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## Accepted Manuscript

Title: THE IMPORTANCE OF DATA STRUCTURE IN  
STATISTICAL ANALYSIS OF DENDRITIC SPINE  
MORPHOLOGY

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Short communication

## THE IMPORTANCE OF DATA STRUCTURE IN STATISTICAL ANALYSIS OF DENDRITIC SPINE MORPHOLOGY

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## HIGHLIGHTS

- Data collection for dendritic spine morphology often results in multilevel data structure.
- The need for implementation of data structure in statistical models is highlighted.
- The use of mixed-effects models to study dendritic spine morphology is proposed.
- Mixed-effects models are superior to commonly used statistical methods for studying dendritic spine morphology.

## ABSTRACT:

Background: Dendritic spine morphology is heterogeneous and highly dynamic. To study the changing or aberrant morphology in test setups, often spines from several neurons from a few experimental units e.g. mice or primary neuronal cultures are measured. This strategy results in a multilevel data structure, which, when not properly addressed, has a high risk of producing false positive and false negative findings.

Methods: We used mixed-effects models to deal with data with a multilevel data structure and compared this method to analyses at each level. We apply these statistical tests to a dataset of dendritic spine morphology parameters to illustrate advantages of multilevel mixed-effects model, and disadvantages of other models.

Results: We present an application of mixed-effects models for analyzing dendritic spine morphology datasets while correcting for the data structure.

Comparison with existing methods: We further show that analyses at spine level and aggregated levels do not adequately account for the data structure, and that they may lead to erroneous results.

Conclusion: We highlight the importance of data structure in dendritic spine morphology analyses and highly recommend the use of mixed-effects models or other appropriate statistical methods to deal with multilevel datasets. Mixed-effects models are easy to use and superior to commonly used methods by including the data structure and the addition of other explanatory variables, for example sex, and age, etc., as well as interactions between variables or between variables and level identifiers.

#### ABBREVIATIONS:

LogOR: log odds ratio

#### KEYWORDS:

Dendritic spines; mixed-effects models; statistics and numerical data; data structure

### 1. BACKGROUND:

Dendritic spines are protrusions of the membrane of dendrites forming the postsynaptic part of a synapse. Spines contain neurotransmitter receptors, organelles and signaling systems and are crucial for the initial assessment of incoming signals from the presynaptic axon end. Spine morphology and density are known to be diverse and dynamic, and abnormalities have been associated to neuropsychiatric disorders (Arnold et al., 2016; Haas et al., 2013; Kim et al., 2013, 2015; Viana da Silva et al., 2016). When studying dendritic spine morphology in fixed tissue e.g. rodent brain slices, researchers have generally employed two approaches to analyze differences in spine morphology. In the first approach, spines are categorized into

classes on the basis of relative spine length and diameter (usually termed: stubby, mushroom, and long thin) (Harris et al., 1992). The distribution of spine classes and differences of specific spine parameters within these classes can then be compared between experimental test groups. The second approach considers all spines without classification and reflects the dynamic and heterogeneous nature of dendritic spines better (Hering and Sheng, 2001).

It is common practice to select several neurons from a few animals for analysis. However, in doing so, the spine parameters investigated form a multilevel or nested structure: spines (level 0) belong to neurons (level 1) and neurons belong to animals (level 2). If statistical tests are applied without taking the clustering (i.e. the effect of a specific neuron and/or mouse on the spine parameters) into account, the independence assumption is violated. This may result in an underestimation of standard errors and ultimately in an underestimation of the p-value, which implies a high risk for false positive results. Underestimation of standard errors is particularly severe for coefficients of explanatory variables that are measured at level 1 or higher, for example the variable genotype in genetically modified mice, which is measured at level 2 (mouse level) (Steele, 2008). Mixed-effects models can deal with this multilevel structure and are the norm in other fields of research, such as cluster randomized control trials. To investigate how original research articles deal with this multi-level data structure when analyzing dendritic spine morphology, we performed a small systematic review of the recent literature. We searched “(dendritic spines[MeSH Terms]) OR dendritic spine[MeSH Terms]” in Pubmed and reviewed 142 articles published in 2016 and 2017 (Pubmed search on August 23<sup>rd</sup> 2017). While the search criteria did not allow the inclusion of all relevant publication in the time period, it selected a highly relevant subpopulation unbiased towards statistical methods used.

