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Altered nocifensive behavior in animal models of autism spectrum disorder: the role of the nicotinic cholinergic system

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Abstract

Caretakers and clinicians alike have long recognized that individuals with autism spectrum disorder (ASD) can have altered sensory processing, which can contribute to its core symptoms. However, the pathobiology of sensory alterations in ASD is poorly understood. Here we examined nocifensive behavior in ASD mouse models, the BTBR T^{+} *Itpr3*^{tf}/J (BTBR) and the fragile-X mental retardation-1 knockout (*Fmr1*-KO) mice. We also examined the effects of nicotine on nocifensive behavior given that nicotine, a nicotinic cholinergic receptor (nAChR) agonist that has antinociceptive effects and was shown to improve social deficits and decrease repetitive behaviors in BTBR mice. Compared to respective controls, both BTBR and *Fmr1*-KO had hyporesponsiveness to noxious thermal stimuli and electrical stimulation of C-sensory fibers,

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Chemical compounds studied in this article

Nicotine (PubChem CID: 89594); Saccharin (PubChem CID: 656582)

Disclosures

All authors have read the journal's authorship agreement and policy on disclosure of potential conflicts of interest and have no conflict of interest to disclose

Authorship

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normal responsiveness to electrical stimulation of Aβ- and Aδ-fiber, and hyperresponsiveness to visceral pain after acetic acid intraperitoneal injection. In BTBR, nicotine at lower doses increased, whereas at higher doses, it decreased hotplate latency compared to vehicle. In a significantly different effect pattern, in control mice, nicotine had antinociceptive effects to noxious heat only at the high dose. Interestingly, these nocifensive behavior alterations and differential responses to nicotine antinociceptive effects in BTBR mice were associated with significant downregulation of α3, α4, α5, α7, β2, β3, and β4 nAChR subunits in several cerebral regions both, during embryonic development and adulthood. Taken together, these findings further implicate nAChRs in behaviors alterations in the BTBR model and lend support to the hypothesis that nAChRs may be a target for treatment of behavior deficits and sensory dysfunction in ASD.

Keywords

nicotine; nicotinic; autism; social behavior; nAChR; BTBR; nociception; nocifensive; pain

1. Introduction

Autism spectrum disorder (ASD) includes a group of neurodevelopmental disorders characterized by social communication and interaction deficits and repetitive, restricted patterns of behaviors, activities, or interests (American Psychiatric Association, 2013; Christensen et al., 2016; Constantino and Charman, 2015; Pedersen et al., 2012; Richards et al., 2015). Some of those restricted patterns of behavior or activities can be related to alterations in response to sensory input, as indicated in the diagnostic criteria for ASD in the Diagnostic and Statistical Manual of Mental Disorders – 5th edition (DSM-5) (American Psychiatric Association, 2013). Caretakers and clinicians alike have long recognized that individuals with ASD have altered responses to sounds, tastes, texture, smells, and/or apparent indifference to noxious stimuli (American Psychiatric Association, 2013; Ausderau et al., 2014a; Ausderau et al., 2014b; Ausderau et al., 2016; Patten et al., 2013). Studies using standardized scales to evaluate sensory phenotypes show that ASD individuals can display a spectrum of sensory response alterations with three predominant patterns. Hyperresponsiveness, a pattern in which ASD individuals show negative response to mild stimulation (Baranek et al., 2006; Ben-Sasson et al., 2009; Green et al., 2015). A second pattern is one of hyporesponsiveness, in which ASD patients show diminished or absent response to stimuli perceived as noxious or known to inflict pain (Ben-Sasson et al., 2009). A third pattern is that of sensory seeking behavior characterized by the pursuit of repeated exposure to given sensory stimuli. While the most prevalent pattern in ASD is that of hyporesponsiveness (Ben-Sasson et al., 2009), a large number of patients actually present a mixed pattern and simultaneously display hyper- and hyporesponsiveness to sensory stimuli(Baranek et al., 2006). Therefore, while it was only recently listed in the diagnostic criteria, sensory dysfunction in ASD individuals has been broadly described (Ausderau et al.,2014a; Ausderau et al., 2014b; Ausderau et al., 2016; Baum et al., 2015; Green et al., 2015; Patten et al., 2013; Tordjman et al., 2009).

While researchers have extensively reported on qualitative patterns of altered sensory processing in ASD, the neurobiological underpinnings of these alterations remain poorly

understood. In a recent study using quantitative sensory testing, researchers showed that high-functioning adolescent with ASD have decreased thermal sensitivity (increased warmth and cold detection threshold) and that their thermal detection thresholds and heat pain thresholds correlated with their intelligence quotients (Duerden et al., 2015). In another study, high functioning ASD children were shown to have increased pain (lower pressure pain thresholds) and touch sensitivities (Von Frey monofilaments) compared to healthy children(Riquelme et al., 2016). Studies using questionnaires to evaluate behavioral responses to sensory stimuli show that in ASD individuals, higher levels of tactile hypo-responsiveness and increased tactile seeking behaviors significantly correlated with increased social impairment and repetitive behaviors (Baker et al., 2008; Foss-Feig et al., 2012; Hilton et al., 2010). Interestingly, others showed that sensory disturbances correlate with severity of ASD-related deficits in children, but not in adolescents or adults (Kern et al., 2007). However, it remains unknown whether these associations between social deficits and sensory dysfunctions are causative or are due to shared underlying pathobiology.

Studies in animal models of ASD have increased our understanding of altered behavior and pain sensitivity in ASD. For example, mice harboring mutations in the *Mecp*2 gene, a model of human Rett syndrome, a disorder which can be associated with ASD, display heat hyporesponsiveness at baseline and in the setting of inflammatory pain(Downs et al., 2010). In humans, mutations in the *SHANK* genes can be associated with ASD (Leblond et al., 2012; Leblond et al., 2014) and mice with null mutations of the *Shank*2 gene have social deficits (Schmeisser et al., 2012; Won et al., 2012) and hyporesponsiveness to mechanical and noxious heat stimuli as well as to inflammatory and neuropathic pain (Ko et al., 2016). Fragile X syndrome, a single gene neurodevelopmental disorder resulting from lack of expression of the mRNA binding protein-encoding the *Fmr1* gene, is the leading known genetic cause of autism in humans. Mice with null mutations in the *Fmr1* gene (*Fmr1*-KO), show social deficits (Spencer et al., 2011) and hyporesponsiveness to inflammatory and neuropathic pain (Price et al., 2007). Here, using different modalities of sensory stimulation, we investigated the nocifensive behavior in BTBR T^{+} *Itpr3*^{tf}/J (BTBR) mice, a well-studied model, which recapitulates core ASD phenotypes (Silverman et al., 2013; Silverman et al., 2015; Silverman et al., 2012; Silverman et al., 2010a) and in the *Fmr1*-KO mouse(Sorensen et al., 2015; The Dutch-Belgian Fragile, X Consortium., 2013). Additionally, we sought to determine whether nicotine, an agent that modulates behavior deficits would also modulate altered nociception in the BTBR model. As others and we have shown that the nicotinic cholinergic system modulates social deficits in the BTBR model (Karvat and Kimchi, 2014; Wang et al., 2015), we investigated whether nicotine, which is known to have analgesic effects, would also modulate nocifensive behavior in the BTBR mouse.

2. Material and methods

2.1. Animals

The investigational protocol was approved by the Children's National Health System Institutional Animal Care and Use Committee and conducted in compliance with the Guide for the Care and Use of Laboratory Animals. Breeding pairs of BTBR T^{+} *Itpr3*^{tf}/J (BTBR) and C57BL/6J (B6, control strain for BTBR) mice were purchased from Jackson Laboratory

(Bar Harbor, ME) and bred in our animal facility. The BTBR strain has been extensively studied and is known to model the core symptoms of ASD (Brodkin, 2008; McFarlane et al., 2008; Price et al., 2007; Silverman et al., 2013; Silverman et al., 2015; Silverman et al., 2012; Silverman et al., 2010a; Wang et al., 2015). A balanced number of age-matched (8–22 weeks) male and female mice were included in BTBR and B6 studies. We also examined the nocifensive behavior in males FVB.129P2-*Pde6b*$^{+}$ *Tyr*$^{c\text{-}ch}$ *Fmr1*^{tm1Cgr}/J (*Fmr1*-KO) (The Dutch-Belgian Fragile, X Consortium., 2013) and FVB.129P2-*Pde6b*$^{+}$ *Tyr*$^{c\text{-}ch}$/AntJ (FVB, control strain for *Fmr1*-KO), both obtained from the Jackson Laboratory and bred in our animal facility. Male *Fmr1*-KO mice recapitulate features of human fragile X syndrome, a monogenic disorder associated with ASD, and display social deficits and hyperactivity (Sorensen et al., 2015). Mice of all strains were group housed (up to five/cage) on a 12h light cycle and had unrestricted access to food and water.

