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Developmental pyrethroid exposure causes long-term decreases of neuronal sodium channel expression

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Abstract

Pyrethroid insecticide use has increased over recent years because of their low to moderate acute toxicity in mammals. However, there is increasing concern over the potential detrimental effects of pyrethroids on developing animals. Most recently, we have shown that developmental exposure to deltamethrin results in long-term neurobehavioral effects. Pyrethroids exert their toxicity by acting on the voltage-gated sodium channel (Na_v), delaying channel inactivation and causing hyperexcitability in the nervous system. Previous *in vitro* studies found that exposure to agents that increase Na^+ in flux, including deltamethrin decreased Na_v mRNA expression. However, it is unknown whether this occurs *in vivo*. To determine whether developmental pyrethroid exposure decreases Na_v mRNA expression, pregnant mice were exposed to the pyrethroid deltamethrin (0 or 3 mg/kg) every three days throughout gestation and lactation. Na_v mRNA expression was measured in the striatum and cortex of the offspring at 10–11 months of age, a time at which behavioral abnormalities were still observed. Developmental exposure to deltamethrin decreased expression of Na_v mRNA in a region- and isoform-specific fashion by 24–50%. Deltamethrin exposure also resulted in the persistent down-regulation of brain-derived neurotrophic factor (Bdnf) in the striatum by 66% but not in the cortex, suggesting a plausible mechanism for some of the associated behavioral effects observed previously. Taken together these data suggest that developmental deltamethrin exposure results in persistent deficits in Na_v and BDNF mRNA expression that may contribute to long-term behavioral deficits.

Keywords

Pyrethroid; Deltamethrin; Sodium channel; BDNF; Neurodevelopmental

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Conflict of interest

The authors declare they have no conflict of interest.

Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.neuro.2016.04.002.

1. Introduction

Pyrethroid insecticides are potent neurotoxic insecticides that account for over 25% of the total annual pesticide use in the world (Casida and Quistad, 1998; Horton et al., 2011; Morgan, 2012). The primary mechanism by which pyrethroids exert their neurotoxic effects in insects involves delaying the inactivation of voltage-gated sodium channels (Na_v) (Narahashi, 1971). This delayed inactivation increases sodium in flux, action potential generation, neuronal excitability and ultimately leads to conduction block, particularly with Type II pyrethroids, convulsions and death, if the dose is high enough (Narahashi, 1996).

Na_v are integral membrane proteins principally composed of a large (~260 kDa) α subunit that forms a central ion-conducting pore and one or more smaller auxiliary β subunits that are important modifiers of channel gating kinetics, cell-surface expression, and cell-cell interactions (Isom, 2001). The mammalian genome contains 10 sodium channel α subunit isoforms and four β subunits isoforms, yielding multiple subunit combinations, some of which are differentially sensitive to the effects of pyrethroids (Meacham et al., 2008; Soderlund, 2012). In an oocyte expression system, deltamethrin has the most pronounced effect on sodium currents through $Na_v1.3/\beta3$ (Meacham et al., 2008) and $Na_v1.6/\beta1 + \beta2$ channels (Tan and Soderlund, 2010). This may be particularly important to the potential developmental neurotoxicity of deltamethrin, as $Na_v1.3/\beta3$ channel complexes are highly expressed in the developing rodent brain (Albrieux et al., 2004) and the $Na_v1.6$ isoform is abundantly expressed in the nodes of Ranvier, dendrites, and synapses (Caldwell et al., 2000).

Although there is a general lack of information in the literature describing the developmental neurotoxicity of pyrethroids (Shafer et al., 2005), developing animals are more susceptible to the acute toxic effect of type II pyrethroids (Sheets et al., 1994). This greater susceptibility has been ascribed to lower metabolic detoxication enzymes compared to adults (Cantalamessa, 1993; Sheets et al., 1994). However, the greater abundance of the highly sensitive $Na_v1.3/\beta3$ channels during neuronal development may also contribute to the increased susceptibility of the developing central nervous system (Meacham et al., 2008). Determining mechanism (s) of the potential developmental neurotoxicity of pyrethroids is particularly important because there is documented exposure of pregnant women and children to pyrethroids (Whyatt et al., 2002; Berkowitz et al., 2003; Heudorf et al., 2004; Bouwman et al., 2006; Barr et al., 2010), and the number of pyrethroid poisonings in children reported to poison control centers has increased recently (Power and Sudakin, 2007). More recently, we reported higher levels of pyrethroid metabolites in the urine of children increased likelihood of ADHD diagnosis (Wagner-Schuman et al., 2015) and that low-level deltamethrin exposure of mice during gestation resulted in hyperactivity and impulsive-like behavior in their male offspring (Richardson et al., 2015).

