always on sideways, never on highways. © 2014 The Author. Published by Elsevier Ltd. on behalf of IBRO. This is an open access article under the CC BY-NC-ND license (http://creativecommons.org/licenses/by-nc-nd/3.0/).

Key words: excitation inhibition, developmental sequence, delivery, intracellular chloride.

Abbreviations: ACSF, artificial cerebrospinal fluid; CFP, cyan fluorescent protein; GAD, glutamic acid decarboxylase; DZP, diazepam; ENOs, Early Network Oscillations; EPSCs, excitatory post synaptic currents; GDPs, Giant Depolarizing Potentials; MSNs, Medium Spiny Neurons; NMDA, N-methyl-D-aspartate; PB, phenobarbital; PSCs, post synaptic currents; SPAs, Synchronized Plateaux in small-cell Assemblies.
INTRODUCTION

GABAergic signals play a crucial role in the generation of behaviorally relevant oscillations by means of specific synaptic connections. The network of GABAergic interneurons is particularly diversified enabling a fine tuning of the activity that principal neurons generate (Freund and Buzsaki, 1996; Buzsaki and Draguhn, 2004). Classically, GABA is presented as the prototype of inhibitory transmitter acting by means of a hyperpolarization and shunting inhibition to reduce on-going activity. This however does not give enough credit to GABAergic currents that are endowed with the unique capacity to inhibit or excite neurons depending on activity levels and intrinsic or extrinsic factors. No other fast-acting transmitter shares this feature of GABA and its twin brother glycine. This is a consequence of the permeability of GABA receptors channels to chloride and other anions and the exquisite capacity of intracellular chloride levels to change and reverse the polarity of the current.

As many other researchers, I have been intrigued in the mid 1970s by the mechanisms and pathological implications of this feature. One obvious line of research was the role of chloride regulation in epilepsies as many antiepileptic agents act by reinforcing GABAergic inhibition and conversely blocking GABA receptors to generate seizures. For me as for many other researchers, understanding epilepsies was an excellent way to better understand how the inhibition operates in physiological conditions: brain pathology has always been an excellent school to understand brain operation provided that these studies are done in parallel. My interest to developmental neurobiology shifted when I moved to head a new laboratory in Paris located in a maternity hospital. Obviously, this was time to look at the GABA/(Cl–) network activity links in health and disease in a developmental perspective since the incidence of seizures is highest early in life. It turned out rapidly that GABA signals and immature networks had been largely ignored. With my colleague E. Cherubini and our students J.-L. Gaiarsa and E. Corradeti, we therefore started from scratch in 1987 performing intracellular recordings of hundreds of hippocampal neurons from fetal to postnatal life (Ben-Ari et al., 1989, 2007). This led to a series of unexpected observations that required more than two decades to comprehend and put in a wider frame. Indeed, as often in science, it became apparent that the GABA sequence is but the visible part of the iceberg, the submerged one pertains to fundamental issues related to the maturation process, the role of activity in brain development, the timing of shifts from immature to adult networks, etc.

The present account illustrates the difficulties of going beyond one’s expertise, crossing across disciplines and taking time to think and elaborate global concepts. This is more difficult today than it was in the past because of the publish or perish agenda, the short-term financial support and grant systems that are incompatible with conceptualization. Thinking «Big» is not fashionable at times when speed is more important than content, technology prevails over depth-oriented research and “big data collection” over concept-oriented research. Yet, having then a relatively stable recurrent public financial support, I was offered favorable conditions to take my time to investigate issues that were initially neither meant to be published in “high profile” journals like Nature or Science nor to novel “translatable” observations with therapeutic perspectives. Surprises as always come from leaving aside the obvious.

To set the scene, I shall first describe briefly the various classical observations made by the pioneers of GABA research before describing the evolution of the questions and hesitations I had during this procedure and how they led me very slowly across three decades to a fascinating direction that included the development of the concepts of “Checkpoint” (Ben-Ari and Spitzer, 2010) and «Neuroarcheology» (Ben-Ari, 2008) and a novel treatment of autism and other developmental disorders (Lemonnier et al., 2012, 2013).

PIONEERING PRE-HISTORICAL TIMES: THE INVENTION OF GABA

At early times, the GABA saga resulted from a conjugated series of observations made primarily in New York, Montreal, Canberra and Harvard. Florey and colleagues isolated GABA from brain extract and Grundfest, Purpura, Kuffler and colleagues showed depressive actions of GABA in various preparations (Purpura et al., 1957, 1959; Kuffler, 1960). The effects of GABA on the spinal cord were described first by Curtis and colleagues in Canberra and synthesized analogs and antagonists in collaboration with J. Watkins (Curtis et al., 1961; Curtis and Watkins, 1965). During these early times, when Journals like Nature were keen to publish fundamental studies in neurophysiology and not primarily short-term genetic stories, K. Krnjevic and colleagues reported the actions of various molecules on cortically evoked inhibition in rabbits, cats or monkeys (Krnjevic and Phillips, 1963; Krmjevic and Schwartz, 1966). Using the newly developed microiontophoretic applications of drugs, K. Krnjevic revealed the inhibitory actions of an agent isolated from cortical material identified as GABA. The description of these crude observations and the depth of deductions made are worth quoting at times when eloquence is not a dominating quality of papers being replaced by sterilized, impersonal style less accounts: «In view of the possibility that GABA is the chemical agent mediating cortical inhibition, an attempt was made to find a selective antagonist of its depressant action on cortical neurons.»
Neither of the agents listed above nor any other of the substances tested was able to block this action. It was concluded that cortical inhibition differs from spinal inhibition in its pharmacological properties; and that our observations are consistent with the possibility that GABA is the cortical inhibitory transmitter (whereas glycine is for the spinal cord) (Kelly and Krmjevic, 1968).

To confirm the crucial role of GABA in cortical inhibition, a selective antagonist was indeed much needed and this was achieved by D. Curtis and colleagues (Bicuculline, a specific GABA antagonist, diminishes basket cell inhibition of hippocampal pyramidal neurons, an inhibition which is not affected by strychnine). The inhibitory transmitter released by basket cells is thus probably GABA (Curtis et al., 1961; Curtis and Watkins, 1965). It became rapidly apparent that the antagonists of GABA are also epileptic agents leading to the still dominant concept of the excitatory/inhibitory balance needed to avoid seizures and hyperactivity. This was to be further reinforced by the discovery of the antiepileptic actions of pro-GABA drugs like the benzodiazepines and phenobarbital (PB). This was the basis for the long-lasting and still dominating excitatory/inhibitory imbalance suggested to be THE central cause of epilepsies and many pathological conditions. Although used here as well, this simplified view fails to fully account for the plethora of roles that GABAergic systems fulfill.

A PERSONAL PILGRIMAGE TO GABA RESEARCH: ADULT GABAERGIC INHIBITION IN HEALTH AND DISEASE

An introduction to GABA: The Medical Research Council unit of Cambridge

Finishing my PhD in France («Unitary cellular plasticity in the amygdala in vivo using a Pavlovian sensory conditioning») (Ben-Ari, 1972; Ben-Ari and Le Gal La Salle, 1972), I decided to make my Post doc in Cambridge. Without really programing it, I started to work on GABAergic signals. Having worked for some times in the amygdala, I started to examine the distribution of glutamic acid decarboxylase (GAD) and other markers making the first detailed description of GABAergic signals in the various nuclei of the amygdala. We used a dissection method, which would seem genuinely prehistoric today intra-cardiac perfusion of the heart with cold artificial cerebrospinal fluid (ACSF) and dissection of the fresh material in the cold room... well dressed to that occasion. Using this method and a Methylene Blue stain of the surface of fresh slices, we could dissect with K. Zigmond and assay GAD even the optic tract or the stria terminalis and its bed nucleus. We provided a rather complete description of the concentrations of GABAergic markers in amygdaloid nuclei (Ben-Ari et al., 1976c,d; Zigmond and Ben-Ari, 1976).

In parallel, with J.S. Kelly, we examined the effects of microiontophoretic applications of dopamine and GABA on amygdaloid neurons using the recently developed technique of iontophoresis of agents with brief currents. We relied on a stereotaxically positioned guide tube, sealed to the skull in order to obtain stable recording conditions. Then, the stereotaxic position of the cells was determined with the position of the microelectrode tracks determined histologically. Dopamine exerted inhibitory actions on the spontaneous activity of neurons but interestingly, neuroleptics like alpha-flupenthixol or pimozide had no significant effect on the dopamine or GABA sensitivity of neurons. We suggested that the effects of neuroleptics on animal behavior may not be explicable simply by a general blockade of dopamine receptors at post-synaptic sites (Ben-Ari and Kelly, 1974, 1976).

With Kanazawa and Dingledine, we compared the actions of micro-iontophoretic applications of GABA and acetylcholine on neurons of the reticular and ventrobasal nuclei of the thalamus (Ben-Ari et al., 1976a,b). Using again in vivo recordings, we found that acetylcholine inhibited reticular neurons but excited ventro-basal neurons selectively, an important observation considering the control by the reticular nucleus of the inflow of information to the specific nuclei of the thalamus. Intravenous perfusion with atropine or picrotoxin blocked respectively the cholinergic and GABAergic inhibitions. The cat’s brains were perfused in Cambridge and the for-malin containers brought with me during my monthly trips to France for histology (leading to interesting animated discussions with custom officers). There were clearly opposite effects of acetylcholine in reticular and ventro-basal neurons in keeping with their involvements in the sleep–waking cycle that were to be uncovered by Steriade and colleagues (Contreras and Steriade, 1997; Steriade, 1997).

Investigating the complex links between GABA and on-going activity in Montreal: la belle province

Already at these early times, it became obvious that as chloride is the main anion player, alterations of (Cl–) could shift the polarity of GABA actions and contribute to epilepsies. Going to learn with K Krmjevic the roles of GABA in relation to epilepsies was an obvious choice. Krecho or KK as called then was one of the genuine experts of GABAergic signals. Having worked with Eccles and Milledi on presynaptic inhibition in the end of the 1950s (Eccles et al., 1959); KK made some of the main descriptions of central inhibition. KK was known for his incredibly hard-working regimen, days starting at eight and finishing seldom before midnight. My task was to elaborate an in vivo preparation in order to perform intra-cellular recordings of hippocampal neurons in anaesthetized rats. Recording neurons for 10–15 min preferably with a reasonable resting membrane potential (negative to –50 mV) was celebrated as this was no simple task. Hippocampal GABAergic inhibitory post synaptic currents (IPSCs) were found to be extremely labile, rapidly reduced by recurrent synaptic activation leading to the conclusion that the plasticity of inhibition is a major factor in epilepsies (Ben-Ari et al., 1980a, 1981a). The reversal of the chloride gradient was readily observed in these conditions, after short episodes of hyperactivity, the IPSC became an «EPSP». Our paper entitled «Lability of synaptic inhibition of hippocampal pyramidal neurons» published by the Canadian Journal of Physiology and Pharmacology was to the best of my knowledge one of
the first descriptions of this fragility of inhibition, stressing the possible links of this property with hyperactivity and seizures. The complex mechanisms underlying the shift of GABA polarity with recurrent activation had to await more appropriate in vitro preparation and molecular approaches: this was achieved two decades later. The Montreal work inaugurated a series of investigations on the basic mechanisms of epilepsies.