2.2. Nicotine trial

We examined the effect of chronic nicotine treatment on nocifensive behavior outcomes in B6 and BTBR mice. After baseline behavior outcome measurements, animals (age-matched male and female) were assigned to receive one of five doses [zero (vehicle), 50, 100, 200, or 400μg/mL, calculated as nicotine free-base] of (−) nicotine (hydrogen tartrate salt, Sigma-Aldrich, St. Louis, MO) in drinking water (Matta et al., 2007). Saccharin (2%, Sigma-Aldrich) was the vehicle for all nicotine solutions (Matta et al., 2007), which were prepared three times/week.

We also examined the effect of nicotine on visceral pain (writhing test, see below) in a different cohort of B6 and BTBR mice. In this cohort, we administered only two doses of nicotine (0 (vehicle) or 100μg/mL) and obtained behavior outcomes (number of writhes) only after treatment as to not repeat acetic acid injections.

2.3. Behavior assays

In order to minimize variability, each category of behavior assay was conducted by the same investigator between 9 AM and 2 PM and always included animals from control and mutant groups. During experiments examining nocifensive behavior, mutant mice and respective controls were examined during each session in order to control for the effect of time. Nocifensive behavior in response to “phasic pain” (Le Bars et al., 2001) was evaluated using thermal and electrical stimuli [hotplate latency, cold plate sensitivity, and current threshold (sensory fiber interrogation) in this order] and to “tonic pain” using the writhing test (Le Bars et al., 2001). Animals undergoing tail flick and the writhing test did not undergo any other nociception assay. Only one of testing paradigms was conducted per day and the investigator conducting quantitative sensory testing was unaware of the animals’ genotype and/or treatment.

2.3.1. Hotplate latency—In order to evaluate nocifensive response to noxious heat, mice were placed on a hotplate (Harvard Apparatus, Holliston, MA) and latency to display of pain-avoiding behaviors (jumping, stomping or repeated lifting or licking of hind or front paws) was measured(Le Bars et al., 2001). The hotplate temperature was chosen according to previous studies of ASD-like mouse models including *Fmr1*-KO and BTBR mice, which

had been performed using a hotplate set at 55°C (Chadman et al., 2008; Ko et al., 2016; Silverman et al., 2010b; Veeraragavan et al., 2012). In order to avoid injury, animals were allowed to stay on the hotplate for a maximum of 30 seconds.

2.3.2. Tail-flick latency—Mice were gently held in a mouse holder and the middle third of the tail was placed over a radiant heat source (Ugo Basile, Varese, Italy) with infrared intensity set at 20%. Latency to tail flick (withdraw) away from the stimulus was measured to the nearest 0.1 s. Withdrawal of the tail stopped the stimulus, the latency to tail flick was recorded automatically, and the cut-off time was set at 15 s. The final tail flick latency was an average of three measurements obtained at least 1 min apart (Le Bars et al., 2001).

2.3.3. Cold Plate Sensitivity—We measured cold plate sensitivity using a Peltier cooler set at 2°C (Harvard Apparatus, Holliston, MA). Mice were observed on the cold plate for 5 minutes and their behavior -recorded. The time animals spent withdrawing from the cold surface (lifting front or hind paws, rubbing of front paws) was recorded with a stopwatch and the total time withdrawing from the cold plate was interpreted as cold plate sensitivity, i.e., the longer the time withdrawing from the cold plate, the higher the cold plate sensitivity (Choi et al., 1994).

2.3.4. Sensory nerve fiber interrogation using sine wave electrical stimulation —In order to evaluate specific somatosensory fibers in models of ASD, we used a sine-wave electrical stimulation paradigm at three frequencies: 5, 250 and 2000 Hz that preferentially stimulate C, Aδ, and Aβ fibers respectively as previously described (Finkel et al., 2012; Finkel et al., 2006; Koga et al., 2005; Spornick et al., 2011). Briefly, electrical stimuli generated by a neurostimulator (Neurotron, Inc, Baltimore, MD) were delivered to the tail of gently restrained mice. Stimuli at different frequencies (5, 250 and 2000 Hz) were delivered at increasing intensities, lasted one second and were set on a 50% duty cycle (each stimulus is followed by a one-second stimulus-free interval). Between stimulations at different frequencies, animals rested for one-minute. Vocalization was the nocifensive behavior outcome and its occurrence prompted termination of the stimulus. For each frequency, the electrical stimulus amperage that elicited audible vocalization or the maximum amperage delivered was defined as current threshold (Finkel et al., 2012; Finkel et al., 2006; Spornick et al., 2011). Current thresholds for each frequency were the average of five consecutive measurements obtained in response to 2000, 250, and 5 Hz sequentially. The current threshold unit of measurement is “unit” (U), which corresponds to 100 times the amperage that elicited audible vocalization.

2.3.5. Writhing test—Mutant mice and respective controls received a single intraperitoneal injection of 0.6% acetic acid diluted in phosphate buffered solution (10μl/g body weight). Animals were video-recorded and abdominal stretching/writhing activity was counted for 30 min following injections as described (Reichert et al., 2001). Mice undergoing the writhing test were euthanized upon completion of the test.

2.4. Nicotinic acetylcholine receptor subunits gene expression during adulthood and embryonic development

Prefrontal cortex, brain cortex, hippocampus, and cerebellum were dissected from adult naïve BTBR, B6, *Fmr1*-KO, and FVB mice. After anesthesia with isoflurane, animals were exsanguinated via cardiac puncture to avoid mRNA contamination during isolation. Prefrontal cortex was obtained by slicing the first 2 mm of frontal cortex using a 1mm coronal mouse matrix (Roboz, Gaithersburg, MD), after carefully removing olfactory bulb. We isolated mRNA using a Trizol method with subsequent purification using the Qiagen mini-kit according to manufacturer's instructions. We used a NanoDrop (Thermo Scientific, Wilmington DE) to quantify mRNA, and 1μg was used to synthesize cDNA (iScript Reverse Transcription Supermix for RT-qPCR, Bio-Rad). Relative gene expressions of nAChR subunits were quantified by realtime polymerase chain reaction using One-Step qRT-PCR Kit in the Abi Prism® 7900 (both from Applied Biosystems, Foster City, CA). Validated TaqMan hydrolysis probes (all from Thermo Scientific) to nAChR subunits (α2, α 3, α4, α5, α6, α7, β2, β3 and β4) were as follows: Mm00460630_m1 (Chrna2), Mm00520145_m1 (Chrna3), Mm00516561_m1 (Chrna4), Mm00616329_m1 (Chrna5), Mm00517529_m1 (Chrna6), Mm01312230_m1 (Chrna7), Mm00515323_m1 (Chrnb2), Mm00532602_m1 (Chrnb3), Mm00804952_m1 (Chrnb4)), and β-actin probe as the reference gene (Mouse ACTB ,#4352663, Applied Biosystems).

For analyses of nAChR expression during embryonic development, male and female BTBR or B6 mice were coupled overnight and separated early the following morning (counted as embryonic day 0.5 (E0.5) if pregnant). At E13, pregnant females (identified by visual inspection) were anesthetized and exsanguinated via cardiac puncture. The uterus was removed and each fetus dissected from its embryonic membranes. Because at this embryonic age it is not possible to recognize individual cerebral areas, all intracranial embryonic material was dissected after carefully removing brain meninges. For each pregnancy, embryonic brains from three to four fetuses were pooled and mRNA was isolated as described above. For each pregnancy, a single fetal pool was analyzed and for each strain, five pregnancies were assayed.

2.5. Statistical analysis

All statistical analyses were performed using STATA 12.0 (StataCorp., College Station, TX). We tested baseline nocifensive behavioral measurement differences using Kruskal-Wallis test or one way ANOVA, as appropriate. In the nicotine trial, we examined the effect of different nicotine doses on each nocifensive behavior outcome using a mixed-effect model. In each model, we introduced interaction terms between time and nicotine dose to assess the effect of treatment on change of outcome from baseline to post-treatment measurements compared to vehicle treatment. We separately examined whether there was a difference of treatment effect comparing BTBR and B6 mice strains by testing the interaction between strain and treatment effect. In all models, the effects of age, weight, or sex were adjusted for when appropriate. Model residuals were inspected and outliers removed for final analyses. P values <0.05 were considered statistically significant. RT-qPCR reactions were quantified and expression ratios (mutant vs. respective controls) were calculated using the Pfaffl method, REST2009 program from Qiagen(Pfaffl et al., 2002).

3. Results

3.1. Nocifensive behavior in BTBR mice

BTBR mice had increased tolerance to noxious heat stimuli as they had higher hotplate and tail flick response latencies (Figure 1A and 1B, χ^2 (df) = 107.2 (1), p<0.001) compared to controls (B6) mice. In addition, BTBR mice had increased tolerance to noxious cold stimuli as they displayed lower sensitivity (less time withdrawing from the cold plate) to the cold plate compared to B6 animals (Figure 1C, χ^2 (df) = 11.4 (1), p<0.001).

We then examined the nocifensive response to sine wave electrical stimulation in BTBR mice. Compared to B6 animals, BTBR had increased tolerance to electrical stimulation at the frequency that stimulate unmyelinated C-fibers (5Hz) as they had higher current threshold in response to 5Hz stimulation (Figure 1D, χ^2 (df) = 32.2 (1), p<0.001). In contrast, BTBR mice had similar sensitivity to 250Hz and 2000Hz electrical stimulations, which recruit myelinated Aδ and Aβ fibers respectively, compared to B6 animals (Figure 1E and 1F).

