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Loss of motoneurons in the ventral compartment of the rat hypoglossal nucleus following early postnatal exposure to alcohol

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Abstract

Perinatal Alcohol exposure (AE) has multiple detrimental effects on cognitive and various behavioral outcomes, but little is known about its impact on the autonomic functions. In a rat model of fetal alcohol spectrum disorders (FASD), we investigated neurochemical and neuroanatomical alterations in two brainstem nuclei, the hypoglossal nucleus (XII_n) and the dorsal nucleus of the vagus nerve (X_{dn}).

One group of male Sprague-Dawley rats (n=6) received 2.625 g/kg ethanol intragastrically twice daily on postnatal days (PD)4–9, a period equivalent to the third trimester of human pregnancy, and another group (n=6) was sham-intubated. On PD18–19, the rats were perfused and medullary sections were immunohistochemically processed for choline acetyltransferase (ChAT) or two aminergic receptors that mediate excitatory drive to motoneurons, α_1 -adrenergic (α_1 -R) and serotonin 2A (5-HT_{2A}-R), and c-Fos.

Based on ChAT labeling, AE rats had reduced numbers of motoneurons in the ventral XII_n (XII_n-v; 35.4±1.3 motoneurons per side and section vs. 40.0±1.2, p=0.022), but not in the dorsal XII_n or X_{dn}. Consistent with ChAT data, both the numbers of α_1 -R-labeled motoneurons in the XII_n-v and the area of the XII_n-v measured using 5-HT_{2A}-R staining were significantly smaller in AE rats (19.7±1.5 vs. 25.0±1.4, p=0.031 and 0.063 mm² ±0.002 vs. 0.074±0.002, p=0.002, respectively). Concurrently, both 5-HT_{2A}-R and c-Fos staining tended to be higher in AE rats, suggesting an increased activation.

Thus, postnatal AE causes motoneuronal loss in the XII_n-v. This may compromise upper airway control and contribute to increased risk of upper airway obstructions and sudden infant death in FASD victims.

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Disclosures

The authors declare no competing interests.

Keywords

Fetal alcohol spectrum disorders (FASD); upper airway control; α -adrenergic receptors; serotonin receptors; sudden infant death syndrome (SIDS)

1. Introduction

The detrimental impact of alcohol consumption during pregnancy on the offspring has been noted in texts written prior to the Common Era (Calhoun and Warren, 2007). However, it is only 40 years ago when two publications prompted a systematic clinical and scientific interest in the effects of prenatal alcohol exposure (AE) on human development (Jones and Smith, 1973; Jones et al., 1973). Since then, numerous studies aimed at investigating the mechanisms by which prenatal AE exerts its detrimental effects. The various pathophysiological consequences of prenatal AE have been subsumed under the diagnostic term of Fetal Alcohol Spectrum Disorders (FASD) (reviewed in Riley et al., 2011), and the prevalence of FASD is estimated to be as high as 2–5% (reviewed by May et al., 2009). Indeed, ethanol is the most commonly abused substance that impacts the human embryonic and fetal development and, in particular, the developing brain. Prenatal AE is also a leading preventable cause of neurodevelopmental disorders, intellectual disability and birth defects (Guerri et al., 2009).

The neurotoxic effects of alcohol on the developing brain depend on the timing and dose of alcohol (West et al., 1994; Guerri, 1998). The brain is especially vulnerable to AE during the initial period of the “brain growth spurt” (West et al., 1994; Guerri, 1998) which in rats occurs between gestational day (GD)18 and postnatal day (PD)9 and in humans coincides with the third trimester of pregnancy (Dobbing and Sands, 1979; Guerri, 1998). During this period, alcohol interferes with the proliferation of astro- and oligodendrocytes, neuronal dendrite arborization, synaptogenesis and progression of programmed cell death (apoptosis) (West et al., 1994; Guerri, 1998; Ikonomidou et al., 2000). The relative immaturity of the newborn rat brain due to a shift of the brain growth spurt to the extra uterine period makes an early postnatal AE in rats an attractive animal model of FASD (Light et al., 1998; Cudd, 2005; Kelly and Lawrence, 2008). The model has been extensively used for investigation of the neurotoxic impact of AE on brain neuroanatomy (Pierce et al., 1993), cognitive functions (Johnson and Goodlett, 2002; O’Leary-Moore et al., 2006; Stettner et al., 2011), motor control (McGough et al., 2009; Stettner et al., 2011), neurotransmitter receptor expression (Volgin, 2008) and sleep-wake behavior (Volgin and Kubin, 2012).