Kainate: the dragon of the epilepsies

Returning to France, I decided to work more on the amygdala and trace some pathways to and from the amygdala. An ingenious method had been developed at these early days to trace functional connections between brain structures. It consisted in the destruction of cell bodies while sparing axons en passant by injecting kainic acid, a powerful excitatory agent in the structure investigated (references in Ben-Ari, 1985). I was interested in tracing amygdala connections by injecting dyes, determining their transport while avoiding the en passant fibers. I was struck already during the first experiment how convulsive kainate was when injected even at homeopathic doses in the amygdala. Long-lasting recurrent seizures and status epilepticus were generated leading to the discovery of a useful model of temporal lobe epilepsies (Ben-Ari and Lagowska, 1978; Ben-Ari et al., 1979). This was followed by a systematic investigation of the seizures generated by central or systemic administrations of kainic acid (Ben-Ari et al., 1980b, 1981b; Ben-Ari, 1985; Nadler, 1979). It was also in accordance with human data pointing to the amygdala as nodal in this form of epilepsy. These studies that led to thousands of investigations using kainic acid to study epileptogenesis (2657 references as of January 2014) have been reviewed elsewhere and need no further development here. Suffice it to stress the contributions of this model in gaining better understanding of the importance of seizures in neuronal loss, the progressive maturation of seizures induced brain damage or the classical seizures induced sprouting of mossy fibers that remained the first and most compelling model of reactive plasticity induced by seizures. This provided a direct demonstration of the Jacksonian concept of “seizures beget seizures” and how this operates. The concepts initiated in these early investigations have been summarized in my highly quoted 1985 review (Ben-Ari, 1985, 2010). These studies made well before the receptors were cloned, provided most of present understanding of basic mechanisms of temporal lobe epilepsies. The demonstration decades later of the shift in granule cells from purely AMPA to mixed AMPA/kainate receptor-mediated synaptic excitation post synaptic currents (EPSCs) after seizures provided a vivid illustration of the importance of reactive plasticity in epilepsies (Epsztein et al., 2005, 2010)… and why important observations require decades to be substantiated. GABAergic signaling was never far from our preoccupations. We and many other groups showed how recurrent seizures in vivo and in vitro led to specific loss of GABAergic interneurons notably the somatostatin-containing dendritic-projecting neurons and the relevance of this failure of inhibition in epilepsies (Hirsch et al., 1999; Cossart et al., 2001; Dinocourt et al., 2003). Interestingly, it turned out that kainatergic synapses also innervate preferentially certain types of neurons underlying their vulnerability to seizures (Cossart et al., 1998, 2001; Ben-Ari and Cossart, 2000). Working on epilepsies is bound to lead you gently to investigate GABAergic signals (also see below).

DISCOVERING THE GABA DEVELOPMENTAL SEQUENCE IN PARIS

Three unexpected observations

In January 1986, I was nominated head of a medical research council unit (INSERM) at the Port-Royal maternity (Paris). This implied shifting my research to brain development and the effects of early insults. A bibliographic search revealed that the developing brain was not much a topic of interest in terms of neuronal activity, we knew next to nothing on the electrical properties of intrinsic and transmitter-gated currents in embryonic and early post-natal neurons. It was assumed then that they should not differ from adults in keeping with the pharmaco-therapeutic approaches that were then similar in the young and adults.

Probably the first suggestion of a developmentally regulated shift of GABA actions was made by Obata (1972) in spinal cord neurons. Applications of GABA or glycine-depolarized 6-day-old chick spinal neurons in culture and hyperpolarized 10-day-old embryos. The authors suggested an ingenious developmental sequence with GABA present first on the somata prior to dendrites as they develop (Obata et al., 1978). Purpura, Schwartzkroin Prince and colleagues made pioneering studies at this period. Intracellular recordings of kitten hippocampus in vivo – an achievement at these early times – showed that inhibition was the predominant form of synaptic activity early on, i.e. that inhibition measured by the polarity of the PSPs preceded excitatory PSPs (Purpura et al., 1968). In contrast, subsequent in vitro studies suggested that excitatory synaptic events were more common in hippocampal tissue from young kittens, and that inhibitory synaptic activity was fairly late in developing in slices of rabbit (Schwartzkroin, 1981; Schwartzkroin and Kunkel, 1982) and rat (Dunwiddie, 1981; Harris and Teyle, 1983) hippocampi (also see Schwartzkroin and Altschuler, 1977).

However, these studies raise a number of issues. The resting membrane potential was strongly depolarizing in these early investigations, this will necessarily impact the determination of the genuine driving force of GABA (see also below and (Tyzio et al., 2003, 2008). Thus, using intracellular recordings, Schwartzkroin and colleagues found depolarizing responses to somatic GABA application and depolarizing GABAergic post synaptic currents (PSCs) in neonatal (P6–10) rabbit hippocampal CA1 pyramidal neurons. The Em was of –53 mV, and the reversal potentials of GABAergic postsynaptic potentials were of –36 and –46 mV, respectively (Mueller et al., 1983, 1984), although a more negative value of –54 mV of the somatic EGABA was also reported (Mueller et al., 1983). The authors suggested that depolarizing GABA inhibits ongoing activity via shunting
mechanisms and that these developmental changes are due to two types of GABA receptors/channels: a hyperpolarizing type permeable to chloride and a depolarizing type permeable to sodium and/or calcium in addition to chloride. An alternative hypothesis was that GABAergic signals differ in dendrites and somata in keeping with parallel investigations (Alger and Nicoll, 1979; Andersen et al., 1980; Mueller et al., 1984). Another study by the same group (Mueller et al., 1984) showed that the depolarizing potential produced in CA1 neurons by stimulation of the stratum radiatum is partially mediated by GABA, which is depolarizing at this stage of development. In sum, there was no consensus on the actions of GABA on immature neurons.

To resolve this controversy, we decided to record neurons starting from late gestation at the end of the second postnatal week. In this aim, we developed slicing and recording procedures to enable stable recordings of intrinsic parameters from embryonic or very immature neurons to wean and adult rodents. Already in our first recordings in 1987 (Ben-Ari et al., 1989), we encountered three unexpected observations (Fig. 1A, B):

(i) Immature neurons generated a bizarre network-driven pattern that we called «Giant Depolarizing Potentials (GDPs)» that seemed similar to interictal activities (Fig. 1A);
(ii) but, GDPs like currents evoked by the GABA analog isoguvacine reversed at membrane potentials that were close to the reversal of GABAergic and transiently blocked by GABA antagonists suggesting that they are mediated by GABA receptors (Fig. 1B);
(iii) in very immature slices, GABA receptor antagonists blocked all on-going currents (Fig. 1A) before generating seizures, in contrast to adult neurons where they triggered recurrent seizures directly (Fig. 1A).

Synaptic currents like those evoked by exogenous GABA had a similar physiological and pharmacological profile excluding a mixed receptor permeable to cations and anions (Fig. 1B). Parallel studies revealed that GDPs like the depolarizing actions of GABA followed a developmental sequence shifting earlier in older structures than younger ones (CA3 than CA1). Collectively, these observations suggested that...
GABAergic signals came first, excited neurons before shifting to inhibition because of a reduction of intracellular chloride (Cl\(^-\)), and that depolarizing GABA is instrumental in the generation of a unique pattern of activity that disappears when the shift has taken place. The presence of GDPs was observed in thousands of recordings since then in neonatal slices and in fact their absence usually signified that the slice was in bad conditions.

Our reaction to these unexpected observations was a mixture of scepticism and conviction that there must be an artifact somewhere that we have underestimated. We made hundreds of recordings, altering the experimental conditions, the recordings tools, the ACSF and almost everything that could be considered... but the results were «disappointing»: GDPs were consistently observed as well as depolarizing/excitatory GABA. In addition, the frequency of GDPs was age dependent suggesting a developmentally regulated sequence. As GDPs were also reduced and even blocked by NMDA receptor antagonists, we thought that somehow glycine – then discovered by Ascher and colleagues to be essential for the operation of NMDA currents (Nowak et al., 1984) – could be involved in this response. In favor of this hypothesis, GDPs were highly sensitive to NMDA receptor blockers and the voltage-dependent Mg\(^{2+}\) block of NMDA currents – that determines the contribution of NMDA currents to ongoing activity – was less operative in immature than adult neurons (Ben-Ari et al., 1988) raising the possibility of a more substantial contribution of NMDA currents to immature patterns than adults (see below). We nevertheless tested this possibility with various approaches including glycine receptor antagonists and other manipulations (Gaiarsa et al., 1990). Although glycinergic signals were later found to be more active in the immature hippocampus than the adult one, this possibility had to be discarded. We concluded that our results must be genuine but were far from understanding the biological advantage of such a complex sequence.

To convince ourselves that these observations are not due to an artifact, with K. Krnjevic and E. Cherubini, we asked P. Ascher, a highly respected expert in ionic currents to evaluate them. We met with an even harder resistance that we encountered earlier – due to an artifact, with K. Krnjevic and E. Cherubini, we directly tested the three rules that seemed to emerge from our recordings.

**GABA a pioneer early operating transmitter**

Does GABA operate before glutamate? Does it really provide the early tonus of activity required to organize early patterns? Our observations were indirect and could be due to other actions of bicuculline – which indeed was found subsequently to be the case. I decided therefore that the only way to prove or disprove this hypothesis was to study the maturation of GABAergic currents in morphologically reconstructed neurons: if GABA is operative first, then the first functional synapses in poorly developed neurons must be GABAergic. At these early days, the sophisticated genetic tools to birth and fate date neurons were not available. We recorded in slices prepared a few hours after birth (P0) hundreds of pyramidal neurons in CA3 or CA1 pyramidal hippocampal neurons and determined their synaptic currents, filled them with biocytin and reconstructed them post hoc. The results were remarkably clear cut (Tyzio et al., 1999) illustrating how old-fashioned experiments can be highly informative.

There were three types of neurons (Fig. 2A):

(i) **Neurons with no synaptic currents at all**, these pyramidal neurons had a small soma and no apical or basal dendrites. They correspond to recently born neurons and constitute at birth the vast majority of pyramidal neurons (80%).

(ii) **Neurons with only GABAergic PSCs**, these were endowed with a larger soma, and only a small apical dendrite that has not reached the distal lacunosum moleculare. These neurons correspond to intermediate developmental stage.

(iii) **Neurons with both GABA and glutamate PSCs** endowed with both an apical dendrite that reaches the lacunosum moleculare and a basal dendrite seen for the first time (Fig. 2A). These correspond to the “oldest” neurons of the population.

Several hundreds of recordings failed to reveal a single pyramidal neuron with glutamate but not GABA currents at birth indicating that GABAergic signals must be operative first: GABAergic synapses require a small apical dendrite to operate whereas glutamatergic PSCs operate when the apical dendrites have reached the distal molecular layer. Therefore, GABAergic synapses are formed first, requiring no specific conditions, in contrast to glutamatergic synapses that require more mature targets. Experiments in culture were to confirm this scenario with an early formation of GABAergic
synapses. Interestingly, axons always started to grow at 6 o’clock and dendrites at 12 challenging the idea that neurites grow in all directions and the one growing fastest will become a dendrite.

This study paved the way to other experiments required to confirm the sequence and to determine whether this sequence is restricted to pyramidal neurons or occurs also in GABAergic interneurons (Hennou et al., 2002; Gozlan and Ben-Ari, 2003). The results were again clear-cut; at P0, there were three types of interneurons:

(i) **Interneurons with no synaptic current** and a limited extension.

(ii) **Interneurons with GABA but not glutamate synaptic currents** endowed with more extended dendritic and axonal arbours.

(iii) **Mature interneurons with GABA and glutamate currents** with extended dendrites and axons.

Therefore, this sequence applies to glutamatergic and GABAergic neurons (Hennou et al., 2002; Gozlan and Ben-Ari, 2003). There was however an important difference: at birth 80% of interneurons had operative GABA and glutamate synapses whereas less than 20% of pyramidal neurons were in that situation. Indeed, extending this approach to embryonic neurons revealed that at E19/20 almost none of the pyramidal neurons had functional synapses, contrasting with the relatively high percentage of interneurons (80%) endowed with both GABA and glutamate signals. Interestingly, dendritic-targeted interneurons appeared to operate before somatic-oriented ones suggesting an additional developmental sequence within the interneuronal population. This
concept was to be generalized by Cossart's group in the lab with more modern genetic fate-mapping tools to show that early-born GABAergic interneurons are also Hub-generating neurons (Picardo et al., 2011) (see below).

Clearly, hippocampal GABAergic interneurons mature first, innervate pyramidal neurons and other interneurons providing most if not all the synaptic currents initially. Interestingly, these observations also suggest that pyramidal neurons innervate GABAergic interneurons before innervating other pyramidal neurons. In keeping with this, although rare, early embryonic GDPs are exclusively mediated by GABAergic receptors. This developmental sequence was also observed in CA1 neurons but with a later onset in keeping with the protracted maturation of these neurons. Therefore, this sequence is developmentally regulated depending on the birth date of the neuron in keeping with basic principles of developmental neurobiology. Recent studies using more sophisticated methods have confirmed this property in some but not all brain structures. Therefore, at least in the hippocampus, in spite of the "long journey" of interneurons (Marin and Rubenstein, 2001), interneurons are first come, first served. Subsequent observations showed that newly born neurons in the adult brain have also immature features including early GABAergic depolarizing actions (Ben-Ari et al., 2007; Mejia-Gervacio et al., 2011; Tao et al., 2012).

