

Probably want to tighten up language we use a lot of things:  
 - within / between  
 - stochastic / biological (some bio is stochastic!)  
 - systematic - uncertainty / variability

May be careful definition early on  
 then stick to one term?

## Variability in reported midpoints of (in)activation of cardiac $I_{Na}$ . ↑

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prob. this  
 when talking  
 about  
 where  
 means  
 one!

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## Abstract

Electrically active cells like cardiomyocytes show variability in their size, shape, and electrical activity. But should we expect cell-to-cell variability in the properties of their ionic currents? In this brief systematic analysis we gather and visualise reports of two important electrophysiological parameters: the midpoints of activation and inactivation of the cardiac fast sodium current,  $I_{Na}$ . We find a considerable variation in reported values between experiments, with a smaller variation reported within experiments (i.e. from cell to cell). We show how the between-experiment variability can be decomposed into a correlated and an uncorrelated component, that the correlated component is much larger, and that it affects both midpoints almost equally. We then review biological and methodological issues that might explain the observed variability, and provide rough estimates of the range of variability induced by each. The within-experiment variability and uncorrelated between-experiment variability match our estimates, but the correlated between-experiment variability is not explained. This leaves room for, but does not prove, a biological variability in  $I_{Na}$  properties.

## Introduction

Variability in electrophysiological properties, between and within subjects, arises at several scales. Electrically active cells such as cardiomyocytes and neurons are present in different numbers (Olivetti et al., 1995), differ in size and shape (Volders et al., 1998), and express different numbers of ion channels (Schulz et al., 2006). But as we continue down the scales, towards small molecules and single atoms and into the realms of chemistry and physics, we may expect biological variability to disappear, and intrinsic stochasticity to be the main cause of variability?

Where do ion channels fit in this picture? Transcription, translation, anchoring, and degradation of ion channel genes can affect the total number of channels in a cell, and hence the maximal conductance of its aggregate (whole-cell) currents. But should we also expect cell-to-cell or intersubject differences in properties that are not governed by channel count, such as voltage-dependence? Ion channel function is known (or suspected) to be modulated by several mechanisms, including localisation, phosphorylation, stretch, and even proximity to other channels (Marionneau and Abriel, 2015; Daimi et al., 2022; Beyder et al., 2010; Hichri et al., 2020). But what is the impact of such mechanisms on variability in baseline currents, measured under controlled experimental conditions?

In this brief systematic analysis we review published patch-clamp data to see if it can help us answer these questions. Specifically, we study published values for the voltage-dependent midpoints of activation ( $V_a$ ) and inactivation ( $V_i$ ) of the human cardiac fast sodium current  $I_{Na}$ , as measured using the whole-cell

does that already  
 need a def. all person?  
 if we can  
 reward.

needs  
 defining?

patch-clamp configuration in cells heterologously expressing SCN5A. We first outline our data gathering and filtering methods, and then show the full data set. As this includes data from different and possibly incompatible experiments, we then split into subgroups according to the most well-reported properties (channel subunits and cell type). We then study correlation between  $V_a$  and  $V_i$  in experiments that measured both, and end with a speculative overview of factors that may explain the observed variability.

As this work is based entirely on summary statistics gathered from the literature (means and standard errors / deviations), we forgo formal statistical analysis (which would be only possible by making several assumptions about the data underlying the summary statistics) and present a visual analysis instead.

## Methods

All expression system data used in this study were collected as part of a previous study on single-point mutations in SCN5A (Clerx et al., 2018). For the current study, we reduced this data set to keep only the wild-type (control) measurements. The systematic process whereby the original data was gathered is detailed below. Although this is not a study into effect sizes, we followed the PRISMA guidelines [REF]

To identify candidate studies, we searched PubMed for “SCN5A mutation” (with the last search occurring in May 2016) and looked in previously published lists of mutations (Napolitano et al., 2003; Moric et al., 2003; Ackerman et al., 2004; Zimmer and Surber, 2008; Hedley et al., 2009; Kapplinger et al., 2010, 2015). Studies identified this way were then scanned to see if they contained measurements of  $V_a$  or  $V_i$ , made in one of three recognised expression system types (see below), along with the number of cells measured and a standard deviation or standard error of the mean. Next, we filtered out studies made at normal or raised body temperatures, but kept studies made at “room temperature” (as stated by the authors) or at any temperature in the range from 18 to 26°C. Because of the considerable effort involved in performing experiments at body temperature, we assumed that studies not mentioning temperature satisfied these criteria and could be included. Similarly, we excluded any studies of mutations under non-baseline conditions (e.g. with stretch, with remodelling, with ischemia, etc.). All data collection and selection was performed by M.C.

