Precision of morphogen gradients in neural tube development

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Morphogen gradients encode positional information during development. How high patterning precision is achieved despite natural variation in both the morphogen gradients and in the readout process, is still largely elusive. Here, we show that the positional error of gradients in the mouse neural tube has previously been overestimated, and that the reported accuracy of the central progenitor domain boundaries in the mouse neural tube can be achieved with a single gradient, rather than requiring the simultaneous readout of opposing gradients. Consistently and independently, numerical simulations based on measured molecular noise levels likewise result in lower gradient variabilities than reported. Finally, we show that the patterning mechanism yields progenitor cell numbers with even greater precision than boundary positions, as gradient amplitude changes do not affect interior progenitor domain sizes. We conclude that single gradients can yield the observed developmental precision, which provides prospects for tissue engineering.
During embryogenesis, tissues specialize, and cells in different subdomains take on different fates. Morphogen gradients can provide the positional information to define the boundaries of distinct progenitor domains (Fig. 1A). According to the French flag model, boundary positions are defined by threshold concentrations. Molecular noise in morphogen production, transport, and decay affects the gradient shape such that the threshold concentration is reached at different readout positions in different embryos. How such variable gradients can encode precise spatial information, and to which degree they are accurate enough to pattern a developing tissue is an open question, despite intense studies.

Given the challenges in visualizing morphogen gradients, detailed studies of gradient precision have only been carried out in a few developmental systems. First and foremost, for the Bicoid gradient in the early *Drosophila* embryo, the Decapentaplegic (Dpp) gradient in the *Drosophila* wing disc, and for the Sonic hedgehog (SHH) and Bone morphogenetic protein (BMP) gradients in the mouse neural tube (NT). In all these systems, the morphogen gradients take on an exponential form:

\[ C(x) = C_0 \exp\left(-\frac{x}{\lambda}\right) \]

with an amplitude \( C_0 \) at the morphogen source at \( x = 0 \) and a decay length \( \lambda \). In the French flag model, domain boundaries are positioned where the gradient concentration reaches a threshold concentration \( C_0 = C(x_0) \), i.e., at

\[ x_0 = \lambda \ln\left(\frac{C_0}{C_{\text{th}}}\right). \]

The gradient amplitude \( C_0 \) and length \( \lambda \) differ between embryos as a result of molecular noise in morphogen production, decay and diffusion. Differences in gradient amplitude and decay length translate into differences in the readout position \( x_0 \), where the threshold concentration \( C_0 \) is reached in each embryo \( i \) (Fig. 1B, top). The overall readout position for the particular threshold concentration \( C_0 \) is the mean position \( \mu_x = \text{mean}(x_{0,i}) \). The positional error is given by the standard deviation (SD) of the readout positions in the different embryos,

\[ \sigma_x = \text{SD}(x_{0,i}). \]

Following this definition, the positional error of two centrally located NT progenitor domain boundaries, the dorsal NKK6.1 boundary and the ventral PAX3 boundary (Fig. 1A), was found to be about 1–3 cell diameters. In parallel, also the gradient variability was measured, which has enabled the discussion of patterning precision in the NT. Due to challenges in measuring morphogen gradients directly, GBs-GFP was used as transcriptional reporter of SHH signaling, and phosphorylated Smad1/3/5 (pSMAD) as a readout of BMP signaling. Close to the source, the positional error increased from a single cell diameter (4.9 μm) to about three cell diameters over time. In the center of the NT, however, the positional error was reported to increase from 1 to 2 cell diameters in early stages to more than 30 cell diameters later on. Combined readout of the imprecise SHH and BMP gradients was proposed to yield the higher precision of the central progenitor boundaries. However, even a combined readout fails after 15 somite stages (SS), i.e., after about 30 h of spinal cord development, and it remained unclear how the precise patterning of the central progenitor domains is achieved.

In this article, we revisit the problem of morphogen gradient readout and patterning precision. We show that the positional error of gradients in the NT has previously been overestimated, and demonstrate that a single morphogen gradient in the NT is sufficiently precise to define the NKK6.1 and PAX3 progenitor domain boundaries with the reported accuracy during the first day of NT development. We further show that technical limitations do not allow for precise gradient measurements in the center of the NT at later stages. We develop a theoretical framework and combine it with numerical computations to estimate gradient variability also at later stages of NT development. Based on the measured variabilities in morphogen production, turnover and diffusion, we infer the variability of morphogen gradients and thus their positional error as it results from molecular noise. The resulting positional accuracy is consistent with the observed precision of the readout boundaries in the mouse NT. The gradients are thus, in principle, sufficiently precise to yield the observed patterning precision. Furthermore, we show that the size of gene expression domains is independent of the activity and variability thereof when defined by the threshold-based readout of a single morphogen gradient. This results in a very robust mechanism to produce precise numbers of progenitor cells.

**Results**

**Positional error of gradients was previously overestimated in the mouse NT.** In principle, a wide range of error metrics could be defined, but in order to address the question whether a gradient is sufficiently precise to define a progenitor domain boundary, the variability of the gradient and of the progenitor domain boundary must be measured with the same metric. Zagorski et al. determined the positional error of the NKK6.1 and PAX3 domain boundaries by first determining their position in individual embryos, and by subsequently determining their standard deviation, \( \sigma_x \) (Eq. (2)). Going forward, we refer to Eq. (2) as the direct error estimation method (DEEM). For the gradient, on the other hand, a different, well-established approach was used, given by the linear error propagation formula

\[ \sigma_x \approx \frac{\partial C}{\partial x} \sigma_C, \]

\[ \text{to estimate the positional error of gradients } C \text{ from their slope, } \frac{\partial C}{\partial x}, \text{ and variance, } \sigma_C^2, \text{ instead of using Eq. (2) directly with gradients. In this indirect way, vertical variability of the gradients (measured by their standard deviation, } \sigma_C = \text{SD}(C(x_0))) \text{ is translated into horizontal variability of the readout position (} \sigma_x = \text{SD}(x_{0,i}) \text{) by multiplication with the inverse slope of the mean gradient (Fig. 1B, C). We now test whether both approaches yield an equivalent result.}