Previous *in vitro* studies found that exposure to compounds that delay Na_v inactivation, such as veratridine and scorpion toxin, decreased Na_v protein levels (Dargent and Couraud, 1990) and mRNA expression (Lara et al., 1996; Magby and Richardson, 2015), possibly as a compensatory mechanism to reduce hyperexcitability in the cells. The mechanism of this down-regulation is not well established, but may include calpain activation. Additionally,

in vitro studies reported that the down-regulation of sodium channels occurs only in brain slices from immature animals and not in slices obtained from adult animals (Dargent et al., 1994). These findings suggest a unique susceptibility of the developing nervous system to agents that increase Na_v influx that is likely independent of differences in detoxication capacity. However, it is not known whether *in vivo* exposure to agents that delay Na_v inactivation, such as pyrethroids, causes similar effects.

In this study, we report that developmental exposure to the pyrethroid deltamethrin results in long-term down-regulation of Na_v mRNA expression *in vivo*. This down-regulation is correlated with decreased brain-derived neurotrophic factor (BDNF) an important growth factor that is regulated by neuronal activity (Lu, 2003; Imamura et al., 2006; Sharma et al., 2008; Hara et al., 2009; Ihara et al., 2012). Thus, developmental exposure to deltamethrin appears to result in long-term changes in Na_v expression, which could contribute to the persistent alterations of neuronal function and long-term behavioral deficits observed in our previous study.

2. Materials and methods

2.1. Chemicals

Deltamethrin was purchased from ChemService (99.5% purity and lot 418–66B; West Chester, PA). All other reagents were purchased from Sigma-Aldrich unless otherwise noted.

2.2. *In vivo* exposure

Male and female C57BL/6J mice were purchased from Jackson Laboratories (Bar Harbor, ME) at 6–8 weeks of age. Mice were maintained on a 12:12 light/dark cycle with food and water available ad libitum. All procedures were conducted in accordance with the NIH Guide for Care and Use of Laboratory Animals and approved by the Institutional Animal Care and Use Committee at Rutgers-Robert Wood Johnson Medical School.

Breeding and deltamethrin treatments were performed as described previously (Richardson et al., 2015). Briefly, pregnant female mice were individually housed and fed 0 or 3 mg/kg deltamethrin dissolved in corn oil and administered in a small amount of peanut butter every 3 days throughout gestation and lactation, starting at gestation day 6 and ending at weaning on PND25. This dosing paradigm was used to reduce handling stress on the pregnant female (Caudle et al., 2005) and the dose administered every 3 days to allow for significant clearance of the compound (Ruzo et al., 1979). This dose of deltamethrin is lower than the developmental NOAEL (12 mg/kg;(Kavlock et al., 1979)) and was determined based on our previous work that demonstrated no overt toxicity to the dam or offspring (Armstrong et al., 2013). Male mice were sacrificed by decapitation at approximately 10–11 months of age (n = 5–7 with each animal representing an individual litter). These mice were littermates of those previously reported to display hyperactivity and impulsive-like behavior at this time point (Richardson et al., 2015). Males were chosen for these experiments to eliminate the possibility of estrogen-related cyclicity, as estrogen levels affect Na_v expression (Hu et al., 2012; Wang et al., 2013), and because of the male predominant behavioral deficits observed following developmental deltamethrin exposure (Richardson et al., 2015). Brains

were removed, and the frontal cortex and striatum dissected on ice, and frozen in liquid nitrogen. Samples were stored at −80 °C until RNA isolation.

