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Basic developmental rules and their implications for epilepsy in the immature brain

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ABSTRACT – The construction of the human brain with its 10^{15} synapses follows a set of complex developmentally and environmentally regulated steps. A series of sequences have been described that are instrumental, in the sense that a failure of any one of them leads to dramatic, life-long consequences. Hence the importance of determining the sequential maturation of neurons, synapses and cortical maps. It is also important to determine how network-driven events become installed, as neuronal activity intervenes in all of these steps and modulates, for better or worse, the outcome. A fundamental consequence of these sequential events is that any disruption will have very different consequences depending on when it occurs, indeed, "when is as important as what". An obvious aspect of these general features is related to seizures. In fact, the developing brain has both a higher incidence of seizures in human and animal models, and experiences seizures that can produce long-lasting consequences that are also stage-dependent.

This seminar and the series of slides presented are an introduction to these issues, summing up several studies made notably by INMED researchers during the last two decades (http://www.inmednet.com). It concentrates on four basic developmental rules: i) the generation by very immature neurons, of very large currents mediated by the activation of receptors in neurons that bear no synapses. This is due to the release of GABA that diffuses to distal sites and acts as a paracrine factor; ii) the excitatory/inhibitory shift of the actions of GABA during development because of a progressive reduction in the intracellular chloride concentration; iii) the sequential formation of GABAergic synapses and networks before glutamatergic ones, implying that, at an early stage, all the excitatory drive will be GABAergic; iv) the presence, at an early stage, of a unique, primitive pattern in all developing structures, this pattern disappears when most GABAergic synapses have shifted to their adult configuration-.Several consequences of these sequences are described including: i) a control of neuronal migration by GABA-acting drugs, and the possibility that migration disorders are also generated by environmental factors that include the effects of GABA-acting agents; ii) If GABA excites immature neurons and inhibits adult one, then GABA-acting agents will also produce different effects on the mother and the embryo; iii) early brain oscillations are generated by the periphery and propagate centrally – notably to the sensory-motor cortex, suggesting that peripherally-generated movements may provide an important signal for the formation of cortical maps, in keeping with the importance of embryonic movements; iv) "seizures beget seizures" in the developing brain. This has now been shown in a triple chamber with the two intact hippocampi that we developed, and with which it has been possible to show that only recurrent seizures that include high frequency oscillations can transform the naïve,



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contralateral hippocampus to an epileptic one that seizes spontaneously. Most interestingly, at an early developmental stage, when GABA excites many neurons and the density of glutamatergic synapses is not sufficiently high, purely glutamatergic seizures cannot lead to long-term consequences, the additional excitatory drive provided by GABAergic synapses is needed. In other words, at that stage, blocking GABA synapses generates seizures, as in adults, but these do not lead to long-term consequences. The mechanisms that underlie these differences is due to the need for high frequency oscillations (> 80 Hz or so), and these can only be generated when GABA synapses are operative in the developing brain: GABA receptor antagonists are ictogenic, but not epileptogenic.To facilitate teaching purposes the paper is published together with supplemental data (as a PowerPoint presentation included in the accompanying DVD), thus allowing an overview of important developmental steps and their implications.
[*Published with supplemental data on CD*]

Key words: neuronal migration, excitatory action of GABA, oscillation, seizure, antiepileptic drug, GABAergic interneuron

There is a paradox as far as epilepsy in the developing brain is concerned. The incidence of seizures is higher early in life and seizures lead to long- term deleterious sequel; yet immature neurons are less vulnerable than adult ones to a variety of insults including anoxo-ischemic episodes and seizures. In other words, seizures are more frequent - indicating that immature networks are endowed with a capacity for high excitability and synchronized events.

These observations lead to a number of conclusions. Firstly, seizures are more readily generated because one or more of the mechanisms that in adults prevent excessive activity, are not available. Secondly, seizures must produce their consequences by altering fundamental processes rather than "simply" causing neuronal lesions. This implies that understanding and treating neonatal and early seizures must incorporate a detailed analysis of the sequences of expression of the principal constituent of cortical networks in order to determine where the missing mechanisms are that fail to prevent seizure generation, and which sequence is affected by hyperactivity leading to permanent alterations of the normal sequence of events. To further complicate the issue, it is essential to take into account that the developing brain is not a small adult brain, but a unique machine with channels, molecules and mechanisms that are entirely or largely absent in adults. Thus, it is not possible to rely on mechanisms operating in adults to unravel the causes of the paradox summarized above. The central issue is to determine when during development, neuronal activity can alter developmental processes *(figure 1/DVD figure 2)*.

Studies performed mostly in Unit 29 of the French medical research council (INSERM) have unraveled several fundamental rules of brain development and have shown that these rules most likely underlie the unique properties of the developing brain as far as epileptogenesis is concerned. This work summarizes the salient features of these observations and, relying on pictures from actual experiments, shows how they explain the high incidence of seizures and their long lasting consequences on the developing brain. We shall divide the sequence into 3 steps:



Figure 1. Nature and Nurture in development & epilepsies.

migration, synapse formation, network construction (*figure 2/DVD figure 3)*.

To evaluate the impact of activity on brain maturation, we shall examine successively the role of the principal transmitters, GABA and glutamate, on essential developmental

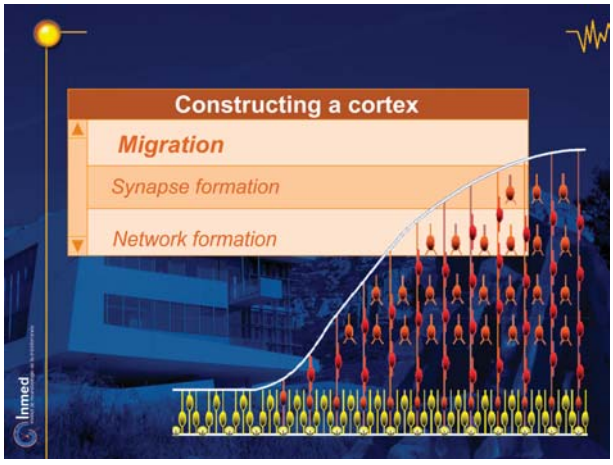


Figure 2. Constructing a cortex.