Prenatal AE predisposes to respiratory disorders, including problems with the neural control of the upper airway that may result in upper airway obstructions (Usowicz et al., 1986). FASD-associated craniofacial dysmorphisms, including midface hypoplasia and microretrognathia that are typically caused by AE during first trimester of pregnancy (Sant’Anna and Tosello, 2006), may exacerbate these problems. Indeed, anatomical predisposition combined with insufficient activation of upper airway muscles during sleep underlies the obstructive sleep apnea (OSA) syndrome in both children and adults (Suratt et al., 1988; Katz and White, 2003). Prenatal AE also has been recognized as a risk factor for sudden infant death syndrome (SIDS) (Iyasu et al., 2002; Burd et al., 2004; Duncan et al., 2008). While the fundamental mechanisms causing SIDS are still debated, a leading hypothesis today is the “brainstem hypothesis”, according to which a major subset of SIDS cases is related to abnormal brainstem functions that result in impaired cardiorespiratory and arousal responses to a transient asphyxia (Kinney, 2009). In support of the brainstem hypothesis, morphologic alterations of brainstem nuclei have been described in post-mortem material from SIDS victims (reviewed in Kinney, 2009) but comparable data for humans

with FASD are scarce. In animal models of FASD, few studies investigated the impact of AE on the brainstem but a reduction of the size of the principal sensory trigeminal nucleus associated with a partial loss of neurons within this region has been reported for different species and AE protocols (Mooney and Miller, 2007; Mooney and Miller, 2009).

Given the important role of the hypoglossal motor nucleus (XIIn) in the control of upper airway patency (Sauerland and Harper, 1976; Remmers et al., 1978; Horner, 2011), our goal was to investigate the impact of early postnatal AE on the size and neurochemical morphology of the XIIn in an established rat model of FASD. For comparison, we assessed the effects of AE on the dorsal motor nucleus of the vagus nerve (Xdn), a nucleus located adjacent to the XIIn that is important for central autonomic control of visceral functions (Kalia and Sullivan, 1982). We found that young adolescent rats subjected to early postnatal AE had reduced numbers of motoneurons in the ventral compartment of the XIIn (XIIn-v) which contains motoneurons that innervate the tongue protruder muscles (McClung and Goldberg, 1999; Lewis et al., 1971; Krammer et al., 1979; Dobbins and Feldman, 1995), whereas the dorsal compartment of the XIIn (XIIn-d) and the Xdn did not differ between the AE and sham-treated rats. A preliminary report has been published (Stettner et al., 2012).

2. Materials and Methods

We used an established rat model of FASD in which rat pups are exposed to alcohol during the early postnatal period (Light et al., 1998; Kelly and Lawrence, 2008; Volgin, 2008) that corresponds to the third trimester of human pregnancy with the regard to the stage of brain development (Dobbing and Sands, 1979). All animal procedures followed the guidelines of the Guide for the Care and Use of Laboratory Animals of the National Institutes of Health and were approved by the Institutional Animal Care and Use Committee of the University of Pennsylvania. Thirteen natural litters from timed-pregnant Sprague-Dawley rats obtained from Charles River Laboratories (Wilmington, MA) were culled and cross-fostered among lactating dams to obtain 8 experimental litters. Each experimental litter contained randomly mixed 10 or 11 male pups derived from 6 or 7 different mothers. The animals were housed on a 12:12 light/dark schedule with lights on at 7 am and *ad lib* access to food and water. The pups were weighed on each day when they were handled and stayed with the dam except the brief periods needed for the treatments. On PD4, each litter was randomly assigned to either AE or sham treatment (non-mixed design, Light et al., 1998). The AE group received alcohol from PD4 to PD9 administered as two intragastric intubations per day (2.625 g/kg per intubation, 11.9% v/v in a custom milk formula, 2 h apart). Two hours after the second alcohol administration, an additional intubation was performed and only milk was given to the AE group to compensate for reduced maternal milk consumption in intoxicated pups. The sham-treated group underwent the same daily routine of intubations, but no fluid was infused (Kelly and Lawrence, 2008). To estimate blood alcohol concentration (BAC), blood was collected on PD4 from a separate group of AE rats (n=8) sacrificed 2 h after the second alcohol intubation. BAC was determined using NAD-ADH reagent (Sigma, St. Louis, MO, USA) and was 341 ± 23 (standard error - SE) mg/dl, consistent with previous results obtained from a similar experimental protocol (O'Leary-Moore et al., 2006; McGough et al., 2009). Because the rats used in this study also served as a control group for another study in which we investigated behavioral effects of early postnatal AE and the potential beneficial effects of treatment with an orexin receptor antagonist (Stettner et al., 2011), all animals in this study received daily intraperitoneal injection of a vehicle (10% (w/v) of (2-hydroxypropyl)- β -cyclodextrin and 10% (v/v) dimethyl sulfoxide (DMSO) in sterile saline) between PD12 and PD14. Although DMSO has a potential to produce apoptosis in some regions of the developing mouse brain and in cultured rat hippocampal neurons (Hanslick et al., 2009), no effect of DMSO on medullary structures have been reported to date. Furthermore, the vehicle treatment would similarly

affect both the AE and sham-treated rats and, therefore, it was unlikely to confound the results described in this report.

