Cleft Palate Is Caused by CNS Dysfunction in Gad1 and Viaat Knockout Mice

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Abstract

Background: Previous studies have shown that disruption of GABA signaling in mice via mutations in the Gad1, Gabrb3 or Viaat genes leads to the development of non-neural developmental defects such as cleft palate. Studies of the Gabrb3 and Gad1 mutant mice have suggested that GABA function could be required either in the central nervous system or in the palate itself for normal palatogenesis.

Methodology/Principal Findings: To further examine the role of GABA signaling in palatogenesis we used three independent experimental approaches to test whether Gad1 or Viaat function is required in the fetal CNS for normal palate development. We used explant cultures to demonstrate that the Gad1 or Viaat mutant palates were able to undergo palatogenesis in culture, suggesting that there is no defect in the palate tissue itself in these mice. In a second series of experiments we found that the GABA_A receptor agonist muscimol could rescue the cleft palate phenotype in Gad1 and Viaat mutant embryos. This suggested that normal multimeric GABA_A receptors in the CNS were necessary for normal palatogenesis. In addition, we showed that CNS-specific inactivation of Gad1 was sufficient to disrupt palate development.

Conclusions/Significance: Our results are consistent with a role for Gad1 and Viaat in the central nervous system for normal development of the palate. We suggest that the alterations in GABA signaling lead to non-neural defects such as cleft palate as a secondary effect due to alterations in or elimination of fetal movements.

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Introduction

The mechanism leading to non-neural developmental defects in mice homozygous for null alleles of the genes Gad1, Gabrb3 or Viaat (Sk:32a1) has been an enigma [1,2,3,4,5]. Each of these genes encodes a component required for GABA neurotransmission including GABA synthesis (Gad1), GABA vesicular transport (Viaat) and the postsynaptic response to GABA (Gabrb3). The most prominent phenotype noted by us and other investigators has been the surprising presence of a cleft palate in neonatal mice homozygous for null mutations in Gad1, Viaat and Gabrb3 [3,4,3,6]. In addition, possible associations between human oral clefts and alleles of Gad1 or Gabrb3 have been reported [7,8,9]. Overall, this literature suggests that understanding the developmental mechanisms that lead to oral clefts in the Gad1, Viaat and Gabrb3 mice might lead to new insights into the origin of human oral clefts, a common congenital disorder.

Several studies have been performed to determine whether GABA signaling is required in the CNS or in peripheral tissues for normal palate formation in Gad1 and Gabrb3 mutant mice. These studies have drawn different conclusions depending on the gene examined and the experimental approach taken. In the case of Gabrb3, two studies support a role in non-neural cell types for normal palatogenesis. In one case, transgenic expression of Gabrb3 from a neuron-specific enolase promoter failed to complement the cleft plate phenotype of a Gabrb3 null mutation, suggesting that non-neural expression of Gabrb3 was required for normal palate formation [10]. Another study showed that palate development was normal in a pan-neuronal-specific knockout of the Gabrb3 gene, again suggesting that Gabrb3 function was required in the palate or other craniofacial structures for normal palatogenesis [11]. In the case of Gad1, previous studies indicated that the cleft palate phenotype of Gad1-/ - pups was due to a lack of fetal oral movements as well as inhibition of palate shelf elevation due to the abnormal position of the tongue between the shelves [12,13]. This analysis of the Gad1 phenotype was consistent with a requirement for GABA signaling in the CNS for normal fetal movement that in turn allows normal palate development. However, several papers have reported GABA, Gabrb3 or Gad1 expression in developing craniofacial structures such as the palate, oral epithelium, tooth placodes or condensations, tooth buds and palate medial edge epithelium [4,10,14,15,16,17]. The presence of Gad1 mRNA or protein as well as GABA in these structures is consistent with a functional role for GABA signaling in the palate. In addition, Gad1 gene expression has been detected in several epithelial placodes as well as in non-neural tissues that are developmental signaling centers, suggesting a role for GABA in developmental processes outside of the CNS [18]. In the case of Viaat, cleft palate and body
wall phenotypes were noted in a previous analysis of a Viaat knockout mouse [5]. However, that report did not examine the mechanisms underlying the non-neural phenotypes in the Viaat mutants.