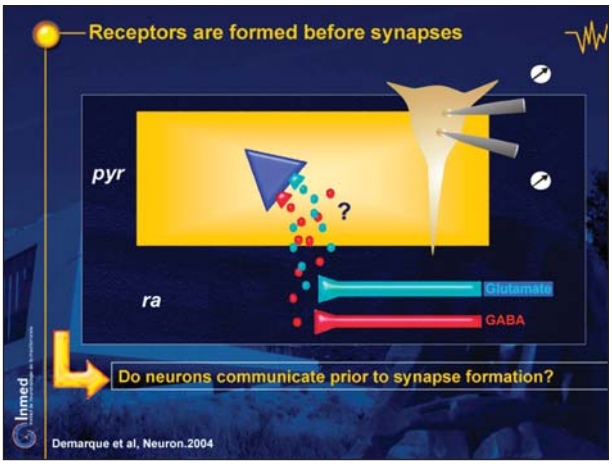


Figure 3. Receptors are formed before synapses. Demarque *et al. Neuron* 2004.

steps, and, in parallel, illustrate how this information impacts on neurological disorders. In the adult brain, glutamatergic neurons constitute the vast majority of neurons, and provide the main source of the excitatory drive. The dense networks of GABAergic interneurons that constitute 10-15% of the neurons on the other hand, provide the source of the inhibitory drive. In spite of this apparent imbalance, interneurons play a crucial role in the generation of behaviorally relevant patterns (Freund and Buszaki, 1996). Various types of interneurons control the fine discharge of the principal cells, and the patterns that networks will generate. In the absence of functional GABAergic neurons and synapses, cortical networks seize and do not generate behaviorally relevant patterns. Let us now examine whether they are operative at early stages and whether they control essential developmental processes.

Neuronal migration

In a wide range of systems, receptors develop before synapses and the obvious question is whether they exert an influence. In other words, does modulation by environmental factors, including neuronal activity, start with synapse formation or does it occur at an earlier stage when synapses are not yet operative?

If transmitters are released and act on immature neurons endowed with receptors but not functional synapses, they should generate a current by activating post-synaptic receptors. With this in mind, we therefore patch-clamp recorded immature neurons in which the conventional, fast synaptic responses cannot be generated and found that indeed, electrical stimuli evoked giant currents that are several order of magnitude larger than conventional synaptic currents (*figure 3; DVD figure 4-6)*. These currents are mediated by the activation of GABA- and glutamate NMDA-type receptors – but not AMPA receptors that are responsible for the majority of fast acting, ionotropic synapses in the adult brain. We also found that there is a tonic release of GABA and glutamate at an early stage, since a GABA receptor antagonist generates a tonic current indicating an ongoing release of GABA (Demarque *et al.*, 2002). The release mechanisms are unique in the sense that they do not depend on essential vesicular release proteins and factors including release proteins and machinery. They can even be generated in animals in which the release machinery has been knocked out. This ***paracrinic*** release of GABA acts on distal neurons to generate large currents prior to synapse formation. At a later stage, this system disappears and is replaced by the more conventional focal action of transmitters. Therefore, neurons communicate prior to synapse formation and there is a non-canonical release of transmitter that can act diffusely on distal sites (also see below).

Transmitter release and neuronal migration

Is transmitter release functional and does it alter migration? We tested the possible role of this paracrine release on migration *in vitro,* using an original preparation in which two hippocampal slices are cultivated next to each other: one of the slices was obtained from a GFP transgenic mouse *in* which all neurons are green, allowing us to follow visually their course and migration on the other, dark slice (*figure 4; DVD figure 7*; Manent *et al.* 2005. We found (*figure 5; DVD figure 8)* that blocking GABA receptors retarded neuronal migration considerably indicating that migration – an activity-dependent process (*DVD figure 6*; Komuro and Rakic) - is modulated by a non-focal, paracrinic action of transmitters, most notably GABA. These and other observations are in line with the extensive

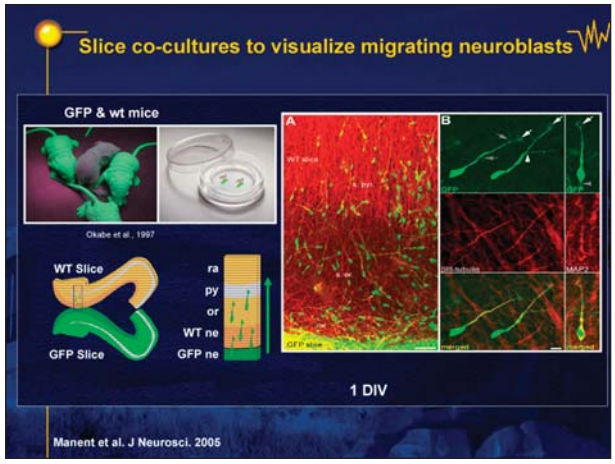


Figure 4. Slice co-cultures to visualize migrating neuroblasts. Manent *et al. J Neurosci* 2005.

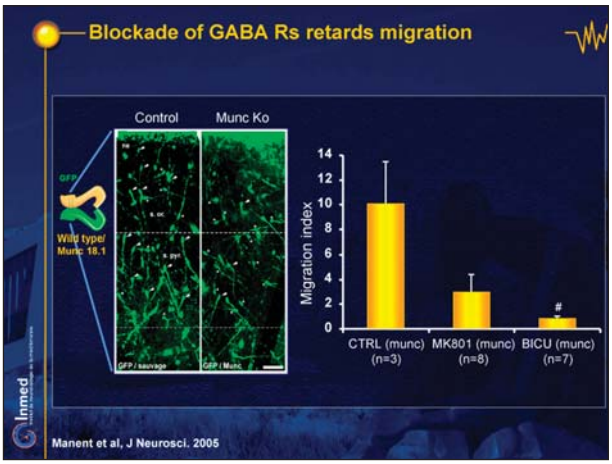


Figure 5. Blockade of GABA Rs retards migration. Manent *et al. J Neurosci* 2005.

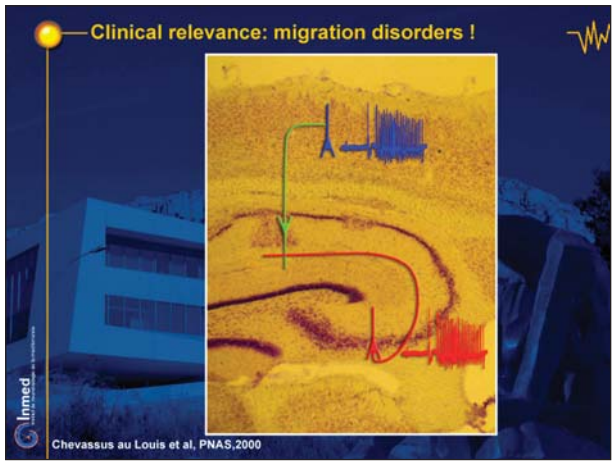


Figure 6. Clinical relevance: migration disorders! Chevassus-Au-Louis *et al. PNAS* 2000.

evidence that GABA is a morphogenic agent that, at an early stage, modulates neuronal migration in addition to synapse formation, and neuronal growth and differentiation. Thus, transmitters, notably GABA, can modulate early developmental processes by means of actions on receptors that are, in essence, extra -synaptic (also see Owens *et al.* 1999, 2002; Represa and Ben-Ari 2005). Therefore, agents acting on GABA receptors can be expected to act on neurons that possess few or no synapses at all. This type of mechanism could be involved in the formation of heterotopic masses and other migration disorders such as those produced in pregnant rats leading to the formation of aberrant connections between the hippocampus and the neocortex (Chevassus-Au-Louis *et al.* 1998 and *figure 5; DVD figure 8*). At any rate, they draw our attention to the possible deleterious actions of drugs acting on GABA receptors, notably benzodiazepines or certain anti-epileptic agents. In keeping with this, preliminary observations suggest that some AEDs indeed affect migration (Manent *et al.* in preparation). These actions are of the utmost importance in view of the part played by migration disorders in human epilepsies. A variety of animal models of these disorders exist, some of them such as the one produced by *in utero* injection of an anti-mitotic agent MAM *(figure 5; DVD figure 8)*. Studies using this model show that the ectopic masses present in the hippocampus are, in fact, neocortical neurons that were trapped in the hippocampus because of the delayed migration. Patch recordings from both the "normal" target of these neurons and their host structure shows that they have connections both with the neocortex and the hippocampus, creating thus an aberrant bridge connecting, via one synapse, two brain structures that are not connected normally *(figure 6; DVD figure 9*; Chevassus-Au-Louis *et al.*, 1998). Seizures will readily propagate from the hippocampus to the cortex.