We found 19 original research articles that report spine morphology parameters in fixed tissue (**Suppl. Table 1**). None of these report the use of mixed-effects models, however one article applied a two-level nested ANOVA, dealing with data structure. Two articles applied their statistical test at spine level without correction, 7 articles dealt with the multilevel structure by averaging the spine data at neurite, neuron or animal level, and 10 did not specify at what level the statistical tests were performed. Averaging data at neurite, neuron or animal level (aggregating data) comes with two problems: one is that by effectively

throwing away information, statistical power of detection is lost. This error is on the conservative side and is the least severe of the two problems. The second and more critical problem is that relying on aggregated data poses the risk of conducting the ecological fallacy, which can give erroneous result to either side by making inference about the spines based on inference drawn for the higher levels, neuron or animal. Unfortunately, as appears from our review, many papers do not provide sufficient details to assess their statistical approach.

Here we apply mixed-effects models to analyze dendritic spines in a dataset of morphology parameters. We compare the results to the commonly used analysis of data at spine level and at different aggregated levels, and we consider assumptions, advantages and disadvantages of all strategies applied.

## 2. METHODS:

### *2.1. Sample preparation and data collection*

Left cerebral hemispheres from wildtype (W mice,  $n = 8$ ) and genetically modified female mice (R mice,  $n = 7$ ) were stained with FD Rapid Golgi-Stain Kit (FD Neurotechnologies, Ellicott city, USA) and cut into 150  $\mu\text{m}$  thick slices (figure S1 in suppl. Methods). Five to nine anterior cingulate cortex (ACC) pyramidal neurons per mouse were identified (Olympus PlanApo 60X; oil-immersion; numerical aperture=1.4) by their prominent apical dendrites and sampled by systematic uniform random sampling. Image stacks (90-105 consecutive images at 1  $\mu\text{m}$  interval) of the selected neurons were captured by optical wide-field microscopy (Olympus BX50, Tokyo, Japan) and newCAST software (Visiopharm, Hoersholm, Denmark). 3D image reconstruction and analysis were performed using Imaris software version 7.6.3 (Bitplane AG, Zurich, Switzerland). Analysis of dendritic spine morphology was performed on reconstructed images using Imaris software version 7.7.1 (Bitplane AG, Zurich, Switzerland).

### *2.2. Statistical analysis*

All statistical analyses were performed using R. Continuous parameters were analyzed using a linear mixed-effects model (lmer function in the R package ‘lme4’ (Bates et al., 2015)) (**Formula 1**). Significant contributions of parameters were calculated using the ‘lmerTest’ package in R. Binomial parameters were analyzed using a generalized linear mixed-effects model (glmer function in the R package ‘lme4’ (Bates et al., 2015)) . Other statistical tests were applied as defined in the R package ‘stats’. We reject the null hypothesis when  $p < 0.05$ . R script is available in **suppl. methods**.

$$(1) \text{Outcome}_{\text{snm}} = \beta_0 + \beta_1 \cdot \text{Genotype}_m + \beta_2 \cdot \text{Level}_{\text{snm}} + \beta_3 \cdot \text{Depth}_{\text{snm}} + u_{\text{nm}} + v_m + \varepsilon_{\text{snm}}$$

Where  $\text{Outcome}_{\text{snm}}$ : spine parameter explained by spine (s), neuron (n) and mouse (m);  $\beta_0$ : intercept of the model;  $\beta_{1,2,3}$ : coefficients for explanatory variables; u: effect of neuron; v: effect of mouse;  $\varepsilon$ : residual error term.