In this study we used three independent experimental approaches to show that the non-neural defects in Gad1 and Viaat mutant mice are due to loss of gene function within the CNS during development. We performed a pharmacological rescue experiment to show that the GABA\(_A\) agonist muscimol can suppress the cleft palate phenotype in Gad1 and Viaat mutant embryos. The ability of muscimol to rescue the cleft palate phenotype of Gad1 and Viaat mutants suggests that multimeric GABA\(_A\) receptors are downstream of Gad1 and Viaat in palate formation, a pathway that is most consistent with GABA signaling in the CNS. In addition, we used a serum free explant culture system to show that oral explants derived from Gad1lacZ\/- and ViaatlacZ\/- embryos were competent to undergo normal palate shelf elevation and fusion when removed from their normal oral context. This experiment provided additional evidence that Gad1 and Viaat function are not directly required for palate formation. A third experimental approach was to inactivate Gad1 specifically in neural precursor cells throughout the CNS. We took advantage of a Gad1 conditional allele to demonstrate that an early neural precursor specific knockout of Gad1 was sufficient to cause a cleft palate phenotype in mutant embryos. Our work strongly supports the idea that the cleft palate phenotype seen in the Viaat and Gad1 knockout mice belongs to a family of fetal defects in non-neural tissues that are caused by a loss of normal fetal movements due to defects in CNS or muscle function during development.

Materials and Methods

Mouse strains

All work with mice conformed to the stipulations of the University of Georgia Institutional Animal Care and Use Committee. The University of Georgia animal welfare assurance number is A3437-01 which expires on 11/30/2011. The ViaatlacZ knockin/knockout allele was generated by gene targeting in ES cells. Genomic clones containing the mouse Viaat locus were isolated from a 129/SvEv lambda phage library. A 5.2-kb fragment immediately upstream of the translation start site was used as a 5’ arm and a 2.8-kb fragment starting downstream of the start codon was used as a 3’ arm. A β-galactosidase reporter/neomycin resistance cassette was placed into an NcoI site in the Viaat locus. This NcoI site includes the start codon of the Viaat gene [19]. After linearization the targeting vector was electroporated into GSI-1 ES cells (obtained from Genome Systems Inc.). Four targeted ES cell lines were injected into blastocysts and one chimera transmitted the knockout allele to the founder offspring. The Viaat knockout mice used in this study had been crossed for 4–10 generations to the C57Bl/6J background. The Gad1lacZ knockin/knockout mice have been described previously [20]. The strain carrying the floxed conditional allele of the Gad1 gene was obtained from Dr. Richard Palmiter. This strain has been backcrossed for 10 generations to the C57Bl/6J background. The strain carrying the floxed conditional allele of the Gad1 gene was obtained from Dr. Richard Palmiter. This strain has been described previously [6]. The Nestin-Cre strain [21] was obtained from The Jackson Laboratory. Fetal mice were genotyped using yolk sac DNA and adult mice were genotyped using tail DNA as template for PCR.

Histological analysis

For paraffin sections, embryos were fixed in Bouin’s solution at 4°C from 3 hours to overnight depending on the embryonic stage and washed in phosphate buffer (pH 7.3) overnight. Then the embryos were then dehydrated in a graded series of ethanol, equilibrated with xylene, embedded in paraffin and sectioned at a thickness of 10 µm. Sections were stained with Mayer’s hematoxylin (Sigma) and cosin solution after dewaxing with xylene, and mounted on slides.

Whole mount lacZ histochemistry was performed on E9.5-14.5 day old heterozygous Viaat embryos. The embryos were fixed in 0.4% paraformaldehyde (PFA), 100 mM sodium phosphate pH 7.3, 2 mM MgCl\(_2\), 5 mM EGTA for 30–60 minutes and then rinsed in 100 mM sodium phosphate pH 7.3, 2 mM, MgCl\(_2\), 0.01% sodium deoxycholate, 0.02% igepal for at least 3 hours and stained in 100 mM sodium phosphate pH 7.3, 2 mM MgCl\(_2\), 0.01% sodium deoxycholate, 0.02% igepal, 5 mM potassium ferricyanide, 5 mM potassium ferrocyanide, 1 mg/ml X-gal at 37°C. After staining, embryos were washed in PBS and postfixed in 4% PFA/PBS for 1 hour at room temperature.

Palate Explant Culture

For the explant cultures we developed a new serum free culture system that combined a serum-free in vitro mouse embryo culture technique [22] with a previously described method for dissecting the palatal region for in vitro culture [23]. E13.5 Mouse embryos were dissected in Knockout DMEM (Invitrogen). The mandibular region including tongue was removed from the embryo head and the brain tissues were dissected out in parallel at the level of the eyes by using an aseptic scalpel. Also, the remaining tissues from the hindbrain and cerebellum including their covering skin were completely removed with fine forceps. The explants were cultured in a rolling bottle apparatus from BTC Engineering (Cambridge, UK) at 37°C in 3 ml of serum-free media per palate in an atmosphere of 95%O\(_2\)/5% CO\(_2\). The culture medium was replaced after 24 hr. After culture for 1 or 2 days, explants were processed for paraffin embedding and H&E staining as described above.