Transmitter uptake systems and early seizures

If transmitters act at this early stage, in a paracrinic manner, then the control of their concentration may play an important role. The concentration of GABA and glutamate in the extracellular space is controlled by a variety of transporters. We therefore tested the effects of blockers of the main transporters on neuronal activity at an early developmental stage E18 to P0 *(DVD figure 10)*. We found that GABA transporters are not active at birth – blockers of this transport do not alter currents generated by GABA. This will allow GABA to diffuse and act on distal sites *(DVD figure 11)*. In contrast, glutamate transporters are operative early on, indicating that the control of glutamate concentration is essential at this early stage to prevent possibly excitotoxic actions of glutamate *(DVD figure 12)*. In keeping with this, glutamate transporter blockade generates large oscillations in hippocampal and neocortical networks *(DVD figure 13)*, and *in vivo icv* injections of a glutamate receptor antagonist in pups generates recurrent seizures with suppression bursts that are reminiscent of the encephalopathies (Demarque *et al.* 2004). Thus, deficiencies in glutamate transport could mediate early, severe epilepsies.

Synapse formation

The formation of synapses *(figure 7; DVD figure 14)* is an important step as it signals a more organized, topical mode of communication and the beginning of network formation with its generation of behaviorally relevant patterns. The two principal transmitter systems that operate are GABA and glutamate, acting on receptors permeable to anions, chloride in particular for the former, and cations,

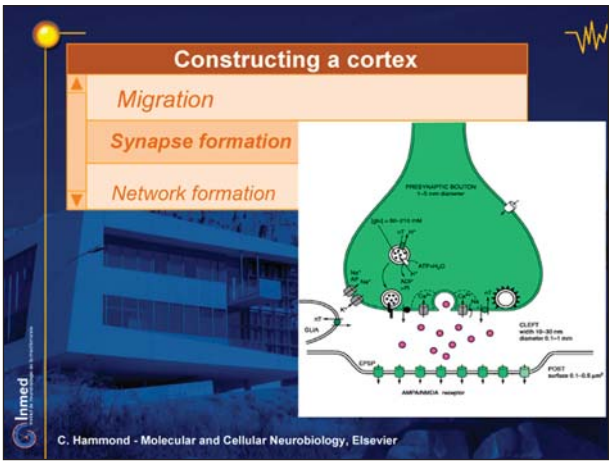


Figure 7. Constructing a cortex.
C. Hammond, *Molecular and Cellular Neurobiology*, Elsevier.

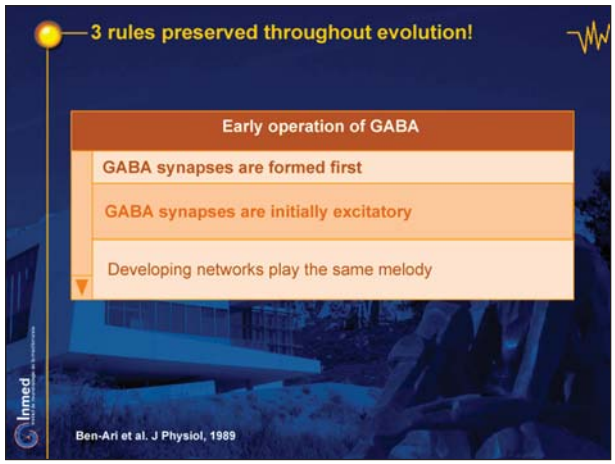


Figure 9. Three rules preserved throughout evolution!
Ben-Ari *et al. J Physiol*, 1989

sodium, potassium and calcium for the latter *(figure 8; DVD figure 15)*. Agents that augment the efficacy of GABAergic synapses are often anti-epileptics and anxiolytics; agents that increase the excitatory drive will induce hyperactivity and cell death *(DVD figure 16)*. This raises the issue of how an equilibrium between these transmitters is reached throughout development *(DVD figure 17)*. This also raises the question of how to develop cortical networks with functional activity at all stages – promote neuronal development and synapse formation yet preserve stability and prevent over excitation that will be excitotoxic, or dominant GABAergic inhibition that will retard developmental functions that are modulated by the increased intracellular calcium that results from the activation of glutamate synapses *(DVD figure 17)*?

In 1989, **three basic developmental rules** *(figure 9; DVD figure 18)* for the maturation of GABA and glutamate systems were unraveled. These present a possible solution to this issue:

i) ***GABAergic synapses precede glutamate synapses*** (*figure 10*; *DVD figure 19*; Ben-Ari 2002; Tyzio *et al.* 1999). In the developing hippocampus, interneurons do not develop hand-in-hand with the pyramidal neurons: GABAergic interneurons and synapses mature first and are operative at a stage when the principal cells are silent (Ben-Ari *et al.* 2004). Interneurons divide prior to the glutamatergic neurons, extend their axons and dendrites prior to the principal cells, establish functional synapses before principal neurons and can generate patterns when principal neurons are essentially inert. We patch-clamp recorded a large sample of CA1 pyramidal neurons at birth and reconstructed them after identifying their PSCs. We found three populations of neurons, silent ones with no PSCs that have few or no dendrites, bigger neurons with

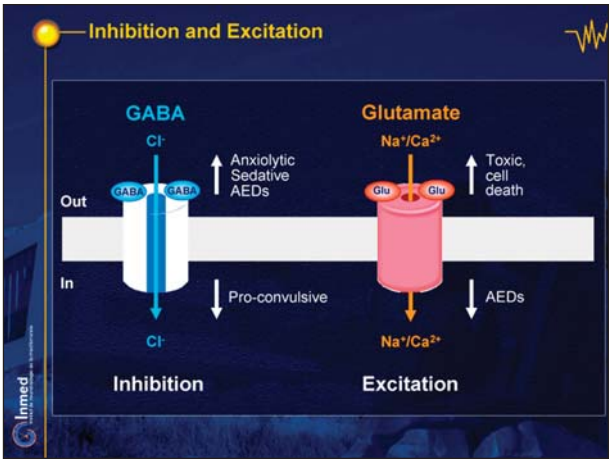


Figure 8. Inhibition and Excitation.

