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Living or dying in three quarter time: Neonatal orchestration of hippocampal cell death pathways by androgens and excitatory GABA

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Abstract

In the immature brain, GABA (γ -aminobutyric acid) is an excitatory neurotransmitter. With development, GABA becomes inhibitory due to the changes in the expression level of chloride transporters, leading to a negative shift in chloride ions. Clinical evidence and studies in animal models indicate greater brain damage in male versus female neonates following a variety of neuronal insults. The recent finding of Nunez and McCarthy suggest a mechanism whereby androgens may endanger male neurons to the possible excitotoxic effects of excitatory GABA in neonates. Given our growing understanding of the excitatory immature GABAergic system and how sex, age and hormone milieu can influence this system, such studies might pave a path for novel clinical treatments to alleviate neonatal risk for brain injury.

Keywords

rat; male; female; γ 2 GABA subunit; cell death; hippocampus

Introduction

The dogmatic understanding of gamma-aminobutyric acid (GABA) is that it is the primary inhibitory neurotransmitter in the adult brain (Ben-Ari et al., 2007). However, since the early studies of the Ben-Ari lab showing endogenous depolarizing currents in neonatal hippocampal neurons (Ben-Ari et al., 1989) that could be traced to GABA (Cherubini et al., 1990), increasing evidence has demonstrated that GABA is excitatory in the developing hippocampus and other brain regions (Mueller et al., 1983; Janigro and Schwartzkroin, 1988; Ben-Ari et al., 1989; Chesnut and Swann, 1989; Cherubini et al., 1990; Luhmann and Prince, 1991). Indeed, an excitatory role for GABA in the developing hippocampus, a brain circuit prone to seizures, is of clinical concern. In the recent article by Nunez and McCarthy, the authors present experimental evidence suggesting that GABA could be, not only excitatory in the developing hippocampus, but excitotoxic as well (Nunez and McCarthy, 2008). Furthermore, the excitotoxic actions of GABA in developing brain are dependent on the sex and hormonal milieu of the animal. This mini-review will examine some of the previous findings on the excitatory

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role of GABA in the developing brain and the possible clinical significance of hormone and sex-specific differences in the system.

GABA is Excitatory in the Developing Brain

There is a fundamental age-dependent difference in the neuronal response to GABA. In the majority of mature hippocampal neurons, GABA is hyperpolarizing (Baker and Kratky, 1967; Fonnum and Storm-Mathisen, 1969; Curtis et al., 1970). In contrast, in immature hippocampal neurons, GABA mediated responses are depolarizing/excitatory (Chesnut and Swann, 1989; Ben-Ari et al., 1990; Cherubini et al., 1990). Unlike most adult neurons, immature neurons maintain relatively high intracellular chloride concentrations ($[Cl^-]_i$), resulting in membrane depolarization following GABA_A receptor activation. This process has been observed in a wide range of neuron types from different brain regions and animal species (Dudel and Kuffler, 1961; Eccles, 1964; Eccles et al., 1966; Gerschenfeld, 1973; Bowery et al., 1975; Schwartzkroin and Altschuler, 1977; Dunwiddie, 1981; Schwartzkroin, 1981; Harris and Teyler, 1983; Leinekugel et al., 1995; LoTurco et al., 1995; Obrietan and van den Pol, 1995; Ye et al., 2004).

A characteristic of mature neurons is that activation of GABA_A receptors results in an inward rectifying Cl^- gradient that hyperpolarizes the neurons. However, in immature neurons, GABA_A receptor activation causes an efflux of Cl^- , resulting in a depolarizing response that could trigger an action potential upon reaching threshold. Such a depolarization can further lead to a dramatic and rapid increase in the intracellular calcium concentration $[Ca^{2+}]_i$, which can be completely blocked by the GABA_A antagonist bicuculline. The $[Ca^{2+}]_i$ rise can also be blocked by cadmium and nimodipine, indicating that the mechanism of Ca^{2+} increase is primarily via L-type voltage operated Ca^{2+} channels (Obrietan and van den Pol, 1995). In addition, as in the study by Nunez and McCarthy, the use of $[Ca^{2+}]_i$ imaging techniques demonstrate that all immature neurons examined thus far show a depolarizing response to GABA (Obata et al., 1978; Alger and Nicoll, 1979; Andersen et al., 1980; Thalmann et al., 1981; Alger and Nicoll, 1982; Connor et al., 1987; Yuste and Katz, 1991; Lin et al., 1994; Leinekugel et al., 1995; Obrietan and van den Pol, 1995; Garaschuk et al., 1998; Kulik et al., 2000; Kuner and Augustine, 2000).