Muscimol rescue experiment

To generate homozygous offspring for the rescue experiments, we intercrossed Gad1 or Viaat heterozygotes. To increase the likelihood that litters were within comparable ranges of gestational age matings were set up at 9pm and the mice were separated at 9am the next day. Muscimol injections were done within the same time window for each set of pregnant dams. This was done to improve the reproducibility of the results between different litters. In the Gad1/- rescue experiment, muscimol was injected into the abdominal cavity of pregnant dams every 24 hours from E13.5 to E15.5. For the Viaat/- rescue experiment, muscimol was injected every 12 hours from E13.5 to E16.0. We used 4 mg/kg of muscimol for each injection. This is a dosage that results in obvious sedation of the mice [24].

RT-PCR

RT-PCR analysis of mRNA expression was performed as previously described [20]. The primer sequences and expected sizes for each gene are as follows:

- **Viaat (578 bp)**: 5’-GTCGAGGGAGACATTCATTACG-3’, 5’-GTACAGCAAGACTAATTGCC-3’
- **Gad1 (302 bp)**: 5’-CTTCGCTCGAACCATCCCTCGAAGC-3’, 5’-GGCGGTTTGTGCTCCTGCCTCCCGTTCT-3’
GAPDH (310 bp) 5′-GTCTCACTTCTGCTGACGTATCGCTC-CACTCAC-3′, 5′-CAATCTGAGTGGATGCTTGGCTTGTATTTTGCG- 
T-3′

Gabrb3 (356 bp) 5′-GAATGAATAGGTTGACGGACG- 
3′, 5′-CAGCGAGGTGTAATTTACTCACTCAG-3′

Ucp-1 (593 bp) 5′-TAGGTTAATAGGTGCTCCTAGGGA-3′, 
5′-CGCTTGGTACTGCTCTGG-3′

Results

Generation of the ViaatlacZ and Gad1lacZ knockin/ knockout mice

We generated mice carrying lacZ-tagged knockin/knockout alleles of Viaat and Gad1 for genetic studies and to facilitate the detection of Viaat or Gad1 expressing cells by β-galactosidase histochemistry. The ViaatlacZ and Gad1lacZ mice add to the existing mouse resources that express lacZ or fluorescent proteins in GABAergic and/or glycinergic neurons (Table 1). The strains listed in Table 1 carry marker gene knockin alleles or BAC transgenes wherein marker gene expression is likely to recapitulate most or all of the expression pattern of the endogenous gene. Additional transgenic lines have been generated with smaller segments of the regulatory regions of these genes [25,26,27]. Previously described knockin/knockout alleles of Viaat and Gad1 were also tagged with marker genes, but expression of the marker from the knockin allele could not be detected [4,5]. Our ViaatlacZ knockin allele was generated by inserting a lacZ coding sequence into the first exon of the Viaat gene (Figure 1A) [19]. Targeted ES cell clones and mice were identified by Southern blot analysis of genomic DNA (Fig. 1B) and RT-PCR analysis showed that Viaat transcripts were undetectable in the homozygous ViaatlacZ offspring (Fig. 1C). A similar strategy was used to generate a lacZ tagged allele of Gad1. The design of the Gad1lacZ allele was described previously [20]. In both strains the neo cassette was removed by crossing the founder mice to a germine Cre deleter strain [28]. The studies described here used mice carrying the neo-deleted lacZ knockin/knockout alleles of Viaat and Gad1.

To validate the expression of the lacZ marker from the ViaatlacZ and Gad1lacZ knockin alleles, we performed an initial analysis of β-galactosidase expression in the knockin mice. Whole mount and section Xgal histochemistry revealed expression patterns in both mouse strains that mirrored our previously published in situ hybridization results (Fig. 1D)[18,19].

Viaat is required for normal palate and ventral body wall development

To define the spectrum of mutant phenotypes in the ViaatlacZ knockout mouse we examined newborn progeny from intercrosses of ViaatlacZ heterozygous mice. The ViaatlacZ/-/- progeny were immediately identifiable due to their lack of movement, hunched posture, and failure to breathe (Fig. 2A). All ViaatlacZ homozygous offspring died at or immediately prior to birth. Examination of the pups showed that the ViaatlacZ/-/- newborns exhibited a cleft secondary palate, umbilical hernia and small bumps under the skin on the dorsal side of the cervical region (Fig. 2B-2F). Histological examination of paraffin sections showed that none of the ViaatlacZ/-/- newborns examined had inflated their lungs at birth indicating an early fundamental defect in respiratory function (data not shown).