To determine the positional error with Eq. (3), an averaged inverse slope needs to be determined from the individual concentration or intensity profiles. Gregor et al. used numerical differentiation of the mean gradient to determine the derivative (personal communication), an approach that we refer to as numerical differentiation error propagation method (NumEPM for short). Bollenbach et al. and Zagorski et al. (personal communication) first fitted an exponential function to the mean gradient (Fig. 1B, bottom). Then, they exploited that for an exponential function

\[ \frac{\partial C}{\partial x}(x) = -\frac{C(x)}{\lambda}, \]

such that \( \frac{\partial C}{\partial x}^{-1} \) follows from the local value of the fitted exponential function and the fitted decay length \( \lambda \). We use the acronym FitEPM to refer to this error propagation method. If these approximations are appropriate, then both the NumEPM and the FitEPM should yield the same positional error as the DEEM. We can test this by applying all three methods to the same set of 10^4 exponential gradients with...
Fig. 1 Estimation of positional error of morphogen gradients in the vertebrate neural tube. A Schematic of opposing noisy SHH and BMP gradients with resulting gene expression domains. The patterning domain is confined between the floor plate (FP) and the roof plate (RP). B Gradients from different embryos (top, different colors) differ in amplitude and decay length. As a result, the threshold concentration $C_\theta$ is reached at different readout positions $x_\theta,i$. The overall readout position, $\mu_x$, is given by the mean of the readout positions, $x_\theta,i$. The positional error, $\sigma_x$, is the standard deviation of the readout positions $x_\theta,i$ (Eq. (2)). At the threshold concentration $C_\theta$, the readout (middle) of the gradient typically assumes its half-maximal value. If the precision of both gradient and readout is measured by the same error metric, there is a 1:1 correspondence between them. Noise in individual gradients can further widen the transition zone between two adjacent domains. With the FitEPM (bottom, Eq. (14)), individual exponential gradients are averaged (red solid line) and then linearly fitted with another exponential (red dotted line), to then apply linear error propagation to estimate the positional error. C In the linear error propagation method, the local tangent (green) to an averaged morphogen concentration gradient (blue) is used to translate the vertical gradient variability, $\sigma_C$, into a horizontal positional error, $\sigma_x$ (black). D Comparison of the previously reported positional error of GBS-GFP (green squares) with the results obtained with 104 randomly drawn gradients that match the reported gradient summary statistics. The positional error is calculated either directly, Eq. (2) (DEEM, black solid line), or with the linear error propagation method, Eq. (3), using the derivative of either the mean gradient (Eq. (13), NumEPM, blue dashed line) or of an exponential function fitted to the mean gradient (Eq. (14), FitEPM, green solid line). See Methods for details. E When applied to sets of exponential gradients with mean and standard deviation of $C_0$ and $\lambda$ identical to those reported for GBS-GFP (green) and pSMAD (red), the FitEPM overestimates the positional error (Eq. (2), black) by orders of magnitude further away from the source. Source data are provided as a Source Data file.
Fig. 2 Single gradients are precise enough to define the central progenitor domain boundaries in the mouse neural tube. A) The GBS-GFP and pSMAD gradients\(^9\) follow an exponential curve only close to the source and then transition to a noise bed, as revealed by fitting a kinked line (black) to the logarithmic intensity with the kink position as free parameter (green square). B) Exponential limit as obtained from the kink position in the GBS-GFP (green) and pSMAD (red) intensity profiles over time. The dorsal NKX6.1 (blue) and ventral PAX3 (black) domain boundaries (extracted from\(^9\)) lie within the exponential part of the gradients only during the first 15 somite stages (dashed line). Later, they fall in the noise-dominated central region (gray). DV position in (A, B) is measured from the ventral end. C, D, E Comparison of the reported positional error evolution of the dorsal NKX6.1 (green) and pSMAD (red) gradients along these domain boundaries. Gradient errors determined either with the FitEPM (Eq. (14), dotted lines) or the DEEM (Eq. (2), solid lines), in units of cell diameters (cd). The shaded region indicates the range into which the gradient positional errors (green, red) would need to fall for the gradients to be precise enough to directly explain the measured domain boundary precision. F, G Rescaling the domains to the mean domain length changes the decay length and introduces an artificial positional error. H, I, J, K After subtraction of the domain scaling error introduced in\(^9\), the gradients’ positional errors (green, red) are sufficiently low to be consistent with the reported dorsal NKX6.1 (blue) and ventral PAX3 (black) boundary errors. Source data are provided as a Source Data file.

\[
C_i(x) = C_0 \exp \left( -\frac{x}{\lambda_i} \right) \tag{4}
\]

different gradient amplitudes \(C_0\), and lengths \(\lambda_i\).

To determine the positional error according to its mathematical definition (Eq. (2)), one needs to determine the positions, \(x_{0i}\), where each gradient reaches the threshold concentration \(C_0\) (Fig. 1B, top). For the purely exponential gradients, there is only a single intersection and the readout position is thus unique. The readout position \(\mu_i\), for a given concentration threshold \(C_0\) is then given by the mean of all \(x_{0i}\), while the positional error at this readout position is given by the standard deviation (Eq. (2)). If this is repeated with threshold concentrations over the entire concentration range, the positional error along the domain is obtained (Fig. 1D, black line). While the NumEPM results in positional errors (Fig. 1D, blue dashed line) close to the DEEM (Fig. 1D, black line), the FitEPM yields much larger positional errors (Fig. 1D, green line), and strongly overestimates the positional error further away from the source (Fig. 1D, E). The difference between the NumEPM and the FitEPM arises from a subtle, but important difference. With the NumEPM, the slope of the mean gradient is obtained by numerical differentiation, whereas in the FitEPM, an exponential function is fitted to the mean gradient to estimate their slope. The arithmetic mean of (different) exponentials is, however, not exponential itself (Fig. 1B, bottom). There is no way to write

\[
\frac{1}{n} \sum_{i=1}^{n} C_{0i} \exp \left[ -\frac{x}{\lambda_i} \right] = C_0 \exp \left[ -\frac{x}{\lambda} \right]
\]

unless all decay lengths are equal, \(\lambda = \lambda_i\). Further away from the source, an exponential function fitted to the mean of exponentials lies below that mean (Fig. 1B, bottom), when fitted without logarithmicizing the data first. The inverse of this underestimated concentration then enters Eq. (3), which is why the positional error in\(^9\) is much higher than the error directly evaluated with Eq. (2) further away from the source.