What might be the developmental mechanism that allows GABA to switch from being excitatory to inhibitory in a nearly ubiquitous fashion? An excitatory to inhibitory switch was first suggested by Obata et al. (1978), who examined GABA effects in spinal neurons. Applications of GABA depolarized 6-day-old chick spinal neurons in culture and hyperpolarized neurons from 10-day-olds (Obata et al., 1978). Schwartzkroin and colleagues followed with reports of GABA depolarizing responses in neonatal kitten and rabbit hippocampal CA1 pyramidal neurons which diminished in 2 to 4 week-old animals (Schwartzkroin and Altschuler, 1977; Dunwiddie, 1981; Schwartzkroin, 1981; Harris and Teyler, 1983).

The higher $[Cl^-]_i$ of immature neurons leads to excitatory actions of GABA in immature neurons (Ben-Ari and Holmes, 2005) as Cl^- flows down its concentration gradient in response to GABA_A receptor activation and the opening of the Cl^- channel. As the neuron matures there is a reduction of $[Cl^-]_i$ relative to the outside of the neuron leading to an inhibitory response to GABA (Kuner and Augustine, 2000). The change in $[Cl^-]_i$ has been shown in every animal species and brain structure investigated to date, suggesting that the excitatory-inhibitory switch has been preserved through evolution (Andersen et al., 1980; Thalmann et al., 1981; Alger and Nicoll, 1982; Kuner and Augustine, 2000). The maturational change in $[Cl^-]_i$ is thought to be primarily controlled by two cation-chloride cotransporters. The Cl^- ion cotransporters, Na-K-2Cl 1 (NKCC1) and K-Cl 2 (KCC2), have been shown to change during neuronal

development and have been implicated in the GABA switch from excitatory to inhibitory (Plotkin et al., 1997; Delpire, 2000; Delpire and Mount, 2002; Payne et al., 2003; Mercado et al., 2004; Gamba, 2005).

The membrane transport protein, NKCC1, controls Cl^- uptake across the plasma membrane internalizing one Na^+ , one K^+ , and two Cl^- (Payne et al., 2003). As a result, NKCC1 transports Cl^- into cells so that the Cl^- equilibrium is more positive than the resting membrane potential, resulting in a GABA-evoked depolarization as Cl^- flows out along the concentration gradient. NKCC1 has been found to be expressed at high levels in immature neurons and decreases during development (Rohrbough and Spitzer, 1996; Plotkin et al., 1997; Fukuda et al., 1998; Sung et al., 2000; Delpire and Mount, 2002; Li et al., 2002; Mikawa et al., 2002; Wang et al., 2002; Payne et al., 2003; Yamada et al., 2004; Dzhalal et al., 2005).

The chloride-transporter, KCC2 is the principal transporter of Cl^- out of neurons. KCC2 extrudes K^+ and Cl^- using the electrochemical gradient for K^+ . As a result, the cell's $[\text{Cl}^-]$ is lower than electrochemical potential equilibrium, due to an active Cl^- transport mechanism that is responsible for extruding Cl^- from the neuron (Misgeld et al., 1986; Thompson et al., 1988). Cl^- extrusion is weak in developing neurons but increases as the cell matures (Misgeld et al., 1986; Luhmann and Prince, 1991; Zhang et al., 1991; Clayton et al., 1998; Jarolimek et al., 1999; Lu et al., 1999; Rivera et al., 1999; Wang et al., 2002; Khirug et al., 2005). Thus, as demonstrated by Rivera et al (1999) the excitatory actions of GABA decrease during development as the expression of KCC2 mRNA increases (Rivera et al., 1999). Using an antisense oligodeoxynucleotide approach to reduce the amount of KCC2 in neurons, these investigators went on to show that there was a corresponding increase in the excitatory GABA response. Correspondingly, over-expressing KCC2 in immature neurons reduces GABA-elicited calcium responses *in vitro* (Stein et al., 2004; Chudotvorova et al., 2005; Lee et al., 2005). Taken together, the results of these studies indicate that the developmental expression of KCC2 is pivotal for development of inhibitory GABA responses.

