Blockade of androgen receptors is sufficient to alter the sexual differentiation of the substantia nigra pars reticulata seizure-controlling network

James G. Heida1, Jana Velíšková1,2, Solomon L. Moshé1,2,3
1 Saul R. Korey Department of Neurology
2 Dominick P. Purpura Department of Neuroscience
3 Department of Pediatrics, Laboratory of Developmental Epilepsy, The Montefiore/Einstein Epilepsy Center, Albert Einstein College of Medicine, Bronx, NY 10461, USA

Received August 14, 2007; Accepted December 16, 2007

ABSTRACT – The substantia nigra pars reticulata (SNR) controls seizures in a sex-specific manner. At postnatal day 15 (P15), SNR infusion of GABAA receptor agonist muscimol have proconvulsant effects in males but not in females. In males, administration of an androgen receptor antagonist flutamide between P0-P2 led to the disappearance of the proconvulsant muscimol effects at P15. Thus, activation of androgen receptors is important for the presence of proconvulsant SNR muscimol responses.

Key words: flutamide, substantia nigra, development, GABA, rat

Males seem to have a higher incidence of seizures than females (Hauser et al. 1993). This is evident early in life as many types of developmentally regulated seizures and syndromes such as febrile seizures, severe myoclonic epilepsy of infancy, Landau-Kleffner syndrome and Lennox-Gastaut syndrome are more predominant in males than females (ILAE, 2006). This may, in part, be due to a differential development of circuits critical in the initiation or control of seizures, which may be regulated by the presence of early-life sex hormones. Of particular interest is the development of the seizure-controlling network located in the substantia nigra pars reticulata (SNR) (Iadarola and Gale 1981, Velíšková and Moshe 2006). In rats, this area has been shown to develop differently in males and females (Velíšková and Moshe 2001, Kyrozis et al. 2006). The presence of sex hormones during development influences the maturation of the SNR in both sexes (Velíšková and Moshe 2001, Galanopoulou and...
Moshé 2003, Giorgi et al. 2007). In males at postnatal day 15 (P15), the SNR has a proconvulsant response to localized micro-infusions of muscimol, while in females at this age, SNR muscimol has no effect on seizure threshold. This male-specific phenotype has previously been shown to depend on the presence of early life testosterone (Velíšková and Moshé 2001, Giorgi et al. 2007). Male rats castrated at P0 (just prior to their natural, postnatal testosterone surge) develop the female phenotype, which can be reversed back to the male phenotype by early postnatal testosterone administration (Velíšková and Moshé 2001, Giorgi et al. 2007). Evidence from female rats also shows that exogenous testosterone treatment can produce the male phenotype (Velíšková and Moshé 2001, Giorgi et al. 2007). Thus, it is apparent that testosterone plays a major role in the development of the proconvulsant SNR phenotype in P15 rats.

Within the brain, androgens such as testosterone and its metabolite dihydrotestosterone (DHT) act primarily on androgen receptors (AR) (Lu et al. 1999, Grisham et al. 2002). AR have been found in many brain regions, and the consequences of their activation via testosterone and DHT have been shown to influence the development of several nuclei such as the nucleus bulbocavernosus in the spinal cord (Breedlove and Arnold 1983) and the amygdala (Staudt and Dorner 1976). Since previous studies have established that the development of the male SNR is critically dependent on the presence of testosterone (Velíšková and Moshé 2001), we used the androgen receptor antagonist flutamide (Lieberburg et al. 1980, O’Connor et al. 1998) to explore the role of ARs in the determination of muscimol-sensitive SNR effects on flurothyl-seizure thresholds.

**Methods and materials**

**Animals**

Male rat pups from timed, pregnant Sprague-Dawley rats (Taconic Farms Germantown, NY, USA) were used in all experiments. All animals were housed under standard environmental conditions with a constant ambient temperature of 23°C (60% humidity) and a 12:12 h light:dark cycle with food and water available ad libitum. During the expected day of birth, animals were checked every two hours in order to ensure that the first treatment of the pups could commence within four hours, a time point before the first postnatal testosterone surge (Weisz and Ward 1980).

**Drug treatment**

After birth, the pups were removed from the home cage for sexing and subsequent treatment. Males were given a bolus subcutaneous injection of the AR antagonist flutamide (10 mg/kg, Sigma Chemicals Inc, St Louis, MO, USA) dissolved in peanut oil beginning at P0 (day of birth). Subsequently, each male pup was treated again on P1 and P2, these first three days being critical to testosterone’s effectiveness in influencing the functioning of the SNR (Giorgi et al. 2007). After the final injection at P2, all animals were left undisturbed with their mothers until P13. In order to rule out any effects of injection stress on seizure threshold, we treated a separate group of males with vehicle solution (peanut oil) in the exact same manner.

**SNR surgery and infusions**

At P13, all male rats (oil- and flutamide-treated) were fitted with a bilateral, 22-gauge cannulae aimed at the SNR, under ketamine-xylazine anesthesia as previously described (Velíšková et al. 1998a). Animals were allowed a 48-hr recovery period before infusions and subsequent seizure threshold testing took place. At P15, animals were randomly assigned to receive either saline or muscimol. Muscimol (100 ng in 0.25 μL) [Sperber et al. 1987, Garant et al. 1995]]; or equal volume saline was infused bilaterally. Correct cannulae location was confirmed histologically on thionin-stained coronal sections.