### 3. RESULTS:

#### 3.1. *Applying mixed-effects models for continuous data*

We analyzed a dataset of dendritic spine morphology parameters from 8 W and 7 R mice that had a multilevel structure: spine (level 0), neuron (level 1) and mouse (level 2) (**Figure 1A**). This dataset contains information on spine length, volume, area, maximum and minimum diameter for the whole spine, as well as the three main spine parts (head, neck and ground). First we focused at the spine length for spines with length  $< 1.4 \mu\text{m}$  ( $n = 23579$ ). To properly deal with the data structure, we applied a strategy that involved a linear mixed-effects model, where level identifiers can be added to explain the data structure. Additionally, this model allows the addition of other explanatory variables (random intercept model), for example sex, and age, etc., as well as interactions between variables or between variables and level identifiers (random slope model). In our example, we made a model for spine length with neuron and mouse as level identifiers (random effects) and genotype as explanatory variable of interest. We furthermore added spine level and spine depth as explanatory variables. Spine level and depth are covariates explaining the position of spines on the dendrite that need to be included in the statistical model to correct for

morphological differences of the dendritic trees of mice with different genotypes (unpublished data). We found that the genotype of the mice had a significant effect on spine length. Spine length from R mice was decreased compared to W mice ( $\beta_{R-W} = -50.4 \text{ nm} \pm 20.3 \text{ nm}$ ,  $p = 0.03$ ) (**Figure 1B**). Furthermore, we could estimate the variance partition coefficients of the model, which estimates the contribution of each level to the total variance. For spine length, neuron and mouse levels contributed for 9.02% and 0.12% to the total variance, respectively. These contributions increased up to 43.35% and up to 8.16% for neuron and mouse levels, respectively for other continuous parameters in our dataset, clearly highlighting the importance of including data structure in the statistical analyses. When we analyzed spine neck volume in the same way, the linear mixed-effects model did not find genotype as a significant contributor to the model ( $\beta_{R-W} = -0.00360 \pm 0.00184 \text{ } \mu\text{m}^3$ ,  $p = 0.07$ ).