Our final data set includes measurements in three different expression systems: HEK293 or tsA201 (both indicated as ‘HEK’), CHO cells, and Xenopus oocytes. A clear statement of cell type was part of the inclusion criteria (see above), so that no missing-data strategy was required. The exact SCN5A  $\alpha$ -subunit expressed in these cells was not always clearly indicated. We found at least four different isoforms, which we labelled: **a**, sometimes known as Q1077 and with GenBank accession number AC137587; **b**, known as Q1077del or GenBank AY148488; **a\***, hH1, R1027Q, or GenBank M77235; and **b\***, hH1a or T559A; Q1077del, no GenBank number (see also Makielski et al., 2003). Missing  $\alpha$ -subunit information was recorded as “ $\alpha$ -subunit unknown”. Finally, we noted whether or not studies stated to have co-expressed  $\beta$ 1 subunit; No information on  $\beta$ 1 coexpression was taken to mean it was not co-expressed.

Some studies we surveyed recorded separate control (wild-type) experiments for each mutant. We shall therefore distinguish between *studies* and *experiments*, where a study can contain several experiments. Such control measurements commonly contained both a  $V_a$  and a  $V_i$  estimate, which we shall regard as part of the same experiment, so that correlations between  $V_a$  and  $V_i$  can be studied.

We shall use the term *within-experiment variability* to describe the changes from cell to cell (as quantified by the standard deviation), and will use the term *between-experiment variability* to refer to differences in reported means — even in cases where those means are reported in the same publication.

The full list of midpoints and references can be found in Supplementary Table 1. A database version of the same data is available for download from <https://github.com/MichaelClerx/ina-midpoints>.