The synthetic, purely exponential gradients (Eq. (4)) were drawn such that the statistical properties (mean and standard deviation) of the gradients are identical to those reported for GBS-GFP and pSMAD\(^9\) (see Methods). Even though the synthetic gradients do not include local noise or deviations from the exponential shape, the positional error obtained with the FitEPM with the synthetic data (Fig. 1D, green line) is very similar to that reported in\(^9\) for the real data (Fig. 1D, green squares). This suggests that it is mainly the computational method that generates the high apparent positional error rather than the additional deviations that are included in the measured, but not in the fitted gradients. We conclude that the accuracy of Eq. (3) hinges critically on the accuracy of the derivative, and that the FitEPM exaggerates the gradient positional error.

Single gradients are sufficiently precise to define the central progenitor boundaries in the mouse neural tube. The measured GBS-GFP and pSMAD gradients differ from the fitted gradients that we considered in Fig. 1 not only in that they are noisy, but also in that they follow an exponential trend only within a certain proximity to the source, and then switch to a shallow noise bed (Fig. 2A). The dorsal NKX6.1 and ventral PAX3 boundaries lie within the exponential part of the gradients only for the first 15 SS (Fig. 2B). After that, the measured GBS-GFP and pSMAD
gradients in9, which arti...art of the preprocessing of the three cell diameters, at the ventral end. Since the NT grows (Fig. 2F, G). The stage-matched gradients that we received from the authors of9 are binned in 5 SS, corresponding to 10 h of the gradients (Fig. 2C)

The measured GBS-GFP and pSMAD gradients9 contain an approximately constant noise bed that dominates the gradients (Fig. 2B). It is not known whether the shallow part of the GBS-GFP and pSMAD gradients afterwards (Fig. 2B) is an approximation of exponential growth or a shallow noise bed that is completely noise dominated. As the same settings were used in all measurements, the decline of the GBS-GFP and pSMAD gradients amplitudes over developmental time9 further restricts the detection range at later times such that also the dorsal NKX6.1 boundary will lie outside the GBS-GFP detection range. Indeed, the gradient noise is equal everywhere in the noise bed, including the region where the detection of an exponential gradient with 8-bit imaging is certainly impossible (Fig. 3B). While 16-bit imaging would be possible, at least the SHH reporter GBS-GFP reporter poses further limits. With its eight concatemerized fragments of a FoxA2 enhancer that contains a GLI binding site22, GBS-GFP necessarily follows the SHH response of FoxA2. FoxA2 requires high SHH concentrations, and its expression is thus restricted to the ventral-most SHH-secreting floor plate and the adjacent p3 domain23. While GBS-GFP extends beyond the p3 domain, presumably because the fragments lack additional negative regulatory elements of the full FoxA2 enhancer, GBS-GFP will have the same strong dependency on GLIA input as FoxA2. Other SHH-responsive genes that do not depend on GLIA input are well known to be expressed at much lower SHH concentrations than FoxA2. Accordingly, the transition to the noise bed could either indicate the limits of 8-bit imaging or the response limits of GBS-GFP. In both cases, technical limitations preclude the detection of exponential gradient profiles in the center of the NT.

But are long-range exponential gradients plausible, and could cells detect such low concentrations? In the developing vertebrate NT, positional information is provided by opposing SHH and BMP gradients24 (Fig. 1A). If each morphogen patterns only onehalf of the domain, the morphogen concentration in the center of the final domain will be about 104-fold lower than at the source. In the bacterial chemotaxis response, adaptation allows cells to sense concentration gradients spanning at least five orders of magnitude, and cooperativity in receptor clusters enables a high gain such that the occupancy of one or two receptors can be sensed25. Whether similar effects are at work also in the NT is

\[ \xi = x/L = 0 \text{ at the ventral end}. \]
unclear, but adaptation in the SHH responsiveness has been noted\textsuperscript{12,20}, and the PTCH1 receptor organizes as dimer of dimers, and each dimer binds one SHH\textsuperscript{26}. Accordingly, it is, in principle, possible that morphogens can be sensed by cells over a $10^5$-fold concentration range (11.5 $\lambda$), which corresponds to about 220 $\mu$m in the mouse NT—enough to cover the entire NT domain with opposing gradients (Fig. 3A). But even if the gradients can be sensed over 11.5 $\lambda$, how precise would the conveyed positional information be? We will now take a computational approach to estimate the gradient variability based on available data.

**Estimation of positional error from statistical gradient properties.** Close to the source, the measured gradients can be approximated well by exponential functions\textsuperscript{9}, and the positional error of the individual gradients. This then allows to predict the positional error at a given point in time (i.e., at a given size of the NT), consistently with the observed readout precision in the mouse NT. Our results retain their validity for general C\textsubscript{0}. Hence, we assume that the ratio C\textsubscript{0}/C\textsubscript{0} follows a log-normal distribution:

$$\frac{C_0}{C_0} \sim \text{Logn}(\mu_0, \sigma^2_0) \iff \ln\left(\frac{C_0}{C_0}\right) \sim N(\mu_0, \sigma^2_0).$$

Here, $\mu_0$ and $\sigma_0$ are the mean and standard deviation of the Gaussian random variable $\ln[C_0/C_0]$. We can use the properties of log-normal distributions\textsuperscript{27} to express $\mu_0$ and $\sigma_0$ in terms of the mean $\mu_0$ and standard deviation $\sigma_0$ of $C_0/C_0$:

$$\mu_0 = \ln \mu_0 - \sigma^2_0/2 \quad \text{and} \quad \sigma^2_0 = \ln \left[1 + \frac{\sigma^2_0}{\mu^2_0}\right]$$