We also examined nocifensive behavior in a model of visceral pain. Over 30 min after intraperitoneal injection of 0.6% acetic acid , BTBR mice showed hyperresponsiveness to visceral pain as they had significantly more writhes compared to B6 animals (Figure 1G, χ^2 (df) = 6.41 (1), p=0.01).

3.2. Nocifensive behavior in a model of fragile X syndrome, a human disease associated with ASD

The *Fmr*1-KO, compared to its control mouse, displayed a pattern of nocifensive behavior similar to the BTBR mouse. Specifically, *Fmr1*-KO mice had increased tolerance to noxious heat as they had higher hotplate [H (df) = 19.66 (1), p<0.001] and tail flick [H (df) = 10.59 (1), p=0.001] latencies compared to FVB control mice (Figures 2A and 2B). *Fmr1*-KO mice also had increased tolerance to noxious cold stimuli as they displayed lower sensitivity to the cold plate compared to FVB animals (Figure 2C, $F_{(1, 20)}$ = 86.83, p<0.001).

Regarding response to electrical stimulation, *Fmr1*-KO mice, compared to FVB animals, had higher current threshold in response to 5Hz stimulation (Figure 2D, $F_{(1, 25)}$ = 6.22, p=0.02), a pattern of hyporesponsiveness similar to that seen in BTBR mice. Further, compared to FVB mice, *Fmr1*-KO animals had similar sensitivity to 250Hz and 2000Hz (myelinated Aδ and Aβ fibers respectively) electrical stimulation (Figures 2E and 2F, p=0.19 and p=0.85 respectively).

Regarding nocifensive behavior in response to visceral pain, *Fmr1*-KO displayed significantly more writhes after intraperitoneal injection of 0.6% acetic acid compared to FVB mice (Figure 2G, χ^2 (df) = 4.093 (1), p=0.04).

3.3. Differential effect of nicotine comparing B6 and BTBR mice

As others and we have shown that, the nicotinic cholinergic system modulates social deficits and repetitive behavior in BTBR mice and as nicotine is known to have analgesic effects, we examined its effects on nocifensive behavior in this model. We first asked whether nicotine

treatment, compared with vehicle, had similar effects on nocifensive behavior comparing B6 and BTBR mice by examining time*treatment* strain interactions in the four-week nicotine trial. Figure 3 shows the comparisons of the effects of nicotine (compared with vehicle) on changes in behavior outcomes (post-treatment compared to baseline) in BTBR (gray bars) versus B6 (white bars) strains (time*treatment*strain interactions). We found that the effect of nicotine, compared with vehicle, indeed varied according to mouse strain. Specifically, compared with vehicle, four-week nicotine treatment at lower doses (50 and 100μg/ml) was associated with significantly greater increases on hotplate latency in BTBR compared to B6 mice (strain*time*treatment interaction $z = -2.42$, $p=0.016$ and $z = -4.47$, $p<0.001$ for 50 and 100μg/ml respectively, Figure 3A). At higher doses, compared with vehicle, nicotine also had a differential effect on nocifensive behavior comparing BTBR and B6 mice. At 200 and 400μg/ml nicotine led to significant decreases in hotplate latency in BTBR, whereas in B6 mice, it lead to no significant changes or increases in hotplate latency (strain*time*treatment interaction $z = 2.05$, $p=0.040$ and $z = 6.12$, $p<0.001$ for 200 and 400μg/ml respectively, Figure 3A). Concerning cold sensitivity, nicotine was also associated with different effect patterns in BTBR compared with B6 mice. Specifically, compared with vehicle, at lower doses (50 and 100μg/ml) nicotine led to significant increases in cold plate sensitivity in BTBR, whereas in B6 mice nicotine was associated with significant decreases in cold plate sensitivity (strain*time*treatment interaction $z = -2.75$, $p=0.006$ and $z = -4.13$, $p<0.001$ for 50 and 100μg/ml respectively, Figure 3B). In contrast, compared with vehicle, nicotine at 400μg/ml was associated with decreases in cold plate sensitivity in BTBR but with no significant changes in B6 mice ($z = 3.26$, $p=0.001$, Figure 3B). Regarding current thresholds, the effects of nicotine on 2000 and 5Hz were similar in BTBR and B6 mice. However in response to 250Hz stimulation, compared with vehicle, nicotine treatment at 200μg/ml was associated with slight increases in current thresholds in BTBR, but to slight decreases in B6, patterns of responses that were significantly different ($z = -2.67$, $p=0.008$, Figure 3C).

3.4. Effects of nicotine on nocifensive behavior in B6 (control) and BTBR mice

Figure 4 shows means±SEM changes (post-treatment vs. baseline) in behavior outcomes in B6 (4A and 4B) and in BTBR mice (4C and 4D) at each (0, 50, 100, 200, and 400μg/ml) nicotine dose (time*treatment interaction). In B6, nicotine only at the higher dose (400μg/ml) increased hotplate latency (Figure 4A, $z = 3.18$, $p=0.001$) compared with vehicle. Regarding cold sensitivity in B6 mice, at lower doses, nicotine decreased (time*treatment interaction at 50 μg/ml, $z = -3.24$, $p= 0.001$ and at 100 μg/ml, $z = -3.48$, $p<0.001$, Figure 4B), whereas at 200μg/ml, it increased ($z = 2.12$, $p=0.034$) cold plate sensitivity compared with vehicle. In addition in B6 mice, compared with vehicle, nicotine significantly decreased 2000Hz (χ^2 (df) = 4.42 (1), $p=0.036$) and produced less of an increase in 250 Hz (χ^2 (df) = 5.86 (1), $p=0.016$) and no changes in 5Hz current thresholds compared with vehicle (data not shown).

In BTBR mice, compared with vehicle, at lower doses, nicotine increased (50μg/ml, $z = 6.69$, and 100μg/ml, $z = 7.19$, both doses $p<0.001$, time*treatment interaction, Figure 4C) whereas, at higher doses ((200g/ml and 400μg/ml) nicotine decreased hotplate latency (both doses, $z = -3.21$, $p=0.001$, time*treatment interaction, Figure 4C). Regarding cold stimuli,

compared with vehicle, at 100 μg/ml, nicotine increased (time*treatment interaction, z = 2.00, p= 0.046, Figure 4D), whereas at 400μg/ml, it decreased (z = −3.84, p<0.001, Figure 4D) cold sensitivity in BTBR mice. In BTBR mice, nicotine had no significant effect on electrical current thresholds in response to sinewave stimulation (data not shown).

In a different cohort of animals, we examined the effect of nicotine on the writhing test and obtained behavior outcomes (number of writhes) only after treatment as not to repeat acetic acid injections. Overall, nicotine-treatment significantly decreased the number of writhes compared to vehicle (t=2.88, p=0.006, main effect of treatment). Specifically, nicotine-treated B6 and BTBR mice had significantly less writhes than saccharine-treated respective controls (p<0.001 for B6 and p=0.007 for BTBR) after intraperitoneal injection of acetic acid (Figure 5). Further, comparing the nicotine effect on visceral pain between the two strains (strain*treatment interaction), there was a trend (t=1.85, p=0.07) suggesting that nicotine was associated with greater decreases in writhes in B6 than in BTBR (Figure 5).

Water intake, mouse weight, and nicotine plasma levels with nicotine doses examined here, have been previously reported in B6 and BTBR mice (Wang et al., 2015). We chose not to evaluate the effects of nicotine on nocifensive behavior in *Fmr*1-KO mice because its effects (if any) on social behavioral deficits in the *Fmr*1-KO strain have not been characterized.

3.6. Expression of nicotinic acetylcholine receptor subunits in ASD mouse models

We then sought to determine whether there were alterations on expression levels of nAChR subunits (α2–7 and β2–4) in prefrontal cortex, hippocampus, cerebellum, and brain cortex in BTBR and *Fmr1*-KO mice compared to their respective controls. BTBR mice had significant downregulation of α5 (p=0.006, Figure 5A) nAChR receptor subunit in prefrontal cortex; α4 (p<0.0001), α5 (p=0.001), and β3 (p<0.0001) in hippocampus (Figure 5B); α3 (p=0.006), α4 (p=0.024), α5 (p=0.002), α7 (p=0.005), β2 (p<0.005), and β3 (p=0.01) in cerebellum (Figure 5C). In addition in brain cortex (excluding prefrontal cortex), BTBR mice had significant upregulation of α3 (p=0.004) and downregulation of β2 (p=0.004) nAChR subunits compared to B6 (Figure 5D). In all adult brain regions examined, α6 nAChR subunit mRNA expression was very low (data not shown).

We then investigated whether in BTBR mice, these differences in brain nAChR subunits mRNA expression were present during embryonic development. We found that, embryonic (E13) BTBR brain had significantly reduced nAChR mRNA levels of α3 (p<0.0001); α4 (p=0.004), α7 (p=0.006), β2 (p=0.007), and β4 (p=0.001) compared to B6 fetuses (Figure 6). In fetal brains, α5, α6, and β3 nAChR subunit mRNA expression was very low at the time examined (data not shown).