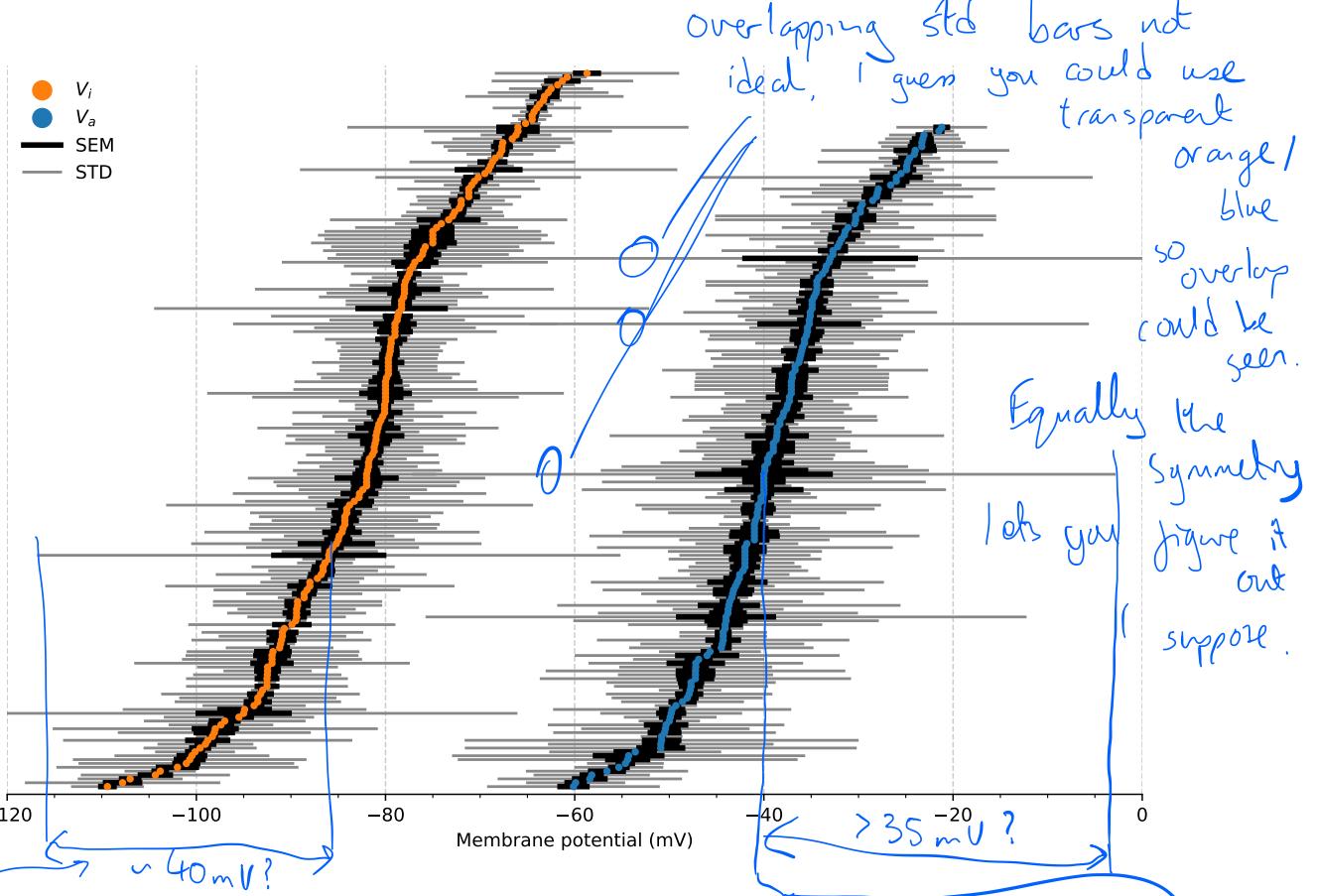


Figure 1: Reported mean midpoints of inactivation ( $V_i$ ) and activation ( $V_a$ ), along with their standard deviation ( $\sigma$ ) and the standard error of the mean (SEM). Vertically, both sets of points are individually ordered from lowest to highest mean membrane potential; correlations between an experiment's  $V_a$  and  $V_i$  are not shown in this figure.

## Results

From 132 publications surveyed, we extracted 172 reports of a (wild-type) midpoint of activation ( $V_a$ ) and 186 reports of a midpoint of inactivation ( $V_i$ ). The obtained means, standard errors of the mean, and standard deviations are shown graphically in Figure 1.

A wide within-experiment variability was observed: standard deviations ranged from 0.4 mV to 22 mV (mean 4.5 mV) for  $V_a$  and from 0.13 mV to 15 mV (mean 3.8 mV) for  $V_i$ . Assuming normality this suggests that 95% of single-cell results in a typical experiment lie in a range of approximately  $\pm 1.96 \times \sigma$  mV.

Substantial between-experimental variability can also be seen in our data set: reported means of  $V_a$  range from -60 to -21 mV (mean -39.2 mV), while means of  $V_i$  range from -109 to -59 mV (mean -81.8 mV).

Interestingly, the SEM for most experiments, which quantifies the degree of certainty in the estimate of the mean, is quite narrow. This suggests that the mean  $V_a$  and  $V_i$  differed significantly between the surveyed experiments, and that one or more confounding factors must exist that cause this difference.

### Subunits and cell type are not the major sources of variability

Cell type,  $\alpha$ -subunit isoform, and  $\beta 1$ -subunit co-expression are known to affect  $V_a$  and  $V_i$ . But do they explain the large between-variability seen in Figure 1?

In Figure 2A, we show the same data as in Figure 1, with experiments using a particular  $\alpha$  highlighted. Data from each of the four subunits spans almost the full range of the observed variability. Notably, the

Significance of the different symbols in B but not A?  
 Thought they might appear elsewhere but can't see anything?

