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Valproic acid disrupts the biomechanics of late spinal neural tube closure in mouse embryos

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ABSTRACT

Failure of neural tube closure in the early embryo causes neural tube defects including spina bifida. Spina bifida lesions predominate in the distal spine, particularly after exposure to the anticonvulsant valproic acid (VPA). How VPA specifically disturbs late stages of neural tube closure is unclear, as neurulation is usually viewed as a uniform 'zippering' process along the spine. We recently identified a novel closure site ("Closure 5") which forms at the caudal extremity of the mouse posterior neuropore (PNP) when completion of closure is imminent. Here we investigated whether distal spina bifida in VPA-exposed embryos involves disruption of Closure 5. Exposure of E8.5 mouse embryos to VPA in whole embryo culture had marked embryotoxic effects, whereas toxic effects were less pronounced in more developmentally advanced (E9) embryos. Only 33% of embryos exposed to VPA from E9 to E10.5 achieved PNP closure (control = 90%). Short-term (8 h) VPA treatment diminished supra-cellular F-actin cables which normally run along the lateral neural folds, and prevented caudal PNP narrowing normally characteristic of Closure 5 formation. Laser ablation of Closure 5 caused rapid neuropore widening. Equivalent ablations of the caudal PNP in VPA treated embryos resulted in significantly less widening, suggesting VPA prevents formation of Closure 5 as a biomechanically active structure. Thus, VPA exposure prevents morphological and biomechanical conversion of the caudal extreme of the PNP during late spinal closure. Closure 5 facilitates neural fold apposition when completion of closure is imminent, such that its disruption in VPA-exposed embryos may lead to distal spina bifida.

1. Introduction

Mammalian primary neurulation is a morphogenetic process whereby the flat neural plate bends to form paired neural folds which become medially apposed and fuse at the dorsal midline, forming a closed neural tube (NT) (Nikolopoulou et al., 2017). Fusion begins at specific initiation points, and is then propagated through a zippering process whereby cellular protrusions at the tips of the neural folds reach across the midline to contact the contralateral side (Rolo et al., 2016). Spinal closure initiates at the hindbrain-cervical boundary (Closure 1) and zippers bi-directionally: rostrally to form the cephalic NT and caudally to form the future spine (Nikolopoulou et al., 2017). The open region of spinal NT, referred to as the posterior neuropore (PNP), transitions from a "spade-like" structure at mid-spinal stages to an elliptical shape with a narrowed caudal extreme when completion of closure is imminent (Galea et al., 2017). This shape change is associated with encircling of the PNP by a supra-cellular F-actin ring. We identified cellular protrusions characteristic of active zippering not only at the main site of closure (Rolo et al., 2016) but also at the PNP's caudal canthus in embryos at the final stage of PNP closure. This suggested active caudal-to-rostral as well as rostro-to-caudal closure when spinal neurulation is completed in the low spine. In support of this finding, laser ablation of the caudal canthus resulted in rapid lateral recoil (*i.e.* widening) of the neural folds (Galea et al., 2017). Hence, a new biomechanically active closure point arises at the caudal extremity of the late-stage closing spinal neural tube, which we have denoted "Closure 5" (Galea et al., 2017).

Although Closure 5 has not yet been directly documented in humans, its existence has been inferred from the clustering of spina bifida lesion in the distal lumbo-sacral spine (Van Allen et al., 1993), at which point zippering has progressed unperturbed along most of the embryonic axis. Evidence for this includes the distal spina bifida caused by *in utero* exposure to the anti-epileptic medication valproic acid (VPA) (Robert and Guibaud, 1982; Van Allen et al., 1993). In mice, exposure to VPA during neurulation also impairs NT closure, but the resulting defects primarily affect the cranial region causing exencephaly (the developmental forerunner of anencephaly) (Nau, 1985; Nau and Loscher, 1986). These teratogenic effects are distinct from VPA's anti-epileptic properties as not all of its anti-epileptic metabolites and analogues cause exencephaly when injected into mice (Nau and Loscher, 1986). Caudal spina bifida similar to that seen in

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humans can be induced in mice by repeated exposure to VPA during mid to late spinal neurulation (three injections on E9) (Ehlers et al., 1992). In cultured rodent embryos, exposure to ~1 mM VPA, which is comparable to concentrations measured in the blood of human patients (Suzuki et al., 2011; Vasudev et al., 2001), causes cranial and/or spinal NT defects depending on the treatment regime (Lampen et al., 1999; Seegmiller et al., 1991). However, embryos from certain mouse strains have been reported to be more sensitive to the teratogenic effects of VPA both *in vivo* (Lundberg et al., 2004) and in culture (Naruse et al., 1988). Here we set out to identify a VPA treatment regime which disrupts PNP closure in cultured CD1 mouse embryos and to use this model to determine whether VPA diminishes Closure 5 formation, as a possible explanation for the distal spina bifida in exposed individuals.

2. Materials and methods

2.1. Embryo culture and treatments

VPA was purchased from Sigma (Cat. No. V0033000) and dissolved with vigorous agitation in neat rat serum. Studies were performed under project license numbers 70/7469 and P8B3095F0 under the UK Animals (Scientific Procedures) Act 1986 and the Medical Research Council's Responsibility in the Use of Animals for Medical Research (1993). Outbred CD1 mice were bred in-house. Embryo culture was performed essentially as previously described by our group (Copp et al., 2000).

For experiments starting at E8.5, mice were mated overnight and the morning a plug was found was considered E0.5. Pregnant females were sacrificed in the morning of E8.5 (~8 somites at the start of culture) and their embryos cultured for 24 h.