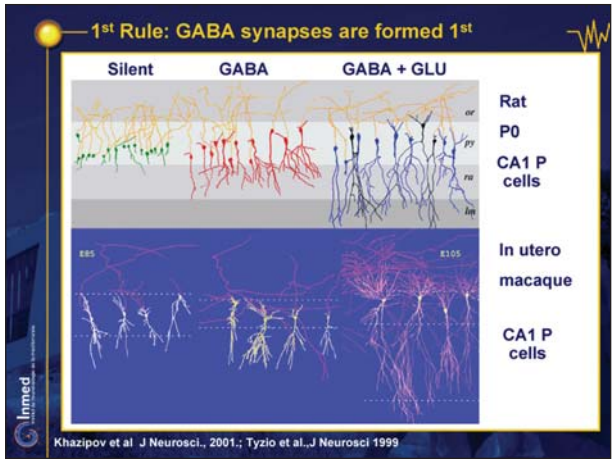


Figure 10. First Rule: GABA synapses are formed first.
Khazipov *et al. J Neurosci* 2001; Tyzio *et al. J Neurosci* 1999.

GABA but no glutamate synapses have a small, apical dendrite, neurons with both GABA and glutamate PSCs have a large, apical dendrite that reaches, for the first time, the distal lacunosum moleculare and a basal dendrite. Therefore, the first synapses are established on the apical dendrites and are GABAergic, neurons must reach the distal *lacunosum moleculare* to have functional glutamate synapses. Since the axons are there already, the critical factor is the degree of maturation of the post-synaptic target: GABAergic synapses are established when axons meet their post-synaptic target, but glutamatergic synapses will not be formed unless the target is more developed. This sequential expression of GABA and glutamate synapses has been confirmed in a wide range of species including primate neurons *in utero* (*figure 10; DVD figure 19*; Tyzio *et al.*, see below,). We patch-clamped CA1 pyramidal neurons in slices obtained from macaque embryos delivered by caesarean section from mid-gestation to two weeks before full-term, and found an earlier formation of GABAergic synapses on pyramidal neurons with a small, apical dendrite and both GABA and glutamate synapses on neurons with extended apical dendrites and basal dendrites. There were no neurons with functional glutamate but no glutamate synapses confirming the earlier formation of GABAergic synapses.

ii) ***GABA inhibits adult neurons but excites immature ones*** *(figure 11; DVD figure 20)*

This is due to a higher concentration of intracellular chloride in immature neurons, which leads to an efflux of chloride when GABA receptors are activated (Ben-Ari *et al.* 1989, Tyzio *et al.* 2003). These gradients have been observed in all species and brain structures studied, indicating that they have been retained throughout evolution (Gao and van den Pol 2001, Owens *et al.* 1999, 2002, Hubner *et al.* 2001). They are also observed in primate neurons *in utero,* and are thus valid for human fetuses and preterm babies (Khazipov *et al.* 2001). The consequence is that when GABA receptors are activated there is an efflux of chloride instead of the usual influx, and a depolarization that will generate action potentials, remove the voltage-dependent magnesium block from NMDA channels leading to an influx of calcium (Ben-Ari *et al.* 1997). This early action of GABA reverses subsequently, with the delayed activation of a chloride co-transporter that exports chloride from the inside to the outside and install the adult concentration of chloride (*figure 12; DVD figure 21*; Rivera *et al.*, 1999). We have suggested elsewhere that this sequence provides a solution to the problem of how to equilibrate, at all developmental stages, excitation and inhibition during brain maturation (*DVD figure 22* and Ben-Ari 2002). Indeed, as the excitatory drive provided by GABAergic synapses is not toxic - as the E rev of GABA is always closer to the resting membrane potential than glutamate (E 50-60 mV as compared to 0 mV). This is also facilitated by the shunting actions of GABAergic synapses that inhibit overexcitation. Thus, GABA will provide a sufficient degree of excitatory drive to raise intracellular calcium via the activation of NMDA channels or voltage-dependent calcium channels, and thus to modulate developmental processes without danger. Also, the longer kinetics of GABAergic as compared to glutamate AMPA receptor-mediated PSCs enables an accumulation of synaptic current in neurons that possess but few synapses. Therefore, during early stages, when the glutamatergic drive is limited, GABA "does the job", exciting neurons and raising intracellular calcium. When the density of glutamatergic synapses is sufficient and a strong inhibition is required to prevent seizure generation, GABA assumes its adult actions following the expression of a transporter that will reduce the intracellular concentration of chloride. This model provides a rational solution to the issue

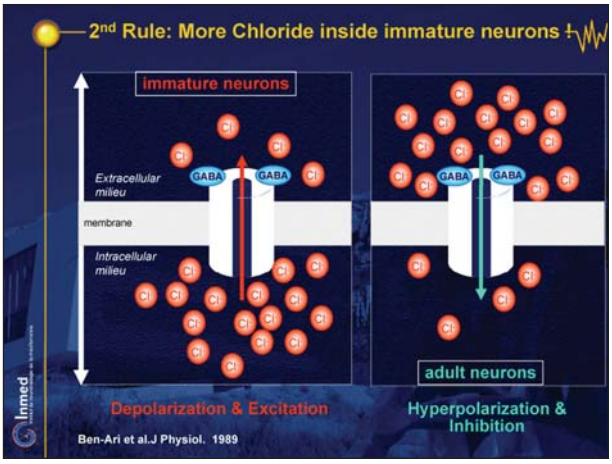


Figure 11. Second Rule: More Chloride inside immature neurons! Ben-Ari *et al. J Physiol* 1989.

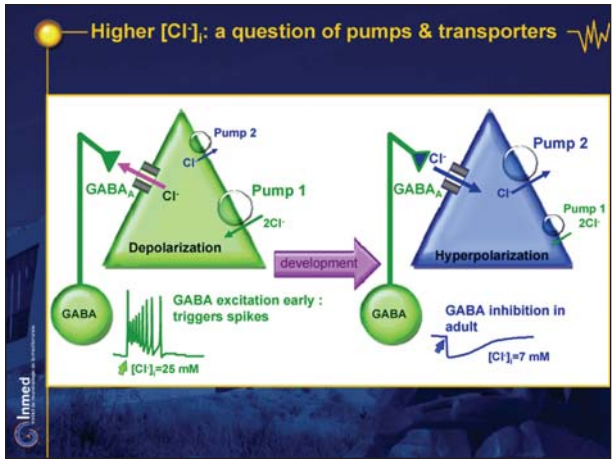


Figure 12. Higher $[Cl^-]_i$: a question of pumps & transporters.