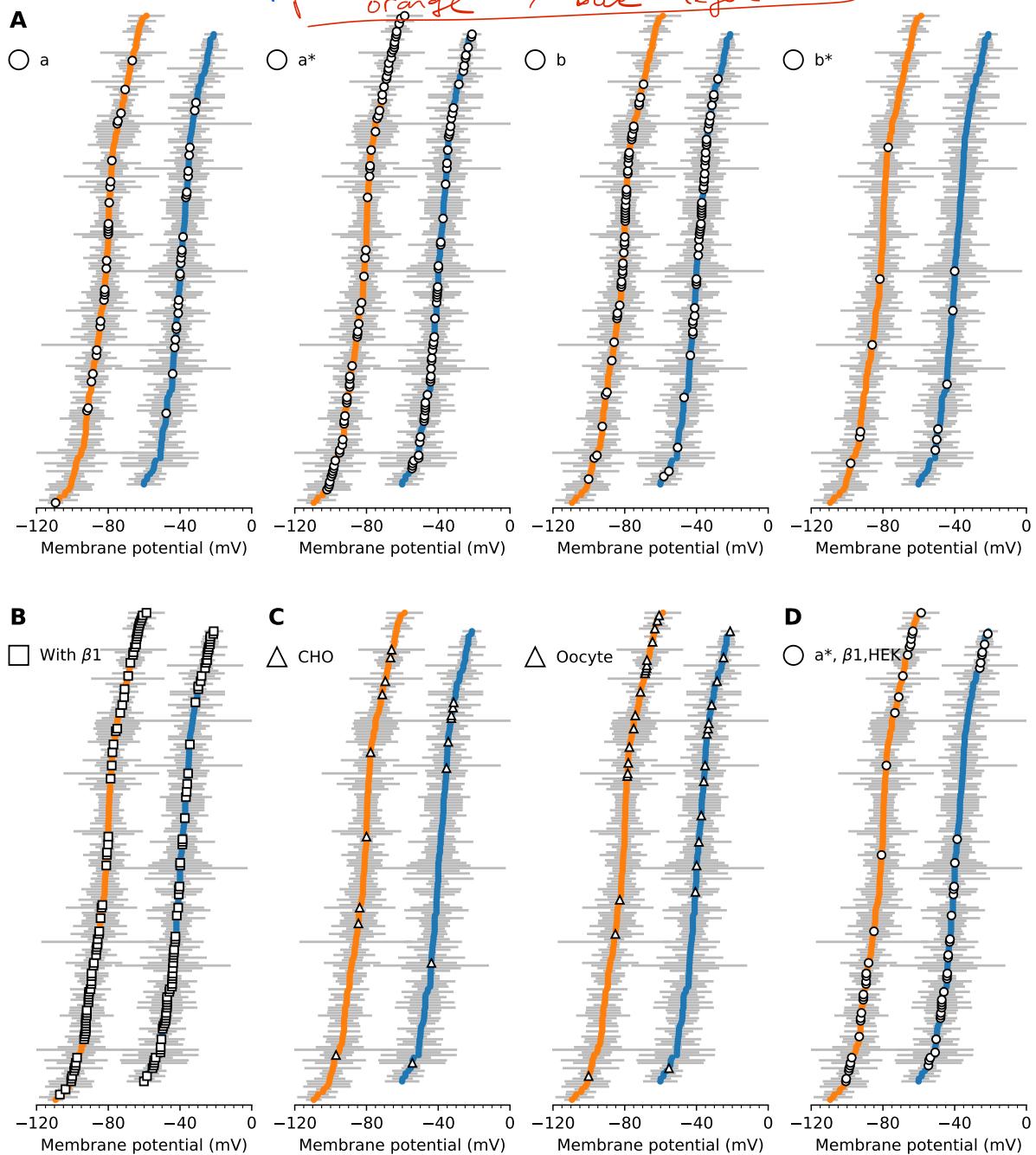


Figure 2: Like Figure 1, but with experiments in particular subgroups highlighted. For clarity, the SEM is not shown. *A*, Experiments using a particular (known)  $\alpha$  subunit. From left-to-right: a (Q1077), b (Q1077del), a\* (R1027Q), and b\* (T559A; Q1077del). The remaining points represent the “subunit unknown” group. *B*, Experiments where a  $\beta 1$  subunit was co-expressed. The remaining points represent experiments where  $\beta 1$  was not co-expressed, or where the authors did not mention co-expression. *C*, Experiments using CHO cells (first panel) or Oocytes (second panel). The remaining points represent experiments in HEK cells. *D*, Experiments in the largest subgroup, with the a\*  $\alpha$ -subunit, co-expressing a  $\beta 1$  subunit, and measured in HEK cells.

impossible to  
do the  
what points  
are left

after you've subtracted all the rest" in your head, so not sure I'd mention that!

You aren't going to like it, but I think I prefer the overlapping histograms you had before<sup>4</sup>, rather than trying to construct them in my head by summing these vertically...