To estimate how domain boundaries behave under variability in the morphogen gradient, we seek to express the expected boundary position $x_g = \mathbb{E}[x_g]$ and its standard deviation $\sigma_g = \text{SD}[x_g]$ as functions of the four gradient parameters $\mu_0$, $\sigma_0$, $\mu_0$, $\sigma_0$. The data for the SHH gradient in the mouse NT suggests that the gradient’s decay length and amplitude are uncorrelated (Pearson’s $R = -0.0061$, Kendall’s $\tau = 0.056$, Fig. 4G). This allows us to exploit the multiplicative properties of two independent random

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**Fig. 4 Statistical properties of the SHH gradient in the developing mouse neural tube.** A-B $\lambda$ is constant over developmental time, consistent with a truncated normal distribution. C-Binning the data into 40 $\mu$m bins reveals that the relative variability in the data drops over time for SHH. D $C_0$ increases as the neural tube expands. The solid line shows an exponential fit $\exp[a + L/\beta]$. E Relative to the growing mean, the variability in the amplitude data is consistent with a log-normal distribution. F Also the relative amplitude variability of SHH declines over time. Data in (C,F) are presented as mean values ± bootstrapped SEM (samples per bin: $n = 31, 63, 22, 36, 11, 12, 6$). G SHH gradient length and amplitude are uncorrelated (Pearson’s correlation coefficient $R \approx 0$, Kendall’s $\tau \approx 0$). Data points are reproduced from\textsuperscript{12}. Source data are provided as a Source Data file.
Putting Eqs. (1), (5) and (6) together, the mean boundary position given by

\[ \mu_x = \mu_\lambda \ln \left( \frac{\mu_0}{\sqrt{1 + \sigma_\lambda^2 / \mu_\lambda^2}} \right) \]  

If there is no variability in \( C_0 / C_0 \) (i.e., \( \sigma_0 = \Delta \sigma_0 = 0 \)), then Eq. (8) reduces to the deterministic case, Eq. (1). The squared positional error follows from combining Eqs. (1), (5) and (7):

\[ \sigma_x^2 = \sigma_\lambda^2 \sigma_0^2 + \sigma_\lambda^2 \sigma_{\mu_\lambda}^2 + \sigma_{\mu_\lambda}^2 \]  

\[ = \left( \mu_\lambda^2 + \sigma_\lambda^2 \right) \ln \left( \frac{\mu_0}{\sqrt{1 + \sigma_\lambda^2 / \mu_\lambda^2}} \right) \left( \frac{\mu_0}{\mu_\lambda} \right)^2 \]  

Notably, \( \sigma_0 \) enters the position and positional error of a domain boundary only through the coefficient of variation \( CV_0 = \sigma_0 / \mu_0 \). Equations (8) and (9) provide direct insight into how the statistical distributions of the gradient length and amplitude impact on the location and variability of the readout position (Fig. 5). The larger \( \sigma_0 \), the smaller \( \mu_\lambda \) (Fig. 5D), i.e., the further the domain boundary shifts upward the concentration gradient, toward the morphogen source. Variability in the decay length \( \lambda \), on the other hand, leaves the mean boundary position unaffected (Fig. 5B), as Eq. (8) is independent of \( \sigma_0 \). A larger mean gradient length or amplitude shifts the boundary downhill, away from the source (Fig. 5A, C). The positional error, on the other hand, depends on both gradient parameters and their scatter in a complicated nonlinear fashion that can even be non-monotonic.

We can further substitute Eq. (8) into Eq. (9) to obtain the positional error as an explicit function of the boundary position:

\[ \sigma_x^2 = \mu_\lambda^2 \left( 1 + CV_0^2 \right) \ln \left( 1 + CV_0^2 \right) + CV_0^2 \mu_\lambda^2 \]  

Equation (10) is by construction identical to the direct way of computing the positional error via Eq. (2) (DEEM) from infinitely many gradients.

The positional error as a function of its readout position \( \mu_\lambda \), as given by Eq. (10), is independent of the mean gradient amplitude \( \mu_0 \). Precise knowledge of the change of \( \mu_0 \) over time (or as a function of \( L \)) is therefore not required to predict the positional accuracy in a noisy morphogen gradient. All that is needed is the variation of the amplitude relative to its mean, \( CV_0 \). This has several beneficial consequences. A practical one is that no absolute measurement of the gradient amplitude is needed from experiments—relative values are sufficient to quantify positional accuracy. Another convenience is that the exact functional relationship used to fit or model the change of \( \mu_0 \) over time or length, be it exponential as in Fig. 4D, linear as in Fig. 5, or any other form, has no effect on the positional accuracy, as long as \( CV_0 \) is given. Third, the fact that the absolute scale of the gradient amplitude is irrelevant implies that positional accuracy is unaffected by temporal changes in morphogen abundance, as long as \( CV_0 \) remains sufficiently low.

### Precision of gradient readout boundaries in the NT.

With Eq. (10), the precision of a domain boundary is fully determined by its relative location in the patterning domain, \( \xi = \mu_\lambda / L \), the domain length \( L \), the mean decay length \( \mu_\lambda \), and the coefficients of variation of the gradient length and amplitude, \( CV_\lambda \) and \( CV_0 \). As estimates for the latter three are known from measurements (Fig. 4, Table 1), we can predict the boundary precision in the growing NT at any point in development, anywhere in the patterning domain. For the reported gradient variabilities, the positional error in the center of the NT becomes as high as 15 cell diameters over time (Fig. 6A, B, black contours). The reported precision of the PAX3 and NKK6.1 domain boundaries (1–3 cell diameters) is more likely to be correct than that of the gradients as the steep boundaries and the concomitant change in the fluorescent signal are much easier to detect. We used numerical optimization to determine the variability at which the positional accuracy of the SSH and BMP gradients together, or one of them alone, would be consistent with the reported positional accuracy of the NKK6.1 and PAX3 domain boundaries. Minimizing the difference between predicted (Eq. (10), color gradient & contour...
lines in Fig. 6C, D) and measured positional errors (data points in Fig. 6C, D) yielded optimal values for the gradient variability, CV₁ and CV₀. Assuming that the boundaries are always defined by the more precise gradient, we can reproduce the boundary precision with CV₁ = 0.08 ± 0.04, CV₀ = 0.23 ± 0.33 for SHH and CV₁ = 0.06 ± 0.04, CV₀ = 0.26 ± 0.16, 95% C.I. for BMP (Fig. 6C, E). Remarkably, the fitted boundary precision to the SHH gradient alone yields similar CV values, CV₁ = 0.05 ± 0.03, CV₀ = 0.30 ± 0.20, 95% C.I. (Fig. 6D, E), challenging the previously proposed idea that opposing gradients serve to increase positional accuracy in the NT⁹.