Indeed, the inward Cl^- transporter NKCC1 and Cl^- extruding transporter KCC2 mRNA and protein levels are inversely expressed during development. NKCC1 expression is highest in early in development and relatively low in mature neurons. In contrast, KCC2 levels are significantly lower during the first two postnatal weeks when compared to adulthood (Yamada et al., 2004; Dzhalal et al., 2005). In sum, these observations provide a molecular mechanism for the shift from GABA mediated excitation to inhibition in developing neurons.

Chloride Gradients are Influenced by Numerous Factors

The timing of the shift in Cl^- gradients depends, not only on maturation, but also on the species, sex, brain structures, activity, and neuronal type (see (Ben-Ari et al., 2007) for review). Since postnatal developmental is a period associated with seizure susceptibility, such sex differences may have important physiological and clinical relevance. The exact trigger underlying the change in Cl^- gradients and the excitatory to inhibitory GABA switch remains controversial. The switch may in part be dependent on activation of GABA_A receptors themselves in an autoregulatory fashion (Ganguly et al., 2001; Fiumelli et al., 2005; Ouardouz and Sastry, 2005) or perhaps a more general repeated neuronal activation might be sufficient. Accumulating data also suggests a role for growth factors and tyrosine kinases in the switch (Kelsch et al., 2001; Aguado et al., 2003; Rivera et al., 2004). However, it is apparent that although there is evidence for multiple mechanisms, they all appear to be time and cell type specific events (Curtis et al., 1970; Dunwiddie, 1981; Schwartzkroin, 1981; Harris and Teyler, 1983; Connor et al., 1987; Ben-Ari et al., 1989; Swann et al., 1989; Luhmann and Prince, 1991; Lin et al., 1994; Feleder et al., 1996; Mitsushima and Kimura, 1997; Han et al., 2002; Moenter and DeFazio, 2005; Dayanithi et al., 2006).

Hormones and Sex can Affect Excitatory GABA Actions

One of the more dramatic findings of a factor influencing the excitatory-inhibitory GABA switch is that shown for the hormone oxytocin during parturition (Tyzio et al., 2006). Oxytocin is a maternal hormone of pregnancy whose release helps trigger labor. Tyzio et al. (2006) have shown that, in the rat, GABA caused a depolarization of hippocampal neurons from animals taken *in utero* and a few days after delivery, but when sampled shortly before, during, and after delivery, there was a dramatic and transient fall in $[Cl^-]_i$ and a corresponding shift in the response to GABA from excitatory to inhibitory and back again. The rapid and transient shift was traced to elevations in circulating oxytocin. Not only did oxytocin produce the shift, but also the administration of the oxytocin receptor antagonist, atosiban, to the dam blocked the shift in fetal neurons. Moreover, administration of the NKCC blocker, bumetanide, overcame the actions of the oxytocin receptor antagonists, suggesting that oxytocin works to rapidly reduce NKCC1 activity leading to a reduction in $[Cl^-]_i$. These results indicate that along with inducing uterine contraction, oxytocin may be a functional neuroprotective agent in the fetus by blocking the potentially excitotoxic effects of GABA during the stressful passage through the birth canal. Indeed, blocking oxytocin receptors in pregnant rats aggravated the effects of hypoxic episodes in fetal neurons. Given such intriguing results, further studies exploring this phenomenon are needed to demonstrate the physiological relevance of the developmental shift of GABA actions (Tyzio et al., 2006).

Gonadal steroid hormones have also been shown to play a role in the timing of the of excitatory-inhibitory GABA switch. Kyrozis et al (2006) has demonstrated that neurons from the female rat switch from excitatory to inhibitory before those of males (Kyrozis et al., 2006). In addition, Giorgi et al (2007) recently showed that the GABA_A receptor agonist muscimol had convulsant effects on juvenile male but not female rats tested on postnatal day 15. Moreover, the authors went on to demonstrate that exposure of female rats to androgens during postnatal days 0–2 was sufficient to produce convulsant responses to muscimol at postnatal day 15 (Giorgi et al., 2007); in essence, a sex-reversal of the sex difference in muscimol responsiveness previously noted. These findings suggest that the endogenous increase in circulating androgens that occurs shortly after birth in male rats prolongs the excitatory actions of GABA.