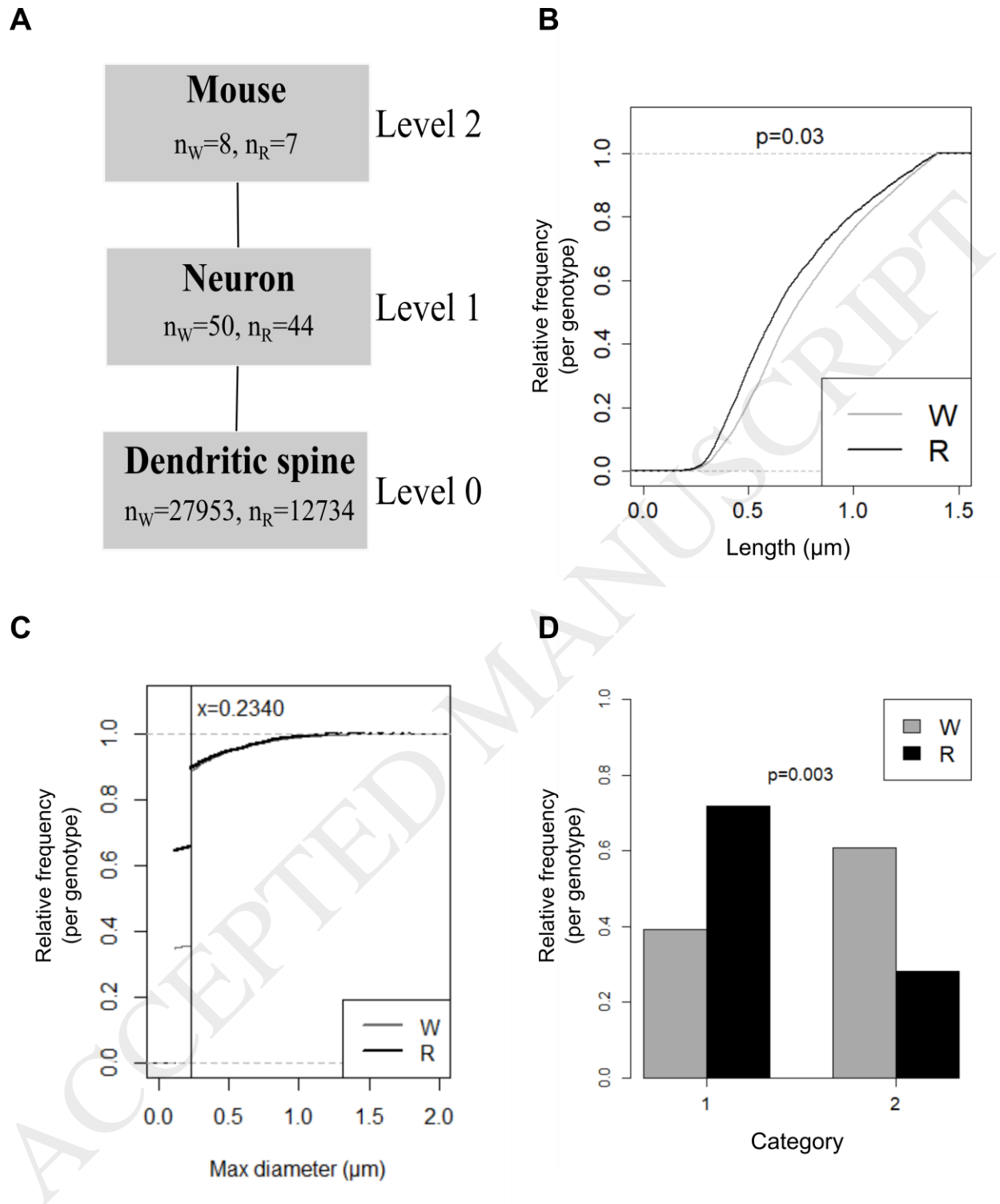


Figure 1: Dendritic spine morphology datasets form multilevel data structures

A) The underlying structure of the dataset analyzed.  $n$ : number of units at each level for wild type (W) or genetically modified (R) mice. B) Comparison of the cumulative frequency of spine length (for spines with

length  $< 1.4 \mu\text{m}$ ) between W ( $n = 16752$ ) and R ( $n = 6827$ ) dendritic spines. P-value as determined using a linear mixed-effects model. C) Comparison of the cumulative frequency of spine maximum diameter between W ( $n = 27182$ ) and R ( $n = 11820$ ) dendritic spines. In contrast to panel B, maximum spine diameter is discrete in the lower range ( $\leq 0.2340 \mu\text{m}$ ) due to limitations of the instrument (cut points at 117.0 nm and 234.0 nm). In the higher range, maximum spine diameter is continuous. D) Comparison of relative frequency of spines per genotype from the low range subset per category (category 1: maximum spine diameter  $< 117.0 \text{ nm}$ , category 2:  $117.0 \text{ nm} \leq$  maximum spine diameter) between W ( $n=24106$ ) and R ( $n=10592$ ) spines. P-value as determined using a generalized linear mixed-effects model.

### *3.2. Applying mixed-effects models for discrete data*

Parameters such as maximum spine diameter behaved in a discrete manner in the lower range (**Figure 1C**), where the measured structure size was close to the voxel size and where relatively large standard errors compared to the measurements were observed. This is an issue intrinsic to the resolution of the microscope and the method used to measure these dendritic spine parameters. In order to analyze these data, we first split the data in two subsets, one containing the continuous data in the higher range (spine maximum diameter  $> 234.0 \text{ nm}$ ,  $n = 4304$ ) and one containing the discrete data in the lower range (spine maximum diameter  $\leq 234.0 \text{ nm}$ ,  $n = 34698$ ). The first subset was suitable to be analyzed with a linear mixed-effects model as described above, and we found that genotype did not contribute significantly to the model for maximum spine diameter for spines in the high range subset ( $p = 0.73$ ). To investigate the spine maximum diameter in the low range subset, we divided the discrete data into two categories (1: value  $< 117.0 \text{ nm}$  and 2:  $117.0 \text{ nm} \leq$  value) and applied a generalized linear mixed-effects model with logit-link for binomial data, with neuron and mouse as level identifiers and genotype, level and depth as explanatory variables. We found that genotype was a significant contributor to the model, and that R mice were less likely to have spines of the higher category compared to W mice ( $\log\text{OR}_{\text{R-W}} = -7.10 \pm 2.39$ ,  $p = 0.003$ ) (**Figure 1D**).