raised above (*DVD figure 22*, Ben-Ari 2002). If GABAergic synapses are formed first, GABAergic interneurons should also develop at an early stage. In the developing hippocampus, interneurons do not develop hand-in-hand with the pyramidal neurons: GABAergic interneurons and synapses mature first and are operative at a stage when the principal cells are silent (Hennou *et al.* 2002, Ben-Ari *et al.* 2004). Interneurons divide prior to glutamatergic neurons, extend their axons and dendrites prior to the principal cells, establish functional synapses before principal neurons and can generate patterns when principal neurons are essentially inert. Most interestingly, the rules for establishing functional GABA or glutamate synapses between an axon and its dendritic target are not identical: GABA synapses are established once the contact is established, whereas glutamate synapses can only be formed when the target neuron has reached a certain degree of maturation. Therefore, in spite of a rather long journey – interneurons are not generated in the close vicinity of the principal neurons, but in the distal ganglionic eminence – interneurons must, for some reason, be at work at an early stage. In parallel studies, we found that already *in utero*, patterns generated exclusively by interneurons can be recorded (Ben-Ari *et al.*, 2004).

iii) ***Developing networks speak only one language*** *(figures 13, 14; DVD figures 23-24)*

We discovered some time ago that the developing hippocampal network has a single pattern – which we referred to as giant depolarising potentials (GDPs) (Ben-Ari *et al.* 1989; Ben-Ari 2001). This pattern is characterised by recurrent bursts with large, polysynaptic currents mediated by GABA and glutamate receptors. Subsequent studies revealed that a similar pattern is present in all developing networks, in all species including the developing primate hippocampus *in utero*. GDPs provide almost all the activity as long as GABAergic synapses are excitatory. The shift from excitatory to inhibitory GABA signals the end of GDPs and the expression of more adult patterns; it is at that stage that a network is sufficiently complex that it can generate behaviorally relevant patterns. GDPs are generated by the combined action of excitatory GABA synapses and large NMDA receptor-mediated synaptic currents. The latter are of particularly long duration in the immature brain consequent to the presence of a subunit of NMDA receptors that confer to neurons very long kinetics. The synergistic action of GABA and NMDA receptors is the core of this pattern, the small depolarization produced by GABA is sufficient to remove the blockade from NMDA receptors and generates the long, poly-synaptic currents notably *via* the activation of an excitatory recurrent loop – the activation of interneurons leading, in return, to an enhancement of the excitation on principal cells and the generation of large synaptic currents. GDPs have now been recorded by a plethora of techniques – including cell-attached and whole cell recordings, as well as field and surface recordings – and preparations including slices and also cultures and *in vivo*. In the intact hippocampus *in vitro* – a preparation developed in the laboratory that has major advantages in comparison to slices – GDPs can be generated by a wide range of sites but they propagate along the developmental gradient – from rostral to caudal sites – indicating a developmental activity gradient (Khalilov *et al.* 1997, 2003). GDPs can be considered as a type of a primitive pattern with little information content that exists to signal activity in developing connections and to promote synapse formation and possibly provide early signals for the construction of operating functional units on the basis of neurons that fire together, wire together". A general developmental curve depicting the maturation sequences in primate hippocampal neurons is shown in *figure 15 (DVD figure 25)*. This figure, that provides the most complete curve available today, is a series of measures (Boltzmann equations to depict the temporal matu-

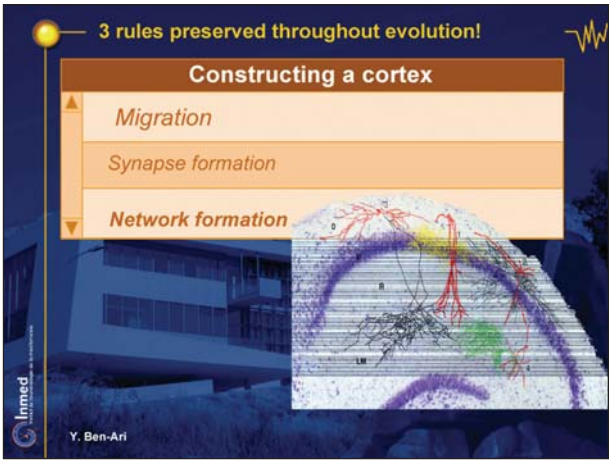


Figure 13. Three rules preserved throughout evolution!

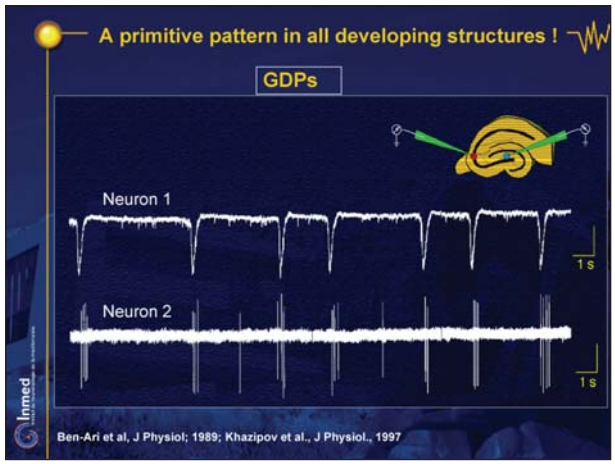


Figure 14. A primitive pattern in all developing structures! Ben-Ari *et al. J Physiol* 1989; Khazipov *et al. J Physiol* 1997.

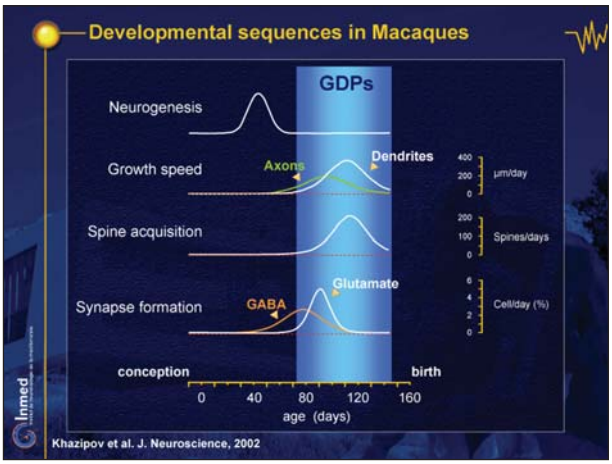


Figure 15. Developmental sequences in Macaques. Khazipov *et al. J Neuroscience* 2002.

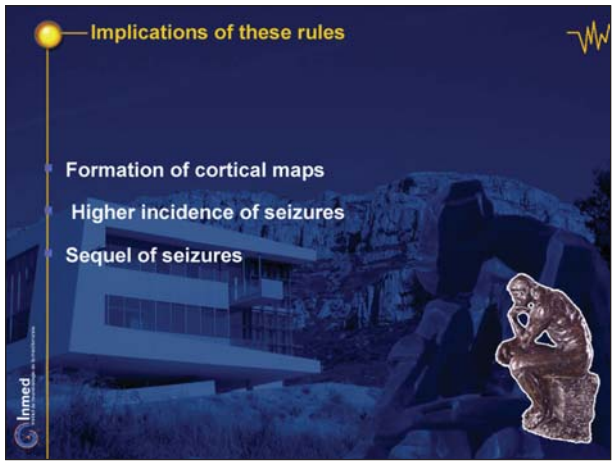


Figure 16. Implications of these rules.