2.3. Quantitative real-time polymerase chain reaction (qPCR)

qPCR was performed as described previously (Richardson et al., 2008; Fortin et al., 2013). Briefly, total RNA was isolated using Qiagen RNeasy mini kits and RNA (0.5 μg) was reverse-transcribed using Superscript II (Invitrogen, Carlsbad, CA). QPCR reactions were performed in duplicate using an ABI 7900HT and SYBR Green (Applied Biosystems, Carlsbad, CA) detection. β-actin was used as the normalizing gene and data were calculated using the $2^{\Delta\Delta Ct}$ method as described previously (Richardson et al., 2008). Primers were designed using the Primer Blast program (NCBI) and are listed in Supplemental Table 1.

2.4. Statistical analysis

All statistical analyses were performed on raw data. Data were analyzed using Student's *t*-test to determine effects on individual isoforms within a single brain region.

3. Results

Developmental deltamethrin exposure did not result in alteration of maternal weight gain during pregnancy nor body weight of the offspring at sacrifice (Supplemental Fig. 1). Likewise, no overt signs of pyrethroid toxicity were observed in the dam or offspring.

3.1. Regional differences in Na_v gene expression in the cortex and striatum

As an initial step in characterizing the effects of developmental deltamethrin exposure on Na_v expression in 10–11 month old offspring, we first assessed whether there were regional differences in Na_v isoform expression. The relative abundance of Na_v isoform expression was $Na_v1.2 > Na_v1.6 > Na_v1.1 > Na_v1.3$, regardless of brain region examined. Comparing the two regions, in the cortex, $Na_v1.2$ (11%) and $Na_v1.6$ (21%) were more highly expressed, while $Na_v1.1$ (40%) and $Na_v1.3$ (54%) were more highly expressed in the striatum (Fig. 1) when normalized to cortex. The relative abundance of β subunits in the cortex was Navβ2 > Navβ1 > Navβ 3> Navβ4 whereas the striatum Nav β2 > Nav β1 > Nav β4 > Navβ3. Comparing the two regions the Navβ2 (50%) and Navβ4 (12x fold) were more highly expressed in the striatum than in the cortex, when normalized to cortex (Fig. 1).

3.2. Developmental deltamethrin exposure reduces expression of Na_v

In the frontal cortex, offspring developmentally exposed to deltamethrin exhibited a significant 30% reduction in $Na_v1.3$ mRNA expression compared to controls at 10–11 months of age (Fig. 2A). There was also a significant reduction (26%) in expression of the β3 subunit in the frontal cortex of exposed mice (Fig. 2B).

In contrast to the isoform-specific down-regulation of Na_v in the cortex, developmental deltamethrin exposure broadly decreased mRNA expression of all the Na_v isoforms assayed in the striatum, as $Na_v1.1$, $Na_v1.2$, $Na_v1.3$, and $Na_v1.6$ were significantly decreased by 24–26% (Fig. 3A). Similar to the α subunits, the β subunits were more broadly affected by

deltamethrin in the striatum, with significant reductions of β1, β3, and β4 by 18–27% (Fig. 3B).

3.3. Developmental deltamethrin exposure reduces BDNF mRNA in the striatum but not the cortex of male mice

Brain-derived neurotrophic factor (BDNF) expression is correlated with neuronal activity (Heese et al., 2000; Lu, 2003; Imamura et al., 2006; Abuhatzira et al., 2007; Sharma et al., 2008; Ihara et al., 2012). To determine whether decreased Na_v expression results in reduced neuronal activity, we measured BDNF mRNA as a proxy for neuronal activity. In mice developmentally exposed to deltamethrin BDNF mRNA in the cortex was not changed. However, in the striatum of mice exposed to deltamethrin, BDNF mRNA was reduced by 66% compared to control animals (Fig. 4).

4. Discussion

Previous *in vitro* studies found that exposure to compounds that delay Na_v inactivation reduces Na_v mRNA and protein (Dargent and Couraud, 1990; Dargent et al., 1994, 1995; Lara et al., 1996; Paillart et al., 1996; Magby and Richardson, 2015), but it is unknown whether this occurs *in vivo*. Here, we report that developmental deltamethrin exposure caused a significant reduction of both Na_v α and β subunit mRNAs in the brain. We also show that developmental deltamethrin results in persistent down-regulation of BDNF mRNA. These data demonstrate that developmental deltamethrin exposure causes long-term alterations in the expression of Na_v and BDNF, both of which are essential to proper brain development and function.