The inferred CV values for the gradients were used to plot the contours in Fig. 6C, D, and they lie near the lowest measured SHH gradient variability (Fig. 4C, F). Visual inspection shows that the reported variability corresponds to gradient profiles with some very short and some very long gradients that are difficult to reconcile with a successful patterning process (Fig. 6F). The variability inferred by us, while still resulting in variable gradients, does not result in such outliers (Fig. 6G). This raises the question whether the reported outliers reflect biological variation or technical problems in reliably measuring the morphogen gradients. Or differently put, how accurate are the reported gradient variabilities?

**Technical limitations in measuring morphogen gradient variability.** According to the reported gradient properties (Table 1), the SHH gradient is only about half as precise as the GBS-GFP gradient (Fig. 7A), even though GBS-GFP is a direct SHH reporter, and the data pre-processing artificially increased its gradient length variability (Fig. 2). We emphasize that this difference is observed already at the earliest developmental timeframe, long before adaptation results in the down-regulation of the SHH-dependent response. The difference may reflect temporal averaging for the GBS-GFP reporter (as would then also be expected for the other gradient readouts), and/or a higher technical variation for the SHH antibody staining than for the GBS-GFP reporter. The antibody staining for SHH in the NT patterning domain is very weak compared to that in the notochord, making gradient measurements challenging.

In support of technical limitations in determining gradient variability, the coefficients of variation are strongly negatively correlated with the intensity of the signal for SHH and GBS-GFP (Fig. 7B, C), though the gradient amplitude increases for SHH and decreases for GBS-GFP and pSMAD over developmental time due to adaptation (Fig. 7D), while the coefficients of variation show the opposite trend (Fig. 7E, F). This suggests that technical limitations at low concentrations artificially increase the reported variability, precluding an accurate measurement of the true gradient variability. We therefore turned to simulations to infer the expected variability based on the reported variability of morphogen production, degradation and transport rates.

**Gradient variability as a result of molecular noise.** In a cellular tissue, the morphogen production, degradation, and transport rates vary from cell to cell. This variability ultimately generates the variability in the steady-state morphogen gradient profiles. We can estimate this variability by simulating a simple reaction-diffusion model on a continuous 1D domain where these parameter values differ randomly from segment to segment (Fig. 8A). To describe the steady-state morphogen profiles, we solve the steady-state reaction-diffusion equation

\[ \frac{\partial C}{\partial x} = D \frac{\partial^2 C}{\partial x^2}, \quad x \in [-L_S, L] \]

on a one-dimensional domain that was split into two subdomains, a morphogen source (\(-L_S \leq x \leq 0\)) and a patterning region (\(0 \leq x \leq L\)). The Heaviside step function \(H\) ensures that morphogen is produced at rate \(p\) only in the source, whereas it...
degrades at a linear rate \( d \) everywhere. Morphogen transport is driven by Fickian diffusion with diffusivity \( D \). With zero-flux boundary conditions \[
\frac{\partial C}{\partial x} \bigg|_{x=0} = 0 = \frac{\partial C}{\partial x} \bigg|_{x=L},
\]
the deterministic solution is given by a concentration profile that follows hyperbolic cosines:

\[
C(x) = \frac{p}{d} \left( H(-x) \left( 1 - \cosh \frac{x}{\lambda} \right) \right)
+ \frac{\sinh \left[ \frac{L_5}{\lambda} \right]}{\sinh \left[ \frac{(L + L_5)}{\lambda} \right]} \cosh \left[ \frac{L - x}{\lambda} \right].
\]

The decay length \( \lambda = \sqrt{D/d} \) depends on the morphogen diffusivity \( D \) and the turnover rate \( d \). The \( \cosh \) is nearly exponential in the patterning domain except for a small deviation in the far end \( x \approx L \) due to the zero-flux boundary. In the infinite size limit \( L \to \infty \), a pure exponential emerges for \( x \geq 0 \):

\[
C(x) = C_0 \exp \left[ -\frac{x}{\lambda} \right] \quad \text{with} \quad C_0 = \frac{p}{d} \left( 1 - \exp \left[ -\frac{2L}{\lambda} \right] \right).
\]

In our simulations, we divided both subdomains into cells of length 4.9 \( \mu \)m, the average cell diameter in the mouse NT\(^2\) (Fig. 8A), and assigned each cell its own value of the three kinetic parameters \( k = p, d, D \), drawn independently from log-normal distributions with prescribed means \( \mu_k \) and coefficients of variation \( CV_k \) (Fig. 8B). Repeating the simulations many times for various \( CV_k \) values yielded independent noisy gradients spanning many orders of magnitude (Fig. 8C), from which we extracted \( \lambda \).
with

\[ \tilde{\mu}_k = \ln \left( \frac{\mu_k}{1 + CV_k^2} \right), \quad \tilde{\sigma}_k^2 = \ln \left( 1 + CV_k^2 \right), \quad k = d, D. \]

Using the expectation value and variance of log-normal distributions,

\[ \mu_k = \exp \left( \tilde{\mu}_k + \frac{\tilde{\sigma}_k^2}{2} \right), \quad \sigma_k^2 = \mu_k^2 \left( \exp \left[ \tilde{\sigma}_k^2 \right] - 1 \right), \]

we find, for single cells,

\[ CV_k^2 = \left( 1 + CV_k^2 \right)^{1/4} \left( 1 + CV_k^2 \right)^{1/4} - 1 \]

(11)

In patterning domains with many cells, \( CV_k \) is lower due to cell averaging. The data from our simulations with \( L = 50 \) cell diameters precisely follows Eq. (11) up to \( CV_{d,D} = 1 \), and in the
case of $d$ also beyond (Fig. 8E, lines), with a small proportionality constant shared by all curves. When all parameters are varied, though, CV$_d$ saturates at about 0.24. Larger values, such as the published CV$_d$ = 0.4 for SHH (Fig. 4C), are unattainable even with extreme molecular variability, suggesting that the reported gradient variability$^{9,12}$ is more technical than biological.