Concerning *Fmr1*-KO mice, nAChR subunits mRNA levels prefrontal cortex and hippocampus were similar to FVB controls (data not shown). However, in cerebellum, *Fmr1*-KO mice had significantly decreased expression of α4 [0.625, (0.246, 1.141) 95% C.I, fold change and 95% confidence interval, p=0.032] and β4 [0.544, (0.277, 1.077) 95% C.I, p=0.023] compared to FVB controls.

4. Discussion

The results of the present study add to the existing literature showing that animal models carrying mutations known to occur in idiopathic and syndromic ASD have associated alterations in nociception and nocifensive behavior. Here we found that both, the BTBR and the *Fmr1*-KO mice on an FVB background had hyporesponsiveness to noxious hot and cold stimuli, and actually, hyperresponsiveness to visceral pain (acetic acid writhing test) compared to respective controls. We also found that nicotine, a prototypical nAChR agonist, which has been shown to modulate behavior deficits in BTBR mice, had differential effects in BTBR and mice with normal sociability. Taken together, these findings suggest that in ASD-like models, there are associated alterations in nocifensive behavior that can vary according to stimulation modality and that cholinergic agents that modulate social and repetitive behavior deficits can also modulate their altered nocifensive phenotype.

We found that compared with respective control strains, BTBR and *Fmr1*-KO mice displayed hyporesponsiveness to noxious thermal stimuli and electrical stimulation of C sensory fibers, similar responsiveness to electrical stimulation of Aβ and Aδ fibers, and hyperresponsiveness to visceral pain following intraperitoneal injections of acetic acid. Interestingly, these results mirror those findings in ASD individuals who display a mixed pattern (sensory hyper- and hyporesponsiveness) of nocifensive response (Baranek et al., 2006). We also found that the antinociceptive effects of nicotine in BTBR mice, were significantly different from the effects observed in control mice. In BTBR, at lower doses (50 and 100μg/ml), nicotine accentuated the hyporesponsiveness to the hotplate test but attenuated the hyporesponsiveness to the cold plate, an effect pattern that was significantly different from that observed in control B6 mice. At higher doses (200 and 400 μg/ml) nicotine decreased hotplate latency, normalizing responsiveness to thermal stimuli, but it decreased cold plate sensitivity, thus accentuating the hyporesponsiveness to noxious cold in BTBR mice. Therefore, these differential responses to the antinociceptive effects of nicotine raise the possibilities that the nicotinic cholinergic system could be altered in ASD and that nicotine, in addition to modulating social deficits and repetitive behaviors (Wang et al., 2015), also modulates the altered nocifensive phenotype in BTBR mice.

The findings that BTBR and *Fmr1*-KO mice have increased writhing after intraperitoneal injection of acetic acid, which models visceral pain, are relevant. While one must be circumspect about extrapolating findings in mice to humans, it is tempting to speculate that these findings in the models of ASD studied here recapitulate those in humans with ASD. In fact, a recent meta-analysis showed that children with ASD experience significant more gastrointestinal symptoms including more abdominal pain than those without ASD (McElhanon et al., 2014). Interestingly, nicotine modulated the nocifensive response in the ASD model reducing the number of writhes in a pattern suggestive that the effect of nicotine was lesser than in BTBR than in control mice. Therefore, these findings suggest that the models studied here are valuable for studies of increased visceral pain in ASD and that the nicotinic cholinergic system might have a role in modulating the nocifensive response to visceral pain in ASD models.

Others have shown that administration of nicotine acutely or chronically can yield nonlinear patterns of dose-effect relationships on animal behavior (Picciotto, 2003; Popke et al., 2000). For example, in rats, acute nicotine administration is associated with an U-shaped dose response in complex cognitive tasks that require accurate time perception while in tasks that evaluate response rates, nicotine yields a bell-shaped dose-effect relationship (Popke et al., 2000). We had previously reported that nicotine administered over four weeks at doses used in the present investigation, also yielded non-linear dose-effect relationships on social and repetitive behaviors in the BTBR and B6 mice (Wang et al., 2015). Thus, the findings of a nonlinear pharmacodynamic profile of chronic nicotine on nocifensive behavior in BTBR and B6 mice are in keeping with our previous results and those of others(Picciotto, 2003; Popke et al., 2000). One could only speculate that these non-linear patterns of nicotine dose-response curves reflect differences in molecular and/or genetic factors leading to different response patterns in BTBR and B6 mice. Alternatively, one could argue that nicotine at different doses activates and/or inhibits different neuronal circuits in the BTBR and B6 brain, thus leading to a non-liner dose-response.

Interestingly, the nocifensive behaviors alterations and the differential responses to nicotine in BTBR mice were associated with significant downregulation of the expression of several nAChR subunits (α3, α4, α7, β2, and β4) during embryonic development (E13) and adulthood. That altered nAChR subunit expression impacts animal behavior, synapse formation, and neuronal architecture has been shown in investigations of mice with null-mutations of specific nAChR subunits (Bailey et al., 2010; Role and Berg, 1996). For example, mice with α5 nAChR subunit null mutations have impaired attentional performance (Bailey et al., 2010), a deficit that is associated with lack of α5-dependent developmental peak in nicotinic signaling early in post-natal life and alterations in dendritic morphology in prefrontal cortex(Bailey et al., 2012). Mice with β2 nAChR subunit null mutations have behavior inflexibility shown by absence of adaptive behaviors and impaired capacity to interrupt ongoing behaviors (Avale et al., 2011; Granon et al., 2003). Interestingly, β2 null mutants also have impaired spatial learning at older age, which is coupled with neocortical hypertrophy, hippocampal pyramidal neuronal loss, astrogliosis, and microgliosis (Zoli et al., 1999). Therefore, taken together, our results and those of others raise the hypothesis that alterations in nAChRs subunits expression might play a role not only on social deficits and repetitive behavior, but also in the associated nocifensive behavior alterations in ASD-like mice.

That nAChRs subunits have a role on nocifensive behavior and on the antinociceptive effects of nicotine has also been previously shown (Bagdas et al., 2015; Damaj et al., 1999; Freitas et al., 2015; Saika et al., 2015; Wieskopf et al., 2015; Xanthos et al., 2015; Yalcin et al., 2011). In studies using nAChR agonists and antagonists as well as mice with nAChR subunits null mutations, researchers have shown that nAChRs containing α3, α4, α5, α6, α7, β2, and β4 subunits are involved in modulating nocifensive behaviors in response to acute noxious thermal stimuli (Damaj et al., 2007; Damaj et al., 1999; Marubio et al., 1999) and in response to inflammatory and neuropathic processes (AlSharari et al., 2012; Bagdas et al., 2015; Damaj et al., 1999; Freitas et al., 2015; Saika et al., 2015; Wieskopf et al., 2015; Xanthos et al., 2015; Yalcin et al., 2011). Others have also shown that mice with null mutations of α4, α5, or β2 have decreased response to the analgesic effects of nicotine, but

intact response to the antinociceptive effects of morphine (Damaj et al., 2007; Marubio et al., 1999). Here we showed that while nicotine had an effect both in B6 (control) and BTBR mice, the effect of nicotine was significantly different in BTBR mice compared to the control strain. Further, these differential effects were observed at different nicotine doses and in response to different stimulation modalities. Therefore, future studies will test the hypotheses that 1) downregulation of nAChR subunits might have contributed to the altered nocifensive behavior phenotype in BTBR mice and 2) different nAChR subtypes modulate the differential responses to nicotine in BTBR compared to B6 mice.

While the mechanisms of altered nocifensive response in BTBR and *Fmr1*-KO mice are incompletely understood, this study supports the use of these two models for investigations of the pathobiology of altered sensory responsiveness associated with ASD. While these two models have entirely unrelated and different genetic abnormalities and backgrounds, it is noteworthy that BTBR and *Fmr1*-KO mice displayed similar nocifensive behavior phenotypes. One emerging principle in ASD is that circuit-level alterations could possibility explain the phenomenon that similar ASD behavior phenotypes could originate from syndromes associated with significant genetic disparities. In fact, researchers have hypothesized that imbalances in excitatory and inhibitory circuits could possibly contribute to the core behavior deficits in ASD (Zikopoulos and Barbas, 2013) and a few studies in BTBR (Han et al., 2014) and *Fmr1*-KO mice (Martin et al., 2014; Olmos-Serrano et al., 2011) support this hypothesis. For example, in BTBR, treatment with negative allosteric modulators of metabotropic glutamate receptor 5, which modulates excitatory neurotransmission, improves social deficits and reduces repetitive behavior (Silverman et al., 2012; Silverman et al., 2010a). Researchers have shown that BTBR mice have reduced GABAergic tone and treatment with agents that modulate excitatory/inhibitory balance, such as positive allosteric modulators of postsynaptic $GABA_A$ receptors and $GABA_B$ agonists or histone deacetylase inhibitors, improves social deficits and repetitive behaviors in these animals (Han et al., 2014; Kratsman et al., 2016; Silverman et al., 2015). One could then speculate that imbalances between excitatory and inhibitory circuits could also contribute to alteration in nocifensive behaviors displayed by BTBR and *Fmr1*-KO mice. Further, as researchers have shown that the antinociceptive effects of nicotine are altered in the setting of decreased GABAergic signaling (Varani et al., 2012), it is also conceivable that imbalances in excitatory/inhibitory signaling in BTBR, could at least in part have contributed to the differential effects of nicotine comparing BTBR and control mice. In turn, these findings in BTBR mice are compatible with the hypothesis that, chronic nicotine, possibly by its interaction with the GABAergic system, modulates nocifensive response as well as social deficits and repetitive behavior.