Measuring Na_v mRNA in the striatum and cortex yielded brain region-specific differences in basal Na_v mRNA expression as well as differences in deltamethrin-induced down-regulation. A recent paper reported that $Na_v1.1$,1.2, and 1.6 protein levels were higher in the striatum compared to the frontal cortex in nerve terminal preparations from rats (Westphalen et al., 2010). Consistent with previous results (Brysch et al., 1991), the frontal cortex expressed more $Na_v1.2$ mRNA than the striatum, which may partially explain why the overall expression of Na_v isoforms in the frontal cortex was not as affected by developmental deltamethrin exposure compared to the striatum. In contrast, the striatum has higher expression of $Na_v1.1$ and $Na_v1.3$ isoforms, which is also consistent with previous studies (Beckh et al., 1989; Shah et al., 2001). $Na_v1.3$ is especially sensitive to pyrethroid modification, whereas the relative sensitivity of $Na_v1.1$ is currently unknown, and Na_v 1.3 is more highly expressed during development in the rodent brain (Albrieux et al., 2004; Meacham et al., 2008). Taken together, these data suggest that the higher expression of pyrethroid-sensitive Na_v isoforms in the striatum, such as $Na_v1.3$ and perhaps $Na_v1.1$, may be important contributing factors to the regional differences observed in the down-regulation of Na_v isoforms following developmental deltamethrin exposure. Further, decreased expression of the corresponding β subunit isoforms likely contributes to the differential effects observed, as levels and isoform specificity of the β subunit have profound effects on Na_v gating kinetics, activity, and mRNA expression (O'Malley and Isom, 2015).

From a mechanistic standpoint, we recently reported that deltamethrin decreased Na_v mRNA through increases in intracellular calcium and activation of calpain (Magby and Richardson, 2015). Importantly, these effects were blocked by inclusion of tetrodotoxin in the culture media, demonstrating the requirement of interaction with Na_v to produce down-regulation. Although this is the first report of which we are aware to report decreased Na_v expression *in vivo* following pyrethroid exposure, deltamethrin and similar compounds that delay Na_v inactivation are not alone in their ability to alter Na_v mRNA expression. There are a number of other models in which Na_v expression is altered *in vivo*. For example, kainate-induced seizures transiently increased Na_v mRNAs encoding for $Na_v1.2$ and $Na_v1.3$ in adult rats (Bartolomei et al., 1997). In animal models, hypoxia causes Na^+-dependent depolarization following the insult (Fung and Haddad, 1997). In turn, hypoxia can either increase (Xia et al., 2000; Zhao et al., 2005) or decrease (Xia and Haddad, 1999; Gu and Haddad, 2001; Zhao et al., 2005) Na_v expression, depending on age, duration of exposure and brain region. Chronic hypoxia in adult animals causes decreased Na_v protein and mRNA expression, which coincides with a net decrease in neuronal excitability (Gu and Haddad, 2001). Thus, there appears to be the potential for developmental deltamethrin to cause long-term alterations in neuronal excitability based on its ability to down-regulate Na_v.