**Flurothyl-seizure thresholds**

To determine if blockade of AR activation by flutamide altered the male SNR phenotype, all animals were subjected to flurothyl-seizure threshold testing. Thirty minutes after SNR infusion of muscimol or saline, the animal was placed in an airtight chamber into which flurothyl was delivered at a constant rate of 40 μL/min. Seizure-onset was measured as the beginning of the first clonic seizure (facial and forelimb clonus with maintained righting reflex). Since the flurothyl flow rate was constant, we calculated the amount of flurothyl needed to induce clonic seizures and expressed the threshold as the amount of flurothyl required to induce seizures. We used a two-way ANOVA, with the main factors consisting of perinatal treatment (flutamide versus oil) and P15 microinfusion (muscimol versus saline). Further analysis involved t-tests. Data are presented as mean ± SEM, significance for all analyses was set at p < 0.05.

**Results**

Two-way ANOVA with main factors perinatal treatment (flutamide versus oil) and P15 microinfusion (muscimol versus saline) did not reveal any significant difference for either of the main factors. However, the interaction of the factors was significant F (1.28) = 9.32; p = 0.005. Since our specific aim was to determine whether flutamide can affect the muscimol-sensitive SNR, we analyzed the groups with perinatal treatment separately, by a t-test and using P15 microinjections as the main factor to identify the source of interaction. Indeed, there was no difference between muscimol and saline SNR microinfusions in the
P0-P2 flutamide-treated animals (figure 1A), but a significant proconvulsant effect of SNR muscimol microinfusions was found in P0-P2, oil-treated rats (figure 1B; p = 0.02).

**Discussion**

We have shown here that in males, early postnatal blockade of AR by flutamide is sufficient to suppress the development of the proconvulsant male SNR phenotype at P15. These results highlight the role of AR activation in the maturation of the SNR network, which is critical for the control of seizures.

During development, testosterone plays a significant role in the masculinization of the nervous system (Breedlove and Arnold 1983, Cooke et al. 1999) including the SNR (Velíšková and Moshé 2001, Giorgi et al. 2007). The sex differences in the SNR involve the maturation pattern of the GABA<sub>α</sub>ergic system (Ravizza et al. 2003) including the GABA<sub>α</sub> receptor α1 subunit mRNA (Velíšková et al. 1998b) and neuron-specific potassium chloride cotransporter KCC2 mRNA expression (Galanopoulou and Moshé 2003), which increases with age in both sexes. However, in the SNR at P15, females have a higher expression of α1 subunit mRNA and KCC2 mRNA compared to males indicating a more mature GABA system (Galanopoulou et al. 2003, Ravizza et al. 2003). Accordingly, at P15, SNR muscimol infusions have a proconvulsant effect in males but not in females or in males castrated at P0. Similarly, postnatal administration of testosterone (or its metabolite DHT) to females or castrated males, leads to proconvulsant effects of SNR muscimol at P15 (Velíšková and Moshé 2001, Giorgi et al. 2007). Later in life, SNR muscimol mediates an anticonvulsant effect, which is already present at P25 in females, but as late as at P30 in males (Velíšková and Moshé 2001). The results of the present study further corroborate these findings and indicate that blockade of AR function in males by flutamide administration at P0-P2 may affect this pattern, thus leading to accelerated maturation of the GABA<sub>α</sub>ergic system. We cannot fully rule out a potential role for estrogen receptor activation in the development of the SNR with this study, which previous data support (Giorgi et al. 2007). The pharmacology and CNS physiology of available, selective estrogen receptor modulators (SERMs) are complex and variable (Bernardi et al. 2003, Zhao et al. 2006, Steyn et al. 2007). A review by Bernardi et al. outlines the complications involved in using SERMs, which include: dual agonist-antagonist actions, differential actions related to the endogenous estrogenic milieu, as well as tissue-specific effects (Bernardi et al. 2003). In view of the aforementioned complications with SERMs, we elected to focus on AR by using flutamide. Therefore, the current data specifically stress the role of the early life activation of AR in the proconvulsant muscimol effects at

Figure 1. Determination of the interaction (two-way ANOVA, p = 0.005) between perinatal treatment (flutamide and oil) and P15 microinfusion (saline and muscimol) on flurothyl seizure thresholds. 

A) Male rat pups exposed to the androgen antagonist flutamide between P0-P2 do not show a proconvulsant response to flurothyl after SNR infusions of muscimol (n = 11) when compared to animals infused with saline (n = 10).

B) Male rats treated with oil (P0-P2) display a proconvulsant response during flurothyl testing after SNR infusions of muscimol (n = 5) compared to saline (n = 6) infused animals. * p < 0.05 compared to saline (unpaired t-test).
P15 and in delaying the maturation of the SNR GABAergic system.

Our data are consistent with previously published reports showing that AR antagonism is sufficient to negate the effects of endogenous or exogenous testosterone on the development of other sexually dimorphic brain structures such as the preoptic area of the hypothalamus (Lund et al. 2000), and the overall function of complex neural systems such as the hypothalamic-pituitary-adrenal axis (Seale et al. 2005). Although there is substantial evidence that neonatal stress can permanently alter the developing brain (Hsu et al. 2003, Andersson and Teicher 2004, Card et al. 2005), the persistence of the proconvulsant muscimol effect in oil)-(vehicle) treated animals clearly shows that the effect of flutamide is specific for the blockade of AR and not related to neonatal stress.

Overall, our study shows that activation of AR is critical for the presence of the proconvulsant SNR muscimol effects at P15, implying that testosterone has a key role in the sexual differentiation of the SNR seizure-controlling network.

Acknowledgements. The authors would like to thank Hong Wong for technical assistance with surgical and histological procedures. This work was supported by the National Institutes of Health Grant NS 20253 and the Heffer Family Medical Foundation. Dr S.L. Moshé is the recipient of the Martin A. and Emily L. Fisher Fellowship in Neurology and Pediatrics.

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