ration of the various functions). Thus, proliferation (taken from the literature) is followed by axon (first) and dendritic extension (then, expressed in µm per day) and spine acquisition. Pyramidal neurons of CA1 have no spines at mid-gestation, but as many as 7000 glutamate synapses a few weeks before delivery. The earlier formation of GABA synapses is described quantitatively in the last curve. GDPs disappear when the density of glutamate synapses is sufficient to generate more complex, behaviorally relevant patterns and GABA assumes its adult actions. An obvious consequence of this early expression of functional GABAergic synapses is that GABA-mimetic agents and anti-epileptic drugs will exert a particularly strong action on the activity-dependent formation of the network (Represa and Ben-Ari 2005). The obvious implications of these gradients are that drugs targeted to a given molecular species will have complex. unexpected actions: they will exert no action on some neurons endowed with different molecular species and act only on that proportion of neurons that have shifted to adult types of molecular species. In addition, the gradient of heterogeneity will depend notably on the species: animals that have an extended period of brain development – such as monkeys and humans– will offer a picture of heterogeneity for an extended period of time thus allowing extrinsic factors to exert a stronger influence than animals in which this period is restricted to a few days.

Implications of these rules

There are several implications and consequences of these rules *(figure 16; DVD figure 26).*

Early activity may participate in the formation of cortical maps

Recordings in chronically implanted pups revealed the presence of sharp waves and tails that are reminiscent of GDPs (*figures 17, 18; DVD figures 27-28*; Leinekugel *et al.* 2002; Khazipov *et al.* 2004). These patterns are generated by GABA and glutamatergic synaptic currents as revealed by whole cell recordings in non-anaesthetized, restrained pups. Most importantly, when these events are recorded in both the periphery and the cortical centre – the sensori-motor cortex – the spindles in the periphery precedes those in the center. Lesions of the spinal cord reveal a large reduction of spontaneous spindles suggesting that the peripherally-generated spindles are the generators of those in the cortical sites. These and other similar observations are in line with the concepts that developing circuits in the periphery and centres communicate *via* a single pattern and that peripheral structures can provide a strong source of modulation of the centres. In a wider perspective, these studies raise the possibility that the programming of the construction of central corti-

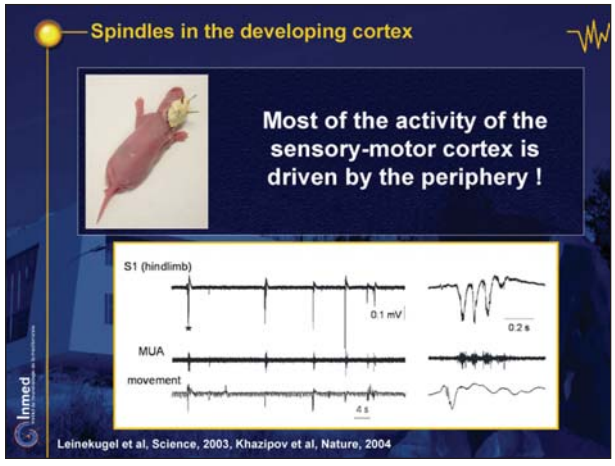


Figure 17. Spindles in the developing cortex. Leinekugel *et al. Science* 2003 ; Khazipov *et al. Nature* 2004.

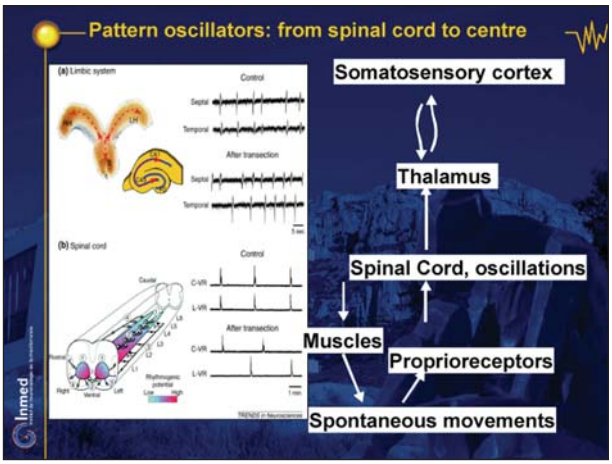


Figure 18. Pattern oscillators: from spinal cord to centre.

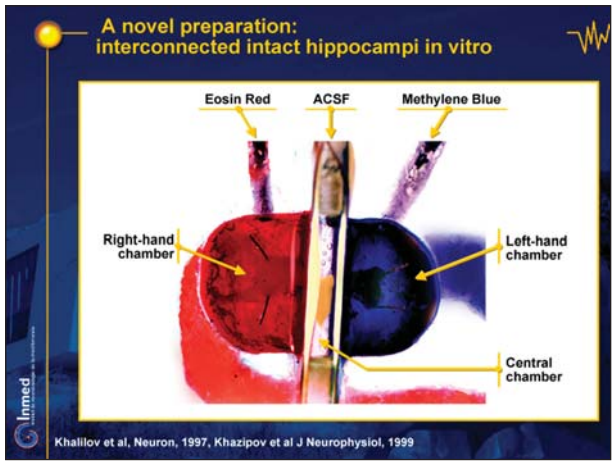


Figure 20. A novel preparation: interconnected intact hippocampi *in vitro*. Khalilov *et al. Neuron* 1997; Khazipov *et al. J Neurophysiol* 1999.

cal organization, including cortical maps and other functional entities, are controlled or at least modulated by peripheral activity. In view of this, a unified pattern of activity in developing structures is perfectly able to contribute to the wiring of distant structures. The alterations produced by aberrant activities such as seizures would produce their deleterious sequelae merely by interrupting these patterns rather than by more specific information-containing signals.

Impact on early seizures

Immature brains have a higher incidence of seizures early on. This is, at least in part, due to the late maturation of a potent inhibitory GABAergic system and the early excitatory actions (*figure 19; DVD figure 29*; Holmes and Ben-Ari 1998). The peak seizure generation in the developing hippocampus (bottom curve) is attained when GABAergic excitation shifts to a dominant inhibition around P 10), glutamate excitatory drive steadily increases, and the importance of GDPs declines, being replaced by more sophisticated patterns that require a relatively elaborated network to function. In keeping with this, seizures are readily generated around the last trimester in primate hippocampal neurons *in utero (DVD figure 30)*.

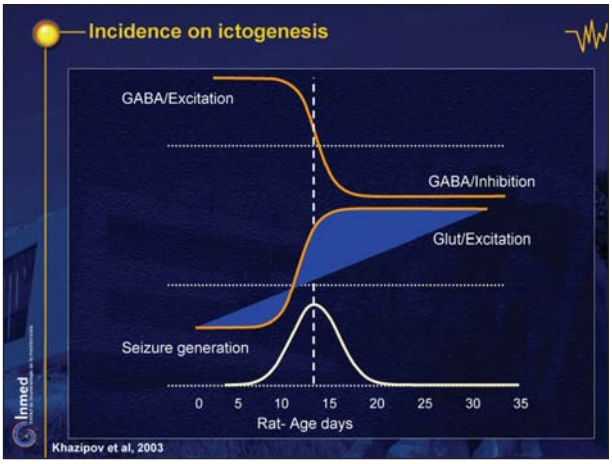


Figure 19. Incidence on ictogenesis. Khazipov *et al,* 2003.