Previous studies found that acute exposure to pyrethroids can rapidly alter BDNF mRNA and protein *in vitro* and *in vivo* (Imamura et al., 2000, 2002, 2005, 2006; Ihara et al., 2012). Studies identifying pyrethroid induced BDNF induction report the induction is likely due to an acute increase in neuronal depolarization and subsequent increase in Ca^{2+} in flux (Imamura et al., 2006; Ihara et al., 2012; Matsuya et al., 2012). In cultured cortical neurons, 1 μM deltamethrin or *in vivo* administration of 25 mg/kg deltamethrin to seven-week old rats caused rapid increases in BDNF mRNA and protein in the cerebral cortex and hippocampus. *In vitro* studies reported that BDNF induction by veratridine was prevented by pre-treatment with permethrin and to a lesser extent deltamethrin (Imamura et al., 2000, 2002, 2005). Imamura et al. (2000) suggested that the repression of BDNF and c-fos is through effects on calcium signaling and inhibition of the transcription factor AP-1 binding in response to permethrin exposure. However, it is possible that down-regulation of Na_v could play a role in reducing activity, BDNF expression and a secondary rise in intracellular Ca^{2+}, because Na_v can be removed from the cell membrane within 15 min of exposure to an Na_v activator and decreased expression of Na_v resulted in reduced excitability and action potential firing in neurons (Dargent et al., 1994; Imamura et al., 2000; Kalume et al., 2007). Because BDNF expression is closely tied to neuronal activity decreased BDNF mRNA in the striatum, but not the cortex of exposed animals, is likely indicative of reduced neuronal activity. Indeed, neuronal activity regulates the transcription of BDNF mRNA, transport of BDNF mRNA and protein, and the secretion of BDNF protein(Lu, 2003). There is likely further connection between the effects on BDNF, as the behavioral profile reported in mice developmentally exposed to deltamethrin (*i.e.*, ADHD-like phenotype including hyperactivity and impulsive-like behavior) is similar to that observed in BDNF heterozygous mice (Monteggia et al., 2007). Further, alterations in BDNF have been linked to ADHD and risk factors for ADHD, including maternal smoking (Yochum et al., 2014). However, a direct link between decreased BDNF mRNA and Na_v mRNA expression remains to be established.

In this study, we chose to focus on mRNA expression because antibodies obtained from several commercial sources did not provide single bands for quantification of Na_v protein levels in mouse brain by western immunoblotting (data not shown). Nonetheless, previous studies reported that Na_v mRNA expression is reflective of changes in sodium channel protein levels, as measured by radiolabeled saxitoxin binding (Xia and Haddad, 1999), neuronal excitability, and electrophysiological recordings of isolated Na^+ currents (Gu and Haddad, 2001). Importantly, the reduction in Na_v was similar in magnitude to previous *in vitro* studies (Dargent et al., 1994). Future studies, including electrophysiological and other protein measurement approaches, are required to definitively correlate mRNA, function and protein levels.