On PD18 and PD19 at the beginning of the lights-off period (8:00–11:30 pm), rats were deeply anesthetized with pentobarbital sodium (100 mg/kg, i.p.; Nembutal, Abbott Laboratories, North Chicago, IL, USA) and were transcardially perfused with phosphate-buffered saline (PBS, pH 7.4) containing 5 ml heparin (1,000 USP units/ml) and 0.003% lidocaine, followed by 4% paraformaldehyde (PFA). At the time of perfusion, the mean body weight of the AE rats was 37 ± 1.1 g (SE) and that of the sham-treated animals 43 ± 1.2 g ($p=0.05$). The medullae were removed, post-fixed in PFA for 72 h at 4°C, cryoprotected in 30% sucrose in PBS for 72 h at 4°C and then cut on a cryostat into 25 μ m transverse sections collected into four series. Separate series of sections were processed immunohistochemically to visualize choline acetyltransferase (ChAT; 1:1,000; catalog symbol: AB144P, Millipore, Temecula, CA, USA), c-Fos (1:250; catalog symbol: sc-52, Santa Cruz Biotechnology, Santa Cruz, CA, USA), and 5-HT (serotonin) 2A receptor (5-HT_{2A}-R; 1:120; catalog symbol: 24288, ImmunoStar, Hudson, WI, USA). Following incubation of the sections with primary antibodies and appropriate biotinylated secondary antibodies, sections were incubated in Vectastatin Elite ABC Kit (Vector Laboratories, Burlingame, CA, USA) and developed with DAB (3,3'-diaminobenzidine) intensified by addition of nickel ammonium sulfate which resulted in a black reaction product (DAB Peroxidase Substrate Kit; Vector Laboratories). The series labeled for c-Fos were then additionally labeled to visualize α_1 -adrenergic receptors (α_1 -R; 1:500; catalog symbol: PA1-047, Thermo Scientific Pierce Biotechnology, Rockford, IL, USA). Following incubation of these sections with primary antibodies against α_1 -R and appropriate biotinylated secondary antibodies, sections were incubated in Vectastatin ABC-AP Kit (Vector Laboratories) and developed using Vector Red Alkaline Phosphatase Substrate Kit I (Vector Laboratories) which resulted in a red reaction product.

Microscopic analysis was performed under bright field illumination at x400 magnification (x10 eyepiece and x40 objective) using an upright microscope (Leica DMLB, Wetzlar, Germany). The sections selected for analysis represented three levels corresponding to 100 μ m anterior, 200 μ m posterior and 500 μ m posterior to the obex according to a rat brain atlas (Paxinos and Watson, 2007). ChAT stained sections were used to count motoneurons in the entire XIIIn (XIIIn-e), the entire Xdn, and the two functionally distinct compartments of the XIIIn, dorsal (XIIIn-d) and ventral (XIIIn-v), and the corresponding motoneuronal densities per mm². Motoneurons were counted when a ChAT-labeled cell profile included the cell body with at least one proximal dendrite. For measurements of cell density and ChAT staining intensity, photographs of the analyzed sections were taken with a digital camera (ProgRes CF^{scan} camera and software; Jenoptik, Jena, Germany). The photographs were then converted to grayscale images (8 bits/channel) and subjected to area and density measurements separately for the XIIIn-e, XIIIn-d, XIIIn-v and Xdn (ImageJ 1.44p software, National Institutes of Health, Bethesda, MD, USA). All cell counts and measurements were done bilaterally, averaged across both sides and expressed as a mean per side. An example of all three ChAT stained sections selected for analysis from one animal is shown in Fig. 1A–C.

The sections double-labeled for α_1 -R and c-Fos were used to count motoneurons in the XIIIn based on a marker other than ChAT, whereas c-Fos staining was used as an indirect measure of motoneuronal activity prior to perfusion. Due to a very high density of α_1 -R⁺ neurons within the Xdn, counting of individual α_1 -R-stained neurons was not possible in this nucleus.

In contrast to the γ -R and ChAT stainings which clearly visualize distinct cell bodies, 5-HT_{2A}-R staining of the XIIIn and Xdn is relatively diffuse (Fay and Kubin, 2000). Thus, 5-HT_{2A}-R stained sections were photographed as described above for the analysis of ChAT staining and analyzed based on densitometric measurements of staining intensity, as described and validated previously (Volgin et al., 2003; Rukhadze et al., 2010). For the Xdn, only the middle of the three sections was subjected to 5-HT_{2A}-R analysis because only at this rostro-caudal level (200 μ m caudal to the obex), 5-HT_{2A}-R labeling accurately delineates the entire cross-section of the Xdn (Fay and Kubin, 2000). Examples of γ -R/c-Fos double staining and 5-HT_{2A}-R staining are shown in Figs. 2A and 2B, respectively.