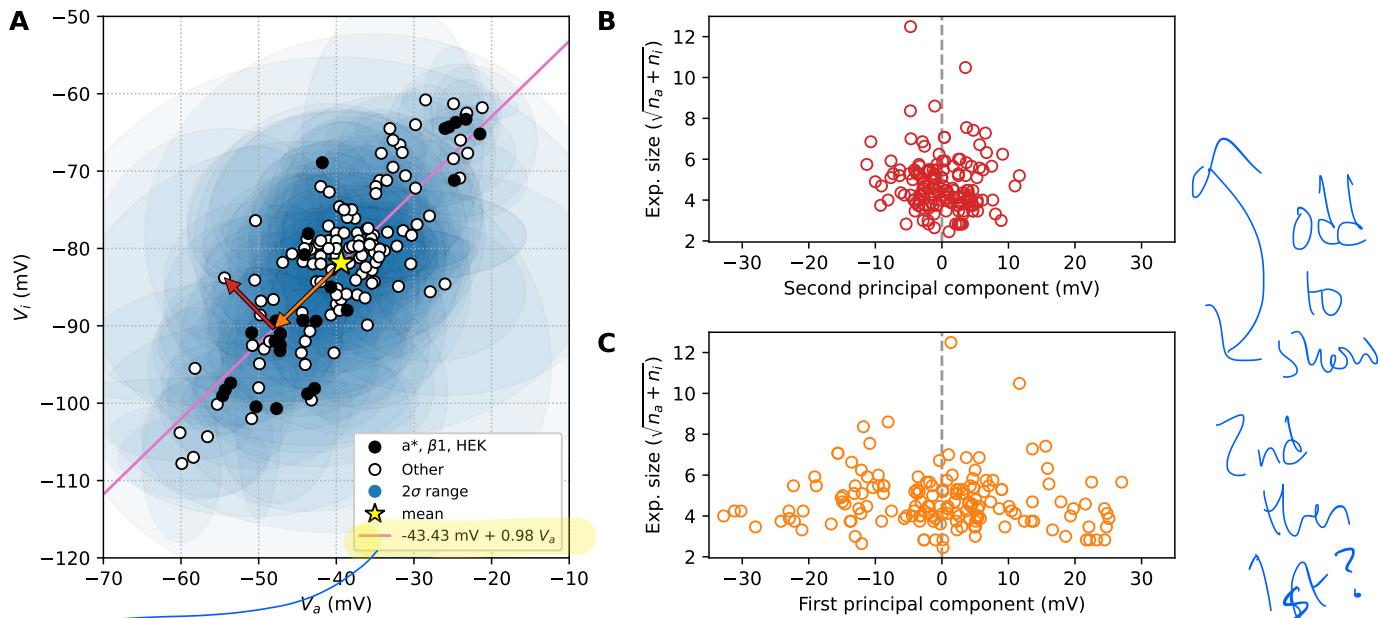


Figure 3: A,  $V_i$  plotted against  $V_a$ , for the experiments that reported both. Experiments from the biggest subgroup ( $a^*, \beta 1$ , HEK) are highlighted as dark dots, with the remainder shown in white. The  $2\sigma$  range around each mean is shown as a blue ellipse. The mean of all points is indicated by a yellow star, and a best fit line through the full data set is shown in pink. For one example point ( $V_a = -54.4 \text{ mV}$ ,  $V_i = -83.8 \text{ mV}$ ) we show the vector from the mean to this point, decomposed into components along the line of best fit (first principal component) and perpendicular to the line of best fit (second principal component). B, The square root of the experiment size as a function of the second principal component, for all points in A. The experiment size is defined as  $n_a + n_i$ , where  $n_a$  is the number of cells tested for  $V_a$  and  $n_i$  is the number tested for  $V_i$ . C, The square root of the experiment size as a function of the first principal component.

"I'm a serious person doing my best" XKCD!

largest subgroup ( $a^*$ ) has the largest span, while the smallest subgroup ( $b^*$ ) has the smallest span. This is consistent with the notion that  $\alpha$ -subunit does not explain the observed variability.

Similar analyses shown in Figure 2B and Figure 2C suggest that  $\beta 1$  co-expression and cell type are not a major source of variability either, although the oocyte results appear clustered near the higher (more depolarised) voltages.

Finally, to see if a combination of these factors explains the spread, we show the largest single subgroup ( $a^* \alpha$  subunit,  $\beta 1$  co-expression, HEK cells) in Figure 2D. As before, the results span almost the entire range observed in Figure 1.

From this visual analysis we can conclude that one or more unknown confounders, other than cell type and subunits, must be the main sources of between-experiment variability in our data set.

$V_a$  and  $V_i$  are strongly correlated

So far we have looked at  $V_a$  and  $V_i$  as independent experiments. We now concentrate on the subgroup of 165 experiments where both a  $V_a$  and a  $V_i$  was reported. These are visualised in Figure 3A.

Each experiment is shown as a black or white dot, with black indicating that this experiment used the  $a^* \alpha$  subunit, co-expressed  $\beta 1$ , and was performed in HEK cells. A linear fit through all means (black and white) was made using unweighted least-squares based linear regression. This had an offset of  $-43.4 \text{ mV}$  and a slope of  $0.98 \text{ mV/mV}$ , with a Pearson correlation coefficient  $R = 0.81$ . The existence

of  
to  
show  
2nd  
then  
1st?  
why  
 $2\sigma$ ?

a bit  
secondary the size of  
the span and notion  
that's consistent with  
more samples from  
same ditto.

again the funnel shape somewhat suggests  
bigger studies would have more consistent answers  
- no big  
confounders?