We compared the ViaatlacZ/-/- newborn phenotype to that of Gad1lacZ/-/- newborns. Nearly all (98%) of the newborn ViaatlacZ homozygotes exhibited umbilical hernias (Fig. 2F, G). Examination of the Gad1lacZ homozygotes revealed a similar, but less frequent (85%) umbilical hernia phenotype that had not been previously reported. Histological sections of ViaatlacZ homozygotes revealed that this defect was indeed an umbilical hernia, not an omphalocele as previously reported for the Viaat homozygous mice [5]. Omphalocele is a severe body wall defect that results in an abnormally large umbilical ring usually exposing the gut and liver [29,30]. In contrast, an umbilical hernia is caused by a failure to retract the umbilical hernia that occurs normally during development [29,30]. These phenotypes suggest that Gad1 and Viaat functions are not necessary for body wall formation per se but are instead required for the retraction of the umbilical hernia during development. In both genotypes, palate shelf elevation fails to occur at E14.5 and all Gad1 and Viaat mutants were born with cleft secondary palates (Fig. 3A-H and data not shown).

We also examined the unusual “bumps” on the dorsal cervical region of the ViaatlacZ/-/- mice (Fig. 2D). These “bumps” were not present in Gad1 mutant newborns. Our analysis indicates that they are clumps of displaced brown fat since they resemble mouse brown fat in H&E stained paraffin sections and RNA extracted from the bumps contained very high levels of Ucp1 mRNA (data not shown). Ucp1 is a specific marker of brown fat [31,32]. In a newborn mouse the major deposit of brown fat is found in the intrascapular region, with additional deposits in various locations in the cervical region [33]. Presumably, the hunched posture and the apparent muscular paralysis exhibited by the ViaatlacZ/-/- homozygotes leads to the displacement of these fat deposits to this abnormal location.

Viaat is not expressed in the developing palate

Previous studies have suggested the possibility that GABA may be functioning as a signaling molecule in non-neuronal tissues during development [10,18,34]. Previous gene expression studies have shown that Gabrb3 receptor subunit and Gad1 mRNAs are expressed in the developing palate and teeth as well as in a variety of other non-neuronal cell types [10,15,16,18,35]. If Viaat function is required in the palate or craniofacial structures it should be expressed in these tissues. To determine whether Viaat mRNA is expressed in the developing palate, we examined the expression of Gabrb3, Gad1 and Viaat in mRNA from dissected palatal shelves from E13.5 and E14.5 day old mice. Using RT-PCR we easily detected Gad1 and Gabrb3 transcripts but could not detect Viaat mRNA in this tissue (Fig. 4). These results indicate that Viaat is not expressed in the palatal shelves at the time of palate shelf elevation and fusion. The lack of Viaat expression in the palate suggests that its function is not required in the palate for its normal development.

Palate explants from Gad1 and Viaat mutants can undergo shelf elevation and fusion

A previous study showed that oral explants made from Gad1 knockout mice could undergo palatal shelf elevation in embryo
In this previous study, the authors also performed exo-utero surgical manipulations that suggested that removal of the tongue was sufficient to allow normal palatogenesis in the $Gad1$ knockout embryos. Building on these previous results, we performed a series of explant experiments to test whether there is a requirement for GABA signaling within the developing palate shelves for normal shelf elevation and fusion in the $Viaat^{lacZ-/-}$ embryos and control littermates. This experiment is a simple way to test whether the mutant palate is capable of undergoing normal morphogenesis when removed from the context of the embryo. We developed an explant culture system that used a previously described dissection protocol to generate explants from the maxillary region of the E13.5 mouse embryos [23]. Explants dissected using this protocol were also used in the previous analysis of palate development in the $Gad1^{lacZ}$ explants [13]. We cultured the explants in a serum free and chemically defined medium that we had previously developed for mouse embryo culture [22]. In this system wild type explants underwent palate shelf formation, growth, elevation and fusion thereby recapitulating the normal events in palatogenesis (Fig. 5A-F). Explants from $Viaat^{lacZ-/-}$ and $Gad1^{lacZ-/-}$ embryos also underwent shelf elevation and fusion in this explant culture system (Fig. 5G-L). The absence of cleft palate in the $Viaat$ and $Gad1$ mutant embryos.