Given the evidence showing a sex or hormone-dependent difference in the timing of neuronal maturation as related to the GABA excitatory-inhibitory switch; the recent articles by Nunez and McCarthy have demonstrated that the degree of GABA_A dependent excitation and the resulting potential for hippocampal excitotoxicity is sex-specific and gonadal steroid hormone modulated (Nunez et al., 2003b; Nunez and McCarthy, 2003, 2004, 2008). In their studies, Nunez and McCarthy demonstrate that, both *in vitro* and *in vivo*, hippocampal neurons of male rats are more susceptible to the damaging effects of a GABA agonist. In addition, the exposure of female neurons to the potent, non-aromatizable androgen, dihydrotestosterone (DHT), allowed GABA induced excitotoxicity similar to that identified in the male. Importantly, all of these effects could be blocked by the co-administration of the androgen receptor antagonist, flutamide, implicating androgen receptor activation as underlying the increased susceptibility to neonatal GABA excitotoxicity. These findings are of particular importance because early in the period where the developing brain will elicit excitatory responses to GABA, there is a rise in androgen levels.

Circulating Androgen Levels Increase Transiently During Development

In humans, the gonad of the male fetus begins secreting testosterone around the 8th week *in utero*. During the 2nd half of fetal life, negative feedback becomes gradually established and this appears earlier in male fetuses than in females. During the perinatal phase, there is a further increase in circulating testosterone over the 1st 6 months of life (Forest et al., 1976). Circulating

androgens, both testosterone and its primary metabolite, DHT, are synthesized and released from the testes during the perinatal period (Sokka and Huhtaniemi, 1995). In contrast to humans, rodents have a slightly different timeline for hormone secretion, but there are similar transient rises in circulating androgens. Testosterone is produced in the Leydig cells starting at E15 of a 22-day gestation period in the rat (El-Gehani et al., 1998). The testosterone level in testes rises slowly from E15–17, with further increases from E18–21. A surge in circulating testosterone is evident during the period of E16–18 in the rat (Weisz and Ward, 1980). Furthermore, in the male rat, a dramatic increase in serum testosterone occurs during the first four hours of postnatal life (Dohler and Wuttke, 1974; Corbier et al., 1983).

The marked increase in testosterone concentrations in perinatal life and the high levels of androgen receptor in hippocampal pyramidal neurons make the hippocampus a obvious target for the effects of androgen postnatally (Kerr et al., 1995; Xiao and Jordan, 2002). One beneficial effect of androgen sensitivity of the developing hippocampus may be to promote spatial learning ability later in life (Isgor and Sengelaub, 1998, 2003). However, the potential for increased spatial learning capacity due to high androgen levels must be tempered by the possible greater endangerment of hippocampal neurons to the excitotoxic effects of GABA. Thus, increases in adult cognitive function could come at a high cost. The carefully choreographed interactions between androgens and excitatory GABA necessary for proper cognitive function could cause an increased susceptibility of the male brain/hippocampus for damage during a developmental period when this same structure is prone to experience a multitude of insults.

In Nunez and McCarthy (2008), the authors implicate the GABA_A receptor $\gamma 2$ subunit as responsible for the androgen dependent increase in GABA excitation (fig. 1). Early in development, GABA_A receptors in hippocampal neurons have low levels of $\gamma 2$ subunit, a subunit that promotes rapid, longer-lasting inactivation (Hutcheon et al., 2000; Bianchi and Macdonald, 2002). Therefore, the higher proportion of receptors that carry this subunit increase the likelihood that there would be a reduction in a GABA response follow an initial excitation (desensitization) (Bianchi et al., 2001). Indeed, Nunez and McCarthy demonstrate that androgen receptor activation results in a down regulation of the $\gamma 2$ subunit and as a consequence, neurons fail to attenuate or desensitize after repeated exposures to the GABA_A receptor agonist muscimol. The end result is cells with enhanced vulnerability to GABAergic excitotoxicity. Taken together, the high expression of the androgen receptor, the rapid rise in androgens in males during early development, the clinical significance for cell loss, and the propensity of the hippocampus to be at the epicenter of seizures makes this brain region an obvious area of study. Nonetheless, such findings may not be specific for the hippocampus. Pibiri et al (2006) have demonstrated a similar androgen dependent down regulation of the $\gamma 2$ subunit can occur in the female cortex, suggesting that the findings reported in the Nunez and McCarthy article might have wide spread implications on how life or death decisions could be imposed upon developing neurons throughout the brain (Pibiri et al., 2006). The ability of neurons to survive, not only following a pathological brain insult, but also during normal developmental processes, is a waltz that appears to be critically orchestrated by gonadal steroid hormones and the sex of the brain in question.