A similar sequence in primate in utero

Are these observations restricted to rodents? We tested this in macaques relying on C-sections in pregnant macaques, slice preparations, physiological recordings and anatomical reconstructions. We succeeded to have access to eight pregnant macaques and to perform this experimental design that provided a reasonable illustration of the events occurring between mid-gestation to a few weeks before delivery. The results were quite striking confirming the presence of three neuronal populations (Khazipov et al., 2001) (Fig. 2B):

(i) Pyramidal neurons with no synaptic currents and no apical or basal dendrites.
(ii) Pyramidal neurons with only GABAergic PSCs endowed with only a small apical dendrite that has not reached the distal lacunosum moleculare.
(iii) Pyramidal neurons with GABA and glutamate PSCs endowed with both an apical dendrite that reaches the lacunosum moleculare and a basal dendrite seen for the first time (Fig. 2B).

The morphological features of neurons were determined in detail including counting all the spines as an indication of the density of glutamatergic synapses. A quantification of these results using Boltzmann equations revealed that at mid gestation, neurons had no spines (and no glutamatergic PSCs) and over 7000 spines and presumably functional synapses a few weeks before birth. GABAergic currents are recorded before glutamatergic ones in poorly arborized immature neurons, the presence of glutamatergic EPSCs requiring long apical dendrites reaching the distal lacunosum moleculare. GDPs are present roughly from mid gestation but are absent shortly before delivery presumably when networks generate more complex patterns (see below). Therefore, the developmental sequences are similar in rodents and primates but at different ages. Pair recordings of interneurons and pyramidal cells revealed that in both rodents (Fig. 3A, B) and macaque (Fig. 3C, D), interneurons and pyramidal neurons are synchronized during GDPs (see below). It is of particular interest to note that embryonic primate networks operate primarily by means of GABAergic neurons initially. This has implications for the use of drugs and neuroactive agents notably on GABAergic signals during pregnancy in humans as they might have different effects on the mother's brain and that of her embryo (see below).

Collectively, these observations provided a detailed scheme of the maturation of synaptic connections at least in the hippocampus where the data available are far more substantial than other brain structures. Subsequent studies were to extend some of these events – particularly the excitation/inhibition shift to a wide range of animal species from worms, turtles to chicken and frogs suggesting that it corresponds to a general evolution conserved property (Ben-Ari et al., 2007). I neither imply that this sequence is identical in all brain structures considering the enormous variability of animal species and brain structures, nor do I imply that all neurons belonging to the same population have the same time-course shifts. Interestingly, if one assumes that in some neurons glutamatergic but not GABAergic currents are functional in utero, it might be interesting to determine which type of inhibition prevents the excitotoxicity that was induced by an exclusively glutamatergic excitatory drive.

Elevated \( \text{Cl}^- \) and depolarizing actions of GABA

In our initial observations, relying on hundreds of intracellular recordings, we reported that the reversal potential of the principal network-driven pattern recorded in immature pyramidal neurons was close to that of GABA (Ben-Ari et al., 1989). We also showed a developmental sequence with a progressive reduction of the reduced during the first post-natal week reaching adult values by the end of the second postnatal week. Electrical stimulation of slices evoked synaptic currents that were reversed like the currents generated by applications of GABA agonists indicating that they are mediated by GABAergic receptors. Because of the considerable importance and plasticity of intracellular chloride, these results could not be sustained without compelling quantitative non invasive measures of \( \text{Cl}^- \), and the resting membrane potential \( (V_{\text{rest}}) \) in immature neurons as developmental alterations of the latter could explain the apparent differences of the former. We started by using various whole-cell recording configurations including the recently developed perforated patch-clamp recording technique to measure \( \text{Cl}^- \), and \( \Delta F_{GABA} \). It became rapidly apparent that these techniques are inadequate to measure \( V_{\text{rest}} \) as the high-input capacitance of immature neurons endowed with few operating voltage- and transmitter-gated currents
produced important leak currents and deviations from the expected values by over 15–20 mV (Tyzio et al., 2003; Ben-Ari et al., 2007).

We therefore decided to implement dual single-channel recordings from the same neuron – single NMDA channels to determine $V_{rest}$ – and single GABA...
channels to determine the driving force of GABA (DF\textsubscript{GABA}): these two values enable then to calculate precisely and unambiguously $E_{\text{GABA}}$. Indeed, NMDA currents reverse at 0 mV, hence by injecting current, it is possible to determine $V_{\text{rest}}$ relying on the current injected to reverse NMDA currents. Using this technique, we found that DF\textsubscript{GABA} shifts progressively during development — whereas $V_{\text{rest}}$ showed a small alteration. The use of other recording or imaging techniques do not provide such unambiguous results; indeed, with all invasive recording techniques including perforated patch, there is a significant shift in the resting membrane potential (Tyzio et al., 2003) and imaging techniques are far from reliable in their estimations of ($\text{Cl}^-/C_0$)\textsubscript{i} levels (see below). We used single GABA or GABA and NMDA channel recordings in other brain structures with quite similar results (Tyzio et al., 2006; Rheims et al., 2008a; Dehorter et al., 2012). It bears stressing that these are average values that are strongly dependent on the amount of activity immediately preceding the measurement: there is to some extent no correct value of ($\text{Cl}^-$)\textsubscript{i} levels as they might be altered by a single burst (also see Marchetti et al., 2005; Yamada et al., 2004; Achilles et al., 2007). However, when large numbers of measures are performed, more elevated levels will be found in immature than adult neurons belonging to the same neuronal population and performed in the same experimental conditions.

This sequence then received considerable support and its mechanistic substrate substantiated with the discovery by Rivera, Kaila, Delpire and many others of the developmental sequence of chloride co-transporters: NKCC1 — the importer found to operate early in development — and KCC2 — the exporter that operates later (Fig. 4) (Lu et al., 1999; Rivera et al., 1999, 2002; Huberfeld et al., 2007; Li et al., 2007; Khirug et al., 2010). The use of selective blockers most notably the diuretic NKCC1 selective antagonist bumetanide (Feit, 1981) that reduces intracellular chloride levels also shifted DF\textsubscript{GABA} (Rohrbough and Spitzer, 1996; Rivera et al., 2002; Dzhala et al., 2005; Huberfeld et al., 2007; Rheims et al., 2008a; Glykys et al., 2009; Khirug et al., 2010; Nardou et al., 2011b; Tao et al., 2012) (Yamada et al., 2004, Achilles et al., 2007). Other investigations showed the importance of KCC2 in specific types of neurons and how these correlate with the actions of GABA (Gulacsi et al., 2003). Collectively, these observations provided formidable support to the developmental sequence of chloride co-transporters and GABA polarity. Interestingly, newly born granule cells in adults have also high [Cl\textsuperscript{-}]\textsubscript{i} levels and depolarizing GABA and have GABAergic synaptic currents before glutamatergic ones (Ge et al., 2005) also see (Carleton et al., 2003; Bordey, 2011; Duveau et al., 2011). We have recently reviewed extensively the large number of similar observations made using various recordings or imaging techniques, preparations and brain structures (Ben-Ari et al., 2007). Here, I shall only stress a number of observations that are particularly relevant to better comprehend frequently raised issues and discuss how these investigations led to novel rules in developmental neurobiology.

**Fig. 4.** Schematic diagram of the developmental alterations of [$\text{Cl}^-$] levels and the polarity of the actions of GABA and the actions of chloride co-transporters. (A) The intracellular [$\text{Cl}^-$] levels are higher in immature than adult neurons. (B) GABA depolarizes and excites immature neurons and inhibits adult ones. The chloride exporter KCC2 I chloride importer is poorly active initially whereas the NKCC1 is highly active in immature neurons leading to different chloride gradients and actions of GABA. Also see Ben-Ari et al. (2007).
GDPs: a primitive synaptic network pattern

The GDPs are probably the most unexpected observation made during these experiments (Fig. 1). GDPs are clearly network-driven, synaptic events that engage a large number of neurons in a synchronous discharge. In an intact neonatal hippocampus preparation (Khalilov et al., 1997a), GDPs propagate from the hippocampus to the septum (Leinekugel et al., 1998). In a triple chamber preparation composed of the two intact hippocampi and their connecting commissures (Khazipov et al., 1999), GDPs propagate from one hippocampus to the other (Khalilov et al., 1999, 2005). GDPs are developmentally regulated appearing a few days after birth, peaking by the end of the first week and disappearing subsequently. Cell-attached and whole-cell dual recordings of interneurons revealed that interneurons fire bursts of spikes triggered by periodical large inward currents during GDPs, (Fig. 3A, B). These are blocked by TTX and by high divalent cations (6 mM Mg$^{2+}$ and 4 mM Ca$^{2+}$) and can be evoked in an all-or-none manner by electrical stimulation in different regions of the hippocampus (Khazipov et al., 1997; Khalilov et al., 1997b). GDPs can be generated by isolated CA3 subfield of the slice confirming their local generation by a small set of interneurons and pyramidal neurons.

GDPs are mediated by GABAA and glutamate receptors, since:

(i) Their developmental time course paralleled that of the excitatory actions of GABA both in terms of the maturation of the neuronal population and the brain region (CA3 before CA1).

(ii) They are readily blocked by glutamate receptor antagonists but at least initially by GABA receptor antagonists.

(iii) Their reversal potential strongly depends on [Cl$^{-}$], and includes at the reversal potential of GABAA an inward component having the same kinetics as a-amino-3-hydroxy-5-methylisoxazole-4-propionate (AMPA) receptor-mediated EPSCs.

(iv) When GABAA receptors are blocked by intracellular dialysis with MgATP-free solution, the remaining component of GDPs reversed near at 0 mV and rectified at membrane potentials more negative than −20 mV, suggesting an important contribution of NMDA receptors in addition to AMPA receptors. When AMPA receptors are blocked, GABAA receptor-mediated depolarization enabled the activation of NMDA receptors presumably via attenuation of their voltage-dependent magnesium block and together generate GDP-like events (see below).

(v) In spite of their apparent similarity to interictal events, comparative studies showed that GDPs have a more hyperpolarized reversal potential than interictal events in keeping with a more significant contribution of glutamatergic currents (Ben-Ari, 2012; Ben-Ari et al., 2012a; Khalilov et al., 1999).

(vi) Extensive investigations particularly by Cherubini and colleagues showed a wide range of molecules and systems that modulate and control the expression of GDPs and showed elegantly how these also control/modulate synapse formation (Mohajerani et al., 2007; Voronin and Cherubini, 2004; Safiulina et al., 2005; Safiulina et al., 2008).

(vii) GDPs differ from other patterns notably the Early Network Oscillations (ENO) in their reversal potential that is less depolarizing suggesting a more important contribution of GABAergic signals (Ben-Ari et al., 2012a; Allene et al., 2008). GDPs are also readily blocked by anoxic conditions whereas ENOs are enhanced by these events (Allene et al., 2008).

The model proposed suggested a synchronous activation of SR-CA3 interneurons by the co-operation of excitatory GABAergic connections between interneurons and glutamatergic connections to interneurons originating from pyramidal cells that would trigger GDPs (Fig. 5A, B) (Ben-Ari, 2001, 2002). The slower rise time of GABAergic than AMPA receptor-mediated currents will synchronize neurons with dispersed kinetics in a population event in comparison to the seizures generated by GABA receptor antagonists and mediated by AMPA receptors. GDP equivalent patterns are observed in vivo (Leinekugel et al., 2002) and in a variety of other in vitro preparations including the intact hippocampal and neuronal cultures (references in Ben-Ari et al., 2007). Similar patterns have also been observed in many immature brain structures including the spinal cord, neocortex, brain stem structures, retina, inner ear (reviewed in Ben-Ari et al., 2007). A picture started to emerge with immature networks endowed with similar singular features that disappear with a well-determined time course to adult time-locked activities. I coined this...
A developmental sequence stating that «developing networks play a similar melody» (Ben-Ari, 2001) implying that in spite of their differences, they share the fact that they have a slow kinetic, are long lasting and have a transient expression and function. Other patterns restricted to immature neurons like the retinal waves have been shown to play important roles in the construction of functional units (Torborg and Feller, 2005; Ackman and Crair, 2014; Mooney et al., 1996; Xu et al., 2011; Ford et al., 2012) and to shift timely to enable vision (Hanganu et al., 2006; Colonnese et al., 2010; Colonnese and Khazipov, 2012). In addition, GABAergic currents play an active role in the generation of retinal waves and their functional consequences (Chabrol et al., 2012). The underlying concept is that brain development required synchronized activities in order to enable heterogeneous neurons to fire and connect together. The developing brain neither needs nor can generate a large repertoire of patterns; GDPS and similar primitive patterns are poorly informative and are not behaviorally relevant unlike their adult counterparts. Studies on preterm babies and immature rodents suggested that immature patterns propagate primarily from the periphery to the centers, do not code sensory information but rather provide an important source of information required to modulate activity dependently the formation of neuronal ensembles (Khazipov et al., 2004; Milh et al., 2007; Colonnese et al., 2010) (Colonnese and Khazipov, 2012; Hanganu et al., 2006). These straightforward electrophysiological experiments were to be confirmed subsequently with more sophisticated tools providing more details on their underlying mechanisms without altering the fundamental scheme.

