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Sanaz Eftekhari *et al.*

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TECHNICAL RESPONSE

DEVELOPMENTAL NEUROLOGY

Response to Comment on "Oxytocin-mediated GABA inhibition during delivery attenuates autism pathogenesis in rodent offspring"

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Bambini-Junior *et al*. questioned whether our treatment in two rodent models of autism has a long-lasting effect into adulthood. In response, we show that bumetanide treatment around delivery attenuates autistic behavioral features in adult offspring. Therefore, the polarity of γ-aminobutyric acid (GABA) actions during delivery exerts long-lasting priming actions after birth.

We are very grateful to Bambini-Junior *et al*. (*1*) for raising the issues of (i) the long-lasting effect on behavior of bumetanide pretreatment in rodent models of autism, (ii) the long-lasting effect on behavior of blocking oxytocin signaling in naïve mothers during the delivery period, and (iii) the sex-dependent response to bumetanide treatment. We are glad to respond with additional experimental observations.

To evaluate whether maternal pretreatment with bumetanide has long-term behavioral effects, we have now evaluated adult behavior in two animal models of autism: rats exposed in utero to valproate (VPA rats) and mice carrying the fragile X mutation (FRX mice). Because VPA rats have been shown to display altered behavior (*2*–*4*), using the social approach-avoidance paradigm (*2*) we show that VPA male adult rats that received bumetanide 1 day before birth (maternal pretreatment) (*5*) display improved sociability (Fig. 1A), spending significantly more time in the social chamber than age matched nontreated VPA rats. Conversely, it has been shown that FRX mice have similar sociability to wild-type (WT) mice in the three-chamber social test (*6*), thereby precluding the use of this paradigm to test the effects of bumetanide. Therefore, we evaluated stereotypical behavior (*7*), and adult FRX male mice displayed a significantly higher number of grooming events (bouts) than WT littermates (Fig. 1B). Maternal pretreatment with bumetanide of FRX mice restored the grooming behavior (Fig. 1B). Therefore, in the valproate and fragile X rodent models of autism, maternal pretreatment with bumetanide 1 day before birth restores control behavioral phenotypes in adult offspring.

We further evaluated the effect of blocking oxytocin receptors with maternal pretreatment of naïve mothers 1 day before delivery with SSR126768A (SSR) (*5*) on adult social behavior in mice and rats (*2*, *8*). We observed that adult rat (Fig. 1C) and mouse (Fig. 1D) offspring exposed to SSR display lower sociability than controls, extending our earlier observations (*5*) to adulthood. Thus, blocking oxytocin signaling in naïve mothers during the delivery period produced adult offspring with altered social behavior.

In addition to our behavioral tests in adult animals, we wanted to verify the presence of network alterations in the developing brain of FRX mice, as we did for the VPA rats (*5*). We therefore performed extracellular intracranial electroencephalographic (EEG) recordings in vivo in the hippocampal CA3 area of head-restrained P14 and P15 WT and FRX mice. We show prominent differences in the oscillatory activity of FRX mice compared with WT (Fig. 1E1). There was a significant decrease in slow oscillatory activity (0.1 to 4 Hz) in comparison with age-matched WT mice. In contrast, fast EEG activity in a wide band of frequencies from 4 to 800 Hz was increased. The most pronounced differences in EEG were observed at the δ (0.1 to 4 Hz) and low γ-band (25 to 40 Hz) frequencies (Fig. 1E2). Maternal pretreatment with bumetanide of FRX mice partly reduced these aberrant oscillations and significantly decreased low γ and fast and ultrafast oscillatory activity in offspring (Fig. 1E1 and 1E2). Hence, FRX mice display an enhanced network oscillation in the hippocampus not observed in age-matched WT mice, and this is restored to physiological values after maternal pretreatment with bumetanide.

Collectively, these results extend our earlier observations suggesting that the priming effects of the polarity of γ-aminobutyric acid (GABA) during delivery are long-lasting, being present in adults, and concern also social behavior components. They also validate our physiological recordings with the behavioral manifestations and the priming effects of bumetanide and oxytocin during the delivery period. The underlying mechanisms remain to be investigated, although it has been suggested that insults during delivery and the early postnatal period lead to long-term behavioral and molecular consequences.

Bambini-Junior *et al*. also criticize the use of males and females in our analysis, highlighting the higher prevalence of autism spectrum disorders in males in humans. However, this male/female difference is one of incidence and not of treatment, because in our clinical trials girls are as efficiently treated as boys by bumetanide (*9*), which suggests elevated intracellular chloride in both. In addition, behavioral differences between males and females are neither systematically investigated nor essential because they do not constitute a condition to identify the autistic syndrome and their underlying mechanisms remain to be determined. In our present experiments, only males were used to test adult behavior (Fig. 1, A and B), and bumetanide pretreatment was efficient in reducing autistic features. Furthermore, our in vivo experiments in FRX mice were performed in a similar ratio of males and females in each group tested, and we observed no difference regarding the sex, suggesting that independently of their sex bumetanide pretreatment restores control phenotypes. Finally, if we assume that bumetanide is less effective in female than in male rodents (or vice versa), we see an overall significant positive effect of bumetanide treatment.

In sum, our study has shown that a pretreatment restricted to the delivery period of autistic rodents attenuates the severity of the syndrome in young and adults, and this is controlled by the polarity of GABA and oxytocin signals at that stage. This raises questions concerning the role of delivery in the GABA excitatory/inhibitory shift and in relation to the emergence of autism. Our priority is to understand these links and the alterations in brain patterns stemming from excitatory GABA.