To examine the effect of the domain length $L$, we also varied the number of cells in the patterning domain from 20 to 200. As expected from the law of large numbers, log-fitting a variable exponential gradient over a longer domain leads to a more robustly fitted slope, such that CV$_d$ $\sim 1/\sqrt{L}$ (Fig. 8F). This allows us to determine the size-dependent proportionality prefactor, resulting in

$$CV_d^2 = \frac{L_0}{L} \left( (1 + CV_0^2)^{1/4} (1 + CV_d^2)^{1/4} - 1 \right)$$

for CV$_D \leq 1$, with fit parameter $L_0 = 6.13 \pm 0.03 \mu m$ (SE). Note that a declining CV$_D$ with increasing $L$ is observed for the measured SHH gradient, but not for the GBS-GFP and pSMAD gradients (Fig. 7E), suggesting that amplitude effects perturbed the latter.

We further find that the fitted decay length starts to drift at moderate CV$_{D,ad}$ (Fig. 8G). If the morphogen diffusivity $D$ or all parameters are noisy, $\lambda$ is underestimated, whereas variability in the degradation rate $d$ alone leads to overestimation of $\lambda$. This further attests to the difficulty in determining morphogen gradient parameters reliably from fitting noisy concentration profiles.

Unlike the decay length variability CV$_0$, the amplitude variability CV$_0$ does not saturate as all cell variabilities are increased, but continues to grow linearly (Fig. 8H). We find CV$_0$ to also grow mildly as the patterning domain lengths (Fig. 8I). Finally, also the fitted amplitude is found to drift as molecular noise increases, proportionally to CV$_{D,ad}$ (Fig. 8J).

With these results, we can infer the physiological range of morphogen gradient variability by plugging in measured CV values. Quantitative data for the two morphogens are only available from measurements in the Drosophila wing disc. For Dpp, the ortholog of mouse BMP4, CV$_d$ = 0.5 has been reported for the degradation rate, CV$_p$ = 0.59 for the production rate, and CV$_D$ = 0.5 for the diffusion coefficient$^{30}$. For Hh, quantitative data is available only for the diffusion coefficient, CV$_p$ = 0.18$^{30}$. Measurements of other morphogens and in other species yield similar CV values$^{31,32}$. Single cell data is available only from cell cultures. The single-cell turnover rate variability of various proteins and transcription factors in mouse embryonic stem cells has been reported to be in the range CV$_d$ = 0.16–0.45$^{33}$. For neural stem cell cultures only bulk measurements are available. Proteome half-life measurements yielded CV$_d$ = 0.21 in mouse and 0.13–0.27 in human$^{34}$. From protein half-life measurements in mouse neurons$^{35}$, one can infer a similar degradation rate variability of CV$_d$ = 0.35–0.5.

Overall, the physiological range of inter-cell CV values appears to be 0.1–1, but most studies report CV $< 0.6$, and all these values likely include some technical noise. At an intermediate value of CV$_{p,ad}$ = 0.3, the biological gradient variability is CV$_d$ = 0.053, CV$_0$ = 0.19 at $L = 100 \mu m$ (CV$_d$ = 0.027, CV$_0$ = 0.20 at $L = 400 \mu m$), which is precise enough to explain the NKX6.1 and PAX3 domain boundary errors by opposing SHH and BMP gradients, or even by SHH alone (cf. Fig. 6C–E). Even when we use a conservative CV value of 0.6 for all three kinetic parameters, the precision of a single morphogen gradient (CV$_d$ = 0.062, CV$_0$ = 0.39) is consistent with the NKX6.1 and PAX3 domain boundary errors (1–3 cells).

We can further use the simulations to estimate the impact of technical limitations on the measured gradient variability. The measured gradients become noisy at about 5% of the maximal value. We can represent this limitation by adding Gaussian white noise $\sim N(0, \eta^2)$ with uniform strength $\eta$ to our simulated gradients in all cells, prior to fitting (Fig. 8K). The observed gradient variability strongly depends on the fitting method (Fig. 8L, M). Fitting the gradients in linear space (lin-fitting) always leads to elevated decay length variability, in particular for white noise exceeding 1%, but even at $\eta = 0$. Fitting the logarithmized gradients (log-fitting) yields significantly lower CV$_d$, but is insusceptible to white noise only as long as $\eta \leq 10^{-3} \cdot \lambda_0$. At stronger white noise levels, we observe a power-law increase CV$_d$ $\propto \eta^{\gamma}$ with exponent $\gamma = 0.188 \pm 0.002$ (SE) and a cross-over with lin-fitting (Fig. 8L). If $\eta$ exceeds a few percent of the amplitude, both methods yield significantly increased CV$_d$. Amplitude variability remains stable with lin-fitting for less than 10% white noise, whereas log-fitting yields mostly larger CV$_0$ values (Fig. 8M).

In summary, our analysis suggests that natural noise in exponential morphogen gradients in the developing NT is sufficiently low to explain the previously reported progenitor domain boundary precision. Thus, both SHH and BMP gradients together—but even a single one of them alone—provide the spatial precision required to define the boundaries lying in the center of the NT with an error of only 1–3 cell diameters. But can morphogen gradients provide even higher patterning accuracy for robust development?