In sum, the three properties of developing neurons and patterns were confirmed and extended in these investigations. A schematic diagram of the development of neurons and patterns in rodents and macaque is presented in Fig. 6. GDPS are depicted in A and their relation to neurogenesis, dendritic growth and spine formation are shown in relation to development from embryos to adults. In B, this is presented in macaque with Boltzmann integrated values from roughly E 40 to a few weeks before delivery. Note that at mid gestation, neurogenesis in the hippocampus is terminated; axons develop prior to dendrites, and GABA before glutamate. Spines acquisition – is almost nil at mid gestation – but reaches 7000 on a single pyramidal neuron before birth. GDPS are present from mid gestation until shortly before delivery. This is also a vulnerable time to epileptogenesis. We then decided to examine in more detail the maturation of brain sequences. Indeed, during evolution, a large variety of communication devices have been used prior to synapses suggesting that GDPS might not be the first pattern in the developing brain.

**Fig. 6.** Schematic diagram of the maturation of the principal markers that have been measured in rodents and primates. (A) GDPS are present after birth and until P10 before shifting to adult patterns, developmental synaptogenesis occurs from E20 to P30 and neurogenesis (in CA3/CA1) terminates before birth, and the peak of dendritic growth and spionogenesis are shown. (B) Similar results in primate (macaque neurons in utero). Boltzmann equations to depict the rate of neurogenesis, rate of growth, first synaptic currents, spines acquisition, and network activities. Note that GDPS are present from mid gestation until shortly before delivery. Adapted from (Khazipov et al., 2001) and (Ben-Ari et al., 2007) -physiological reviews.

**A tri-phasic developmental sequence of brain patterns**

This approach became possible when the development of suitable tools enabled to measure with imaging techniques the activity of large neuronal ensembles in embryonic and early post-natal slices. Indeed, using a two photon system, with Cossart and her team, we began to investigate the mechanisms underlying the generation of GDPS and the type of activities that preceded their occurrence. The development of suitable mathematical tools by several ingenious experts (Cossart et al., 2003; Crêpel et al., 2007; Bonifazi et al.,...
enabled the measure of the activity of hundreds of neurons simultaneously while labeling many neurons in the slice and then performing targeted patch recordings from neurons suspected to play a central role in their generation. We discovered a developmentally regulated triphasic sequence during development (Crepel et al., 2007), GDPs constituting the final stage of the maturation of patterns (Fig. 7A). Initially at an early embryonic stage, neurons only bear voltage-gated non-synaptic calcium channels. These are intrinsic and unaltered by a cocktail of synapse and transmitter antagonists or blockers. Similar calcium currents are generated at an early stage in a wide range of animal species and brain structures and their frequency and parameters have been shown to control the phenotype of neurons (Borodinsky et al., 2004; Spitzer and Borodinsky, 2008). Subsequently, during the delivery period, the first synchronized patterns are observed under the form of Synchronized Plateaux in small Assemblies (SPAs). These events are a non-synapse intrinsic large calcium plateaux generated by gap junctions and also insensitive to synapse or transmitter-gated antagonists. Then, GDPs are observed, these are the first synapse-driven pattern in the developing hippocampus (Crepel et al., 2007). These three patterns have a well-defined developmental sequence, shifting from one to the other and during transitional periods (Fig. 7B), GDPs and SPAs can be recorded in the same neuron, the former inhibits the latter (Crepel et al., 2007). There is a developmental sequence, with functional synapses most likely inhibiting the earlier intrinsic non-synapse-driven pattern; this is initially transient but this shift becomes permanent after repeated GDPs, possibly by shutting off gap junctions. A similar sequence was then observed in other brain structures including the neocortex (Allene et al., 2008), hypothalamus (Gao and van den Pol, 2001), striatum (Dehorter et al., 2011) and early calcium currents also in the substantia nigra (Ferrari et al., 2012) associated with an early release of dopamine. Extensive investigations on the mechanisms of generation and propagation of GDPs (58 references on GDPs in PubMed – notably by Cherubini, Kaila and co-workers (references in our review (Ben-Ari et al., 2007)).

The development of genetic fate-mapping devices enabled to better define the type of neuron involved in synchronizing the early patterns. Indeed, an intriguing issue is that of the behavior of neurons which develop first: are these programed to play an important synchronizing role, a sort of chief conductor of developing symphonies? In an attempt to determine these issues, Cossart and colleagues investigated the fine patterns of neurons involved in the generation of GDPs and noted that GABAergic interneurons play a role of Hub generators, their activity orchestrating that of the entire network (Bonifazi et al., 2009; Picardo et al., 2011). Then, they showed that these orchestrating neurons are always GABAergic and born earlier than other neurons (interneurons or pyramidal cells) with other specific features. Collectively, these and other studies of emerging networks indicate that the developmental sequences of ionic currents and brain networks include also an age- and fate-dependent maturation of neuronal types. Neurons are not equal during maturation and here again GABAergic interneurons appear to serve a role on orchestrating patterns that they will also have later.
Therefore, the excitatory/inhibitory GABA shifts are associated with a complex timing of events that stands at the core of the development of functional neuronal units. When evaluating the importance of the GABA depolarizing actions, it is important to take into account all these features that collectively draw the picture of a global coherent scheme of the succession of events taking place. Without the sequential development of other ionic currents, brain patterns and chloride co-transporters, this observation is a curious one; with these additional properties, this becomes a fundamental feature of brain development.

CONFIRMING AND EXTENDING THE DEVELOPMENTAL SEQUENCES

As often in science, the general developmental scheme suggested by these investigations raised important questions including the possible biological significance of the sequences, their underlying consequences on the firing of immature neurons, the alternative sources of inhibition operating in the absence of inhibitory GABAergic tone and their general biological relevance. Light would often come from other basic experiments.

Delivery is associated with an abrupt E to I shift mediated by oxytocin

This remains probably the most unexpected, compelling and direct proof of the fundamental role and biological relevance of excitatory GABA in developmental neurobiology. Using the single-channel recording techniques, we – in particular R. Tyzio who developed the double single-channel recording techniques from the same neuron (Tyzio et al., 2003) – started to reconstruct the entire developmental sequence of \((\text{Cl}^-)/\text{C}_0\) levels starting from embryos to adults. We expected to find a progressive possibly exponential decline of \((\text{Cl}^-)/\text{C}_0\) levels from embryos to adults. Indeed, we observed elevated \((\text{Cl}^-)/\text{C}_0\) in embryonic neurons and depolarizing DF GABA that declined to adults with an asymptotic curve. However, when we examined in more detail the sequence, we discovered an abrupt and dramatic shift of \(\text{DF}_{\text{GABA}}\) and \(E_{\text{GABA}}\) during the delivery period roughly a couple of hours before and after delivery (Fig. 8). This shift was dramatic as such \((\text{Cl}^-)/\text{C}_0\) levels were never seen before or after that stage and transient as the levels expected from the developmental curve were reached a few hours after delivery.

This curiosity required a biological explanation to be validated and understood. We reasoned that as delivery is associated with the release of a plethora of hormones and other signals that control essential processes, one of these might trigger this abrupt shift for reasons that had to be understood. Oxytocin was a reasonable choice as in addition to triggering labor, oxytocin exerts important roles in social communication between the mother and her baby and even analgesic effects that may imply GABAergic mechanisms (see below). We tested the effects of a selective antagonist of oxytocin receptors (developed to delay early labor) and the results were amazingly straightforward: administration of the antagonist \(\text{in vivo}\) (to the mother) or \(\text{in vitro}\) (to the newly born slice) blocked completely the chloride shift and the excitatory actions of GABA (Tyzio et al., 2006)!

The oxytocin receptor antagonist had no effects on neurons 2 days after delivery indicating that the endogenous oxytocin levels in the slice are significant during the peak of the hormonal effects. Stated differently, in addition to triggering labor, oxytocin reduces dramatically and transiently the levels of neuronal intracellular chloride in newly born pups for a short period. Most intriguingly, oxytocin also modulated the generation of network oscillations activating the large calcium plateaux – the SPAs – present during delivery. Clearly, delivery is associated with a set of mechanisms dedicated to enable a smooth transition of brain activities during this complex event.

![Fig. 8](https://example.com/image.png)

**Fig. 8.** Delivery is associated with an abrupt reduction of \((\text{Cl}^-)/\text{C}_0\) levels. (A) Delivery-related transient perinatal loss of the GABA-mediated excitation. (A) Responses of CA3 pyramidal cells recorded in cell-attached mode to the GABA agonist isoguvacine. Below, summary plot of the proportion of cells excited by isoguvacine during the perinatal period. There is a transient loss of the excitatory effect of isoguvacine near term. Red corresponds to the fetuses whose mothers received SSR126768A. [E21 corresponds to the early phase of delivery (1–2 h before birth); P0 is the day of birth; pooled data from 146 neurons.] (B) Summary plot of the onset of Anoxic Depolarization (AD) in control, in the presence of the oxytocin receptors antagonists atosiban (AT, 5 mM, fetal intracardial perfusion and SSR126768A (1 mg/kg to the mother), and after further addition of bumetanide (10 mM). Each circle corresponds to one hippocampus (n = 82 intact hippocampi at E21). Error bars indicate SEM. Note that the ADs occurred earlier when oxytocin receptors are blocked and control levels are obtained when bumetanide is added. From Tyzio et al. (2006).
We examined the biological significance of this abrupt shift: why do neurons need strong inhibitory GABA and low levels of chloride during the delivery period? We reasoned that strong inhibitory GABA would lead to reduced network activity and enhance neuronal resistance to anoxic episodes as many other and we had shown in various experimental conditions. In my earlier experiments with K. Krnjevic and E. Cherubini in France, we had shown that during the delivery period, neurons are extremely resistant to anoxic episodes; long-lasting durations of anoxic and reduced glycemic conditions barely affect neuronal properties (Krnjevic and Ben-Ari, 1989; Krnjevic et al., 1989). Specifically, when an adult and an immature hippocampal slice are placed in the same chamber, oxygen deprivation abolish ed synaptic transmission in 5 min in the former and over 1 h in the latter. Yet, applying the oxytocin receptor antagonist strongly reduced this resistance thereby rendering neurons more susceptible to damage (Fig. 8B) (Tyzio et al., 2006). Therefore, the hormone is also neuro-protective. This was not all.

Elegant studies by Lagercrantz and colleagues (Bergqvist et al., 2009) have shown that babies delivered by C-sections felt more pain than vaginal delivered ones raising the possibility that oxytocin – known to have also analgesic actions – might be involved in this reaction. Curiously, pain reactions of the newborn during delivery had not been investigated although they might be of some importance. We therefore tested the hypothesis that the reduction of (Cl\(^-\)) levels and associated decrease of on-going activity (see below) might impact pain threshold (Mazzucca et al., 2011). Using a thermal tail-flick assay, we observed that pain sensitivity is two-fold lower in newborns than 2 days later. Oxytocin receptor antagonist strongly enhanced pain in new-borns but not 2 days later whereas the hormone reduced pain sensitivity at both ages suggesting an endogenous analgesia by oxytocin restricted to the delivery period. Pain vocalization produced by whisker pad stimuli were also attenuated in de-cerebrated animals. Oxytocin reduced intracellular calcium levels and depolarizing actions of GABA in trigeminal neurons, suggesting that these effects are mediated by (Cl\(^-\)) levels. The diuretic and specific NKCC1 chloride importer antagonist bumetanide that reduces (Cl\(^-\)) levels exerted the same effects on pain and intracellular chloride in pain pathways indicating that the effects of the hormone are indeed mediated by the polarity of GABA actions.

Therefore, in addition to triggering labor and delivery, oxytocin also acts as a painkiller during this highly vulnerable period and its actions are mediated by the excitatory/inhibitory developmental sequence. A plethora of reactions unique to that day take place to accommodate the massive alterations that must occur within a few hours. This abrupt shift during delivery and the amazing mechanisms elaborated by Mother Nature not only validated our model but stressed unambiguously its biological relevance. An incredible unexpected illustration of the importance of the delivery shift was to come later at the end of this journey with our studies on autism.

