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Design-based stereological study of the guinea-pig (*Cavia porcellus*) cerebellum

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21 **Abstract**

22 Guinea pigs have proved useful as experimental animal models in  
23 studying cerebellar anatomical and structural alterations in human  
24 neurological disease; however, they are also currently acquiring increasing  
25 veterinary interest as companion animals. The morphometric features of the  
26 normal cerebellum in guinea pigs have not been previously investigated  
27 using stereology. The objective of the present work was to establish normal  
28 volumetric and quantitative stereological parameters for cerebellar tissues  
29 in guinea pigs, by means of unbiased design-based stereology. Cerebellar  
30 total volume, grey and white matter volume fractions, molecular and  
31 granular layers volume fractions, cerebellar surface area, Purkinje cellular  
32 and nuclear volumes, and the Purkinje cell total count were stereologically  
33 estimated. For this purpose, cerebellar hemispheres from six adult male  
34 guinea pigs were employed. Isotropic, uniform random sections were  
35 obtained by applying the orientator method, and subsequently processed for  
36 light microscopy. The cerebellar total volume, the white and grey matter  
37 volume fractions, and the molecular and granular layer volumes were  
38 estimated using the Cavalieri's principle and the point counting system.  
39 The cerebellar surface area was estimated through the use of test lines;  
40 Purkinje cellular and nuclear volumes were analysed using the nucleator  
41 technique, whereas the Purkinje cell total count was obtained by means of

42 the optical disector technique. The mean  $\pm$  standard deviation (SD) total  
43 volume of a guinea-pig cerebellar hemisphere was  $0.11 \pm 0.01 \text{ cm}^3$ . The  
44 mean volumetric proportions occupied by the grey and white matters were,  
45 respectively,  $78.0 \pm 2.6\%$  and  $22.0 \pm 2.6\%$ , whereas their mean absolute  
46 volumes were found to be  $0.21 \pm 0.02 \text{ cm}^3$  and  $0.059 \pm 0.006 \text{ cm}^3$ . The  
47 volumes of the molecular and granular layers were estimated at  $112.4 \pm 20.6$   
48  $\text{mm}^3$  and  $104.4 \pm 7.3 \text{ mm}^3$ , whereas their mean thicknesses were calculated  
49 to be  $0.184 \pm 0.020 \text{ mm}$  and  $0.17 \pm 0.02 \text{ mm}$ . The molecular and granular  
50 layers accounted for  $40.7 \pm 3.9 \%$  and  $37.4 \pm 1.8 \%$  of total cerebellar  
51 volume, respectively. The surface area of the cerebellum measured  $611.4 \pm$   
52  $96.8 \text{ mm}^2$ . Purkinje cells with a cellular volume of  $3210.1 \mu\text{m}^3$  and with a  
53 nuclear volume of  $470.9 \mu\text{m}^3$  had a higher incidence of occurrence. The  
54 mean total number of Purkinje cells for a cerebellar hemisphere was  
55 calculated to be  $253,090 \pm 34,754$ . The morphometric data emerging from  
56 the present study provide a set of reference data which might prove  
57 valuable as basic anatomical contribution for practical applications in  
58 veterinary neurology.

59

60 **Keywords:** Guinea pig, cerebellum, stereology, neuroanatomy, nervous  
61 system.

62

## 63 **Introduction**

64           The involvement of the cerebellum in motor coordination, balance  
65 and motor learning has been long and widely recognized (Brooks, 1984;  
66 Llinás and Welsh, 1993; Baillieux *et al.*, 2008; Lee *et al.*, 2015); however, a  
67 growing body of evidence involving neuroanatomical, neuroimaging and  
68 clinical studies indicates that it plays a significant role in non-motor  
69 behavioral-affective and cognitive functions, as well (Schmahmann and  
70 Caplan, 2006; Booth *et al.*, 2007; Molinari *et al.*, 2008; Cantalupo and  
71 Hopkins, 2010; Koziol *et al.*, 2011; De Smet *et al.*, 2013; Roostaei *et al.*,  
72 2014).