Are there Clinical Implications for Excitotoxic GABA?

GABAergic synapses are the first formed, and during early development GABA is the primary excitatory factor in the brain (Tyzio et al., 1999). The immature brain is prone to seizure, and there are important age-dependent differences in the efficacy of GABA_A receptor acting anticonvulsants. In children, the incidence of seizures is highest early in life, particularly during the first few postnatal months (Hauser, 1992b, a). A large number of pathological processes could lead to seizures including inborn errors of metabolism, genetic factors, hypoxic-ischemic

insults or congenital brain abnormalities. Excessive GABA excitation is central to the pathogenesis of damage following hypoxia–ischemia, (Andine et al., 1991; Nunez et al., 2003a, b) seizures (Dzhala et al., 2005; Khalilov et al., 2005), fetal alcohol exposure (Galindo et al., 2005) and early anesthetic exposure (Young et al., 2005). Critical periods of seizure susceptibility have also been documented in animal models. Thus, in the postnatal rat hippocampus, there is a bell-shaped age-dependence of susceptibility to various epileptogenic agents and conditions including kainic acid exposure (Albala et al., 1984; Tremblay et al., 1984; Khalilov et al., 1999), electrical stimulation (Moshe et al., 1981), hypoxia (Jensen et al., 1991), and fever (Holtzman et al., 1981; Baram et al., 1997). The developmental changes in GABAergic responses could help explain the higher incidence of seizures of immature neurons (Baram and Hatalski, 1998; Holmes and Ben-Ari, 1998; Swann and Hablitz, 2000; Holmes et al., 2002)). In the adult brain, the hyperpolarizing and inhibitory actions of GABA may prevent the generation and propagation of seizure activity (Miles and Wong, 1987; Freund and Buzsaki, 1996), and many antiepileptic drugs act by enhancing GABAergic transmission. In contrast, the depolarizing actions of GABA enhance excitability and excitotoxicity in the immature brain. Thus, care should be taken with GABA-enhancing drugs which would have a limited anticonvulsive result or even a proconvulsive action in neonates, whereas GABA_A receptor antagonists might be useful to exert anticonvulsive effects in several seizure models (Dzhala and Staley, 2003; Khalilov et al., 2003; Khazipov et al., 2004; Dzhala et al., 2005; Khalilov et al., 2005). Furthermore, given the results from Nunez and McCarthy, a patient's sex and age are also critical variables for consideration of treatment type.

Both clinically and in animal models, newborn males are more sensitive to brain injury. They suffer greater damage than females to insults of the same magnitude and often experience more deleterious outcomes (Hindmarsh et al., 2000; Lauterbach et al., 2001; Reiss, 2004; Johnston and Hagberg, 2007). Given that repeated muscimol exposure induces excitotoxic cell death of hippocampal neurons *in vivo* and *in vitro* due to excessive calcium influx (Nunez and McCarthy, 2008), the results of the studies by Nunez and McCarthy, if translated to the clinic, may implicate the need for novel sex-specific therapeutic intervention of male neonates at risk for brain injury.

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Figure 1.

Diagram demonstrating a (A) mature neuron with relatively low intracellular chloride concentration resulting from increased expression of chloride transporter KCC2 and a reduction in NKCC1. (B) An immature neuron conversely has higher intracellular chloride concentrations resulting from increased level of NKCC1 and a reduced expression of the chloride transporter KCC2. In addition, the immature neuron expresses the $\gamma 2$ subunit of the GABA_A receptor allowing for inhibition of GABA_A receptor activation after repeated stimulation (C) Androgen receptor activation results in a decreased expression of the $\gamma 2$ subunit of the GABA_A receptor in immature neurons and a failure to desensitize to repeated activation of the GABA receptor.