The GABA/NMDA cooperation: a ménage à trois of receptors

Does GABA also activate calcium currents and by this mechanism also exert trophic actions? Indeed, it has long been known that GABA exerts trophic actions often mediated by molecular cascades triggered by a rise of intracellular calcium (Meier et al., 1983; Represa and Ben-Ari, 2005). The next step was obviously to better investigate whether and how would depolarizing GABAergic signals be involved in these actions. GABA could increase intracellular calcium by two classical mechanisms: activation of NMDA receptor/channels and/or voltage-dependent calcium currents. We found that they both operate (Fig. 9B). Indeed, using a wide range of imaging and physiological recordings, we reported that the depolarization produced by GABA is more than sufficient to remove the Voltage-dependent Mg\(^{++}\) block of NMDA channels thereby producing large intracellular calcium influx (Leinekugel et al., 1995, 1997). In fact, the combination of this depolarization and the large and long-lasting NMDA-mediated EPSCs is the main generating mechanism of GDPS and other immature oscillations: the developing brain talks a lot primarily because of a combined depolarizing GABA and long-lasting NMDA currents initiating synaptic plasticity (see below) and the generation of oscillations that modulate activity dependently the formation of functional units (Safuilina et al., 2006, 2010). This may turn out to be more important than spike generation as was shown later with the demonstration that the threshold of spike generation differed in different neuronal populations stressing the importance of intrinsic currents in immature neurons (Rheims et al., 2008a,b). We also found that the depolarizing GABA also activated voltage-gated calcium currents although the nature of the events in which NMDA or Calcium currents are involved deserves more investigation. In a review, I called this situation a “ménage a trois of transmitters” speaking of GABA, AMPA and NMDA currents in the developing brain acting together to excite (Ben-Ari et al., 1997). This stands in contrast to the adult situation where activation of GABAergic currents reduces the likelihood of activating NMDA currents, synaptic plasticity and long-term potentiation. The cover page of this TINS review included following a suggestion of the chief editor of TINS a picture taken from the film of the French film director François Truffaut entitled Jules et Jim with a vivid and genuine “ménage a trois”. Therefore, the depolarizing GABA currents combined with long-lasting NMDA currents serve a crucial role in the synchronization of immature neurons to fire together and wire together. The GABA excitatory/inhibitory sequence is but one of the multiple facets of the maturation of brain activities.

Dual actions of GABA: the yin and the yang

Does the depolarizing action of GABA also lead to the generation, of action potentials? Is GABA genuinely “excitatory”? The threshold of spike generation is not readily reached by the depolarization produced by GABA. Indeed a simple calculation shows that even
when depolarizing in many neurons, a stronger depolarization is needed. It remained therefore to determine whether and how the depolarizing actions of GABA are sufficient to generate action potentials. In many neurons, cell-attached evoked responses were partly blocked in immature neurons by glutamatergic receptor antagonists and fully blocked when GABA receptor antagonists were added (Fig. 9A). We investigated these issues in neocortical neurons comparing the spike threshold and GABA currents in neurons of layers 5/6 and layers 3 to take into account the different developmental stages of these neurons, the former being older than the latter (Rheims et al., 2008a,b). Using non-invasive single N-methyl-D-aspartate and GABA channel recordings to measure both $E_m$ and $DF_{GABA}$ in the same neuron, we found that GABA strongly depolarizes pyramidal neurons and interneurons in both deep and superficial layers of the immature neocortex (P2–P10). Yet, GABA generated action potentials in layer 5/6 (L5/6) but not L2/3 pyramidal cells consequently to a more depolarizing resting potential of L5/6 pyramidal cells and a more hyperpolarized spike threshold. Interestingly, GABA generated more readily action potentials in interneurons of both layers because of a more suitable $E_m$ and spike threshold. GABA transiently drove oscillations generated by L5/6 pyramidal cells and interneurons that propagated to more superficial layers. These were readily blocked by the NKCC1 co-transporter antagonist bumetanide that reduced [Cl$^-$]i levels, GABA-induced depolarization, and network oscillations. However, a combined activation of GABA and NMDA receptors resulted in a significant increase of [Ca$^{2+}$]i fluorescence. Therefore, the high intrinsic excitability of L5/6 pyramidal neurons and interneurons provide a powerful mechanism of synapse-driven oscillatory activity in the rodent neocortex generated by GABAergic depolarization. The dual role of GABA has also been investigated in relation to neuronal migration acting as “go” and “stop” signals and in relation to epilepsies (Colonnese et al., 2010; Hanganu et al., 2002; Heck et al., 2007; Kanold and Luhmann, 2010; Lapray et al., 2010).

Interestingly, the depolarization produced by GABA can also activate the sodium non-inactivating current ($I_{Nap}$) leading to the generation of bursts. This activation is of particular interest considering the strong dependence of this current on the kinetic of the depolarizing event (Valeeva et al., 2010). These observations suggest that the activation of depolarizing GABA AND voltage-gated currents can indeed generate action potentials. Indeed, in parallel to studies on kainatergic synapses in the epileptic hippocampus (Epsztein et al., 2010; Artinian et al., 2011), this current was found to be activated by currents with a slow rise-time kinetics like the ones present in immature GABAergic currents. Collectively, these observations stress the importance the sequential maturation of voltage and transmitter-gated ionic currents. These are not only neuronal type and age but also sex dependent (see below).

In an elegant study, van den Pol and colleagues used outside-out single patches as sensors of the response to GABA and showed that growth cones release packets of GABA that can be «seen» at some distance illustrating the importance of this paracrine mode of communication (Gao and van den Pol, 2000). This group also showed the importance of the timing of GABA and glutamate interactions and how GABA could either block or augment the

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**Fig. 9.** GABA potentiates the activity of NMDA receptors in the neonatal hippocampus. (A) Synaptically elicited response in neonatal CA3 pyramidal neurons (P5) recorded in cell-attached configuration. (a) In control conditions, electrical stimulation elicited a burst of five action potentials. The number of action potentials was slightly affected by AMPA receptor antagonist CNQX (10 μM) (b) and strongly reduced by further addition of the NMDA receptor antagonist APV (50 μM) (c). (d) The remaining response was blocked by the GABAA receptor antagonist bicuculline (10 μM). (B) A CA3 pyramidal neuron (P5) was loaded extracellularly with the Ca$^{2+}$-sensitive dye fluo-3 AM, and the slice was continuously superfused with the voltage-gated Ca$^{2+}$ channel blocker D600 (50 μM). Focal pressure ejection of a GABAA-receptor agonist, isoguvacine (100 μM), or bath application NMDA (10 μM) had no effect on [Ca$^{2+}$]i fluorescence. However, a combined activation of GABA and NMDA receptors resulted in a significant increase of [Ca$^{2+}$]i fluorescence. (C) Schematic presentation of the interactions between GABAA and NMDA receptors in immature neurons. From Ben-Ari et al. (2007).
excitatory glutamatergic drive in a time-dependent manner (Gao and van den Pol, 2001). Collectively, these observations stress the importance of taking into account the different agenda of developing networks and the heterogeneity of neurons at early developmental stages. This implies a different conceptual investigation of developing neurons incorporating the fact that contrary to adult neurons, two neurons belonging to the same population meant to act together later differ completely at an early stage. Also, the actions of GABA are highly plastic depending ultimately on the amount of activity preceding the measure: a single burst might alter a strongly hyperpolarizing event to a depolarizing and even excitatory one. Pushing a little bit this notion, one might say that measurements of resting (Cl\(^-\)) levels is not possible as this is too volatile!

Therefore, determination of the dynamic control of (Cl\(^-\)) levels in relation to activity is instrumental in order to understand the GABA developmental sequence. This parameter is not readily measured. The most straightforward way is to augment artificially (Cl\(^-\)) levels and then determine the speed with which control (pre-stimulation) levels are restored as an indication of the relative efficacy of NKCC1 and KCC2. We developed a technique based on perforated patch recordings with repeated focal applications of GABA to measure every few minutes \(D_F^{GABA}\) and the ongoing activity of the co-transporters; then a large depolarizing pulse is applied to augment (Cl\(^-\)) levels and the time course of the recuperation to pre-stimulation applications of GABA is determined. We found (Nardou et al., 2011b) that the recuperation to pre-stimulation levels took several minutes in immature neurons and tens of seconds in adult ones in the same conditions. Similarly, in epileptic neurons where KCC2 is clearly degraded, tens of minutes were required (Nardou et al., 2011b) (and see below). Therefore, immature neurons require longer periods than adults to regulate (Cl\(^-\)) levels and return to pre-stimulation levels.

**Is there a stop signal to organize the shift?**

In the model proposed, immature neurons require large network-driven patterns to interconnect and construct functional units. Yet, at some well-defined stage, these must shift to enable the generation of behaviorally relevant patterns with their time-locked currents. But how and when are they interrupted to shift? Hammond and her team in the lab decided to answer to this question relying on the most appropriate brain structure to that effect: the striatum. In adults, the GABAergic Medium Spiny Neurons (MSNs) compose over 95% of the striatal population and are usually quite silent with a highly hyperpolarized resting membrane potential (over -85). This is required in order to enable the generation of coherent targeted movements by the incoming motor cortex signals. Indeed, in Parkinson disease, the striatum is highly active generating synchronized currents that are thought to play an important role in akinesia and other symptoms (Brown, 2006; Hammond et al., 2007; Rivlin-Etzion et al., 2010). Therefore, the striatum constitutes a suitable target to investigate this issue as immature MSNs are expected to generate GDPS and be silenced timely to enable targeted movements. Using a two-photon microscope, enhanced oscillatory activity was found in immature striatal slices that are reminiscent of GDP until P8-10 (Dehorter et al., 2011); these disappeared abruptly consequently to the activation of voltage-gated currents and a reduction of the NMDA immature currents (Fig. 10A). Interestingly, this is also the stage when the pups shift abruptly from immobility or crawling to targeted movements (Fig. 10B) (Dehorter et al., 2012). Therefore, the timing of the shift is programed specifically to occur when most needed. It is likely that other systems have similar time dependence to accommodate development and more adult requirements. Indeed, similar abrupt changes have been reported in various sensory systems such as the inner ear (Wong et al., 2013) and the retina with the classical retinal waves (Mooney et al., 1996; Torborg and Feller, 2005; Xu et al., 2011; Chabrol et al., 2012).

Summing up, the GABA developmental sequence is associated with other alterations that converge to generate a depolarizing-occasionally excitatory-signal, activating NMDA receptors and calcium currents leading to large calcium influxes and various forms of synaptic plasticity. In sum, the unique property of GABA (and glycine signals) to shift polarity in a variety of conditions is particularly suited for developing neurons that require synchronized activities in large neuronal ensembles.

**CHALLENGING THE GABA E TO I SEQUENCE: MUCH ADO ABOUT NOTHING!**

Science advances by trial and errors and challenging fundamental discoveries can fuel novel concepts raising more questions. Our developmental sequence remained undisputed for over two decades before being curiously and severely challenged by three former INMED students (C. Bernard, P. Bregestovski and Y. Zilberter) and by Staley and colleagues.

**The ketone bodies challenge by Zilberter, Bregestovski and Bernard**

In Marseille, P. Bregestovski (Brest), Y. and T. Zilberter were by far the most vehement claiming that the GABA depolarization in slices is «an artifact» for metabolic reasons. The concept was that as maternal milk is enriched in ketone bodies, observations obtained in glucose-perfused immature slices are due to energy deprivation since glucose (10 mM) cannot replace ketonergic metabolism (Rheims et al., 2009; Holmgren et al., 2010). While still at INMED, the Zilberter’s, Brest and colleagues found that GABA did not depolarize immature neurons in the presence of a ketone body metabolite BHB, lactate or pyruvate – in addition to glucose – (Rheims et al., 2009; Holmgren et al., 2010). As repeated internal discussions failed to convince them of the limitations of their results, we resolved to repeat these experiments using the same setups, drugs, animals and experimental conditions and a wide range of tools extending from single-channel recordings to dynamic two-photon microscopy. This large cooperative work with many
different INMED teams infirmed completely their results: ketone bodies metabolites had no effects on the actions of GABA (Tyzio et al., 2011) and the GABA-dependent maturation of hippocampal networks. Then, using a wide range of preparations, techniques, animal species and brain structures, eight other independent laboratories infirmed their observations that to the best of our knowledge have not been confirmed by a single other team casting severe doubt on their validity (references in (Ben-Ari et al., 2012b) also see (Gomez-Lira et al., 2011)). In addition, the Zilberter’s and colleagues used abnormally high concentrations of pyruvate that are only found in pathological conditions and provide in fact a signature of damage and inflammation (hundreds of references in PubMed, see (Ben-Ari et al., 2012b)). Using reliable measures – notably mitochondrial pH – Kaila and co-workers demonstrated that these effects are due to acidosis (Ruosuvuori et al., 2010) in contrast to the
indirect evaluations made by Bregestovski and Bernard (2012). Clearly, this criticism was a dead end based on irreproducible results and intrinsic contradictions.