5. Conclusions

Based on the data presented here, we conclude that alterations of neuronal activity due to deltamethrin exposure during the perinatal period have long lasting impacts on Na_v expression. Whether or not these changes result in alterations of neuronal excitability and functional alterations in neurotransmission is currently under investigation. Because of the importance of BDNF to neuronal development and its role in long-term potentiation it is vital to fully understand how pyrethroid exposure down-regulates BDNF mRNA. In light of previously reported association between pyrethroid exposure and ADHD (Wagner-Schuman et al., 2015) and the observation of ADHD-like behaviors in animals exposed to deltamethrin (Richardson et al., 2015), determining whether or not alterations in Na_v expression, manifesting as changes in neuronal excitability or the down-stream effects of excitability on neuro-transmitter or neurotrophin systems, could play a role in this phenotype could provide new insight into the pathophysiology of behavioral dysfunction.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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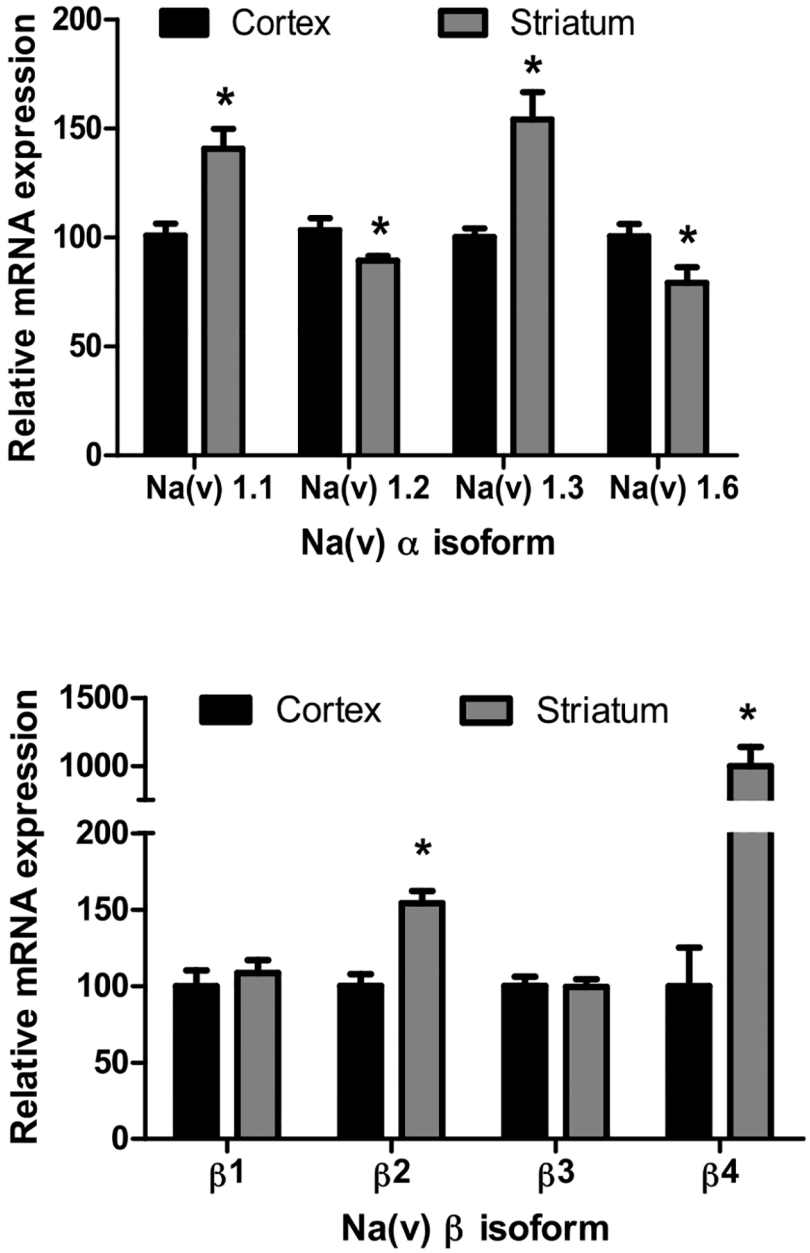

Fig. 1.

Regional Differences in Basal Expression of α subunit Sodium Channel mRNA in Frontal Cortex and Striatum. Data represent expression of Na_V mRNA in the (Top) α subunits (Bottom) β subunits. Regional differences in basal isoform expression comparing cortex to striatum using the cortex as the normalized value. Data represent $2^{\Delta\Delta Ct}$ analysis of qPCR data expressed as percentage of cortex values. * = $p < 0.05$, n = 5–7. Error bars represent SEM.