We note some limitations of this study. Some might argue that the nociception evaluation modalities examined here are reflexive in nature and therefore incompletely evaluate the influence of the effect of cholinergic agents on behavior domains that are relevant to pain perception and sensitivity such as anxiety, fear, and memory (Vierck and Yezierski, 2015). In fact, researchers have shown that changes in cholinergic tone can yield discrepant results in reflexive and operant escape evaluations of nociception (Vierck et al., 2016). Therefore, it is important that future studies determine the effect of nicotine on operant escape from nociceptive thermal stimulation in BTBR and control mice to further our understanding of

the role of the cholinergic system on altered nociception in animal models of ASD. In addition, some might argue that by not correcting for multiple comparisons, the alpha error rate in the analysis of the mRNA levels data could have been inflated. We posit that by using robust and powerful tools for the analysis of behavior data (mixed-effects model (Liu et al., 2010; Wainwright et al., 2007)) and mathematical approaches based on a pair wise fixed reallocation randomization test to analyze the mRNA data (Pfaffl, 2001) added rigor to the analytical approach and minimized inflation of the alpha error rate.

Nevertheless, the results of the present study support the hypotheses that BTBR and *Fmr1*-KO mice model the nocifensive behavioral responses described in ASD. Therefore, they could be valuable for studies of the pathobiology of the altered sensory responses in ASD. The findings of altered expression of nAChR subunits both, during development and adulthood in ASD-like mice, support the hypothesis that altered nicotinic signaling might have a role in the pathobiology of ASD. Additionally, this study lends further support to the hypothesis that nAChR-directed interventions have the potential to, not only modulate social behavior deficits, but also to modulate the observed altered nocifensive response in autistic-like mice. Lastly, the present results can inform future studies seeking to determine what specific nAChR subtypes are involved in the modulation of nocifensive response in ASD.

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Abbreviations

ASD	Autism spectrum disorder
BTBR	BTBR T^{+} *Itpr3*^{tf}/J
FVB	FVB.129P2-*Pde6b*$^{+}$ *Tyr*$^{c\text{-}ch}$/AntJ, control strain for *Fmr1*-KO
B6	C57BL/6J, control strain for BTBR
Fmr1-KO	FVB.129P2-*Pde6b*$^{+}$ *Tyr*$^{c\text{-}ch}$ *Fmr1*^{tm1Cgr}/J
nAChR	nicotinic acetylcholine receptor
RT-qPCR	real-time reverse transcription polymerase chain reaction

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Highlights

1. Sensory alteration in autism spectrum disorder is associated with disease severity
2. Autism-like mouse models (BTBR) display altered nocifensive behaviors
3. BTBR have significant alterations in the expression of nicotinic receptor subunits
4. Nicotine modulates behavior deficits and decreases repetitive behavior in BTBR
5. Nicotine also modulates nociception alterations in BTBR mice

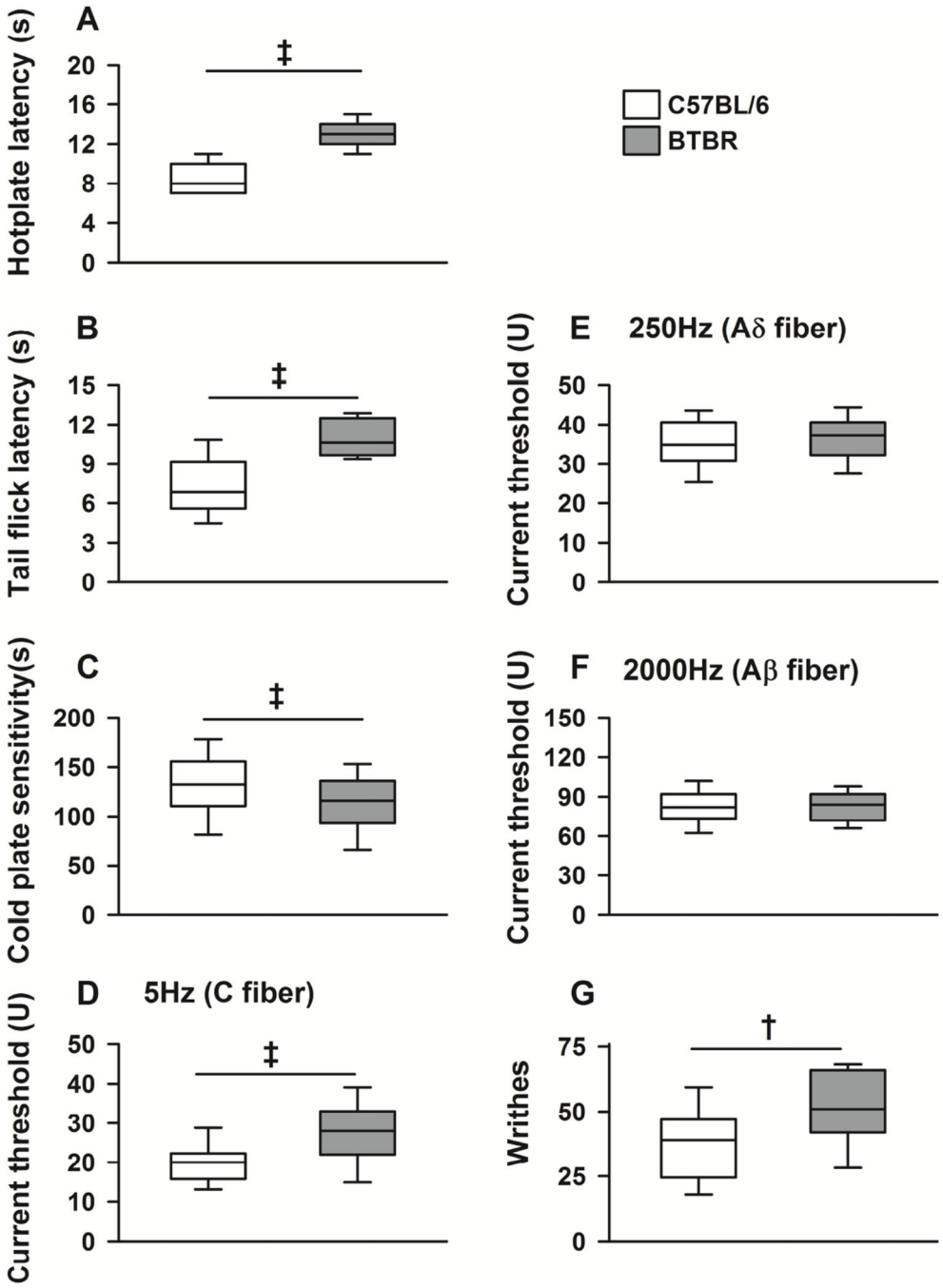

Figure 1. Nocifensive behavior phenotype in C57BL/6 (B6) and BTBR mice

Box plots show median, interquartile range, and 5th and 95th percentiles for behavior outcomes in B6 (white) and BTBR (gray) mice. P values refer to comparisons of baseline measurement between strains. BTBR mice had higher hotplate (A, $p<0.001$) and tail flick response latencies (B, $p<0.001$) compared to controls (B6). C, BTBR mice had lower sensitivity (increased tolerance) to the cold plate ($p<0.001$) compared to B6 animals. D, BTBR had higher current threshold (increased tolerance) to electrical stimulation at frequencies (5Hz) that stimulate unmyelinated (C-fiber) sensory nerve fibers ($p<0.001$), but

similar current thresholds in response to 250Hz (E, p=0.30) and 2000Hz (F, p=0.70) electrical stimulations, which stimulate Aδ and Aβ fibers respectively, compared to B6 animals. G, in a model of visceral pain (over 30 min after an intraperitoneal injection of 0.6% acetic acid), BTBR mice significantly more writhes compared to B6 animals (p=0.008). Baseline measurements were obtained before any nicotine treatment. Age-matched and balanced numbers of male and female mice were included. N ≥14 (male and female mice) per genotype for each outcome measurement. † indicates p≤0.01, and ‡ p≤0.001.