The injury challenge by Staley and co-workers

Although they contributed to some important initial observations on the roles of chloride co-transporters and the depolarizing actions of GABA, Staley and co-workers generated a second front suggesting that the GABA depolarization is due to injury in the preparation of young slices (Dzhala et al., 2012). Using glucose perfused slices and primarily chlomeleon imaging techniques, they suggested that in immature neurons “increases in [Cl⁻], correlated with disruption of neural processes and biomarkers of cell injury…” These data support a more inhibitory role for GABA in the unperturbed immature brain, demonstrate the utility of the acute brain slice preparation for the study of the consequences of trauma, and provide potential mechanisms for both GABA-mediated excitatory network events in the slice preparation and early post-traumatic seizures”. The damage underlies the depolarizing actions of GABA in slices, less so in intact preparations and is largely restricted to the surface of slices. Interestingly, one of the leading co-authors of this publication – R. Khazipov – published another paper (Valeeva et al., 2013) showing that “the excitatory actions of GABA in hippocampal slices during the first post-natal days are not due to neuronal injury during slice preparation, and the trauma-related excitatory GABA actions at the slice surface are a fundamentally different phenomenon observed during the second post-natal week”. So the challenge concerns not the embryonic, delivery and early post-natal period but only the exact date at which this shift takes place (rather after P5 not P7 or else). In a developmental neurobiology perspective, the debate on the exact date of the shift is futile considering the variability of experimental conditions, neuronal type and heterogeneity of neurons, etc. It is quite difficult to see why would a vibratome damage neurons starting from P5 and not a couple of hours before or after! Here also, there is a wide range of observations that cannot be reconciled with the injury explanation (Ben-Ari et al., 2007; Tyzio et al., 2014) (DeFazio et al., 2002) including the fact that depolarizing GABA has been observed in intact hippocampal preparations, in various adult preparations and present in adult slices of autistic animal models but not if these have been treated with a diuretic during the delivery period (Ben-Ari et al., 2007; Tyzio et al., 2014 and below).

It is important to stress that the chlomeleon technique is primarily a pH measure providing at best a ratiometric qualitative indication of chloride levels (Ben-Ari et al., 2012b) and requiring elementary controls that were not performed by Staley and co-workers (Glykys et al., 2009, 2014a; Dzhala et al., 2012). The heterogeneity of chloride levels (from 1 to 120 mM) with this technique is an order of magnitude above that observed with more reliable electrical techniques in slices prepared by expert teams. The imaging scanning speed is hundred times slower than that assessed with electrical recordings and the separation of yellow fluorescent protein (YFP) and cyan fluorescent protein (CFP) signals with a band pass for yellow (510–540 nm) is debatable, differing from the original one used by Kuner and Augustine (2000). It is manifest that the validity of challenging the observations made with direct electrical recordings with an imaging technique is questionable (also see below).

The slice artifact explanation of Bernard and Bregestovski

This study was nevertheless welcomed by Bernard and Bregestovski who rushed to publish a review edited by their team member Y. Zilberter entitled: “Excitatory GABA: how a correct observation may turn out to be an experimental artifact” (Bregestovski and Bernard, 2012). This review amalgamates irreproducible results, ignores ones that cannot be reconciled with their suggestions, refers to observations that are incompatible with one another and attest a limited understanding of developmental processes. For example, Bregestovski and Bernard rely on Staley’s suggestions of an injury explanation to challenge the GABA excitatory actions (Dzhala et al., 2012) ignoring that these experiments were made in glucose perfused “slices” that according to them are artifact suffering from insufficient metabolic supply, raising the paradox of two incompatible explanations for a single phenomenon. Bernard and Bregestovski also rely on the Wang and Kriegstein (2011) who showed that in utero and post-natal administration of bumetanide leads to important malformations in off-springs suggesting that the diuretic ought not to be administered during pregnancy. Relying on this study, Bregestovski and Bernard conclude that the GABA developmental sequence is valid until delivery – but not after. Yet, had they read carefully the study of Wang & Kriegstein, they would have noticed that the most dramatic effects of bumetanide are observed when the diuretic is injected during the first week post natal suggesting that the post natal depolarizing actions of GABA are essential. Curiously, Bernard and colleagues have in almost parallel studies stressed the importance of GABA excitatory sequence actions (Quilichini et al., 2012)!

The paper of Bregestovski and Bernard (2012) was heavily publicized leading to a paradoxical situation where referees of publications dealing with GABA signaling in developing neurons imposed to discuss this challenge. Grants of studies based on excitatory actions of GABA on immature neurons were rejected! With 12 other experts in studying GABA signaling, we decided that a detailed reply was mandatory (Ben-Ari et al., 2012b). We refuted point by point these allegations that failed to meet the minimal requirements required for a scientific debate, namely reproducible observations, reliable suggestions and correct literature reviewing. The conclusion of this disagreeable moment is that scientific debates are useful only when relying on serious investigations, reproducible results, convincing arguments and if possible a novel concept. This was clearly not the case here.

The impermeant Anion challenge by Staley and co-workers!

But the story did not end there! Staley and workers pursued their fascination by the chlomeleon Technicolor technique to change 180° their views on the actions of
GABA and the roles of (Cl\(^{-}\)), levels (Glykys et al., 2014a). Staley and co-workers informed their numerous pioneering observations on depolarizing GABA in immature neurons and the effects of bumetanide (Dzhala and Staley, 2003; Dzhala et al., 2005, 2008, 2010; Glykys et al., 2009), modeling KCC2 functions and NKCC1 stoichiometry (Brumback and Staley, 2008) and the role of slice damage (Staley and Proctor, 1999; Dzhala et al., 2012). Now, slices are not damaged and NKCC1 and KCC2 have little impact on (Cl\(^{-}\)), levels: the polarity of GABA actions is determined by vaguely defined intracellular impermeant anions (Glykys et al., 2014a). This is an amazing study with no experimental controls, investigations relying exclusively on “specific” KCC2 antagonists that remain to be thoroughly investigated and lack of effects of bumetanide – that previously was found by the same group to have a major effect on (Cl\(^{-}\)), levels of immature neurons. There are other major caveats in this study. Thus, the authors described the lack of effects of bumetanide on neurons that have low (Cl\(^{-}\)), omitting to stress that in these neurons bumetanide has anyhow no effect. Comparing (Cl\(^{-}\)), levels before and after 30-min applications of bumetanide is inadequate as the changes have plenty of time to fade. Astonishingly, the authors neither compared deep and superficial layers nor even referred to their own paper published 2 years earlier where elevated (Cl\(^{-}\)), and excitatory GABA were primarily due to surface injury (Dzhala et al., 2012). Contrary to the earlier study, slices are now not damaged and the reasons for these alterations are not explained. Refuting the extensive direct recordings with the clear cut regulation of GABAergic synaptic currents by chloride co-transporter blockers relying on a pH qualitative imaging technique with a poor, low and slow sensitivity to chloride is doomed to fail. Ad minima, the authors should have compared in real time in a set of neurons the alterations of (Cl\(^{-}\)), with their imaging and more reliable single GABA channel recordings. Without these comparisons, these observations remain a distorted, reflection of the dynamic synaptic events occurring at a fast time course in real life. The theoretical and experimental basis of this study has been recently elegantly challenged (Luhmann, 2014; Voipio et al., 2014) see also the reply by (Glykys et al., 2014b).

Summing up, the GABA developmental sequence is now widely accepted because of the convergence of observations obtained in a wide range of issues and preparations. Indeed, it is important in this debate to take into account these apparently disparate observations that converge including the sequence itself with the oxytocin-mediated abrupt shift during delivery, the neuronal type and age dependence of the sequence, the alterations according to the sex of the animal, the fact that GABA usually does not depolarize adult neurons that are far more susceptible to lack of energy supply, the presence of the sequence in chicken, insects and frogs that to the best of our knowledge do not rely on maternal milk, the confirmation of the sequence in intact preparations, neuronal cultures and in vivo, the chloride co-transporter sequential development that provide a superb mechanistic substrate to the concept, (Ben-Ari et al., 2007) the difference between normal and albinorodents (Barmashenko et al., 2005) and the long-term effects of bumetanide in animal models of autism where this shift is abolished (see below). Admittedly, several Whys and Hows are not clear but the model is validated and its conservation during evolution is still an interesting issue to be determined.

**PATHOLOGICAL IMPLICATIONS OF THE SEQUENCE**

The developing brain differs from the adult one in its susceptibility to insults. Thus, the incidence of seizures is highest early in life raising the possibility that the lack of a powerful GABAergic inhibitory element contributes to that feature. Anoxic insults during the delivery period issue are also an important issue as there are among the most frequent cause of severe lifelong deleterious sequelcs. Curiously, immature post natal neurons are during the first 2–3 days after delivery extremely resistant to anoxic episodes; recordings made form adult and immature slices placed in the same chamber showed a dramatic difference in the duration of anoxic episodes required to abolish synaptic transmission (Cherubini et al., 1989; Crepel et al., 1992). As in addition, a plethora of neurological and psychiatric disorders originate during the embryonic and/or early post natal period, it seemed important to determine the mechanisms underlying these differences: Are the unique features of the developing brain and notably the excitatory actions of GABA a contributing factor? We set to investigate these issues somewhat returning to our early-preferred domain of interest: epilepsies.

**The GABA shift and epilepsies**

It was obvious from very early studies that we performed in vivo (see above) that recurrent activation of synaptic inputs to pyramidal neurons shifted the actions of GABA from inhibitory to excitatory because of an accumulation of chloride and a failure of neurons to cope with the large fluxes of chloride associated with high frequency GABAergic PSCs. Other observations indicate that KCC2 is highly susceptible to seizures, readily inactivated and internalized by recurrent – not single (Khirug et al., 2010) – seizures (Woo et al., 2002; Nardou et al., 2011b; Lee et al., 2007, 2011) and partial knock out of KCC2 also reduces the threshold of seizures (Khalilov et al., 2011) also see (Huberfeld et al., 2007; Pellegrino et al., 2011). We used the triple chamber that we had developed to investigate these issues (Khalilov et al., 1997a; Khazipov et al., 1999) (Fig11). This chamber composed of the two intact neonatal hippocampi interconnected with the commissures that are placed in 3 independent chambers has several unique advantages versus other in vitro preparations. Indeed, here it is possible to perfuse the 2 hippocampi with different liquids enabling to generate seizures with a convulsive agent and check the consequences of the recurrent seizures on the other hippocampi that have not received the convulsive agent. It is also the only in vitro preparation in which the 2 hippocampi can be reversibly disconnected after a predetermined number of propagated seizures in...
order to determine the consequences of a defined number of seizures on the electrical properties of the naive hemisphere. Using this preparation, Khalilov and colleagues showed that after a single seizure, the contralateral neurons remain free of spontaneous seizures after the disconnection (Khalilov et al., 2003, 2005; Nardou et al., 2011b). In contrast, the propagation of many recurrent seizures from the stimulated to the naive hippocampus transforms it to an epileptogenic structure that generates seizures after being disconnected from the kainate-treated hippocampus: it has become epileptic. This preparation also allows to investigate in good conditions the alterations required by a network to become spontaneously epileptic. These were found to include elevated $[\text{Cl}^-]_i$, excitatory GABA and an internalized KCC2; suggesting a return to an immature state. Using our dynamic approach method (see above) to measure the dynamic of $[\text{Cl}^-]_i$, levels regulation, we found that epileptic neurons had difficulties to export chloride as they required several tens of minutes to return to pre-stimulation values in comparison to the tens of seconds of naive age-matched neurons (also see Barmashenko et al., 2011).