73           Design-based stereological techniques allow to efficiently acquire  
74 accurate and precise quantitative estimates of three-dimensional  
75 morphometric features of whole organs from measurements made on two-  
76 dimensional sections, by making use of statistical sampling and stochastic  
77 geometry principles (Boyce *et al.*, 2010).

78           Most stereological investigations on the cerebellum involving  
79 laboratory animals have been carried out on mice (Woodruff-Pak, 2006;  
80 Woodruff-Pak *et al.*, 2010; Wittmann and McLennan, 2011; Kennard *et al.*,  
81 2013; Song *et al.*, 2014), rats (Korbo *et al.*, 1993; Larsen *et al.*, 1993, 2000;  
82 Ragbetli *et al.*, 2007; Sonmez *et al.*, 2010) and rabbits (Akosman *et al.*,  
83 2011; Selçuk and Tıpırdamaz, 2020), but also on domestic animals such as

84 cats (Sadeghinezhad *et al.*, 2020), pigs (Jelsing *et al.*, 2006) and chicks  
85 (Tunç *et al.*, 2006). Apart from a stereological study performed on prenatal  
86 and neonatal guinea-pig cerebella following experimentally-induced  
87 intrauterine growth restriction (Mallard *et al.*, 2000), the morphometric  
88 features of the normal cerebellum in adult animals of this species have not  
89 been previously investigated using stereological techniques.

90 Guinea pigs (*Cavia porcellus*) have proved useful as experimental  
91 animal models in studying cerebellar anatomical and structural alterations  
92 in human neurological disease (Lev-Ram *et al.*, 1993; Furuoka *et al.*, 2011;  
93 Čapo *et al.*, 2015; Bennet *et al.*, 2017; Cumberland *et al.*, 2017), partly due  
94 to their high degree of neurological maturity at birth in relation to the short  
95 gestation period (Altman and Das, 1967; Hargaden and Singer, 2012; Silva  
96 *et al.*, 2016), which is important for clinical studies in human medicine.  
97 Indeed, the brain of newborn guinea pigs is singularly mature, and  
98 postnatal cerebellar neurogenesis is minimal in this precocial species  
99 (Altman and Das, 1967). It was observed that, as early as 45 days through  
100 gestation, cerebellar layers in guinea-pig fetuses were distinct and well  
101 developed, with easily identifiable Purkinje cells, and with the white and  
102 gray matters well differentiated both macro- and microscopically (Silva *et*  
103 *al.*, 2016). Moreover, cellular proliferation events in the cerebellum, unlike  
104 other rodents, are complete at birth in the guinea pig (Lossi *et al.*, 1997).

105           Recently, however, increasing interest has been addressed toward the  
106 clinical features, pathological changes and therapeutic resolution of  
107 neurological disorders of guinea pigs held as pet animals (Hollamby, 2009;  
108 Hawkins and Bishop, 2012). Most incidences of naturally-occurring  
109 cerebellar pathology reported in the literature for pet guinea pigs have an  
110 infectious etiology. Reported aetiological agents are, for instance, the  
111 lymphocytic choriomeningitis virus, leading to cerebellar hypoplasia with  
112 acute destruction of cortex folia and necrosis of granule and Purkinje cells  
113 (Monjan *et al.*, 1971; Hawkins and Bishop, 2012); *Toxoplasma gondii*,  
114 inducing granulomatous meningoencephalitis, foci of necrosis, and chronic  
115 cysts in the central nervous system (Brabb *et al.*, 2012; Gentz and  
116 Carpenter, 2012); and *Baylisascaris procionis* larvae, causing progressive  
117 multifocal encephalomalacia and eosinophilic granulomatous inflammation  
118 of the cerebellum, midbrain and brainstem (Van Andel *et al.*, 1995).