Figure 1. Targeted disruption of the $Viaat$ gene in mice. (A) A schematic representation of the $Viaat$ wild-type genomic locus (Genomic Structure), the targeting vector (Targeting Construct), and the mutant locus (Targeted structure) are shown. A $LacZ$ sequence was inserted into an Ncol site at the $Viaat$ start codon. The lox sites that flank the Neo resistance cassette are indicated with two arrowheads in the region immediately 3’ to the lacZ sequence. The locations of the 5′ (5′ Probes) and 3′ (3′ Probes) flanking probes used in characterizing the $Viaat$ allele are indicated above the map of the wild type $Viaat$ locus. The positions of the restriction sites used in the Southern blot analysis of genomic DNA from ES cells are indicated by single letters. Restriction sites are indicated as follows: H (HindIII), Nc (Ncol), A (Asp718) and N (NotI). (B) Southern blot analysis of genomic DNA from a wild type parental ES cell line (+/+ ) and a targeted ES cell line used to generate the $Viaat^{lacZ}$ mouse (+/−) are shown. In genomic DNA digested with Asp718 the 3’ flanking probe hybridized to a 15.5 kb wild type fragment and a 10.9 kb from the targeted allele (Asp718 panel) and in genomic DNA digested with HindIII this probe hybridized to a 12.1 kb wild type band and a 9.8 kb mutant band (HindIII panel). (C) RT-PCR analysis of cDNA from wild-type (+/+), heterozygous (+/−), and mutant embryo brain at E16.5. For each genotype RT-PCR was performed on RNA that had been reverse transcribed (− lanes) and RNA that had not been incubated with reverse transcriptase (−) to control for non-specific amplification. GAPDH primers were used as a positive control. (D) Localized expression of β-galactosidase activity in $Viaat^{lacZ}$ and $Gad1^{lacZ}$ heterozygotes. The panels from left to right show β-galactosidase activity in E11.5, and E14.5 $Viaat^{lacZ}$ embryos and in E12.5 and E14.5 $Gad1^{lacZ}$ embryos.

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after two days reproduces the previous results seen with the Gad1 -/- explants and shows that palates from the ViaatlacZ mutants are also able to undergo palatogenesis in this system. In total we tested 5 ViaatlacZ mutant explants from 3 different litters and 3 Gad1lacZ mutant explants derived from 3 different litters. Wild type and heterozygous explants from these litters served as controls. In all cases the ViaatlacZ and Gad1lacZ mutant explants underwent palatal shelf outgrowth and elevation in culture. In both the mutants and controls all of the palatal shelves fused to some extent with most of the explants exhibiting nearly complete fusion. There was no difference in the overall extent of fusion observed in control and mutant explants. Normal palate shelf elevation in the explants is consistent with there not being a requirement for GABA signaling within the palate for normal palatogenesis. However, on their own the explant experiments cannot completely exclude a possible role for GABA in the palate shelf itself.

The GABA agonist muscimol suppresses the cleft palate phenotype in the Viaat and Gad1 homozygous embryos. The presence of a cleft palate phenotype in Gad1, Viaat and Gabrb3 mutant mice suggests that the three genes function in a GABA signaling pathway required for palatogenesis. If this signaling pathway

Figure 2. Viaat mutants exhibit cleft palate and umbilical hernia. (A) Gross morphology of a ViaatlacZ (-/-) mutant at E18.5 with hunched posture as compared to a wild type E18.5 littermate (+/+). (B, C) Examination of E17.5 embryos showed that all of the ViaatlacZ mutants exhibited a cleft palate (C) as compared to wild type (B). (D) Viaat mutants have small subcutaneous bumps on the dorsal side of cervical region. (E, F) Compared to wild type (E) nearly all of the ViaatlacZ mice exhibited an umbilical hernia (F). (G) Gad1lacZ mutant E17.5 mice also displayed a similar hernia phenotype. doi:10.1371/journal.pone.0009758.g002

Figure 3. Viaat mutants exhibit delays in palate shelf elevation. (A-D) H&E stained coronal sections showing normal palatogenesis in wild type E13.5, E14.5, E15.5 and E18.5 mice. (E-H) Coronal sections of ViaatlacZ homozygous mutants at E13.5, E14.5, E15.5 and E18.5, showing a failure of palate shelf elevation in the mutants. P, palate; PS, palatal shelf; OC, oral cavity; T, tongue. doi:10.1371/journal.pone.0009758.g003
and Gad1lacZ in the CNS for normal development we examined the phenotype of mice with a CNS-specific knockout of Gad1. Because palate shelf elevation and fusion occur between E13.5 and E14.5 we chose to inactivate Gad1 throughout the CNS in stem cells and progenitors well before these events in palate morphogenesis. To inactivate Gad1 in the early CNS we used a well-characterized transgenic line (NesCre) that expresses Cre under the control of the Nestin regulatory sequences [21]. The NesCre strain expresses Cre exclusively in neural precursors starting at E9.5 days and by E11.5 days the expression of Cre activity is widely and uniformly distributed throughout the nervous system [21,41,42,43]. This strain is known to lack any detectable cre expression in the craniofacial region [44]. The floxed allele of Gad1 used in our experiments has been described previously [6,43].