Statistical analysis was conducted using SigmaPlot 12.0 software (Systat, San Jose, CA, USA). Normality of the distributions was tested using the Shapiro-Wilks test. All values are presented as the means $\pm$ SE. Unless stated otherwise, the analysis was conducted using the average values across all three analyzed rostro-caudal levels in each animal. For normally distributed measures, we used unpaired Student's t-tests for comparison between the treatment groups. To assess the interaction between the treatment (two levels) and rostro-caudal level of the sections (three levels), we used two-way, repeated-measures ANOVA that was then followed by Holm-Sidak-corrected post-hoc comparisons. For not normally distributed measures, we used the Mann-Whitney U-test. Differences were considered significant at $P < 0.05$.

3. Results

3.1. Effect of AE on the XII and dorsal vagal nuclei, as assessed by ChAT staining

AE led to a significant reduction of the number of ChAT-positive (ChAT⁺) motoneurons in the XIIIn-e when compared to sham treatment (65.9 ± 1.4 motoneurons per section and side vs. 73.6 ± 2.6 , $p = 0.026$; $n = 6$ rats per group), whereas there was no difference for the Xdn (63.2 ± 1.7 vs. 64.8 ± 5.5 , $p = 0.82$). The reduction of cell numbers in the XIIIn occurred predominantly in the XIIIn-v (35.4 ± 1.3 vs. 40.0 ± 1.2 , $p = 0.022$) because it was not significant in the XIIIn-d (30.5 ± 1.1 vs. 33.5 ± 2.8 , $p = 0.33$) (Fig. 1D). In addition to the reduced number of ChAT⁺ motoneurons in the XIIIn-v, the area of the XIIIn-v tended to be smaller in the AE compared to the sham-treated rats (0.066 ± 0.003 vs. 0.074 ± 0.002 mm², $p = 0.062$) whereas there was no effect of AE on the area of the XIIIn-d (0.067 ± 0.003 vs. 0.066 ± 0.002 mm², $p = 0.69$), XIIIn-e (0.133 ± 0.004 vs. 0.139 ± 0.004 mm², $p = 0.28$) or Xdn (0.050 ± 0.002 vs. 0.047 ± 0.002 mm², $p = 0.23$) (Fig. 1E). AE did not alter the packing density of ChAT⁺ motoneurons (Fig. 1F) or the intensity of ChAT staining within any region of interest (Fig. 1G).

For all measures, a two-way, repeated-measures ANOVA was performed to evaluate whether, in addition to the treatment, the rostro-caudal level of the section influenced the outcome and/or interacted with the treatment. With this approach, the effect of AE on the number of ChAT⁺ motoneurons in the XIIIn-v also was significant ($DF_{1,2,10}$, $F = 5.0$, $p = 0.049$), whereas the effect of AE on the area of the XIIIn-v was absent ($DF_{1,2,10}$, $F = 2.5$, $p = 0.14$). As expected from the rostro-caudally varying shape and size of the XIIIn and Xdn (Dennison et al., 1981; Aldes, 1995), the rostro-caudal level of the section had a significant influence on the number of ChAT⁺ motoneurons, the area, and the packing density ($DF_{1,2,10}$, $F = 4.0$ – 227.2 , $p = 0.035$ – <0.001), but not on the optical density of ChAT staining ($DF_{1,2,10}$, $F = 3.0$ – 3.5 , $p < 0.051$ – 0.076). However, and importantly, the interaction between the treatment and level was not significant, indicating that AE similarly affected the XIIIn at all three levels.

3.2. Effect of AE, as assessed by α_1 -R and 5-HT_{2A}-R staining

The effect of AE on the motoneuron count and area, especially in the XII-v revealed by counts of ChAT-stained cells was confirmed by the analysis of staining for α_1 -R and 5-HT_{2A}-R. The number of α_1 -R⁺ motoneurons within the XIIIn-v was significantly reduced in AE rats when compared to sham treatment (19.7 ± 1.5 motoneurons per section and side vs. 25.0 ± 1.4 , $p=0.031$; $n=6$ rats per group) (Fig. 2C). As with ChAT staining, AE did not significantly alter the mean number of α_1 -R⁺ motoneurons within the XIIIn-e (36.8 ± 2.4 vs. 42.5 ± 2.6 , $p=0.14$) or the XIIIn-d (17.1 ± 1.3 vs. 17.5 ± 1.3 , $p=0.81$) (Fig. 2C). It is noteworthy, that the proportion of α_1 -R⁺ neurons relative to the number of ChAT⁺ neurons did not differ between AE and sham-treated rats (XIIIn-e: $56.9 \pm 4.9\%$ vs. $58.2 \pm 5.3\%$, $p=0.86$; XIIIn-d: $57.0 \pm 5.0\%$ vs. $54.1 \pm 9.2\%$, $p=0.78$; XIIIn-v: $60.0 \pm 6.7\%$ vs. $62.9 \pm 3.1\%$, $p=0.72$). This suggests that, although not all motoneurons had a detectable α_1 -R staining, ChAT and α_1 -R labeling provided two independent and mutually complementary estimates pointing to a partial loss of XII motoneurons in the XIIIn-v.