**Precision of progenitor domain size and progenitor number.**

In the vertebrate NT, the domain boundaries define the size of the different progenitor domains, which are formed as a result of different readout thresholds, as stipulated by the French flag model$^1$ (Fig. 9). Two domain boundaries located at $x_1$ and $x_2$ are the result of a morphogen readout at thresholds $C_1 = C(x_1)$ and $C_2 = C(x_2)$. As noted in$^3$, the length of a domain is given by

$$\Delta x = x_2 - x_1 = \lambda \ln \frac{C_2}{C_1} - \lambda \ln \frac{C_0}{C_1} = \lambda \ln \frac{C_1}{C_2}.$$  

Notably, it is independent of the location in the entire patterning domain, and also independent of the gradient amplitude $C_0$. Assuming that the domain width perpendicular to the $x$ axis remains roughly constant along $x$, this paradigm provides a very robust mechanism to preserve the gene expression domain.
volume (and thus, the number of progenitor cells) during development. A change in the gradient amplitude \( C_0 \) shifts both domain boundaries by the same distance, such that its size remains unchanged. The domain length is determined only by \( \lambda \), which is stable over developmental time (Fig. 4A), and by the readout threshold ratio \( C_F/C_2 \). Only the very first and last progenitor domains in the pattern are affected by a transient amplitude \( C_0 \), as one of their boundaries is given by the end points of the entire patterning domain, \( x = 0 \) and \( x = L \).

Even in a probabilistic setting with variable gradients, the expected domain length \( \delta \) is unaffected by a change in amplitude, if \( C_1, C_2 \) and \( CV_0 \) are constants:

\[
\mu_\delta = \mathbb{E} [\Delta x] = \mu_1 \ln \left( \frac{C_1}{C_2} \right).
\]

To quantify the variability of \( \Delta x \) in a noisy gradient, we can calculate the variance

\[
\sigma_\delta^2 = \text{Var} [\Delta x] = \text{Var} [x_1] + \text{Var} [x_2] - 2 \text{Cov} [x_1, x_2] = \sigma_x^2 + \sigma_{C_0}^2 + 2 \mu_x \sigma_{x_2} - 2 \mathbb{E} [x_1 (x_1 + \Delta x)].
\]

After some elementary algebra, assuming again independence of \( \lambda \) and \( C_0 \) and using Eqs. (8), (9) and (12), all terms involving the amplitude cancel out, and we find the remarkably simple form

\[
\sigma_\delta^2 = \text{CV}_\delta \mu_\delta = \sigma_1 \ln \left( \frac{C_1}{C_2} \right).
\]

The inaccuracy of the size of a progenitor domain therefore scales with its size itself, with \( \text{CV}_\delta = \text{CV}_\lambda \) as the proportionality constant. For an exemplary domain size of \( \mu_\delta = 50 \mu m \) and a coefficient of variation \( \text{CV}_\lambda = 0.05 \), this results in a domain size error \( \sigma_\delta \) as low as half a cell diameter, regardless of how far away from the source the domain lies. Strikingly, unlike their spatial boundary positions, the size of the gene expression domains is completely independent of variability in the gradient amplitude. The NT patterning mechanism thus yields progenitor cell numbers with even greater accuracy than boundary locations. We emphasize that only a single morphogen gradient is required to achieve this high patterning precision.

**Discussion**

High patterning precision is pivotal for robust development. We now show that the precision of morphogen gradients in the NT is far greater than previously appreciated. A single gradient—rather than the combined readout of SHH and BMP—is sufficient to generate the observed accuracy of the progenitor domain boundary positions also in the center of the mouse NT. We further show that the size of the morphogen-dependent tissue subdomains, that are not bordering the patterning domain edges, is even more precise than the individual boundary positions, because inaccuracies from amplitude variability cancel out. As a result, progenitors can be produced in more accurate numbers than previously anticipated.

These insights provide additional perspectives on gradient-based patterning and on NT development in particular. Mutual information, provided by the simultaneous readout of multiple gradients\(^{8,36,37}\), enhances patterning precision in the early Drosophila embryo\(^{38}\), and has been proposed to apply also to the mammalian NT\(^{9,39}\). While such a mechanism remains possible, given the co-regulation of central and ventral NT genes by SHH and BMP\(^{9,40}\), our finding that the boundaries of the progenitor domains in the NT can be accurately defined by a single gradient over the entire patterning period suggests a simpler patterning mechanism. This provides further prospects for tissue engineering.

The previous mathematical analysis artificially increased the reported gradient variabilities in the NT (Figs. 1 and 2), and we argue that technical limitations further inflate the reported gradient variabilities (Fig. 7). In light of the challenges in detecting exponential gradients far from the source (Fig. 3) and in measuring their variability reliably, we have developed computer simulations to estimate gradient variability based on the reported variability in morphogen production, degradation, and diffusion (Fig. 8). We find that the reported high variability of the decay length \( \lambda \) cannot arise from natural noise in these parameters alone, as it saturates at lower values than previously measured for SHH, GBs-GFP and pSMAD, at high noise levels. Our simulations confirm that for the reported molecular noise levels, the observed precision of the central progenitor boundaries is achieved even with a single gradient for the entire duration of the developmental process. Considering that the reported molecular noise levels are likely also elevated by technical errors, an even higher precision of the domain boundaries appears plausible.

Measuring the morphogen production, decay and transport rates is challenging, but still easier than the detection of low morphogen concentrations. It thus offers a complementary approach to estimating gradient variability. Current measurements of the morphogen production, decay and transport rates represent bulk measurements at the tissue level, and not yet in the mouse NT. Going forward, it will be valuable to obtain data on the single-cell variability of morphogen production and degradation rates. Currently, such data is available only from cell culture systems, but yields similar variabilities as bulk data. The reported CV values are in the range 0.1–1 across all species analyzed, including mice, flies, zebrafish, and humans. As the reported variability includes technical errors, these values present upper bounds. Even for a relatively pessimistic value of 0.6 for production, degradation and diffusion, we find that the gradient imprecision is 1–3 cells over several hundred of micrometers, providing sufficiently accurate positional information to pattern a large domain. Local fluctuations can be reduced further through spatial and temporal averaging\(^{5,41,42}\). Moreover, in zebrafish, NT progenitor boundaries are sharpened by cell sorting\(^{43,44}\).