To generate offspring with a CNS specific inactivation of Gad1 we crossed Gad1lox-/-; NesCre mice to Gad1lox+/+ mice. To confirm that Gad1 had been inactivated specifically in the CNS of the Gad1lox+/+; NesCre/+ offspring from this cross we used RT-PCR to measure Gad1 mRNA levels in RNA isolated from dissected palatal shelves and brain from E14.5 embryos. The RT-PCR analysis showed that the amount of Gad1 mRNA in the palatal shelves of the CNS specific Gad1 knockout embryos (Gad1lox+/+; NesCre) was equivalent to controls, while Gad1 mRNA levels in the CNS were greatly reduced as compared to the control embryos (Fig. 6). Examination of Gad1lox+/+; NesCre embryos at E17.5 showed that the cleft palate and the body wall phenotypes were present in the CNS-specific knockout. In total, we found a complete cleft palate in 9 out of the 11 Gad1lox+/+; NesCre E17.5 embryos that we examined. This cleft palate phenotype was identical to that seen in Gad1 mutant fetuses and newborns. We also found that 6 of the CNS specific E17.5 Gad1lox+/+; NesCre/+ exhibited an umbilical hernia. This genetic test demonstrated that Gad1 function was required within the CNS for normal development of the non-neural tissues affected in the Gad1 knockout.

**Discussion**

There have been numerous observations published over the last 25 years that implicate GABA signaling in the normal development of non-neural tissues [2,3,4,10,11,14,18,34,46,47,48]. A role for GABA signaling in non-neural tissues is plausible given the fact that GABA and other neurotransmitters have well-documented roles in the development of tissues and cell types outside of the CNS. A striking and prominent example of this was a recent report demonstrating that activation of GABA_A receptors alters embryonic stem cell proliferation in cell culture as well as cell proliferation in mouse blastocyst stage embryos [48]. These cell types exist prior to the earliest formation of the CNS, clearly demonstrating a role for GABA signaling in a completely non-neuronal context. In addition, serotonin, dopamine, acetylcholine, epinephrine and norepinephrine each play important roles in the development, maintenance and/or function of organs and tissues that are outside of the CNS [49,50,51,52,53,54,55,56]. In these cases receptors for the transmitters are expressed on the non-neural cell types that respond to them [30,52,57,58]. Viewed from this perspective the idea that GABA may be a signaling molecule in developing cell types outside of the nervous system is a reasonable hypothesis. In this study, we designed a set of experiments to test the hypothesis that GABA is required in non-neural cell types for the normal development and morphogenesis of the palate and retraction of the umbilical hernia.

In one set of experiments we found that muscimol was able to suppress the cleft palate phenotype in the Gad1 and Viatat mutant mice. The ability of muscimol to suppress or rescue the non-neural

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**Figure 4. The developing palatal shelves do not express Viatat transcripts.** RNA from dissected E13.5 and E14.5 brain tissue (Brain) or E13.5 and E14.5 dissected palate shelves (Palate) was analyzed by RT-PCR using primers corresponding to the Viatat, Gad1, Gabrb3 and Gapdh coding sequences. For each tissue and stage RT-PCR was performed with cDNA (+) or with RNA that had not been reverse transcribed (−) to control for non-specific amplification. Gapdh was used as a positive control.

|        | E13.5 | E14.5 |
|--------|-------|-------|
| Brain  | +     | +     |
| Palate | -     | +     |
| Brain  | +     | +     |
| Palate | +     | +     |

is dependent on functional GABA_A receptors, as suggested by the phenotype of the Gabrb3 knockout mouse, then specific pharmacological agonists of GABA_A receptors might suppress or “rescue” the phenotypes seen in Gad1lox-/- and Viatatlox-/- embryos. We tested whether the specific GABA_A agonist muscimol can suppress the non-neural phenotypes of the Gad1 and Viatat homozygous embryos. We chose to use muscimol as an agonist because it is a potent GABA_A receptor agonist that crosses the placental barrier and it binds to the same binding pocket on pentameric GABA_A receptors as GABA [36,37]. Since muscimol binds to a pocket formed by an alpha and a beta GABA_A subunit in a multimeric GABA_A receptor, any rescue of the Gad1lox-/- or Viatatlox-/- mutant phenotypes by muscimol would suggest that multimeric GABA_A receptors were the downstream target of Gad1 or Viatat function [36,38,39,40].