As with ChAT-staining, the mean area of the XIIIn-e measured on the basis of the 5-HT_{2A}-R staining was significantly reduced in AE compared to sham-treated rats (0.131 ± 0.005 vs. 0.148 ± 0.003 mm², $p=0.012$). Again, this reduction occurred mainly in the XIIIn-v (0.063 ± 0.002 vs. 0.074 ± 0.002 mm², $p=0.002$) because it was not significant in the XIIIn-d (0.068 ± 0.003 vs. 0.074 ± 0.003 mm², $p=0.31$). Also as with ChAT staining, the area of the Xdn did not differ between the AE and sham-treated rats (0.080 ± 0.004 vs. 0.081 ± 0.005 mm², $p=0.90$) (Fig. 2D).

5-HT_{2A}-R staining intensity was higher in the AE than in sham-treated rats. The density of 5-HT_{2A}-R immunostaining was significantly increased in the XIIIn-e (150.0 ± 4.9 vs. 131.1 ± 6.3 arbitrary units, $p=0.039$). The effect was caused by the difference in the XIIIn-v (155.5 ± 4.4 vs. 135.0 ± 6.1 , $p=0.022$) because it was not significant in the XIIIn-d (144.6 ± 5.4 vs. 127.1 ± 6.5 , $p=0.066$). No difference was detected in the Xdn (111.9 ± 6.4 vs. 96.2 ± 5.1 , $p=0.082$) (Fig. 2F).

Two-way, repeated-measures ANOVA with the treatment and rostro-caudal levels as the two factors confirmed the significant effect of AE on the number of motoneurons in the XIIIn-v ($DF_{1,2,10}$, $F=6.6$, $p=0.031$) based on α_1 -R staining, and the area of this compartment based on the 5-HT_{2A}-R staining ($DF_{1,2,10}$, $F=11.1$, $p=0.008$). As with ChAT staining, the impact of the rostro-caudal level on these measures was highly significant ($DF_{1,2,10}$, $F=19.4$ and 130.2 , $p<0.001$ for both), but the interaction between the treatment and brain section level was not significant for any of these measures.

3.3. Effect of AE on c-Fos expression in α_1 -R-positive XII motoneurons

AE rats tended to have an increased percentage of α_1 -R⁺ motoneurons that were c-Fos⁺, but the increase was not significant within any of the regions (XIIIn-e: $33.1 \pm 4.0\%$ vs. $22.0 \pm 6.0\%$, $p=0.15$; XIIIn-d: $24.7 \pm 5.4\%$ vs. $15.9 \pm 4.3\%$, $p=0.25$; XIIIn-v: $40.3 \pm 4.8\%$ vs. $26.4 \pm 6.9\%$, $p=0.12$; Fig. 2E).

4. Discussion

We used a rat model of FASD to investigate whether early postnatal AE alters the chemical neuroanatomy of the XIIIn and Xdn. Our main finding is that the numbers of motoneurons in the XIIIn-v, but not in the XIIIn-d or the adjacent Xdn, are significantly reduced following AE. In addition, α_1 -R⁺ motoneurons in the XIIIn-v of rats in the AE group tend to have elevated activity, as assessed by c-Fos immunostaining, and increased staining intensity for the excitatory 5-HT_{2A} receptors.

4.1. Rat model of FASD and its limitations

In the present study, we did not have a control group that did not receive intragastric intubations because most neuroanatomical and/or behavioral measures previously obtained from this model did not differ between sham-intubated and non-intubated control groups (e.g. Pierce et al., 1993; Johnson and Goodlett, 2002; O'Leary-Moore et al., 2006). Accordingly, many recent studies using a similar rat model did not use a non-intubated control group (McGough et al., 2009; Monk et al., 2012; Tiwari et al., 2012). Thus, although our study does not fully exclude the possibility that neurochemical and neuroanatomical measures of the two studied brainstem nuclei in a non-intubated control group would differ from those in entirely unhandled rats our main finding that early AE *per se* causes a reduction of motoneuron numbers in XII-v remains valid despite this potential limitation.