For experiments starting at E9, mice were mated during the day, and noon of the day a plug was found was considered E0. Pregnant females were sacrificed in the morning of E9 (~15 somites at the start of culture) and their embryos cultured for 8 h or 24–36 h as indicated.

At the end of culture, embryos were imaged in their yolk sac using a Leica DFC490 mounted on a Zeiss Stemi SV-11 stereomicroscope, dissected out of their extraembryonic membranes and fixed in 4% PFA. PNP images were then captured using the same setup to analyse PNP dimensions and embryo lateral images were captured to measure dorsal length as a curved line from the otic vesicles to the caudal tip, using Fiji (Schindelin et al., 2012).

2.2. Wholemount staining and confocal microscopy

Embryo whole-mount staining with Alexa Fluor™-568 conjugated phalloidin, DAPI and far red CellMask™ was as previously described (Galea et al., 2017). Images were captured on a Zeiss Examiner LSM880 confocal using a 20×/NA1.0 Plan Apochromat dipping objective. Embryos were typically imaged with X/Y pixel sizes of 0.59 μm and Z-step of 1.0 μm (speed = 8, bidirectional imaging, 1024 × 1024 pixels). Images were processed with Zen2.3 software and visualised as maximum projections in Fiji.

2.3. Laser ablation

Closure 5 laser ablations were performed as previously described using a MaiTai laser (SpectraPhysics Mai Tai eHP DeepSee multiphoton laser, 800 nm wavelength, 100% laser power, 65.94 μs pixel dwell time, 1 iteration). Reflection images of live embryo PNPs were obtained using a 10×/NA0.5 Plan Apochromat dipping objective (633 nm laser wavelength). PNPs were imaged before and immediately after ablation, taking approximately 3 min to capture each Z-stack.

2.4. Statistical analysis

Comparisons between two groups were by Student's unpaired *t*-test accounting for homogeneity of variance in Excel or in SPSS (IBM Statistics 22). Comparison of multiple groups was by one-way ANOVA with *post-hoc* Bonferroni in SPSS. Linear regression F-test was in OriginPro 2016 (Origin Labs). Multivariate analysis for serial PNP width or change in width measurements were by linear mixed models in SPSS accounting for the fixed effects of treatment and percentage of PNP length in repeated measures from each, with a *post-hoc* Bonferroni. Graphs were made in OriginPro 2016 (Origin Labs) and are represented as box plots or as the mean ± SEM when several groups are shown per measurement level. $p < 0.05$ was considered statistically significant.

3. Results

3.1. Closure 5 forms when completion of PNP closure is imminent

The PNP of mouse embryos transitions from a “spade-like” morphology at mid-spinal levels to an elliptical shape when completion of closure is imminent and Closure 5 has formed (Fig. 1A). An F-actin cable is visible running along the neural folds at early stages, and this encircles the PNP at late somite stages (Fig. 1A) (Galea et al., 2017). Consequently, at early stages the F-actin cable does not reach the caudal limit of the PNP, but from the ~21 somite stage the cable forms a purse string-like structure around the PNP (Fig. 1B,C and data previously reported (Galea et al., 2017)). The F-actin cable reached the caudal limit of the PNP in 90% (10/11) of embryos with ≥21 somites, but only 10% (1/14) of embryos with ≤20 somites analysed in the present study.

3.2. VPA exposure retards embryonic development and disrupts PNP closure

The neuro-teratogenic and embryotoxic effects of VPA vary in different mouse strains (Naruse et al., 1988) and gestation ages (Kao et al., 1981), but culture in ~1 mM VPA has previously been reported to cause NTDs in cultured embryos (Kao et al., 1981; Naruse et al., 1988; Seegmiller et al., 1991). In pilot studies, culture of E8.5 CD1 embryos in 1 mM VPA caused clear evidence of embryo toxicity, namely absence of active yolk sac circulation in 7/8 embryos compared with 1/9 vehicle-treated embryos (X^2: $p = 0.002$). All embryos treated with 0.5 mM VPA had visible yolk sac circulation at the end of culture, but treatment delayed embryo development as evidenced by a smaller somite number after 24 h of treatment (Fig. 2A,B) and reduced embryo dorsal length at similar somite stages (Fig. 2C). Despite these clear toxic effects, 0.5 mM VPA did not significantly alter PNP dimensions in embryos which achieved similar somite stages (Fig. 2D,E). Hence, VPA diminishes embryo development during early neurulation, but has no detectable effects on spinal neural tube closure.

Embryos were next cultured for 24 h from a later gestational age (E9), and were found to be less sensitive to the effects of VPA: all embryos cultured in 1 mM VPA (which was toxic for E8.5 embryos) had visible yolk sac circulation at the end of culture (Fig. 3A). This treatment delayed somite number increase (Fig. 3A,B), but did not significantly change embryo dorsal length relative to somite stage matched control embryos (Fig. 3C). PNP length and width could not be compared between groups as most control embryos achieved developmental stages >25 somites and consequently completed PNP closure. A significantly smaller proportion of 1 mM VPA-treated embryos achieved PNP closure within the same time frame (Fig. 3D). However, this comparison is confounded by treated embryos being less developmentally advanced than controls.

In order to compare VPA-treated and untreated embryos at similar developmental stages, cultures were extended to 36 h such that the majority of embryos in both treatment groups achieved ≥25 somites. Of these, 90% of control embryos achieved PNP closure whereas only 33% of 1 mM VPA treated embryos completed PNP closure (Fig. 4A,B).