*see comment on putting bounds on regression maybe!*

of such a strong correlation, strongly suggests the existence of a confounding factor that shifts both  $V_a$  and  $V_i$ . The fact that the line of best fit almost has slope 1 further suggests that both measurements are *could plausibly be* shifted by the same amount.

We can attempt to remove this confounding influence by projecting each point onto the line of best fit. This allows us to decompose the difference between each  $(V_a, V_i)$  measurement and the group mean into a component along the line of best fit, and a component perpendicular to the line of best fit (i.e. principal component analysis, or PCA). An example for a single point is shown by the arrows drawn in [Figure 3.A](#)). The component along the line of best fit (the first principal component) will then contain the correlated between-experiment variability, while the component perpendicular to the line (the second principal component) represents the remaining between-experiment variability once this correlated effect is accounted for. The first and second component are plotted in [Figure 3.C](#) and [Figure 3.B](#). As expected, the first principal component shows a much larger variability than the second, confirming the idea that most variability is due to a correlated effect.

Finally, we test if either type of variability diminishes with increasing number of cells tested. To this end, we define an “experiment size” as the number of cells  $n_i$  tested to measure  $V_i$ , plus the number of cells  $n_a$  tested to measure  $V_a$ . In [Figure 3.B](#) we plot the square root of this quantity ( $\sqrt{n_a + n_i}$ ) as a function of the second principal component to create something akin to a “funnel plot”. Interestingly this shows no clear sign that the uncorrelated variability diminishes with increasing  $n$ , while the correlated variability in [Figure 3.C](#) does appear to go down with increased cell count.

## Discussion

*Cleaver to write as -60 V<sub>a</sub> < -20 etc.?*

We reviewed 172 reported  $V_a$  and 186 reported  $V_i$ , gathered from 133 publications, and found both within-experiment and between-experiment variability. The within-experiment variability was around 4 mV in both  $V_a$  and  $V_i$  on average, but could be as large as 15 mV. Between experiments,  $V_a$  varied over a range of 40 mV, while  $V_i$  varied within a 50 mV bracket. Experiments differed in the expressed  $\alpha$  subunit, co-expression of the  $\beta 1$  subunit, and cell type, but the large between-experiment variability cannot be explained by ~~a combination~~ of these factors. In the 165 experiments providing both  $V_a$  and  $V_i$ , we found a significant correlation with a slope almost equal to 1. Decomposing the between-experiment variability into a correlated and uncorrelated component, we saw that only the correlated component is reduced with increasing cell count. What could explain these observations?

The existence of within-experiment variability is known to every experimenter, and is the reason why each experiment in this study reports its findings as a mean and a SEM (or STD). The existence of between-study or between-lab variability is also indirectly acknowledged by the studies we review: each study provided a new wild-type recording instead of using a value from the literature. Some studies measuring multiple mutants have gone even further, and accounted for *between-experiment* variability by performing a paired “control” wild-type measurement for every studied mutant. For good examples see [Kapplinger et al. \(2015, 27 reported wild-type values\)](#) or [Tan et al. \(2005, 15 reported wild-type values\)](#). The [Tan et al. \(2005\)](#) paper provides the only direct acknowledgement of between-experiment variability we found, citing “seasonal variation in current characteristics” as a reason for their paired study design. However, the wild-type values reported in [Tan et al. \(2005\)](#) and [Kapplinger et al. \(2015\)](#) differ by at most 11 mV and 7 mV respectively — well short of the 40 and 50 mV ranges seen in [Figure 1](#). The full extent of between-experiment variability then, is still somewhat surprising.

Nice idea to summarise. Maybe explain "Correlated" more clearly "affects  $V_a$  and  $V_i$  by some amount"

Table 1: Sources of uncertainty in  $V_a$  and  $V_i$ , along with estimates of the systematic (between-experiment) and stochastic (within-experiment) effects. The systematic uncertainty is further divided into a component that affects  $V_a$  and  $V_i$  equally and an uncorrelated component.

	Correlated $\Delta_V$	Uncorrelated $\Delta_V$	Stochastic $\sigma_V$	Quality of estimate
Temperature	4 mV		0.5 mV	?
Time since rupture		15 mV	1.25 mV	???
Liquid junction potential	12 mV			
Series-resistance compensation	10 mV		2.5 mV	??
Voltage-step protocol differences		10 mV		??
Stretch			1 mV	???
Sum	26 mV	25 mV	3.0 mV	

Should be any  
"uncertainty"  
in some of these  
as such?