Using this preparation, we also determined the minimal requirements that must be met in propagated seizures in order to transform naive neurons to epileptic ones. It turned out that the seizures must contain elevated frequencies – above 40 Hz – to produce their effects (Khalilov et al., 2003, 2005; Nardou et al., 2011b). Interestingly, GABAergic signals are essential for the generation of elevated frequencies and the formation by seizures of an epileptogenic mirror focus. This was directly shown by applications of GABAzine or bicuculline to the naive hippocampus that prevented both the occurrence of high frequencies and the transformation of the naive hippocampus to an epileptic one. Therefore, the hyperactivity produced by blocking GABA signals is in fact not epileptogenic because GABAergic currents are essential for the generation of high-frequency events that are indispensable for the long-term effects of seizures.

### Bumetanide, chloride co-transporters and epilepsies: a complex story

If GABA excites epileptic neurons, reducing $[\text{Cl}^-]_i$ levels to restore hyperpolarizing actions of GABA seemed a reasonable antiepileptic strategy. Activating KCC2 or reducing NKCC1 to respectively enhance its removal or reduce its import can achieve this. Selective KCC2 agonists were not available then (Gagnon et al., 2013) and the inactivation and internalization of KCC2 by hyperactivity suggested that this approach might not be efficient. We shifted to investigate the antiepileptic properties of the highly selective NKCC1 antagonist bumetanide (Feit, 1981). This drug has been used for several decades to treat edema and hypertension with limited side effects – essentially a diuresis and hypokalemia. In vitro studies confirmed its powerful actions in restoring strong hyperpolarizing and inhibitory actions. The effects of bumetanide in different animal models yielded contradictory results. In some, bumetanide reduced acute and chronic seizures in others it failed (Dzhala et al., 2005; Kilb et al., 2007).

Using the triple chamber, we decided to compare its actions on the transformation by propagated seizures of a naive network to an epileptic one and on a mirror focus (Nardou et al., 2009, 2011b). Applying bumetanide to one hippocampus and kainate to the other did not prevent the generation of kainate-induced seizures, their propagation would...
to the contralateral hippocampus, and the formation of an epileptogenic mirror focus. However, bumetanide reduced DF GABA, the excitatory action of GABA in epileptic neurons and blocked spontaneous epileptiform activity in the mirror focus. Therefore, although bumetanide does not prevent formation of the epileptogenic mirror focus suggesting that in addition to NKCC1, other mechanisms contribute to increase DF GABA in epileptic neurons.

Koyama and colleagues showed an interesting illustration of the developmental links between the actions of GABA and epilepsies (Tao et al., 2012). Using a model of febrile seizures to elicit temporal lobe epilepsies later in life, they showed that the aberrant migration of neonatal-generated granule cells persists into adulthood. Bumetanide-like RNAi-mediated knockdown of NKCC1 prevented this aberrant migration, rescued the granule cell ectopia, susceptibility to limbic seizures and development of epilepsy. Thus, febrile seizures produce persistent immature GABAergic signals associated with an architectural signature of pathological conditions with enhanced NKCC1 acting as a triggering factor. This investigation is also in keeping with the depolarizing actions of GABA on newly born granule cells again emphasizing the importance of the excitatory/inhibitory shift during development including in an adult environment.

**PB and diazepam (DZP) to treat epilepsies: another twist**

If GABA excites epileptic neurons, molecules acting on GABA might produce paradoxical effects on epileptic neurons. PB and DZP are extensively used as first- and second-line drugs to treat acute seizures in neonates and their actions are thought to be mediated by increasing the actions of GABAergic signals. PB and DZP have been used for decades to treat epilepsies with positive effects in some types of infantile epilepsies and a failure even paradoxical aggravating actions in other types of epilepsies. This heterogeneity of actions might be due to the plasticity of the polarity of GABA that has been reported by many teams (Huberfeld et al., 2007; Balena and Woodin, 2008; Blaesse et al., 2009; Balena et al., 2010; Khirug et al., 2010). Other studies have however suggested an enhanced efficacy of GABA mimetics when combined with Bumetanide (Ozhalia et al., 2005; Brandt et al., 2010). We used the triple chamber to test the role of excitatory GABA following single or repeated seizures (Nardou et al., 2009). We found that genetic invalidation of NKCC1 (NKCC1 Kos) did not prevent the formation by seizures of an epileptogenic mirror focus suggesting that NKCC1 is not required for the epileptogenic process (see preceding chapter). Also, when applied to one hippocampus from the start, PB blocked the seizure, prevented the formation by seizures of an epileptogenic mirror focus and the GABA polarity shift. In contrast, when applied after several propagated seizures, PB aggravated the epileptiform activity (Fig. 11A–C). Therefore, the actions of PB are conditioned by the history of seizures prior to its first application raising important conceptual and clinical issues.

To get better insights in these actions, we repeated these experiments with DZP and compared them to PB (Nardou et al., 2011a). Using the three-compartment chamber, kainate was applied to one hippocampus and DZP (or PB) to the contralateral one after the formation by propagated interictal and ictal activities of an epileptogenic mirror focus. We found that in contrast to PB, DZP aggravated propagating seizures from the start, and failed to prevent the formation by propagated seizures of a mirror focus. PB reduced and DZP increased the network-driven GDFs suggesting that they might exert other different actions. In keeping with this, PB but not DZP reduced field potentials generated by AMPA/kainate receptor-mediated EPSCs, in the presence of GABA and NMDA receptor antagonists. The following observations suggest that PB exerts direct actions on AMPA/kainate receptors:

(i) a reduction of AMPA/kainate receptor-mediated currents generated by focal applications of glutamate;
(ii) a reduction of the amplitude and the frequency of AMPA but not NMDA receptor-mediated miniature excitatory postsynaptic currents;
(iii) an increase of the number of AMPA receptor-mediated EPSCs failures evoked by minimal stimulation.

These effects persisted in mirror foci. Therefore, the enhanced efficacy of PB versus DZP is due to a reduction by AMPA/kainate receptors-mediated EPSCs in addition to the pro-GABA effects. These additional actions might confer an advantage of PB over DZP in the treatment of neonatal seizures.

Relying on the initial success of bumetanide in experimental conditions, a European clinical trial was initiated on 2-day-old babies with encephalopathic seizures that are refractory to PB (www.nemo.eu). This trial was stopped because of the poor effects of bumetanide and important side effects of the antibiotic treatment on the auditory system. The development of GABA (and glycine) has been investigated in depth (Milenkovic and Rübsamen, 2011; Witte et al., 2014) and suggest that the immature hearing system is vulnerable to bumetanide because of the roles of NKCC1 in the endolymph at an early stage (also see www.nemo.eu). In addition, it is possible that the antibiotic regimen contributes to these side effects and/or the fact that babies were enrolled only once PB was found to have no effects indicating a possible down regulation of KCC2. If so, then the usefulness of bumetanide might be limited to older babies/children and/or early usage of the diuretic before the recurrent seizures down regulated KCC2.

**Bumetanide : a plethora of beneficial actions but ...**

The story did not end here. If GABA excites epileptic neurons, then a similar situation might also occur in other pathological situations. Studies using a plethora of animal models of other pathologies have confirmed a similar sequence (Huberfeld et al., 2007; Boulenguez et al., 2010; Khirug et al., 2010; LüScher et al., 2012; Reid et al., 2013). The use of bumetanide to treat all these disorders is however challenged by complications and side effects that might occur in specific conditions including menopause women where long-lasting treatment with
bumetanide increase the renal calcium excretion and alters the diurnal rhythm of plasma Parathyroid hormone thereby affecting bone formation (Rejmark et al., 2006). Also, continuous administration of the diuretic to the mother and pups from E18 to postnatal periods exerts pathological actions including sensory deficits and schizophrenic-type behaviors stressing the importance of the depolarizing actions of GABA in utero and early post-natal life and its trophic role (Wang and Kriegstein, 2011). As stressed elsewhere (Ben-Ari and Tyzio, 2011), the use of bumetanide during pregnancy is only justified in pathological conditions when chloride levels might be elevated and then restoring normal levels might be useful.

Epilepsies and the heterogeneity of pyramidal neurons

A basic assumption of studies on pyramidal neurons of the hippocampus – say of the CA3 region – is that they are homogeneous. Yet, these neurons have different birth dates and might belong to different stem cells. Studies on epilepsies provided some indications that this is indeed the case. In adult hippocampal slices, blocking GABAergic signals efficiently generates seizures. Furthermore, in a classical experiment, Miles and Wong (Miles and Wong, 1983) also see (Miles et al., 1988; Wittner and Miles, 2007) showed that stimulation of a single CA3 pyramidal neuron in the bicuculline-treated slice triggers all- or none-synchronized paroxysmal events an effect mediated by the wide range of recurrent glutamatergic excitatory collaterals. Yet, curiously, a similar protocol failed to do so in an immature slice, although seizures were generated. Using the two-photon microscope and targeted patch-clamp recording of identified neurons, the apparent dilemma was resolved by uncovering a sub-population of early-generated glutamatergic neurons that impacts network dynamics when stimulated in the juvenile hippocampus (Marissal et al., 2012). This population displayed characteristic morpho-physiological features in the juvenile and adult hippocampus. This study illustrates the heterogeneity of apparently homogeneous glutamatergic neurons rooted in their different temporal embryonic origins. In addition, although functional hub neurons are exclusively GABAergic as far as GDPRs are concerned, some early-born glutamatergic pyramidal neurons are capable of contributing to the generation of synchronized events in the absence of GABAergic signals. Therefore, from a developmental viewpoint, the heterogeneity of neuronal populations must be taken in consideration in epilepsies and most likely also other pathogenic conditions.

AUTISM: AN INCREDIBLE AND UNEXPECTED CONFIRMATION OF THE SEQUENCE AND ITS POTENTIALS IN TREATMENT OF AUTISM

Things often happen when unexpected and not even desired. I was about to retire and was giving lectures and wide audience talks on my research. Giving a talk to the national meeting of association of parents of autistic children, I described the developmental sequence, the role of GABA/intracellular chloride and why in various pathological conditions, intracellular chloride is elevated, GABA excites and BZ can produce paradoxical reactions. The audience was highly interested but one of the doctors treating autistic kids in Brittany – Dr. E. Lemonnier – drew my attention to the fact that autistic children have often-paradoxical reactions to Valium and other GABA-acting agents. Dr. Lemonnier asked me if I would agree to help him making first a small pilot study using bumetanide to block selectively the chloride importer NKCC1. I agreed and he had the ingenious luck to find the appropriate doses of bumetanide to use. He obtained rapidly the authorization from the ethics committee considering that this drug has been used for almost four decades including in babies with little or no side effects. He selected children and adolescents (3–11 years old) with autism of various severities since we could not select a particular type of autism as we might lose important information. The results of this open pilot study revealed an amelioration of the severity (Lemonnier and Ben-Ari, 2010).

This led to a double-blind randomized investigation on 54 children with autism with a regimen of 3 months bumetanide (1.5 mg daily) or placebo followed by wash out. Again the results were significant with the conventional evaluation tools but also by the parents stressing that the children are more “present” (Lemonnier et al., 2012). The side effects were restricted to the usual diuresis associated in a minority of children with hypokalemia treated readily with K+ gluconate syrup. Publishing this result was no simple matter with the usual suspects refusing to admit or even consider that a simple diuretic might work when all the sophisticated genetic-driven therapeutic trials had failed or were less promising. The paper was finally accepted by Translational Psychiatry, receiving very little attention from the media, but not from parents as many – over 80 – continued to rely on this treatment some for over 3 years by now. This also led to the usual avenue when trying to develop a new drug, including search of investors, patents, start up etc. A financial investment (an investment company linked to the Simons foundation) provided us with the opportunity to initiate a large European approved multi centric randomized trial (80 children 2–18 years old) that is now under way to be finalized during the spring of 2015. In the meantime, pilot examinations suggested that the treatment might be useful in Fragile X (Lemonnier et al., 2013). Parallel investigations showed that in eight Asperger adolescents, long-term bumetanide treatment ameliorated visual communication and recognition of emotive figures (Hadjikhani et al., 2013). This also enhanced the activation of brain regions involved in social and emotional perception during the perception of emotional faces. These observations converge to suggest that bumetanide might have beneficial effects in the treatment of autism.

Yet, whether indeed (Cl−), levels are high in cortical neurons of patients with autism remained an open question particularly as bumetanide binds to Albumin and has a poor blood–brain barrier permeability. Clearly,
Experimental investigations were needed to test these issues in animal models of autism. We used the Valproate in utero rat (Kuwagata et al., 2009; Williams and Casanova, 2011; Williams et al., 2001) and the Fragile x mice models, the latter being the most frequent genetic disorder associated with autism traits and a frequently used model to test therapeutic approaches to autism (Bear et al., 2004; Padmashri et al., 2013). We first observed with single GABA channel recordings, elevated (Cl⁻) levels and a depolarized GABA that with both cell attached and field recordings was converted to excitatory actions of GABA. These effects were blocked by bumetanide.