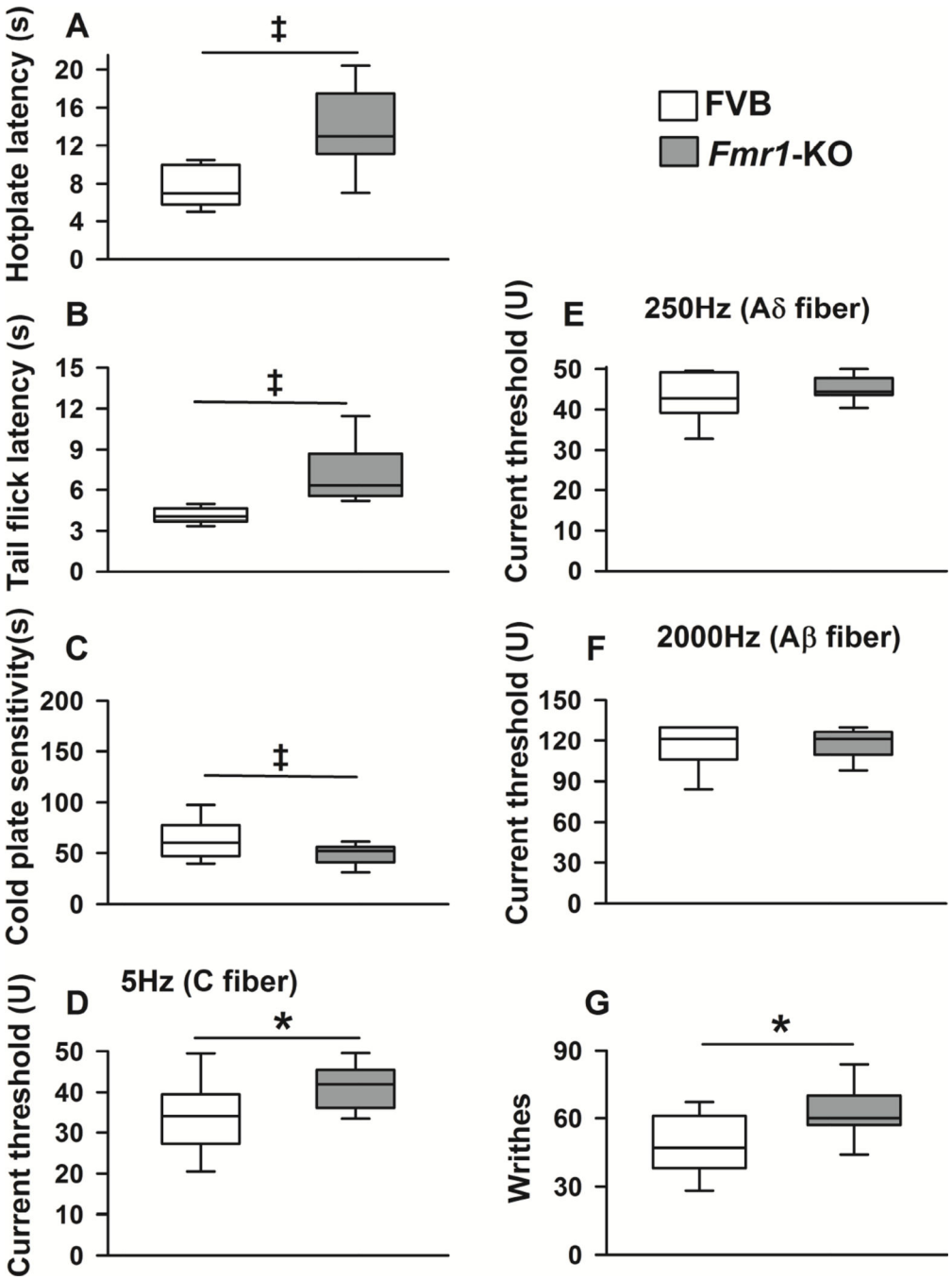

Figure 2. Nocifensive behavior phenotype in FVB and *Fmr1*-KO mice

Box plots show median, interquartile range, and 5th and 95th percentiles for behavior outcomes in FVB and *Fmr1*-KO mice. P values refer to comparisons of baseline measurement between strains. *Fmr1*-KO mice had increased tolerance to noxious heat as they had higher hotplate (**A**) and tail flick (**B**) latencies compared to FVB control mice (both p≤0.001). C, *Fmr1*-KO mice had increased tolerance to noxious cold stimuli as they displayed lower sensitivity to the cold plate compared to FVB animals (p=0.001). D, *Fmr1*-KO mice, compared to FVB animals, had higher current threshold in response to 5Hz

stimulation (p=0.02). In contrast, compared to FVB mice, *Fmr1*-KO animals had similar sensitivity to 250Hz (E, p=0.19) and 2000Hz (F, p=0.85) electrical stimulation, which recruit myelinated Aδ and Aβ fibers respectively. G, *Fmr1*-KO mice also displayed significantly greater number of writhes after an intraperitoneal injection of 0.6% acetic acid compared to FVB mice (p=0.024). Age-matched males were investigated as only males have the behavior phenotypes of interest. N≥6 mice (males only) per group for each outcome measurement. indicates $p<0.05$, † indicates $p\leq0.01$, and ‡ $p\leq0.001$.

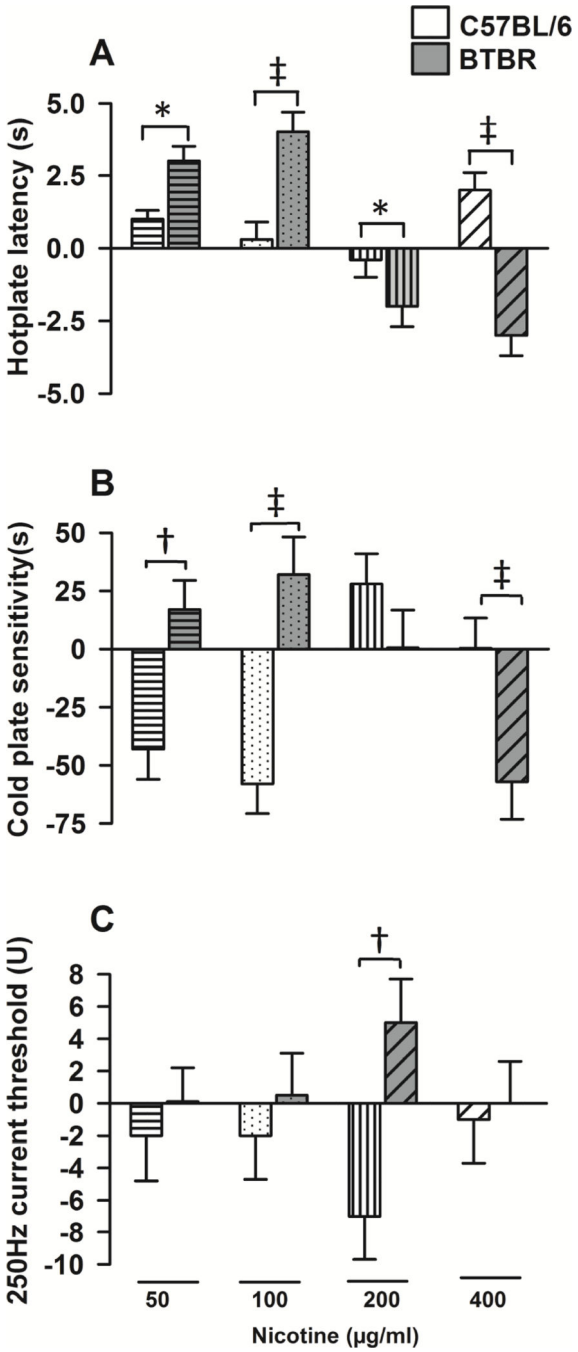

Figure 3. Differential effect of nicotine in C57BL/6 (B6) and BTBR mice

Bars represent (means±SEM) nicotine effect compared with vehicle on behavior outcomes changes from baseline in B6 (white background bars) mice and BTBR (gray background bars). P values reflect refer to comparisons of the effect of nicotine vs. vehicle on changes from baseline between the two (BTBR vs. B6) strains (strain*time*treatment interactions). The nicotine doses indicated at the bottom of the figure apply to all panels. A, compared with vehicle, four-week nicotine treatment at lower doses (50 and 100µg/ml) was associated with significantly greater increases (from baseline) on hotplate latency in BTBR than in B6

mice (A, strain*time*treatment interaction p=0.016 and p<0.001 for 50 and 100μg/ml respectively). Compared with vehicle, at 200 and 400μg/ml nicotine led to significant decreases in hotplate latency in BTBR, whereas in B6 mice, it lead to no significant changes or increases in hotplate latency (A, strain*time*treatment interaction p=0.040 and p<0.001 for 200 and 400μg/ml respectively). B, concerning cold sensitivity, nicotine was also associated with a different pattern of effect in BTBR compared with B6. Compared with vehicle, at lower doses (50 and 100μg/ml) nicotine led to significant increases in BTBR, whereas in B6 mice nicotine was associated with significant decreases in cold plate sensitivity (B, strain*time*treatment interaction p=0.006 and p<0.001 for 50 and 100μg/ml respectively). Nicotine at 400μg/ml, compared with vehicle, was associated decreases in cold plate sensitivity in BTBR but with no significant changes in B6 mice (B, p=0.001). C, in response to 250Hz stimulation, compared with vehicle, nicotine treatment at 200μg/ml was associated with slight increases in current thresholds in BTBR, whereas in B6, to slight decreases in current thresholds in significantly different patterns (p=0.008). N≥10 (male and female) mice for each nicotine dose. *indicates p<0.05, † p≤0.01, and ‡ p≤0.001.