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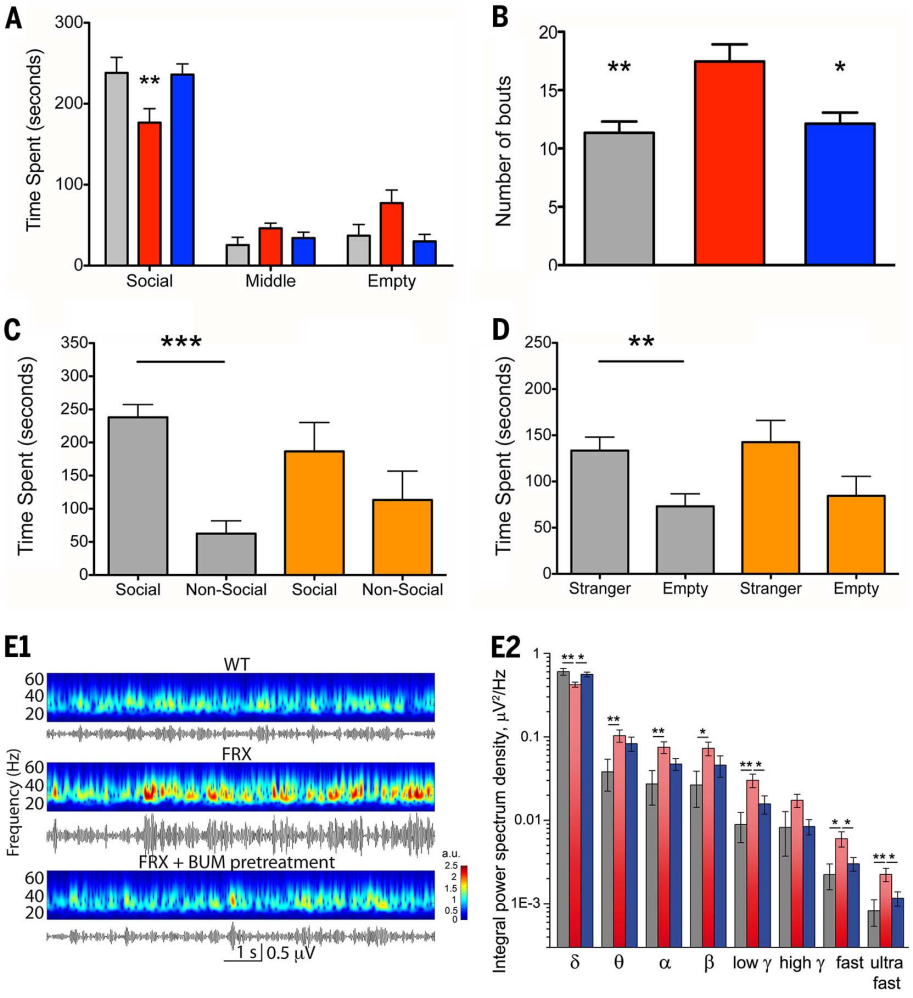

Fig. 1. Maternal pretreatment with bumetanide improves adult aberrant behavior and brain oscillations of animal models of autism. (**A**) Maternal pretreatment with bumetanide improves social behavior in VPA 4.5-month-old rats. Control (gray), VPA (red), and VPA rats with maternal bumetanide pretreatment (blue). $^{**}P < 0.01$, two-way analysis of variance (ANOVA) and Bonferroni post hoc test. (**B**) FRX mice (red, 2.5 months old) display a significantly higher number of grooming events (bouts) than wild-type littermates (gray). Maternal pretreatment with bumetanide on FRX mice (blue) restores the number of bouts to control levels. $^{*}P < 0.05$, $^{**}P < 0.01$, one-way ANOVA with Tukey's multiple comparison post hoc test. (**C**) The 4.5-month-old rat male offspring that received SSR126768A maternal pretreatment (orange) present a reduced social interaction compared with controls (gray). $^{***}P < 0.001$, Mann-Whitney test. (**D**) SSR-treated mice (orange, 2.5 months old) display lower sociability compared with controls (gray). $^{**}P < 0.01$, Mann-Whitney test. (**E**) EEG recordings in vivo were made in the CA3 area of the hippocampus of head-restrained mice. (**E1**) Representative wavelets of CA3 pyramidal layer EEG recordings from P14 and P15 WT, FRX, and FRX mice with maternal bumetanide pretreatment after band-pass filtering from 25 to 40 Hz (low γ-band). Corresponding traces are shown under each wavelet. (**E2**) Integral power spectrum density of δ (0.1 to 4 Hz), θ (4 to 7 Hz), α (7 to 12 Hz), β (12 to 25 Hz), low γ (25 to 40 Hz), high γ (40 to 100 Hz), fast (100 to 400 Hz), and ultrafast (400 to 800 Hz) oscillations band components of EEG revealed by filtering and Fourier transform analysis. WT (gray) versus FRX (red): For β and fast oscillations, $^{*}P < 0.05$; for δ, θ, α, low γ, and ultrafast oscillations, $^{**}P < 0.01$. FRX (red) versus FRX with maternal bumetanide pretreatment (blue): For δ, low γ, fast, and ultrafast oscillations, $^{*}P < 0.05$. One-way ANOVA *F* test. Data presented as means ± SEM.

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