Due to regulatory feedback such as the up-regulation of the SHH receptor PTCH\(^{1,3,22,45}\), the degradation process may not be linear, and the mean rates of morphogen production, degradation and diffusion and their variability may not be constant along the DV axis. Assuming uniform linear rates, our simulations imply that the morphogen gradients remain exponential over a wide patterning distance in the NT (Fig. 8). In case of nonlinear decay, morphogen gradients will no longer be exponential, but follow a power law\(^3\). It is straightforward to generalize our computational method to non-uniform rates and nonlinear processes. However, current measurement methods are not sufficiently precise to distinguish between exponential and power law gradients as the difference is observed mainly far away from the source where concentrations are low. An important question remains how cells can reliably detect very low morphogen concentrations, and what role adaptation plays in this process\(^7,12,20,25\).

We developed a formalism (Eq. (10)) to estimate the positional error along the entire patterning axis from the gradient variability close to the source. Gradient variabilities have been reported also for other patterning systems, including Dpp and Wg in the Drosophila imaginal discs\(^{13}\). Our formalism could thus be applied also in other developmental systems. We emphasize, however, that the accuracy of our formalism hinges on the accuracy of the measured gradient variabilities. Given how challenging it is to visualize morphogen gradients, technical errors are to be expected from such measurements. Based on the molecular noise simulations, we expect higher gradient precision also in other developmental systems.
More than 50 years since the publication of the French flag model\(^1\), it remains a matter of debate how morphogen gradients are read out\(^2\). Recent experiments support a threshold-based readout of the BMP gradient along the zebrafish dorsal-ventral axis\(^46\). Our finding that single noisy gradients provide a much more robust positional patterning mechanism than previously appreciated resolves the long-standing conundrum of how the observed patterning precision can be achieved with a simple threshold-based readout of a single gradient. This opens avenues for modelers and tissue engineers in recapitulating NT patterning in silico and in bio-engineering approaches. The presented formalism is not limited to vertebrates or flies, but applies directly also to other morphogen-dependent patterning systems in which morphogen transport is essentially diffusive.

**Methods**

**Error estimation methods.** The synthetic gradients (Eq. (4)) were produced by drawing independent pairs of random parameters \(\lambda\) from a truncated normal and \(C_0\), from a log-normal distribution such that their statistical properties (mean and standard deviations) were identical to the reported ones. In the DEEM, the positional error is estimated directly according to Eq. (2) at a given readout threshold \(C_0\) (Fig. 1D, E). We computed the set \(X_i\) of positions where a gradient \(C_i\) crosses \(C_0\) using linear interpolation between discrete gradient points. For the synthetic gradients, there is only one such point \((X_i = [x_i])\), whereas noisy gradients can have several. For such noisy gradients, we use the middle point of the transition zone

\[
x_{\text{mid}} = \min X_i + \max X_i
\]

as the readout position for each gradient \(i\). Alternative averaging methods such as the arithmetic mean or median yield similar results for the data analyzed. The location and positional error follow as

\[
\mu_i = \text{mean}\{x_{\text{mid}}\} \quad \text{and} \quad \sigma_i = \text{SD}\{x_{\text{mid}}\},
\]

taken over all embryos \(i\). Repeating this procedure for different \(C_0\) yields a list of \((\mu_i, \sigma_i)\) pairs.

In the NumEPM\(^3\) (Fig. 1D), the derivative in Eq. (3) is evaluated numerically as the slope of the mean gradient \((\langle C(x) \rangle = \text{mean}(C(x))\) (personal communication). For the synthetic (purely exponential) gradients (Eq. (4)), the derivative of the mean gradient can be directly determined from the gradient parameters as

\[
s_e = \frac{\partial C}{\partial x} \quad \text{and} \quad \sigma_e = \sqrt{\frac{1}{n} \sum (C(x) - \mu_e)^2 / \sigma_e^2}.
\]

In the FitEPM\(^9\) (Fig. 1D, E), a different approach is taken, in that an exponential function is fit-lined directly (i.e., without prior log-transformation) to the mean gradient \((\langle C(x) \rangle\) (personal communication). This yields the parameters

\[
\bar{A} = \arg \min \{ \langle C(x) \rangle - \langle C(x) \rangle^2 \}
\]

The slope is then estimated from the derivative of this fitted exponential:

\[
s_e = \frac{\partial \langle C(x) \rangle}{\partial x} \quad \text{and} \quad \sigma_e = \frac{\bar{A}}{C_0 \exp\left(-x_h/\bar{A}\right) \sigma_e}.
\]

**Numerical optimization of gradient variability.** We determined the gradient parameter variability \(\text{CV}_L\) and \(\text{CV}_R\) in Fig. 6 by fitting Eq. (10) to the progenitor domain boundary errors with MATLAB\'s nonlinear least-squares curve fitting routine lsqcurvefit.

**Inference of error bars for the positional boundary error.** In Fig. 6E, we inferred the uncertainties (error bars) associated with the positional error of the domain boundaries assuming that the boundary positions are normally distributed. In this case, the standard error of the standard deviation \(\sigma_n\) is given by \(SE(\sigma_n) = \sigma_n / \sqrt{2n - 1}\) where \(n\) is the sample size\(^47\).

**Simulation of gradient variability from molecular noise.** The reaction-diffusion equation was solved with MATLAB\'s boundary value problem solver bvp4c with absolute and relative error tolerances of \(10^{-10}\). At each interface between two adjacent cells, continuity of the morphogen concentration \(C\) and its flux \(-D(C/\partial x)\) was imposed.
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R.V. and D.I. conceived the study, analyzed the data, and wrote the paper, R.V. developed the theory and simulations and produced the figures.

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