119           In light of the above-listed scientific evidence, the objective of the  
120 present work was to establish normal volumetric and quantitative  
121 stereological parameters for cerebellar tissues in adult guinea pigs, by  
122 means of unbiased design-based stereology (Gundersen and Jensen, 1987;  
123 West, 1993; Boyce *et al.*, 2010). Specifically, the present study was  
124 designed to estimate cerebellar total volume, grey and white matter volume  
125 fractions, molecular and granular layers volume fractions [by using the

126 Cavalieri 's principle (Gundersen and Jensen, 1987)], estimate the  
127 cerebellar surface area (Schmitz and Hof, 2005), the total number of  
128 Purkinje cells [by employing the optical disector method (Gundersen,  
129 1977; Sterio, 1984)], and the mean Purkinje cellular and nuclear volumes  
130 [through the use of the nucleator method (Gundersen *et al.*, 1988b)] in the  
131 guinea pig.

132         The morphometric data emerging from the present study provide an  
133 accurate set of reference data potentially valuable as basic anatomical  
134 contribution to the field of veterinary neurology in order to help  
135 implementing the development of the diagnosis and treatment of nervous  
136 diseases in the guinea pig.

137

## 138 **Methods**

### 139 **Animals and tissue preparation**

140         Six adult male pet guinea pigs, weighing  $569 \pm 64.9$  g, which  
141 spontaneously died of diseases other than those affecting the nervous  
142 system, were used for our research purposes following owners' permission.  
143 The animals did not present a history of neurological disease nor displayed  
144 pathological alterations of nervous tissues.

145         According to Directive 2010/63/EU of the European Parliament and  
146 of the 22 September 2010 Council on the protection of animals used for



147 scientific purposes, the Italian legislation (D. Lgs. n. 26/2014) does not  
148 require any approval by competent authorities or ethical committees, as this  
149 research did not influence any therapeutic decisions.

150 Guinea-pig cerebella were excised from the neurocranium in their  
151 entirety, each was divided into two halves, and then immersed in a 4%  
152 phosphate-buffered formaldehyde solution to enable tissue fixation. One  
153 hemisphere of each cerebellum was randomly chosen and weighed on a  
154 digital laboratory scale. The cerebellar hemispheres were routinely  
155 processed for light microscopic examination and subsequently embedded in  
156 paraffin.

#### 157 **Tissue sampling and stereology**

158 The orientator method (Mattfeldt *et al.*, 1990; Nyengaard, 1999) was  
159 applied to obtain isotropic, uniform, random sections. In essence, each  
160 cerebellar hemisphere was embedded in a paraffin block, which was placed  
161 at the center of a circle with 90 equidistant divisions along the perimeter. A  
162 random number between 0 and 90 was looked up and the paraffin medium  
163 was cut along a line parallel to the direction of the selected number. The  
164 block was placed on its cut surface at the center of a second circle, with 96  
165 nonequidistant divisions along its perimeter. The paraffin was cut along a  
166 line parallel to the direction of a random number ranging from 0 to 96, and  
167 the block was finally re-embedded in paraffin while placed on its cut

168 surface (Fig. 1). Consecutive 25 micrometer-thick sections were cut with a  
169 microtome at uniform constant intervals with a random start and until  
170 exhausting the organ. Every 25th section was collected using the principle  
171 of systematic uniform random sampling (Gundersen and Jensen, 1987), in  
172 order to acquire 12 to 15 sections per animal. Sections were then stained  
173 with Cresyl violet 0.1 % stain solution. A slide scanner (Optic lab H850,  
174 Plustek) was employed for capturing images from sections in order to  
175 enable the subsequent estimation of volumes and surface areas. A  
176 microscope (CX40, Olympus, Germany) equipped with an oil immersion  
177 objective ( $\times 100$ ), connected to a microcator (MT12, Heidenhain, Traunreut,  
178 Germany) and a digital camera (MB-225) was utilized for the estimation of  
179 Purkinje cells total cellular and nuclear volumes. Geometrical probes,  
180 necessary for the stereological analysis of each structural feature  
181 represented in each section (West, 1993), were produced using a dedicated  
182 software (ImageJ; <https://imagej.nih.gov>).