### *3.3. Comparison to alternative analysis methods*

Next, we compared the results obtained using the mixed-effects models to alternative analysis methods, i.e. analyzing parameters at spine level (no aggregation), or aggregating spine information into the neuron or mouse levels before analysis (**Table 1**). Analyses at spine or neuron level would transform these lower levels into the study units, despite them being mutually dependent entities within the true study units, the mice. That problem would be solved by aggregating data all the way to mouse level. However, both aggregating approaches (to neuron and to mouse level) reduce statistical power by reducing the number of units analyzed and they suffer from the additional problem that there are effects of the covariates depth and level that both act on the spines (level 0). Ignoring important covariates, such as level and depth in our model, when performing statistical tests at neuron or mouse level can lead to incorrect estimates and inference.

To illustrate what difference it makes, we reanalyzed the spine parameters performing statistical tests at spine, neuron and mouse level and compared to the results as obtained previously using the mixed-effects models (**Table 1**). To aggregate at higher levels, we took the median per neuron ( $n = 90$ ) or per mouse ( $n = 15$ ) respectively. As predicted, analyses at spine level grossly underestimate the p-value resulting in false positive results, e.g. for spine length  $p=1.2e-62$ . Both aggregation approaches possess less statistical power compared to an analysis at spine level. Consistent with that, these tests turn out less significant (neuron level, e.g. for spine length  $p=0.003$ ) and not significant (mouse level, e.g. for spine length  $p=0.09$ ) p-values for spine length, neck volume and maximum diameter. Even though the analysis at mouse level is the most correct with respect to the data structure, the low power can produce false negative results. By comparing the mouse level results with the results of the mixed-effects model, we observed that the mixed-effects model is able to detect statistically significant differences while accounting for data structure.

We then compared the observed effect sizes (i.e. the difference between genotypes) of the different statistical tests. For example, for spine length, we observed that for analyzes at spine, neuron and mouse level the effect size was rather similar (median difference between  $-78.2$  and  $-85.3$  nm), however the effect size as calculated by the mixed-effects model was only  $-50.4$  nm. This difference indicates that the part of variation of spine length could be explained by either the data structure or the included covariates,

emphasizing the importance of correcting for data structure and essential covariates. When we analyzed spine length without Level and Depth as explanatory variables, a difference between genotypes of -55.1 nm ( $p=0.02$ ) is observed, demonstrating the considerable effect of data structure.

ACCEPTED MANUSCRIPT

Table 1: Comparison of statistical analyses of dendritic spine morphology data.

Spine parameter	Mixed-effects model (variables included: genotype (shown), Level, Depth)		Mixed-effects model (variable included: genotype)		Spine level		Neuron level		Mouse level	
	$\beta_{R-w}$	p-value	$\beta_{R-w}$	p-value	Median <sub>R-w</sub>	p-value	Median <sub>R-w</sub>	p-value	Median <sub>R-w</sub>	p-value
Length (nm)	-50.4	0.03	-55.1	0.02	-81.7	1.2e-62 #	-85.3	0.003 #	-78.2	0.09 #
Neck volume ( $\mu\text{m}^3$ )	-3.6e-03	0.07	-3.7e-3	0.06	-3.3e-3	1.3e-43 #	-2.1e-3	0.03 #	-3.0e-3	0.12 #
Max. diameter (low range, nm)	-7.10 *	0.003	-7.18 *	0.003	-117	<2.2e-16 §	-117	0.0003 §	-117	0.06 §
Comments	(+) data structure (+) covariates		(+) data structure (-) no covariates		(-) inter-dependent samples (-) no covariates		(-) inter-dependent samples (partly corrected) (-) no covariates (-) risk for ecological fallacy		(+) independent samples (-) low sample size (-) no covariates (-) risk for ecological fallacy	

The column ‘mixed-effects model (variables included: genotype (shown), Level, Depth)’ summarizes the results as obtained in paragraphs 3.1 and 3.2 for the parameters spine length, neck volume and maximum diameter. Results are shown only for the variable ‘genotype’. The column ‘Mixed-effects model (variable included: genotype)’ summarizes results from mixed-effects models excluding the variables Depth and Level. For analyses using mixed-effects models, we report the coefficient ( $\beta_{R-w}$ ) and p-value for the variable ‘genotype’.