Seizures can lead to long lasting consequences

In vivo studies have documented extensively the possible long-term actions of seizures at an early stage. As shown earlier, seizures such as those produced by administration of kainic acid during the first week of post-natal life are not associated with brain damage (Tremblay *et al.*, 1984). Various convulsive agents lead to long-lasting consequences including persistent changes in neuronal excitability (*DVD figure 31*; Villeneuve *et al.* 2000). One way of mediating these long term actions could be a reduction in the seizure threshold after an episode of severe seizures; In other words, in this model, "seizures would beget seizures" as first suggested by Gower in the early nineties. This concept has however never received direct confirmation. We recently developed a unique preparation in which this property can be tested readily *in vitro* (*figure 20; DVD figure 32*; Khalilov *et al.* 1997, 2003).

The preparation consists of a triple chamber that can accommodate the two intact hippocampi and their connecting commissure in three independent compartments. It is possible to apply a convulsive agent to one hippocampus and allow a given number of seizures to propagate to the other hippocampus. We can then block the connections reversibly and determine whether the hippocampus has been transformed by seizures. We found that a series of brief seizures generated by the kainate in one hippocampus generated seizures that propagated to the other site leading to a transformation of the naive hippocampus to an epileptic one that generates spontaneous and evoked

seizures *(figure 21; DVD figures 33-35)*. A number of recurrent seizures are required for this effect- five to 10. Most interestingly, the epileptogenic mirror focus is truly epileptic as even 48 hours later, it generates spontaneous ictal events, and when slices are prepared from the intact hippocampus, it generates high frequency ictal events: this is to the best of my knowledge, the only example of an ictal event generated in hippocampal slices. In contrast to interictal events that are readily generated in slices – including human slices obtained after resections from epileptic patients- ictal events are not generated.

We therefore used this preparation both to determine what the minimal requirements for the transformation to take place are - what the mechanisms of induction are - and what the mechanisms mediating the expression of the property are? Examining the pattern of oscillations in the propagated seizures, we observed that very high frequency events are present –80 to 120 Hz or more in the kainite-treated hippocampus *(DVD figure 36)*. Initially, the seizures that propagate to the other side generate only low frequency events. After the propagation of several, high frequency events from the other side, the naïve hippocampus also becomes progressively capable of generating high frequency events and this signals that the naïve hippocampus has become epileptogenic *(DVD figure 36)*. If high frequency oscillations are required for "seizures to beget seizures", then conditions that prevent the generation of these oscillations should also prevent the formation of a mirror focus. We found that blocking either GABA or NMDA receptors by applications of selective antagonists exclusively in the naïve side, the seizures still propagate from the other side, but they do not contain high frequency events *(DVD figure 37)*. Applications of kainate to one hemisphere that generate high frequency events in the treated but not the naïve side, fail to transform the naïve side to an epileptic one. In other words, functional GABAergic receptors are required for "seizures to beget seizures". Without these high frequency events, seizures are not epileptogenic, and most likely will not induce long-term consequences on target structures. Interestingly, blocking GABA receptors in the kainite-treated side also led to a blockade of the long-term effect locally: the kainite-treated side had no high frequency oscillations and did not generate seizures after repeated applications; the other side that did not receive kainate but which was stimulated by low frequency recurrent seizures coming from the treated side, did generate high frequency oscillations and became epileptic (Khalilov *et al.* 2005). Low frequency seizures can generate high frequency events in the contralateral side provided that GABA and NMDA receptors are operative there. This transformation of a naïve hippocampus to an epileptic one is thus analogous to a form of long-term plasticity – an alteration of synaptic plasticity, the activation by high frequency currents of GABA receptors leading to the activation of NMDA receptors, and this synergistic action leads to the increased synaptic efficacy.

Interestingly, patching neurons from the epileptic hippocampus, we found that GABA again excites neurons: the seizures induced a long-term transformation of the inhibitory actions of GABA to excitatory, most likely because of a loss of the capacity to remove and regulate chloride *via* co-transporters *(figure 22; DVD figure 38)*. To some extent, epileptogenesis recapitulates ontogenesis (*DVD figure 39*, Khalilov *et al.* 2005, Cohen *et al.* 2002), and seizures re-induce a return to immature neurons with an excitatory GABA – except that now with the high density of glutamate synapses, seizures will be generated. Therefore, the expression of the epileptogenic properties is, in fact, due to a persistent accumulation of chloride, the seizures producing a loss in the efficacy of neurons to remove the excess chloride produced during the seizures.

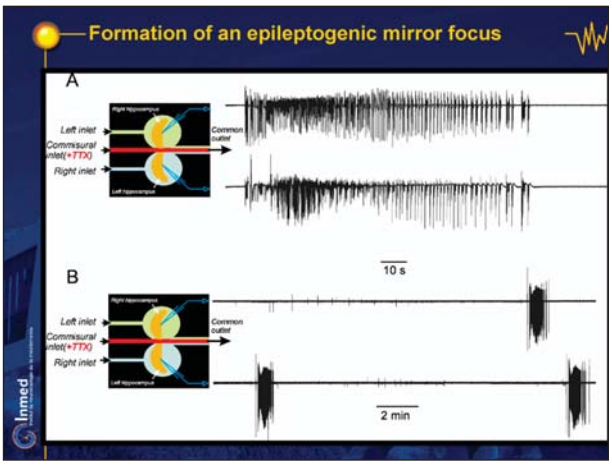


Figure 21. Formation of an epileptogenic mirror focus.

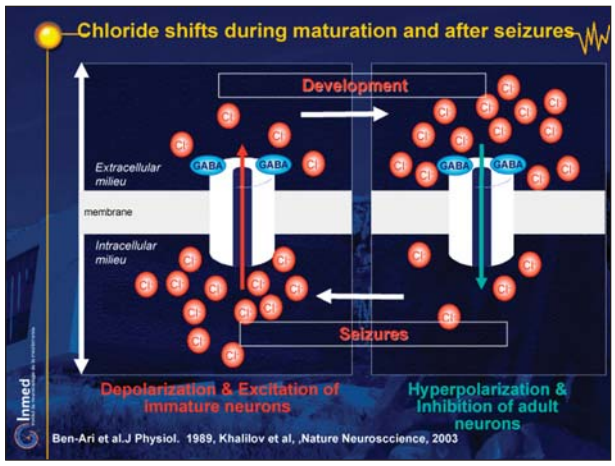


Figure 22. Chloride shifts during maturation and after seizures. Ben-Ari *et al. J Physiol* 1989 ; Khalilov *et al.*, *Nature Neurosccience*, 2003.

This loss of efficacy of the transporters may be more readily triggered by high frequency oscillations.