We injected muscimol into pregnant Viatatlox-/- or Gad1lox-/- mice that had been mated with Viatatlox-/- or Gad1lox-/- males respectively. Multiple muscimol injections from E13.5-E16 days were performed and the resulting offspring were removed at E17.5 days for examination. IP injection of muscimol into the pregnant dams did suppress the development of cleft palate in Viatatlox-/- and Gad1lox-/- mice but did so to different extents in the two genotypes. Muscimol injection suppressed the formation of cleft palate in about 20% of the Viatatlox-/- homozygous mutant embryos and in about half of the Gad1lox-/- embryos that we examined. This cleft palate phenotype was identical to that seen in Gad1 mutant fetuses and newborns. Since muscimol binds to a pocket formed by an alpha and a beta GABA_A subunit in a multimeric GABA_A receptor, any rescue of the Gad1lox-/- or Viatatlox-/- mutant phenotypes by muscimol would suggest that multimeric GABA_A receptors were the downstream target of Gad1 or Viatat function [36,38,39,40].
phenotypes in the Gad1 and Viaat knockouts suggested that tonic activation of multimeric GABA_A receptors was sufficient to provide normal function. The ability of muscimol to rescue suggests that it can substitute for the signal that is normally generated via the activity of the Gad1 or Viaat genes. The simplest explanation for the rescue is that the muscimol was acting on multimeric GABA_A receptors in the developing CNS. On the other hand, if muscimol was acting on non-neural cells then GABA_A receptor subunit transcripts would be expressed in these cells, presumably in cells adjacent to Gad1 expressing cells.

Although we detected Gabrb3 transcripts in palate RNA by RT-PCR we have not been able to detect localized expression of the Gabrb3 mRNA by in situ hybridization. In addition, surveys of gene expression in the developing mouse palate have not detected the expression of any GABA_A receptor subunits besides the Gabrb3 subunit [16,35]. Therefore, the rescue of the cleft palate phenotype in the Viaat and Gad1 mutants by muscimol is consistent with the requirement for GABA signaling within the CNS for normal palate development. However, this point requires

Table 2. Frequency of rescue of cleft palate by muscimol in Gad1-/ and Viaat-/- mice.

| Genotype | PBS Muscimol (Viaat) | Muscimol (Gad1) |
|----------|---------------------|-----------------|
| +/+      | 0/19 1/21           | 1/10            |
| +/-      | 1/46 0/34           | 1/31            |
| --/--    | 19/19 16/20         | 7/14            |

The frequency of cleft palate is expressed as the number of E17.5 day mice exhibiting a cleft palate over the total number examined for that injection condition. The frequency of cleft palate in Gad1-/- E17.5 mice is 100%. In the case of the Viaat mutant mice we have examined over 200 newborn or late gestation embryos and the cleft palate phenotype is present in 100% of the offspring.

*PBS injected controls were Viaat +/- dams from Viaat^{lacZ} heterozygote intercrosses.

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Figure 5. In vitro fetal palate explant culture. (A-F) Wild-type palates from Swiss Webster (SW) embryos at E13.5 were dissected and cultured for 2 days. (A-C) View of the oral surface of explants prior to culture (A) and after 1 day (B) or 2 days (C) of culture. (D-F) sections through the palate explants in shown in panels A-C. (G-I) Normal palatogenesis of the Viaat heterozygous (+/-) (G) and homozygous (-/-) (H) explants after 2 days in culture. Palate explants from the Gad1 mutant embryo at E13.5 also developed normally during culture for 2 days (I). (J-L) Sections of the palate explants shown in panels G-I. doi:10.1371/journal.pone.0009758.g005

Table 2. Frequency of rescue of cleft palate by muscimol in Gad1-/ and Viaat-/- mice.
additional verification and support via a systematic analysis of GABA receptor expression within the palatal shelves to confirm that muscimol rescues the Gad1lacZ and ViaatlacZ mutant phenotypes by acting on receptors in the CNS.