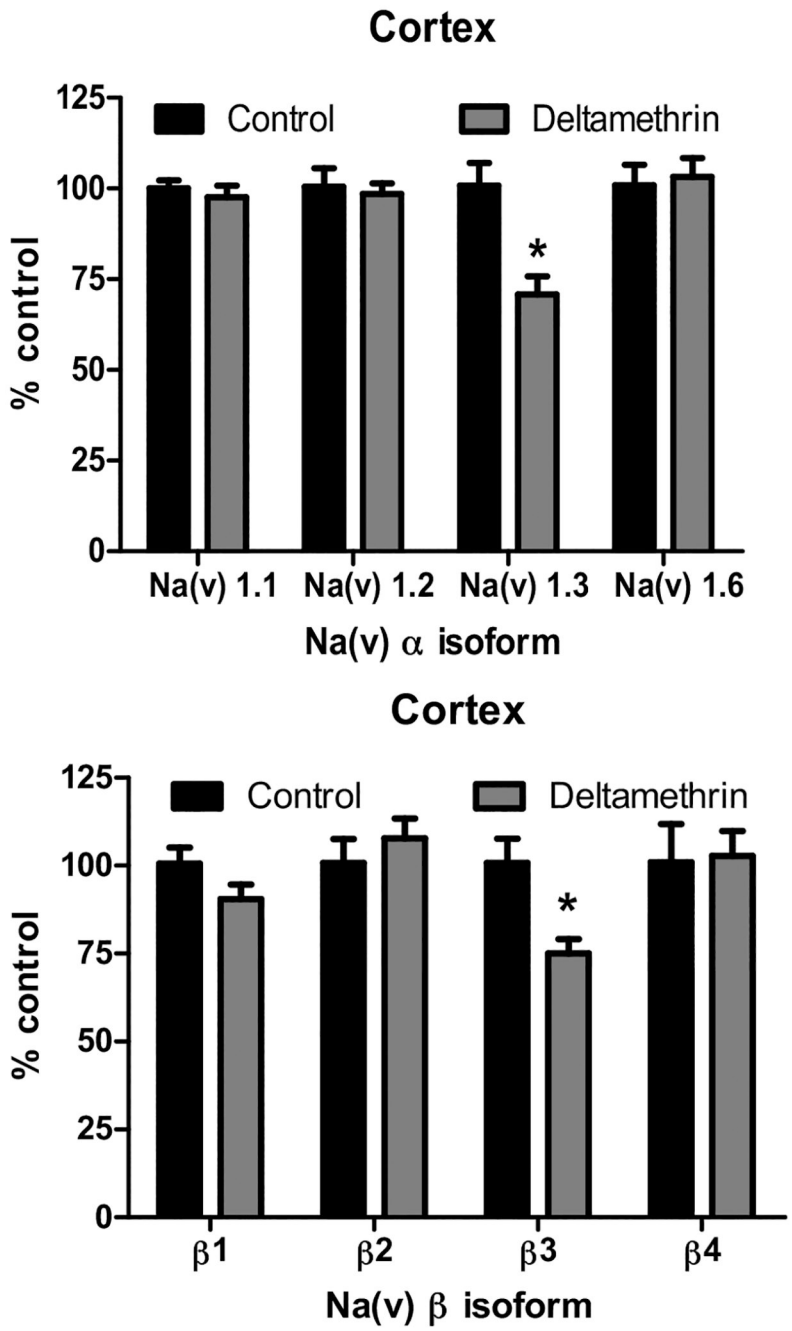

Fig. 2.

Expression of Na_v mRNA in the Frontal Cortex Following Developmental Deltamethrin Exposure. (Top) Cortex, α isoforms. (Bottom) Cortex, β isoforms. All isoforms are compared to control value for that region. Data represent $2^{\Delta\Delta Ct}$ analysis of qPCR data, expressed as percentage of control. Treatment-related differences for each isoform was determined via Student's *t*-test * = $p < 0.05$, n = 5–7. Error bars represent SEM.