Rat pups exposed to alcohol on PD4–9 had lower body weight on PD18–19 when compared to sham-treated group, reflecting a growth lag that was also described in other studies (Johnson and Goodlett, 2002; O'Leary-Moore et al., 2006; Volgin, 2008; McGough et al., 2009). Accordingly, a growth disadvantage of the AE group could theoretically contribute to our findings. However, it is unlikely that a moderate undernourishment limited to the early postnatal period would selectively contribute to a motoneuronal loss that affected only the ventral compartment of the XIIIn. Furthermore, our counts of XII motoneurons well correspond to the counts obtained by others from adult rats and actually tend to be somewhat higher, as one would expect considering that, in those other studies, XII motoneurons were counted after their retrograde labeling from different muscles of the tongue or from the XII nerve, whereas our counts were based on ChAT staining and did not depend on successful retrograde labeling. Thus, our average total counts for XIIIn-e were 66–74 motoneurons per 25 μ m-thick section, whereas Altschuler et al. (1994) counted 28–41 motoneurons per 35 μ m section, and Dobbins and Feldman (1995) reported 42–47 per 50 μ m section for sections located near the obex level. Our average counts for the XIIIn-d (31–34 motoneurons per section) similarly tend to be higher than 20–23 motoneurons per 50 μ m section found following retrograde labeling of XII motoneurons from the tongue with only the lateral branch of the XII nerve left intact (McClung and Goldberg, 1999). This further supports our interpretation that AE caused a differential loss of XII motoneurons preferentially occurring in the XIIIn-v.

4.2. Mechanisms of AE interference with motoneuronal development

The development of the XIIIn follows the general principles of brain development. During early gestation (before GD15 in rats), prospective XII motoneurons proliferate and migrate to the region of the XII nucleus. After GD15 but before birth, cell death (apoptosis) of XII motoneurons occurs, which reduces the XII motoneuronal pool by about 35% (Friedland et al., 1995). Another process completed at birth is the somatotopic organization of the XIIIn, with functional and morphological separation of the ventral and dorsal compartments of the nucleus (Sokoloff, 1993; Berger et al., 1996). During the initial period of the brain growth spurt that in rats corresponds to the early postnatal period until approximately PD14, XII motoneurons undergo numerous morphological changes, including a marked reduction of the complexity of the dendritic tree (Nunez-Abades et al., 1994). It is of note that Xdn neurons migrate and develop significantly later than XII motoneurons (Altman and Bayer, 1980) which may be related to their insensitivity to AE applied during PD4–9.

We used a rat model of FASD with AE applied on PD4–9 which produces an intoxication pattern comparable to binge drinking (Light et al., 1998; Cudd, 2005; Kelly and Lawrence, 2008). We found that early postnatal AE caused a reduction of both the number of motoneurons within the XIIIn-v and the area of the XIIIn-v. In contrast, these measures were not significantly altered in the XIIIn-d or the Xdn. A previous study of the effect of AE on

the rat brainstem reported reduced numbers of neurons in the XIIIn when alcohol was administered during the neuronal generation period (GD12 or 13) (Mooney and Miller, 2007). In other studies, AE during early gestation caused hindbrain and cranial nerve malformations (Dunty, Jr. et al., 2002), and a reduction of motoneurons in trigeminal and facial nuclei but not in the XIIIn (Mooney and Miller, 2007). However, in macaque models with AE spanning nearly the entire intrauterine period with one exposure per week, no alteration of morphometric measures in the cranial motor nuclei, including the XIIIn, have been noted (Mooney and Miller, 2009). In our study, the magnitude of alcohol-induced reduction of XIIIn-v motoneuron numbers was quantitatively similar to the reduction of the XIIIn-v nucleus area, as measured based on either ChAT- or 5-HT_{2A}-R-staining (11.5% less ChAT⁺ neurons vs. 10.1% smaller ChAT⁺ area or 14.9% smaller 5-HT_{2A}-R-stained area), whereas the packing density of ChAT⁺ neurons and optical density of the ChAT-staining did not differ between the alcohol- and sham-exposed rats. Collectively, this suggests that any additional morphological changes at the level of individual motoneurons (e.g., changes in cell body size or dendritic morphology), if present, must have been relatively less prominent than the loss of motoneurons *per se*. Thus, our study extends the earlier findings in the XII nucleus to a different AE exposure protocol and a different developmental period.