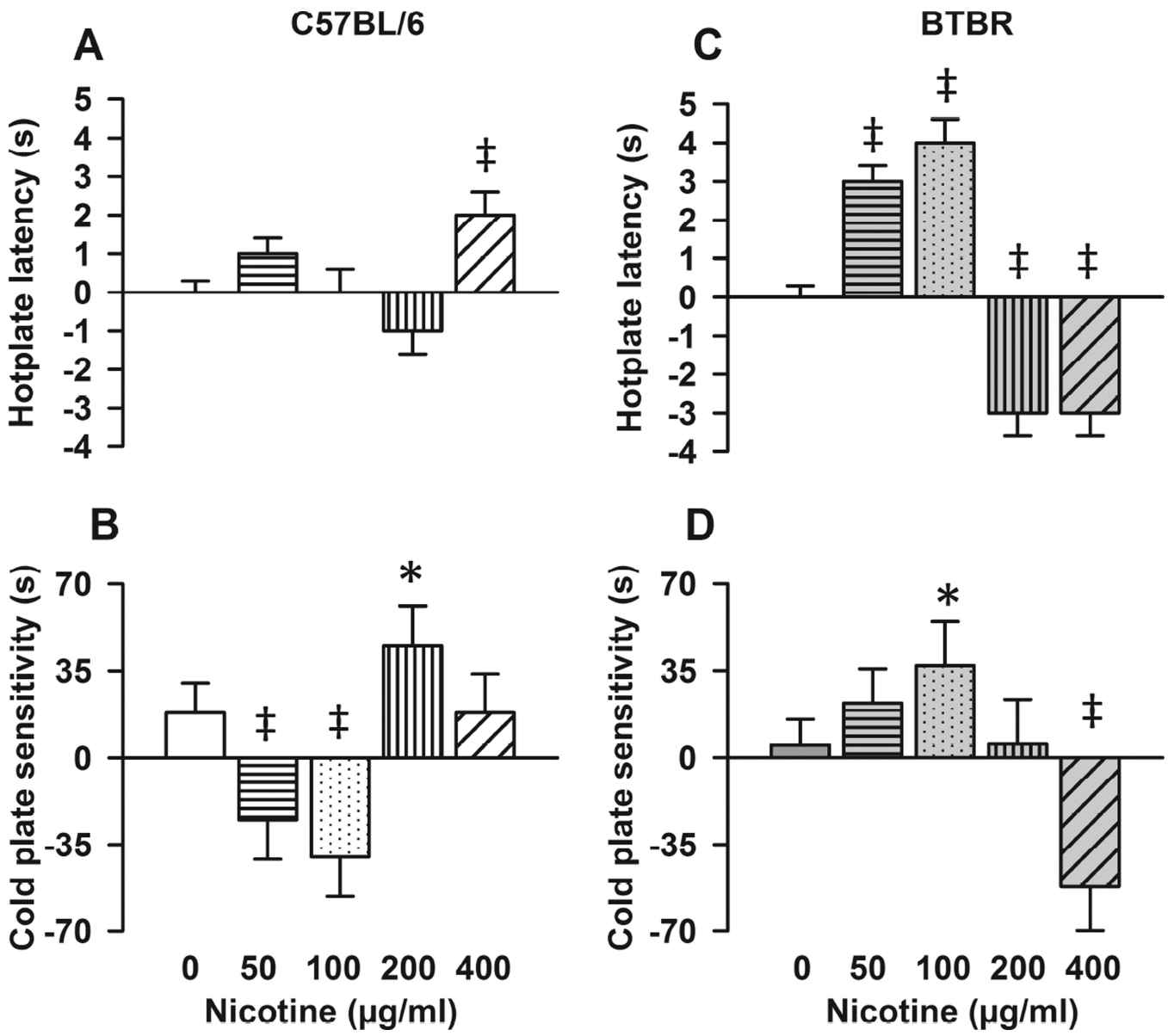

Figure 4. Effect of nicotine on nocifensive behavior in *C57BL/6 (B6)* and BTBR mice

Bars represent (means±SEM) changes (post-treatment vs. baseline) in behavior outcomes in B6 (A and B) and in BTBR mice (C and D) at respective nicotine doses. P values reflect time (post nicotine vs. baseline) and treatment (vehicle vs. nicotine) interactions within indicated mouse strain. A, in B6 mice, nicotine only at the higher dose (400µg/ml) increased hotplate latency compared with vehicle (p=0.001). B, compared with vehicle, at lower doses (50µg/ml and 100µg/ml), nicotine decreased (B, time*treatment interaction, p≤0.001), whereas at 200µg/ml, it increased (p=0.034) sensitivity to cold plate in B6 mice. C, in BTBR mice, compared with vehicle, lower nicotine doses increased (time*treatment interaction, both p<0.001) and higher doses (200g/ml and 400µg/ml) decreased hotplate latency (both p=0.001, time*treatment interaction). D, compared with vehicle, at 100 µg/ml, nicotine increased (time*treatment interaction, p= 0.046), whereas at 400µg/ml, it decreased

($p<0.001$) sensitivity to cold plate in BTBR mice. N≥10 (male and female) per nicotine dose. *indicates $p<0.05$, † $p\leq0.01$, and ‡ $p\leq0.001$.

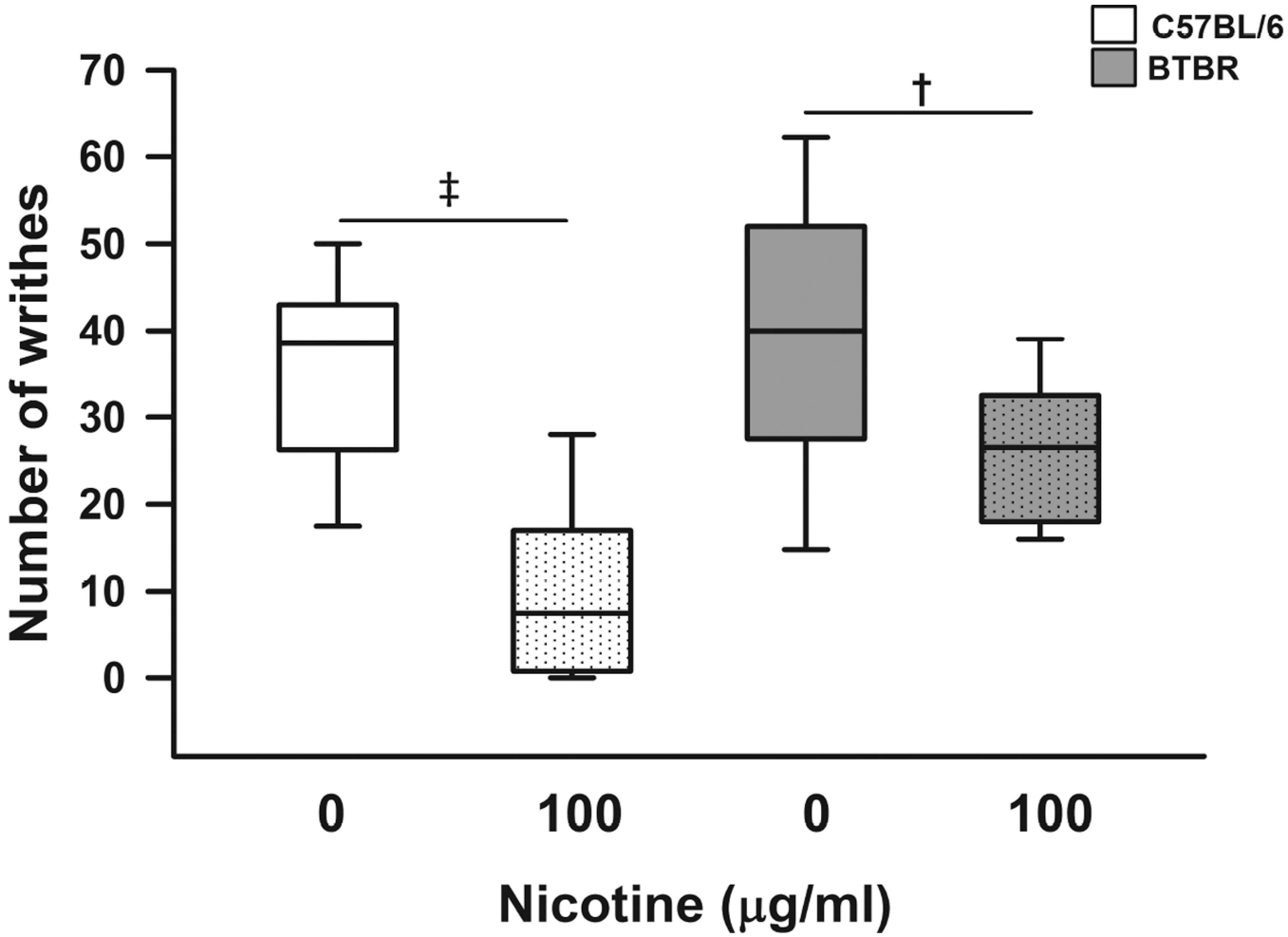

Figure 5. Effect of nicotine on visceral pain in *C57BL/6 (B6)* and BTBR mice

Box plots show median, interquartile range, and 5th and 95th percentiles for number of writhes after acetic acid intraperitoneal injection in B6 (white) and BTBR (gray) mice. P values indicate comparisons between treatments (nicotine 0 or 100μg/ml) within respective strains. Nicotine-treated B6 and BTBR mice had significantly less writhes than saccharine-treated respective controls ($p<0.001$ for B6 and $p=0.007$ for BTBR). N=14 (age matched balanced number of male and female) mice per group. † indicates $p\leq0.01$, and ‡ $p\leq0.001$.