In conclusion, these observations suggest (*DVD figure 40*) that seizures are not necessarily epileptogenic – they can occur recurrently, without sequelae, if they do not include high frequency oscillations that require both operative GABA and NMDA receptors. The most likely explanation for this requirement is that the excitatory drive provided by GABA is required at this stage as the density of glutamate synapses may not suffice: a small but decisive activation of excitatory GABA, activates NMDA receptors by removing the voltage-dependent magnesium blockade leading to a large calcium influx, and a sequence of events that leads to a permanent consequence. This observation suggests that it may be possible to evaluate the epileptogenic capacity of seizures in children by evaluating their intrinsic, dominating, oscillatory frequencies.

GABA is both a pioneer and an essential actor in early development. It is operative first; it excites neurons at a time when neurons have few synapses, and it has a paracrine action on immature neurons, modulating neuronal migration. Then it acts in coordination with NMDA receptors stimulating developing networks intervening in the construction of cortical networks and exerting a wide range of trophic actions. Clearly, because of this plethora of actions and its unique properties, this transmitter is able to act very differently according to the conditions. It seems ideally suited to the job of regulating the complex functional sequences involved in brain development. And last but not least, GABA is greatly involved in early seizures, both in their induction and in their long-lasting consequences: seizures do not transform a naïve network to one that seizes unless GABA synapses are operative. Clearly, the effects of GABA-acting drugs on developing brains must be re-evaluated in the light of this information. □

References

Ben-Ari Y. Excitatory actions of GABA during development: the nature of the nurture. *Nat Rev Neurosci* 2002; 3: 728-39.

Ben-Ari Y, Cherubini E, Corradetti R, Gaiarsa JL. Giant synaptic potentials in immature rat CA3 hippocampal neurones. *J Physiol* 1989; 416: 303-25.

Ben-Ari Y, Khalilov I, Represa A, *et al.* Interneurons set the tune of developing networks. *Trends Neurosci* 2004; 27: 422-7.

Ben-Ari Y, Khazipov R, Caillard O, *et al.* GABAA, NMDA and AMPA receptors: a developmentally regulated "ménage à trois". *Trends Neurosci* 1997; 20: 523-9.

Ben-Ari Y. Developing networks play a similar melody. *Trends Neurosci* 2001; 24: 353-60.

Chevassus-Au-Louis N, Congar P, Represa A, Ben-Ari Y, Gaiarsa JL. Neuronal migration disorders: heterotopic neocortical neurons in CA1 provide a bridge between the hippocampus and the neocortex. *Proc Natl Acad Sci USA* 1998; 95: 10263-8.

Cohen I, Navarro V, Clemenceau S, *et al.* On the origin of interictal activity in human temporal lobe epilepsy *in vitro*. *Science* 2002; 298: 1418-21.

Demarque M, Represa A, Becq H, *et al.* Paracrine intercellular communication by a Ca2+- and SNARE-independent release of GABA and glutamate prior to synapse formation. *Neuron* 2002; 36: 1051-61.

Demarque M, Villeneuve N, Manent JB, Becq H, Represa A, Ben-Ari Y, Anikstejn L, *et al.* Glutamate transporters prevent the generation of seizures in the developing rat neocortex. *J Neurosci* 2004; 24: 3289-94.

Freund T, Buszaki G. Interneurons of the hippocampus. *Hippocampus* 1996; 6: 347-470.

Gao XB, van den Pol AN. GABA, not glutamate, a primary transmitter driving action potentials in developing hypothalamic neurons. *J Neurophysiol* 2001; 85: 425-34.

Hennou S, Khalilov I, Diabira D, Ben-Ari Y, Gozlan H. Early sequential formation of functional GABA(A) and glutamatergic synapses on CA1 interneurons of the rat foetal hippocampus. *Eur J Neurosci* 2002; 16: 197-208.

Holmes GL, Ben-Ari Y. Seizures in the developing brain: perhaps not so benign after all. *Neuron* 1998; 21: 1231-4.

Hubner CA, Stein V, Hermans-Borgmeyer I, *et al.* Disruption of KCC2 reveals an essential role of K-CI transport already in early synaptic inhibition. *Neuron* 2001; 30: 515-24.

Khalilov I, Esclapez M, Medina I, *et al.* A novel *in vitro* preparation: the intact hippocampal formation. *Neuron* 1997; 19: 743-9.

Khalilov I, Holmes GL, Ben-Ari Y. *In vitro* formation of a secondary epiletogenic mirror focus by interhippocampal propagation of seizures. *Nat Neurosci* 2003; 6: 1079-85.

Khalilov I, Le Van Quyen M, Gozlan H, Ben-Ari Y. Epileptogenic actions of GABA and fast oscillations in the developing hippocampus. *Neuron* 2005; 48: 787-96.

Khazipov R, Esclapez M, Caillard O, *et al.* Early development of neuronal activity in the primate hippocampus *in utero*. *J Neurosci* 2001; 21: 9770-81.

Khazipov R, Sirota A, Leinekugel X, *et al.* Early motor activity drives spindle bursts in the developing somatosensory cortex. *Nature* 2004; 432: 758-61.

Leinekugel X, Khazipov R, Cannon R, *et al.* Correlated bursts of activity in the neonatal hippocampus *in vivo*. *Science* 2002; 296: 2049-52.

Manent JB, Demarque M, Jorquera I, Pellegrino C, Ben-Ari Y, Anikstejn L, Represa A. A noncanonical release of GABA and glutamate modulates neuronal migration. *J Neurosci* 2005; 25: 4755-65.

Owens DF, Kriegstein AR. Is there more to GABA than synaptic inhibition? *Nat Rev Neurosci* 2002; 3: 715-27.

Owens DF, Liu X, Kriegstein AR. Changing properties of GABA(A) receptor-mediated signaling during early neocortical development. *J Neurophysiol* 1999; 82: 570-83.

Represa A, Ben-Ari Y. Trophic actions of GABA in neuronal development. *Trends Neurosci* 2005; 28: 278-83.

Rivera C, Voipio J, Payne JA, *et al.* The k+/Cl- co-transporter KCC2 renders GABA hyperpolarizing during neuronal maturation. *Nature* 1999; 397: 251-5.

Tremblay E, Nitecka L, Berger ML, Ben-Ari Y. Maturation of kainic acid seizure-brain damage syndrome in the rat. I. Clinical, electrographic and metabolic observations. *Neuroscience* 1984; 13: 1051-72.

Tyzio R, Ivanov A, Bernard C, *et al*. Membrane potential of CA3 hippocampal pyramidal cells during postnatal development. *J Neurophysiol* 2003; 90: 2964-72.

Tyzio R, Represa A, Jorquera I, *et al*. The establishment of GABAergic and glutamatergic synapses on CA1 pyramidal neurons is sequential and correlates with the development of the apical dendrite. *J Neurosci* 1999; 19: 10372-82.

Villeneuve N, Ben-Ari Y, Holmes GL, Gaiarsa JL. Neonatal seizures induced persistent changes in intrinsic properties of CA1 rat hippocampal cells. *Ann Neurol* 2000; 47: 729-38.