## Sources of uncertainty

In this final section we discuss factors, in addition to those shown in Figure 2, that may cause between-experiment and/or within-experiment effects. Our goal is to estimate the expected level of *uncertainty* in the data, so that we may arrive at an estimate of the remaining (biological?) variability once this has been subtracted (Mirams et al., 2016). We will make some coarse, order-of-magnitude estimates of the expected uncertainty that each factor induces. For systematic effects we shall estimate a "bias", expressed as a voltage difference  $\Delta_V$ , while for stochastic effects we shall estimate a standard deviation  $\sigma_V$ . This distinction is important when making a worst-case scenario estimate of the *combined* factors: we shall simply sum the bias estimates  $\Delta_{V,\text{total}} = \Delta_{V,1} + \Delta_{V,2} + \dots$ , but treat the combined stochastic effect as a sum of random variables for which  $\sigma_{V,\text{total}}^2 = \sigma_{V,1}^2 + \sigma_{V,2}^2 + \dots$ . An overview of the estimates made in this paragraph is shown in Table 1.

The measurements in our data were made at "room temperature", defined by the different authors as anywhere between 18°C to 26°C. Nagatomo et al. (1998) recorded a shift in the midpoint of activation of +0.43 mV per °C, and a +0.47 mV shift for inactivation, although no such shifts were observed by Keller et al. (2005) and both studies used HEK cells. Assuming a 0.5 mV per °C shift and bath temperature ranging over 8°C, this could lead reported midpoints to vary over a range of 4 mV between experiments, but with a similar deviation for both  $V_a$  and  $V_i$ . Within studies, temperature was typically within a 1 or 2° bracket, leading to an estimated  $\sigma_V$  of 0.5 mV. *treat  $\Delta V$  all as positive, even if they are negative!*

Hanck and Sheets (1992) measured  $I_{Na}$  in Purkinje cells and noted a correlation between  $V_a$  and  $V_i$  and time since the patch was ruptured, with both midpoints drifting towards more negative values at a rate of approximately -0.5 mV per minute. A study by Abriel et al. (2001) looked for, but did not find evidence of a similar time-dependent drift in HEK cells. In other studies the time since rupture is not usually reported (or measured). We will assume here that the cells used to measure  $V_a$  and  $V_i$  were subjected to a series of different experimental protocols lasting no more than 30 minutes, and that setting up and switching between protocols was fairly consistent, with a standard deviation of 2.5 minutes (in other words, 98% of the time the protocol was started somewhere in the same 10 minute window). This leads to a maximal  $\Delta_V$  of 15 mV (with no correlation between  $V_a$  and  $V_i$ ) and a  $\sigma_V$  of 1.25 mV.

Liquid junction potential (LJP) errors arise when a liquid-liquid interface arises or changes after the recorded current has been 'zeroed' during a voltage-clamp experiment (e.g. by breaking the seal), and are usually corrected for by subtracting a calculated LJP offset. Systematic errors in this process, or failing to correct entirely, could lead to a systematic (and correlated) difference between the applied and intended  $V_m$  of up to 12 mV (Neher, 1992).

In addition to these confounding factors, we should consider that  $I_{Na}$  is characterised by fast time scales and large current amplitudes, both of which cause problems for membrane potential control in voltage-clamp experiments (Sherman et al., 1999; Lei et al., 2020; Montnach et al., 2021). The striking

I think these numbers need linking more clearly,  
obvious to you but not reader!

Or some do both in same cells  
some  $V_a$  first  $V_i$  last  
and others do vice-versa?

correlation in Figure 3.A could certainly be read as an indication of systematic differences between desired and obtained membrane potentials. Using a combination of experiments and simulations, Montnach et al. (2021) show that errors up to 20 mV can easily be incurred if overly large currents are measured. They recommend keeping peak currents below 7 nA (or below 3.5 nA on automated systems) to obtain an error in  $V_a$  or  $V_i$  of less than 10 mV. There is no obvious way to translate this to a systematic and stochastic error, so we will simply guess a correlated  $\Delta V$  of 10 mV (corresponding to the extra error from large currents) and a  $\sigma_V$  of 2.5 mV (so that 4 sigmas span the range of the expected error when currents are appropriately sized).

presumably they recommend a peak current AND a peak series resistance? yea! I guess magnitude systematic but series resistance stochastic? On top (also varying w/ study) different protocols? (did you do that for the nA in A/B?)

as with other currents (Vandenberg et al., 2012; Clerx et al., 2019), the protocols used to determine the steady states of activation and inactivation are not perfect, so a steady state is not always reached. As a result, the obtained values will depend on the length of the steps in the voltage protocol (as well as on the rates of activation and inactivation, so that this is a possible explanation for the temperature dependency seen by Nagatomo et al. (1998)). Here we use a  $\Delta V$  of 10 mV as an estimate for the between-experiment difference that this might explain - based on what? Simulated IV curves under different protocols?