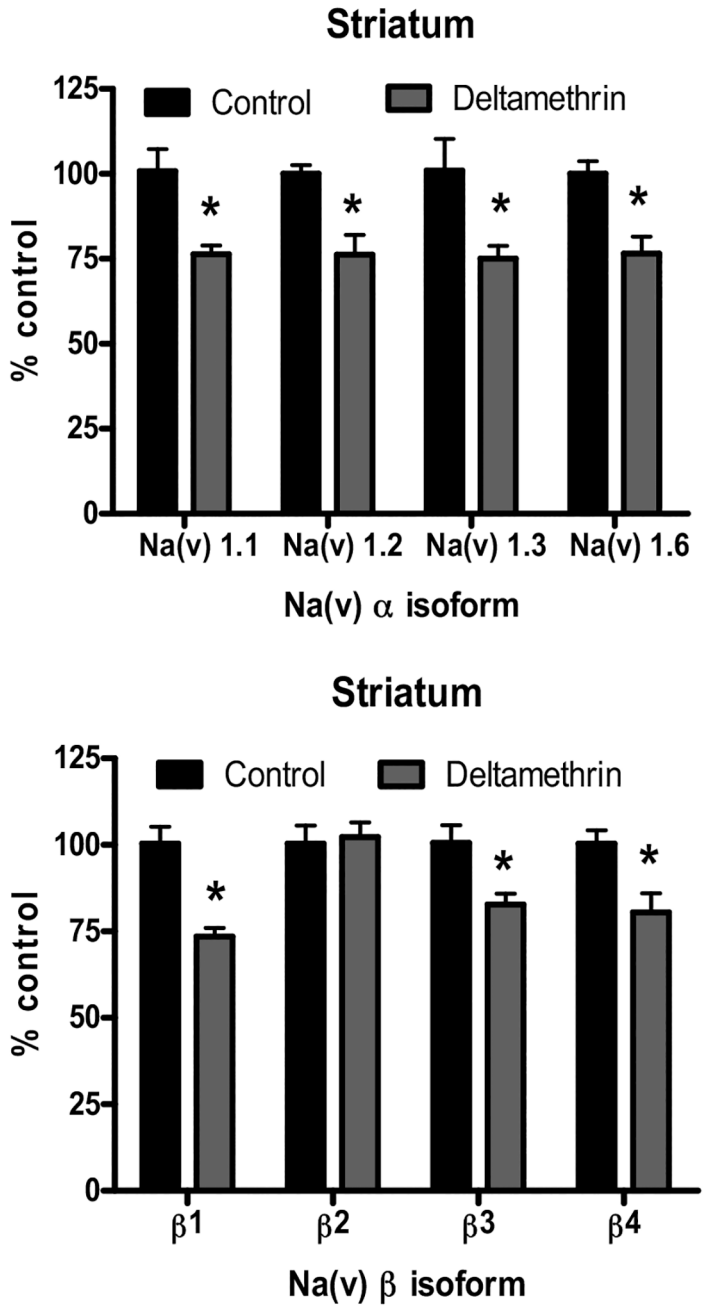

Fig. 3.

Expression of Na_v mRNA in the Striatum Following Developmental Deltamethrin Exposure. (Top) Striatum, α isoforms. (Bottom) Striatum β isoforms. All isoforms are compared to control value for that region. Data represent $2^{\Delta\Delta Ct}$ analysis of qPCR data, expressed as percentage of control. Treatment-related differences for each isoform was determined via Student's *t*-test * = $p < 0.05$, n = 5–7. Error bars represent SEM.

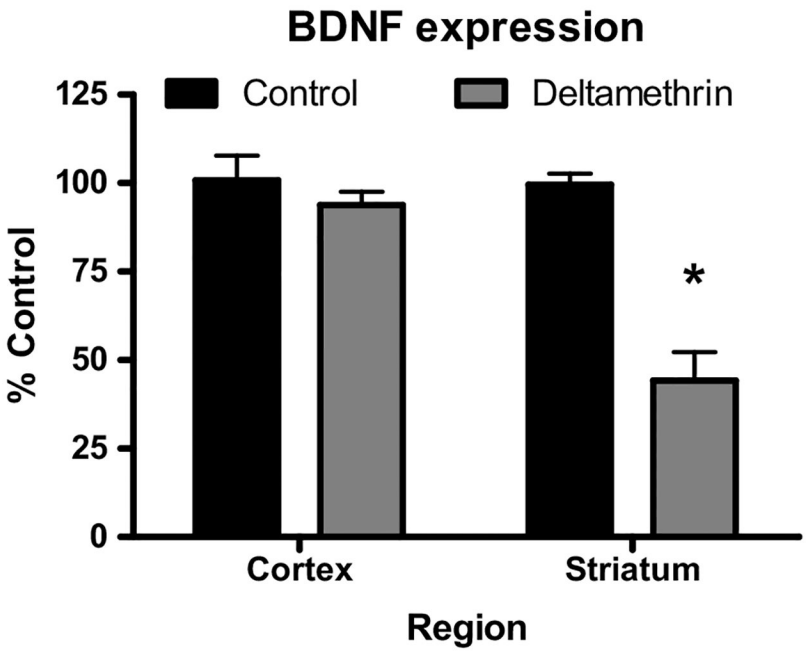

Fig. 4.

Developmental Deltamethrin Reduces BDNF mRNA in the Striatum following Developmental Deltamethrin Exposure. Cortical and Striatal BDNF mRNA expression. BDNF mRNA was compared to control values for that region. Data represent $2^{\Delta\Delta Ct}$ analysis of qPCR data, expressed as percentage of control. Treatment-related differences for each region was determined via Student's *t*-test * = $p < 0.05$, n = 5–7. Error bars represent SEM.