The results of our explant culture experiments and the CNS specific knockout of Gad1 are also consistent with a requirement for GABA signaling in the CNS for normal palate and body wall development. Our explant experiments repeated the results previously obtained with Gad1 mutant explants [13] and demonstrated that Viaat mutant oral explants can also undergo palate shelf elevation and fusion in culture. The ability of the Viaat mutant oral explants to undergo palate elevation and fusion which occur during E13.5-E14.5. The authors did not monitor Gabrb3 mutant oral explants to undergo palate elevation and fusion which occur during E13.5-E14.5. This is a particular concern since the authors were relying on the inactivation of two Gabrb3flox alleles in the offspring. Unfortunately, the authors did not monitor Gabrb3 transcript levels in the CNS and palate of the SynCre Gabrb3flox/Gad1flox mice or the extent of Cre mediated recombination of the Gabrb3flox allele in these experiments [11].

GABA signaling in the CNS is required for fetal movement and developmental processes that depend on fetal movement

Fetal akinesia during human or rodent development has been shown to cause defective development of several non-neural tissues and structures in the fetus. For example, it has been shown that normal lung development requires spontaneous fetal breathing movements in humans and mice [66,67,68]. In humans developmental defects caused by fetal akinesia include multiple pterygium syndrome (OMIM #265000 and #253290) and the fetal akinesia deformation sequence (FADS; OMIM #208150). The phenotypes associated with these syndromes include pulmonary hypoplasia, craniofacial defects, joint contractures, limb defects, skeletal defects, webbing of the skin (pterygia) and cardiac defects. Studies have suggested that in some cases FADS is found in infants born to mothers who have myasthenia gravis, an autoimmune disorder that affects acetylcholine receptors in the neuromuscular junction [69,70]. Recent work has tied several human FADS and multiple pterygium syndrome cases to mutations in nicotinic acetylcholine receptors as well as mutations in RAPSN a protein that is associated with acetylcholine receptors [71,72,73]. This demonstrates that defects in neuromuscular junction components can lead to non-neural developmental defects. These reports provide genetic evidence that mutations in genes required for neuromuscular function can cause a spectrum of non-neural defects.

Within this context, the craniofacial and body wall phenotypes found in Gad1 and Viaat knockouts can be understood as additional examples of non-neural phenotypes caused by changes in fetal CNS function that in turn lead to changes in fetal muscle tone or fetal movements. Previous work has shown that fetal movements during palate formation are impaired in the Gad1 mutant mice [12]. Although the phenotypes are secondary defects caused by the primary defect of disrupted GABA signaling in the CNS, they are fully penetrant developmental defects that are similar to some of the most common developmental defects seen in humans. To develop a comprehensive understanding of the origins of defects in human development it is important to understand the diverse mechanisms, both primary and secondary, that can cause or
increase the likelihood of such defects. We feel that the phenotypes seen in the Gad1 and Viaat homozygous mice are part of a larger spectrum of developmental defects and disorders that are caused by impaired or absent fetal movement. Our work suggests that disruptions of GABA signaling during development could interfere with any one of several developmental processes that depend on fetal movement.

In this context is intriguing that there are correlations between the timing of initial fetal movements in mice or humans and the initiation of palatogenesis. In mice, palate elevation and fusion occur at the same time as the first fetal movements are detected [12]. In humans, movements start at 7–9 weeks, with breathing and hiccup like movements initiating at 8–9 weeks [74]. This coincides with the time of palate shelf elevation during the 8th week of human development [75]). The similarity in timing of human palate elevation and the initiation of fetal movements suggests that alterations in GABA signaling could interfere with human palate development or the development of any other structure or tissue that develops after the initiation of fetal movements. So far, genetic studies have detected weak associations between Gad1 or Gabrb3 alleles and human oral clefts [7,8,9]. Perhaps the weak association of Gad1 and Gabrb3 with oral clefts is due to the fundamental requirement for normal GABA neurotransmission for late fetal or neonatal viability. This would eliminate individuals who are homozygous for null or strong loss of function alleles of Gabrb3 or Gad1 from the populations sampled for these studies.

In conclusion, our work provides multiple lines of evidence that the non-neural developmental defects in Gad1 and Viaat mutant mice are due to a requirement for GABA signaling in the CNS during mouse fetal development. The non-neural defects in these mice are most likely due to defects in fetal movement caused by CNS dysfunction and appear to be part of a spectrum of defects caused by abnormalities in fetal movements. Our results help to clarify the mechanism leading to cleft palate in the Gad1 and Viaat knockout mice and suggest that defects in fetal movement caused by alterations in fetal neuronal GABA signaling may lead to similar defects in humans.

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Author Contributions

Conceived and designed the experiments: BGC. Performed the experiments: WJO JJW RS. Analyzed the data: WJO JJW BGC. Wrote the paper: WJO BGC.

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