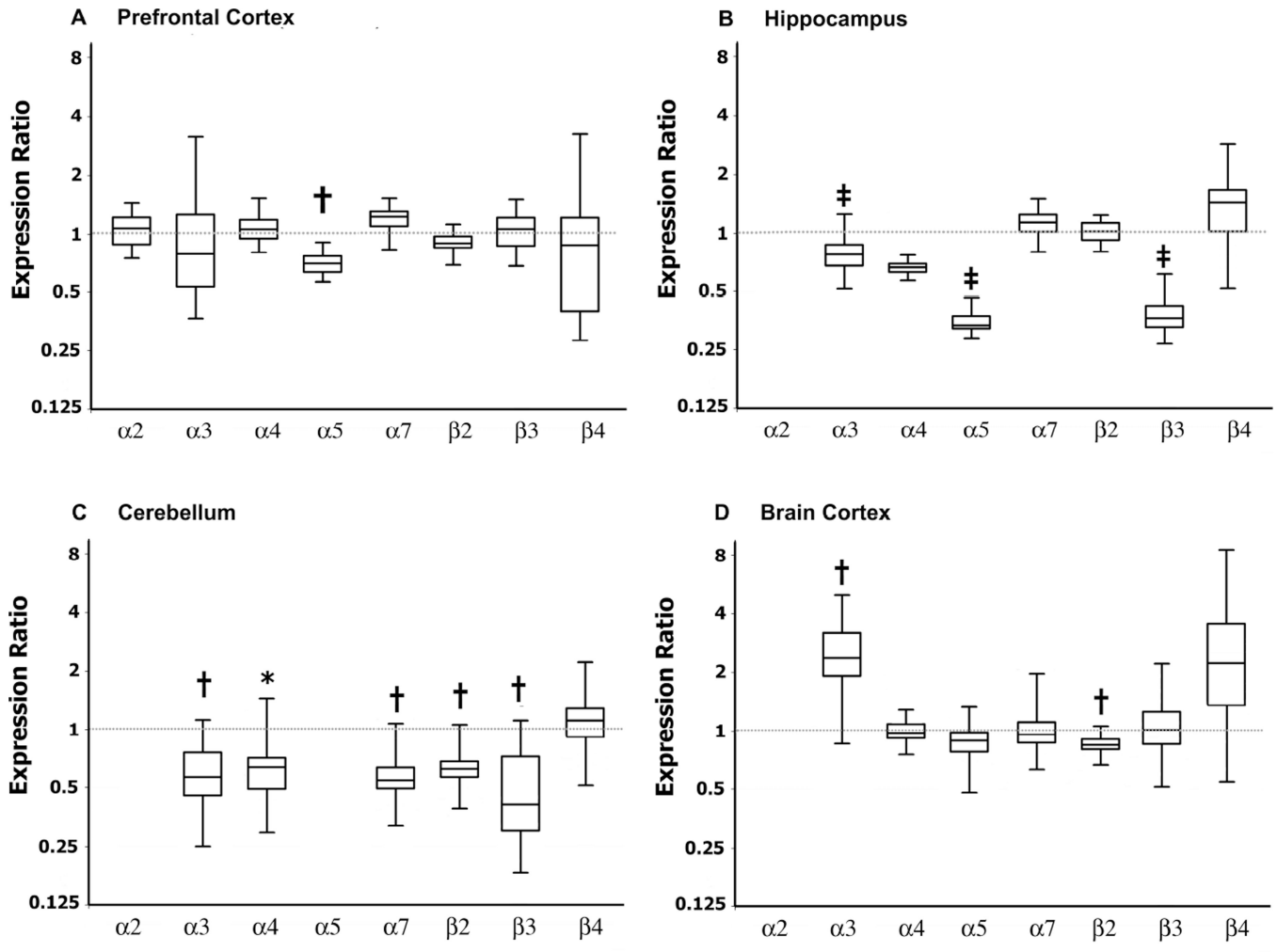

Figure 6. Expression of nicotinic acetylcholine receptor (nAChR) subunits in adult BTBR mice

Box plots show median and interquartile range of observations and whiskers represent minimum and maximum observations. P values refer to indicated gene expression ratios comparing BTBR and B6 (shown by the dotted line = 1). Compared to B6 mice, BTBR had significant downregulation of mRNA levels of α5 (A, p=0.006) nAChR subunit in prefrontal cortex; α4 (p<0.0001), α5 (p=0.001), and β3 (p<0.0001) in hippocampus (B); α3 (p=0.006), α4 (p=0.024), α5 (p=0.002), α7 (p=0.005), β2 (p<0.005), and β3 (p=0.01) in cerebellum (C). In addition in brain cortex, BTBR mice had significant upregulation of α3 (p=0.004) and downregulation of β2 (p=0.004) nAChR subunits compared to B6 mice (D). N= 6 mice per genotype. *indicates p<0.05, † p≤0.01, and ‡ p≤0.001,

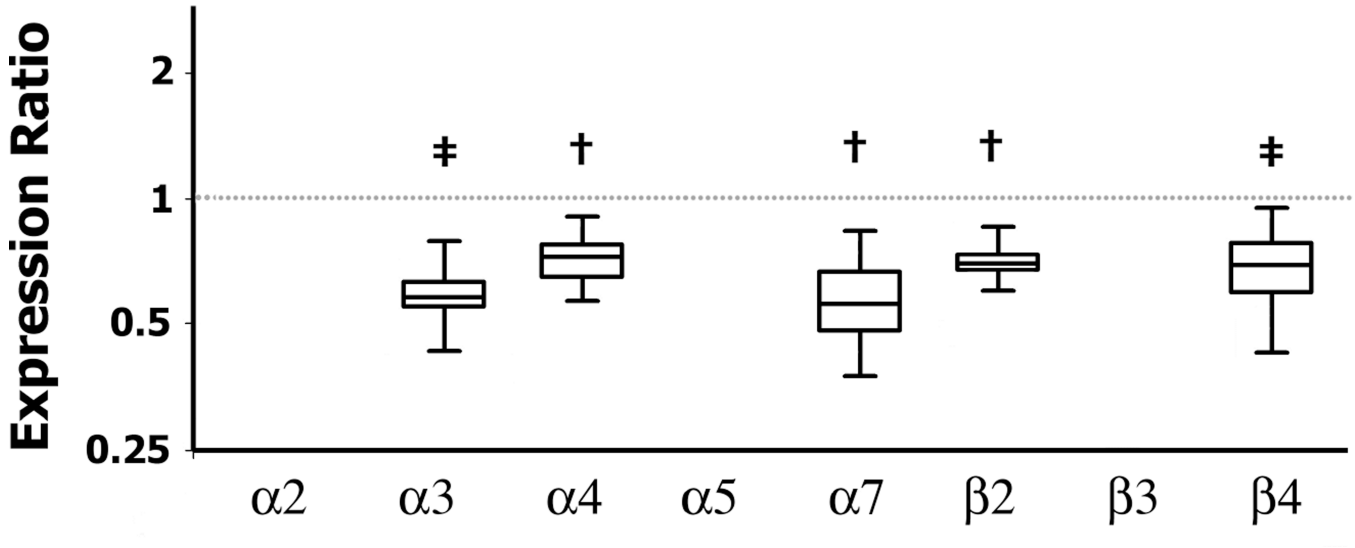

Figure 7. Expression of nicotinic acetylcholine receptor (nAChR) subunits in BTBR mice during embryonic development

Box plots show median and interquartile range of observations and whiskers represent minimum and maximum observations. P values refer to indicated gene expression ratios of fetal brain comparing BTBR and B6 (shown by the dotted line = 1). Fetal brains were collected from pregnant B6 and BTBR mice on E13. In BTBR fetal brains there were significant downregulation of α3 ($p<0.0001$), α4 ($p=0.004$), α7 ($p=0.006$), β2 ($p=0.007$), and β4 ($p=0.001$) nAChR mRNA levels compared to B6 fetuses. N= 5 pooled pregnancies per genotype. * indicates $p<0.05$, † $p\leq0.01$, and ‡ $p\leq0.001$.