Columns 'Spine level', 'Neuron level' and 'Mouse level' summarize results from analyses using not-aggregated data, and the analyses using aggregated data at neuron and mouse level, respectively, reporting median difference between genotypes ( $\text{median}_{R-W}$ ) and p-value. Because (transformed) data for analyzes at spine, neuron and mouse level are not normally distributed, median values and non-parametric test results are reported. For analyzes at neuron and mouse level, the median values per neuron ( $n = 90$ ) or mouse ( $n = 15$ ) respectively were used as input. \*) logOdds ratio; #) p-value as calculated by Wilcoxon Rank Sum test; §) p-value as calculated by Fisher's Exact test.

#### 4. DISCUSSION:

Here we present data to explain the importance of data structure in statistical analysis of dendritic spine morphology parameters. We propose to use statistical methods for analyzing these parameters that takes the multilevel structure of the data into account, resulting in the correct estimation of standard errors, between-group variance and p-value. We showed that by not including the multilevel structure in the analysis, statistical tests are at high risk for producing false positive, but that by aggregating into higher levels to handle the data structure is at risk for producing false negative results.

The (generalized) linear mixed-effects model is a versatile framework that allows for utilization of all available data while respecting the inherent structure, hereby maintaining the statistical power associated with large sample sizes without violating the independency assumption. Furthermore, these multivariate models allow for adding additional explanatory variables and interactions between variables and levels, improving the accuracy of the model. For this, we used readily available and easy to use R packages. Alternatively, statistical software like SAS ([www.sas.com](http://www.sas.com)), SPSS (<https://www.ibm.com>) and STATA ([www.stata.com](http://www.stata.com)) all have options for multilevel data analysis, and there are dedicated software packages for multilevel data, such as MLwiN (Charlton et al., 2017). The statistical framework of mixed-effects models presented here can be applied very generally as the study design requires. For example linear or nonlinear growth models (Steele, 2014) can be applied to longitudinal (repeated measures) studies or a cumulative link mixed model (Christensen, 2015) can be used for ordinal data. We should furthermore note that improved detection methods and image resolution would result in continuous data for most parameters measured in this study, which would of course reduce the need for the transformation of the data into binomial or ordinal data in dendritic spine morphology studies. Bitplane already improved the measuring method for small structures like diameter in newer versions of the Imaris software (version 8.0 or higher) by changing the method from "diameter from cross-section" or "shortest distance to border" to "linear interpolation". However, it is likely that the ambitions of scientists will increase with increasing resolution, and even smaller structures will be of interest to investigate.

The issue of a multilevel data structure is not unique to the study of dendritic spine morphology parameters. It exists in many fields of biological and biomedical research, e.g. electrophysiological recordings in brain slices or single cell gene expression experiments measure parameters for several cells from a few animals, and measurements of parameters from several nephrons from a few animals. Furthermore, proper analysis using multilevel modelling is already the norm when dealing with e.g. cluster randomized control trials and in fields outside of biomedicine, such as social sciences, yet inferior statistical analyses are still commonly applied when studying dendritic spine morphology. In our review, only 1 paper corrected for data structure using a two-level nested ANOVA (Spencer et al., 2017). Likewise, in a recent paper that did not match the search term in our restricted review Kondratiuk *et al.* employed nested Gaussian mixed models to analyze dendritic spine morphology parameters while correcting for the data structure (Kondratiuk et al., 2017). We highly encourage other researchers to follow similar approaches.

#### CONCLUSION:

In this report, we show the importance of correcting for data structure in order to avoid false positive results when working with multilevel data such as morphology parameters obtained by analyzing dendritic spines from mouse brains. We strongly advice to employ statistical models that can fully deal with multilevel data, while the commonly used analysis methods employing data aggregation are at (higher) risk of erroneously estimated effects and false conclusions. Statistical models that include data structure, such as mixed-effects models, are already the norm in other research fields and we advocate for the standardization of the use of such models when studying dendritic spine morphology.

## DECLARATIONS:

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## AUTHOR CONTRIBUTIONS:

VP, JG, APR, JRN and JHC designed the study. VP and APR collected samples and data. VP, JG, and JHC performed statistical analysis. VP, JG, JRN, ADB and JHC interpreted the results. VP wrote the first draft of the manuscript. All authors contributed to the finalization of the manuscript.

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