It is of note that selective loss of motoneurons affecting only some motor nuclei or their subcompartments also occurs in neurodegenerative disorders, including the amyotrophic lateral sclerosis (ALS) (DePaul et al., 1988; Gizzi et al., 1992), Huntington's disease (Calabresi et al., 1998), and tauopathies such as Alzheimer's and Parkinson's disease (Braak et al., 2006). In ALS, tongue protrusive movements are impaired earlier than retropulsive movements (Kawai et al., 2003). This corresponds to our finding of a greater impact of AE on the XII-v that innervates the protrudors than on XII-d that innervates the retractors. Interestingly, among the XIIIn, trigeminal, facial and oculomotor nuclei, motoneurons that express the $\alpha 2$ subunit of GABA_A receptor subunit are more vulnerable to degeneration than motoneurons expressing the $\alpha 1$ subunit. In the XIIIn, $\alpha 2$ subunit is present nearly exclusively in the XIIIn-v (Lorenzo et al., 2006). Thus, a differential expression of GABA_A receptor subtypes and different organization of inhibitory inputs may contribute to the selective loss of XIIIn-v motoneurons following AE during brain development.

4.3. Potential functional implications for upper airway control in SIDS and FASD

It has been proposed that AE might interfere with XIIIn development and might be of prognostic relevance for the predisposition to upper airway obstructions in infants with FASD (Usowicz et al., 1986) and contribute to an increased risk of SIDS (Iyasu et al., 2002; Burd et al., 2004; Duncan et al., 2008; Kinney, 2009). However, the consequences of AE for the development of the XIIIn and other brainstem structures have, so far, attracted only limited attention. Most human brain imaging studies assessing the impact of prenatal AE did not include the brainstem (reviewed in Lebel et al., 2011), and postmortem findings in FASD victims included gross brainstem abnormalities in only a few cases (reviewed in Roebuck et al., 1998). Studies in animal models have demonstrated that exposure of the developing brain to ethanol can reduce the neuronal numbers in some brain regions. The reduction could be caused by inhibition of the early gestational cell proliferation, increased subsequent apoptosis, or both (reviewed by Mooney and Miller, 2009). The potential of ethanol to induce apoptotic cell loss in the developing brain is especially prominent in a window of high vulnerability coinciding with the brain growth spurt (Ikonomidou et al., 2000). During this period, significant developmental changes of the neurochemical profile of neurons in the brainstem, including the XIIIn and Xdn, may occur. A critical period has been identified in rats on PD12–13 during which there is a re-configuration of serotonin receptors in the XIIIn (Volgin et al., 2003), and a transient imbalance occurs between inhibitory and excitatory drives (Liu and Wong-Riley, 2005; Liu and Wong-Riley, 2008). Since these

neurochemical changes were detected in the XIIIn and other brainstem nuclei involved in respiratory and upper airway control, these systems may be particularly vulnerable to endogenous and exogenous stressors during this critical period.

The XIIIn innervates the muscles of the tongue. Because of the tongue's critical position in the upper airway, XII motoneurons play an important role in the central control of upper airway patency when upper airway anatomy is compromised and in the coordination of breathing and alimentary functions (Sauerland and Harper, 1976; Remmers et al., 1978; Travers and Jackson, 1992; Horner, 2011). The XIIIn-v contains motoneurons that innervate protruder muscles of the tongue (Lewis et al., 1971; Krammer et al., 1979; Dobbins and Feldman, 1995; McClung and Goldberg, 1999). Activation of these muscles results in a forward movement of the tongue, which reduces upper airway resistance, thereby protecting the upper airway from obstruction (Sauerland and Harper, 1976; Remmers et al., 1978; Brennick et al., 2001).

A loss of motoneurons in the XIIIn-v may interfere with upper airway control and contribute to an increased risk of upper airway obstruction and SIDS in infants with FASD. Indeed, altered development and maturation of XII motoneurons have been previously proposed to play a role in SIDS. One study reported a reduced number of XII motoneurons and reduced size of the XIIIn in victims of SIDS, with no changes in the Xdn (Konrat et al., 1992). Subsequent studies confirmed these observations (Ottaviani et al., 2006; Lavezzi et al., 2010), but different compartments of the XIIIn were not separately analyzed. In addition to the XIIIn, morphological abnormalities in other brainstem regions important for respiratory and upper airway control and central chemosensitivity have been described in post mortem material from SIDS victims (Matturri et al., 2000; Biondo et al., 2004; Lavezzi and Matturri, 2008). In contrast, with the exception of the raphe nuclei (Paterson et al., 2009), data for the corresponding brainstem regions in FASD do not exist.

4.4. AE effect on the aminergic control of XII motoneurons

Activity of XII motoneurons is importantly driven by pontomedullary noradrenergic and serotonergic neurons which have axonal projections to the XIIIn (Aldes et al., 1992; Manaker and Tischler, 1993; Rukhadze and Kubin, 2007). The endogenous aminergic control of XII motoneurons is mainly mediated by the excitatory adrenergic α_1 -R (Volgin et al., 2001; Fenik et al., 2005; Chan et al., 2006) and serotonergic 5-HT_{2A}-R (Okabe et al., 1997; Zhan et al., 2002; Volgin et al., 2003). The activity of pontomedullary noradrenergic and serotonergic neurons is state dependent, maximal during wakefulness and lowest or absent during REM sleep (Aston-Jones and Bloom, 1981; Trulson and Trulson, 1982; Fenik et al., 2002). An adult-like diurnal organization of sleep-wake pattern develops around PD18 in rats (Jouvet-Mounier et al., 1970; Frank and Heller, 1997). Interestingly, in our study, α_1 -R⁺ motoneurons showed signs of increased activation by aminergic inputs in AE rats, as suggested by a higher percentage of c-Fos⁺ motoneurons among those positive for α_1 -R and increased levels of 5-HT_{2A}-R staining in the XIIIn. These findings suggest a compensatory increase of activity in XII motoneurons in the face of their partial loss.