Stretch induced by deliberate pressure applied to oocytes has been shown to shift midpoints of activation by more than 10 mV (Banderali et al., 2010). If we assume that smaller amounts of pressure are applied accidentally, we might expect some within-experiment variability as a result, e.g. a  $\sigma_V$  of 1 mV. - not obvious if it's something like osmotic pressure due to pipette sol<sup>n</sup> could end up correlated?

Bath and pipette solutions vary between experiments, but we know of no estimates of how this affects  $V_a$  or  $V_i$ . Endogenous currents are known to be present in expression systems, which may further interfere with measurements of  $V_a$  and  $V_i$  (Zhang et al., 2022). Finally, several factors regulate  $I_{Na}$  in cardiomyocytes (Marionneau and Abriel, 2015; Daimi et al., 2022). While some of these mechanisms may be highly specialised, many pathways have multiple targets so that we might expect some forms of biological regulation even in cells non-natively expressing sodium channels. We make no estimate for the influence of these factors.

Do you mean other things in sol<sup>n</sup>? Can these guesses explain our observations?

Using the speculative estimates given above, we might expect a 3 mV standard deviation for within-experiment variability. This is only slightly smaller than the observed average standard deviation of 4 mV, but significantly less than the highest observed standard deviation of 15 mV.

Our pessimistic estimates for uncorrelated between-experiment variability summed to 25 mV, which is only slightly more than observed in Figure 3.B. The estimate for correlated between-experiment variability however, is less than half what is observed in Figure 3.C.

When experiment size was plotted against the correlated and uncorrelated between-experiment variability, the correlated component appeared to decrease with increasing cell count. This is somewhat unexpected, as the correlated variability is most easily explained by some systematic error in voltage control or read-out, which would depend on experimental set up and not change from cell to cell. A possible explanation is that studies with larger cell counts took place over a longer time, allowing experimental variability (or even seasonal biological variability) to creep in. you'd be lucky if variability creeping in made more consistent estimates!

I guess Teun would add a  $V_{off}$  (like in the artefact model) such that 95% within  $\pm 5$  mV to  $\sigma_V$ .

Expression system measurements of the midpoints of activation ( $V_a$ ) and inactivation ( $V_i$ ) show a wide within-experiment and between-experiment variability. Possible explanations for the within-experiment variability include differences in series resistance compensation from cell-to-cell, and to a lesser degree (and with much less certainty) temperature, pressure, and maybe the time since the membrane was

ruptured. Between-experiment variability is unexpectedly large, far exceeding the range that may be expected from previous studies that performed multiple measurements. This variability can be split into a correlated and an uncorrelated component, of which the correlated component is hardest to explain. Although correlated between-experiment variability seems most easily explained by systematic differences in the intended and applied voltage (experimental variability), this type of variability appears to be reduced when experiment size is increased, which is not consistent with this notion. A biological explanation for this variability — e.g. that cells maintain a difference between  $V_a$  and  $V_i$  required to make  $I_{Na}$  function — seems unlikely, given that these experiments were all in cells that did not express SCN5A natively.

It is possible that a future systematic review may be able to address these questions in more detail, although that may require an improvement in reporting of meta-data e.g. following the principles laid out in Quinn et al. (2011). Structured sharing of data and meta-data, i.e. by depositing data and meta-data in structured databases instead of papers and supplements, would be particularly useful. Sharing of individual cell results — even whole current traces — has become technically trivial in the preceding decade, and may also facilitate a far more detailed meta-analysis than was possible here (using only summary statistics such as the mean and standard error). Direct investigations in  $I_{Na}$  variability are also an exciting prospect, although the nature of the question may require a long-term coordinated effort by multiple labs.

The experimental record leaves room for, but provides no direct evidence of, biological variability in the midpoints of activation and inactivation of  $I_{Na}$ .

regulation as one thing happens to channel shifts both at once.

Funding i.e. cells might not have to, channel could do that itself... and almost certainly will with temp. for one.

## Competing interests

1. mean and std. might hide a lot of

## Author contributions

skew and non-Normality want to see full within expt. distn of  $V_a$ ,  $V_i$

2. Any covariance (if  $V_a$ ,  $V_i$  measured in same cells commonly?)

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