But this was not all. We then investigated the entire GABA developmental sequence and found to our surprise that it was abolished already from the embryonic–delivery shift stage: neurons have the same DFGABA in embryonic and post-natal neurons (Fig. 12 H). Bumetanide like the hormone oxytocin restored the correct hyperpolarized driving force of GABA. Therefore, at least hippocampal neurons “behave” as if they remained immature with
bumetanide- and oxytocin-sensitive high (Cl⁻) levels and excitatory GABA.

Perhaps a more astonishing observation stemmed when we started to correct the (Cl⁻) shift only during delivery. We asked the following question: might the oxytocin-mediated delivery shift be a signal that resets the clock leading to long-lasting consequences if it fails. This turned out to be instrumental. Administration of bumetanide in drinking water to the mother during a period of 24 h – shortly before and after delivery – restored the complete developmental sequence: ΔF GABA followed the naïve sequence not only during delivery but also several weeks later (Figs. 12A–F and 13A, D). This treatment also restored GABA inhibitory actions at birth (Fig. 13G, H) and several weeks later (Fig. 13B, C, E, F). Other electrical signatures of networks in animal models of autism including enhanced glutamatergic activity and in vivo increase of brain oscillations in the gamma and higher band frequency were also attenuated (Fig. 13). The treatment also ameliorated behavioral signatures of autism including vocalization – produced in pups following separation from the mother – but also sociability and grooming tests (Tyzio et al., 2014). In

Fig. 13. Maternal pretreatment with bumetanide before delivery switches the action of GABA from excitatory to inhibitory in offspring in VPA and FRX rodents at P15. (A) Average values of ΔF GABA measured in hippocampal CA3 pyramidal neurons at P15 in control (black), VPA (red), and VPA rats pretreated with bumetanide (blue). Note that pretreatment with bumetanide shifts ΔF GABA from depolarizing to almost isoelectric level. (B) Effects of isoguvacine (10 mM; black bars) in rats: Representative traces of spontaneous extracellular field potentials recorded in hippocampal slices at P15 in control, VPA, and VPA rats pretreated with bumetanide (BUM) shortly before and during the delivery period. Corresponding time courses of spike frequency changes are shown under each trace. (C) Average histograms of normalized spike frequency in rats. Isoguvacine (hatched bars) decreased the spikes frequency in control rats (to 38.9 ± 5.1%; gray); increased it in VPA rats (to 213.5 ± 16.3%; red); and decreased it in VPA rats pretreated with bumetanide (to 82.8 ± 10.7%; blue). (D) The same as in (A) for mice. Wild-type mice (WT, black), FRX mice (red), and FRX mice pretreated with bumetanide (blue). (E) The same as in (B) for FRX mice. (F) The same as in (C) for FRX mice. Wild-type mice (decreased to 67.9 ± 6.1%; gray); FRX mice (increased to 165.8 ± 13.5%; red); FRX mice pretreated with bumetanide (decreased to 80.8 ± 8.2%; blue). Data are presented as mean ± SEM. **P < 0.01; ***P < 0.001. Reproduced from Tyzio et al. (2014). (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)
parallel, we observed that the administration of an antagonist of oxytocin receptor to naïve pregnant rats during the same restricted period produced “autistic features” including depolarizing and excitatory GABA and autistic behaviors. Therefore, the polarity of GABA during delivery exerts long-term effects on brain and behavior and these actions involve oxytocin signals stressing the links between GABA/oxytocin and delivery.

This observation raises formidable questions that are pertinent to major public health issues. As ASD is “born” in utero (Courchesne et al., 2007, 2011), how can a manipulation during delivery correct the programmed sequels? Are the genetic effects of Fragile X also reversible by bumetanide? This issue also applies to programed C-sections, pre-term delivery and complications during delivery that are associated with a higher incidence of ASD. They also raise the fundamental conceptual issue of how in utero insults can led to protracted brain disorders and possibly an attenuation of their deficits by processes at work during and shortly after delivery. Is it possible that delivery exerts a priming effect on brain development? These issues are neither restricted to ASD nor to GABA. Since many ionic currents follow developmental sequences, it might reasonably be expected that the interruption of other sequences leads to other lifelong deleterious sequels that can be reduced by selective antagonists. In a more general perspective, this is another example of the persistence of “immature” currents in neurons that have not adequately performed their assigned programs (see below).

**LINKING GENES AND ENVIRONMENTAL EVENTS: THE CHECKPOINT AND NEURO-ARCHEOLOGY CONCEPTS**

Initially centered on GABA developmental sequences, our investigations revealed a bigger picture that bears relevance to the links between genes and environment and to the role of activity in brain maturatation. Indeed, it is clear that the developing brain is highly active, immature neurons generating ionic currents patterns that differ from adults. Stated differently, the developing brain is very talkative but its language differs from the adult’s. The brain requires enhanced activity to construct its maps and functional units and these specific requirements must timely shift to enable the generation of the behaviorally relevant oscillations. These developmental sequences provide the type of activity needed at various developmental stages and enable neurons at very different developmental stages to fire and wire together. Contrary to the construction of an engine (a car or a plane that are “silent until the end”), the brain is active during its construction and the activity it generates is not that which will be used once finished.

The checkpoint concept (Ben-Ari and Spitzer, 2010) suggests that genes and activity operate in series and as in all biological operations; a feedback control is needed and activity does that job (Fig. 14). CNS development requires intermediate stages of differentiation that provides information needed for gene expression. In this model, embryonic neuronal functions constitute a series of phenotypic signatures. In keeping with this, extensive
investigations have shown that procedures and agents that perturb ongoing activities during maturation produce long-term effects. We have suggested that the expression of different embryonic features at different developmental stages provide that control. This has been observed at various essential developmental steps with a proliferation checkpoint, a migration checkpoint, axon guidance checkpoint and neurotransmitter specification checkpoint. An illustration is provided by the nice experiments of Spitzer and colleagues showing that immature neurons generate specific patterns of calcium currents and when these are disturbed they can modify the neurons phenotype (GABAergic instead of glutamatergic) (Borodinsky et al., 2004; Spitzer and Borodinsky, 2006). The GABA/chloride checkpoint is yet another important event of a long chain of informative alterations that signal the developmental stage of a neuron and whether it has successfully implemented its maturation program. This is by no means restricted to GABA and chloride, parallel developmental sequences have been reported in a wide range of systems with similar alterations of ionic currents – from long-lasting to shorter kinetics – and shifts of network-driven patterns – such as in sensory systems (Mooney et al., 1996; Zhou and Zhao, 2000; Torbørg and Feller, 2005; Zhang et al., 2010; Milenković and Rübsamen, 2011; Xu et al., 2011; Ford et al., 2012).

What if things go wrong? From an architectural standpoint, errors in the construction of a building can be unraveled years later and the mechanisms underlying the damage uncovered years later by analyzing the elements that have led to the collapse. The damage – the brain disorder – can be manifested long after the initial insult has taken place depending on the “use” of brain networks and a second hit/insult. Yet, it is likely that the initial insult leaves a trace of the generating event in time and space. I have suggested that neurons that fail to perform their assigned targets and are either misplaced or misconnected remain “frozen” in an immature state that corresponds to the stage at which serious essential developmental steps with a proliferation checkpoint, a migration checkpoint, axon guidance checkpoint and neurotransmitter specification checkpoint. An illustration is provided by the nice experiments of Spitzer and colleagues showing that immature neurons generate specific patterns of calcium currents and when these are disturbed they can modify the neurons phenotype (GABAergic instead of glutamatergic) (Borodinsky et al., 2004; Spitzer and Borodinsky, 2006). The GABA/chloride checkpoint is yet another important event of a long chain of informative alterations that signal the developmental stage of a neuron and whether it has successfully implemented its maturation program. This is by no means restricted to GABA and chloride, parallel developmental sequences have been reported in a wide range of systems with similar alterations of ionic currents – from long-lasting to shorter kinetics – and shifts of network-driven patterns – such as in sensory systems (Mooney et al., 1996; Zhou and Zhao, 2000; Torbørg and Feller, 2005; Zhang et al., 2010; Milenković and Rübsamen, 2011; Xu et al., 2011; Ford et al., 2012).

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In this neuro-archeology concept (Ben-Ari, 2008), alterations of developmental sequences lead to a persistent electrical or architectural signature of the timing of the failure. In this perspective, developmental disorders are due at least in part to the persistent expression of immature currents and oscillations in the adult brain that perturb the operation of well-developed functional networks. This concept has been confirmed in many migration disorders including Double cortex, heterotopic nodules, SRPX2 mutations, where misplaced neurons keep immature features thereby perturbing normo-functioning oscillations (Ackman et al., 2009; Salmi et al., 2013; Falace et al., 2014). This concept suggests that the use of specific antagonists /blockers of immature currents in an adult brain might be a useful strategy to block the perturbing activity without altering adjacent networks that have performed adequately their programs. In this model, the persistence of immature currents and aberrant activities and networks is the ultimate cause of disorder. Gene therapy – replacing the mutation by the correct gene – is unlikely to cure as it will not correct the aberrant activities generated by misplaced and mismatched neurons and produce in the crowded adult brain migration and reconnection with the correct targets. Thus, introduction of the Double Cortin gene in neurons that failed to migrate following the invalidation of that gene, partly improved the default migration during the first few days post natal but not later (Manent et al., 2009). Therefore, to understand and treat brain disorders, it is essential to determine how brain patterns are deviated by the mutation and environmental insult. This coupled with early diagnosis and the use of selective drugs that block the perturbing activities might provide novel therapeutic avenues.

CONCLUSIONS

One conclusion of this journey is that studies initiated to check how neuronal activity develops at early developmental stages unraveled a plethora of issues, observations, and novel queries and concepts. They have also led to possible innovative therapeutics to treat disorders that are at present orphan of treatments. As most if not all-ionic currents follow developmental sequences, our observations with GABAergic signals might have major implications. It is possible that antagonists of other immature voltage-gated and or transmitter-mediated currents will tomorrow constitute a common strategy to block immature perturbing currents without altering the normally developed adult currents. Studies on voltage-gated ionic currents notably calcium currents will be useful to test this hypothesis. Therefore, to understand and treat developmental disorders, it is instrumental to understand developmental sequences and how they are deviated by genetic mutations of environmental insults. The translation of newly identified mutations to therapeutic agents is conditioned by a better understanding of the cascade of events that this mutation induces. In sum, studies on developmental disorders must embrace a genuine developmental perspective.

A second conclusion that remains to be better understood is the functional advantage of this complex developmental Excitatory /Inhibitory developmental sequence. Why do immature neurons need elevated intracellular chloride? One possibility is that this is an evolutionary conserved feature aimed at equilibrating a smaller sum of intracellular negative charges in developing systems. But this speculative hypothesis will have to explain why many peripheral neurons have in adulthood low levels of intracellular chloride.

Finally, the fundamental issue of delivery and its impact on the developmental sequence of ionic currents remains to be better understood. This is by itself a major topic considering the complicated events occurring during delivery that have been ignored until recently as far as the impact they have on neuronal and network activities. Indeed, delivery is associated with a major stress reaction associated with elevated catecholamine (Lagercrantz and Bistoletti, 1977; Faxelius et al., 1983; Greenough et al., 1987; Hillman et al., 2012) and cortisol levels that are not met in adults even under conditions of severe stress. These are meant to enable delivery to perform its major functions including the acquisition of
immunological and microbion features – acquired during delivery – but also the shift to aerobic oxygenation. Interestingly, GABA accelerates lung maturation and promotes chloride extrusion in lungs (Chintagari et al., 2010). Yet, elevated catecholamine augments intracellular chloride and produces an inhibitory to excitatory shift of the actions of GABA (Inoue et al., 2013). A speculative but highly interesting possibility is that oxytocin and other hormones compensate the possible deleterious effects of catecholamine on central neurons during delivery. In this perspective, evolution has enabled to both produce stress needed for peripheral essential functions and provide the clues to avoid the deleterious actions of this stress on neurons. Whatever the correct explanation, we cannot omit from our agenda a better understanding of the alterations during delivery and their impacts.

Acknowledgments—I am indebted to many of my students and colleagues who have worked on these programs. Financial support of Inserm – and various public agencies (ANR, EU, OSEO) – for financial support.

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(Accepted 1 August 2014)

(Available online 26 August 2014)