4.5. Conclusions

We found reduced numbers of XII motoneurons in the XIIIn-v in a rat model of FASD and evidence suggestive of a compensatory activation of the remaining motoneurons through α_1 -R and 5-HT_{2A}-R receptors located in the XIIIn. A reduction of XII motoneurons might impair the control of upper airway patency which, in turn, may contribute to an increased predisposition to upper airway obstructions in infants with FASD, and an increased risk of SIDS in this population.

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Abbreviations

AE	alcohol exposure
ChAT	choline acetyltransferase
DMSO	dimethyl sulfoxide
FASD	fetal alcohol spectrum disorders
GD	gestational day
PD	postnatal day
SIDS	sudden infant death syndrome
5-HT_{2A}-R	serotonin 2A receptor
1₁-R	alpha-1 adrenergic receptor
Xdn	dorsal nucleus of the vagus nerve
XII_n	hypoglossal nucleus
XII_n-e	entire hypoglossal nucleus
XII_n-d	dorsal compartment of the hypoglossal nucleus
XII_n-v	ventral compartment of the hypoglossal nucleus.

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Highlights

- Rats were exposed to alcohol during the early postnatal period.
- Alcohol caused motoneuronal loss in the hypoglossal nucleus, but not in the dorsal vagal nucleus.
- The loss of hypoglossal motoneurons may interfere with upper airway control.
- Impaired upper airway control may elevate the risk of sudden infant death syndrome in FASD.

Figure 1.

Reduced numbers of XII motoneurons in the ventral compartment of the hypoglossal nucleus (XIIIn-v), but not in the nucleus of the vagus (Xdn) in AE rats, as determined by choline acetyltransferase (ChAT) immunostaining. A–C: brain sections from one animal representing the three rostro-caudal levels that were analyzed in this study; the antero-posterior levels are approximately 500 μ m caudal (A), 200 μ m caudal (B), and 100 μ m rostral (C) to the obex. Scale bar in A also applies to B and C. For clarity, only the left side of each section is shown, with the outlines of the relevant structures shown on the opposite side. D: number of ChAT⁺ neurons. E: areas of the studied nuclei and XIIIn subregions. F: density of ChAT⁺ neurons. G: optical density of ChAT staining. The bar graphs show the

means based on 6 measurements per group obtained by averaging within each animal across both sides and the three analyzed rostro-caudal levels. Abbreviations: 3V, third ventricle; cc, central canal; Xdn, dorsal nucleus of the vagus; XIIIn-d, dorsal compartment of the XII nucleus; XIIIn-v, ventral compartment of the XII nucleus; XIIIn-e, entire XII nucleus.

Figure 2.

α_1 -adrenergic receptor ($\alpha_1\text{-R}$)/c-Fos double labeling and serotonin 2A receptor (5-HT_{2A}-R) immunostaining reveal reduced numbers of XII motoneurons in the ventral compartment of the XII nucleus and suggest increased activation of the remaining XII motoneurons in AE rats. A and B: examples of brain sections stained for $\alpha_1\text{-R}$ /c-Fos and 5-HT_{2A}-R, respectively. Scale bar in A also applies to B. The inset in A shows an enlarged image of the boxed region in the main photograph with a group of $\alpha_1\text{-R}^+$ motoneurons of which one has a c-Fos⁺ nucleus (arrow). C: number of $\alpha_1\text{-R}^+$ motoneurons. D: areas of the investigated regions, as measured based on 5-HT_{2A}-R staining. E: percentage of c-Fos⁺ cells among all $\alpha_1\text{-R}^+$ motoneurons. F: optical density of 5-HT_{2A}-R staining. The bar graphs show 6th

means based on 6 measurements per group obtained by averaging within each animal across both sides and the three analyzed rostro-caudal levels. Abbreviations: 3V, third ventricle; cc, central canal; Xdn, dorsal nucleus of the vagus; XIIIn-d, dorsal compartment of the XII nucleus; XIIIn-v, ventral compartment of the XII nucleus; XIIIn-e, entire XII nucleus. In D, Xdn_{middle} denotes average data for a single level at which 5-HT_{2A}-R staining is present throughout the Xdn.