### 183 **Estimation of total and fractional volumes**

184         The accurate estimation of cerebellar total volume was made  
185 possible by employing cerebellar weight and transforming it into a volume,  
186 and by applying the Cavalieri's estimator, taking therefore into account  
187 tissue shrinkage. Cerebellar shrinkage secondary to histological processing  
188 allows to obtain unbiased stereological estimations insensitive to

189 processing-dependent tissue deformations (Dorph-Petersen *et al.*, 2001).

190 The estimation of total volume starting from the weight of the  
191 cerebellum, was performed using the following formula:

$$192 \quad V (\text{cerebellum}) = W (\text{cerebellum}) / \rho,$$

193 where  $\rho$  refers to the weight-to-volume ratio of cerebellar tissue.

194 The estimation of the total volume of the cerebellum through use of the  
195 Cavalieri principle was conducted by using the test point system (Fig. 2)  
196 and following the equation below (Howard and Reed, 1998):

$$197 \quad V = \Sigma P \cdot SSF \cdot T \cdot (a/p)$$

198 where  $\Sigma P$  is the total number of points hitting the structure; SSF (1/25)  
199 represents the section sampling fraction; T (25  $\mu\text{m}$ ) is the section thickness  
200 and  $a/p$  (465,267  $\mu\text{m}^2$ ) refers to the area per point.

201 The fractional volume ( $V_v$ ) of cerebellar structures including white  
202 matter, grey matter, molecular and granular layers, was estimated using the  
203 following formula (Gundersen *et al.*, 1988a):

$$204 \quad V_v (\text{structure}) = \Sigma P (\text{structure}) / \Sigma P (\text{cerebellum})$$

205 where  $\Sigma P$  (structure) is the number of points hitting the white matter, gray  
206 matter, molecular and granular layers, and  $\Sigma P$  (cerebellum) is the number  
207 of points hitting the cerebellum.

208 Lastly, in order to estimate the volume accounted for by each  
209 structure, each volume fraction was multiplied by the total volume of the

210 cerebellum.

### 211 **Estimation of surface area**

212 The surface density ( $S_v$ ) of the cerebellum was estimated by using  
213 test lines (Fig. 2b), and by employing the following formula (Howard and  
214 Reed, 1998):

$$215 S_v = 2 \cdot \sum l / (\sum P \cdot l/p)$$

216 Where  $\sum l$  represents the total number of intersections between the outer  
217 surface of the cerebellum and the test lines,  $\sum P$  refers to the points hitting  
218 the molecular layer,  $l/p$  (658  $\mu\text{m}$ ) was the length of each test line associated  
219 to each point of the test grid.

220 Consequently, for estimating the surface area, surface density was multiplied  
221 by the volume of the molecular layer.

222 In addition, the thickness ( $T$ ) of the molecular and granular layers was  
223 calculated using the following formula (Andersen *et al.*, 2012):

$$224 T (\text{layer}) = V (\text{layer}) / S (\text{layer})$$

225 where  $V$  is the volume and  $S$  is the surface area of each layer.

### 226 **Estimation of Purkinje cell total count**

227 The optical disector method was employed for the estimation of the  
228 Purkinje cell total number, and a motorized stage designed by Department  
229 of Anatomy, Faculty of Veterinary Medicine, of the University of Tehran,  
230 Tehran, Iran, was employed for the purpose. The microscopic fields were

231 selected by moving the microscope stage in the x and y directions for a  
232 constant distance spanning the entire section thickness. The unbiased  
233 counting frame principle was applied for counting the cells. The Purkinje  
234 cells whose nucleolus was located inside the counting frame or crossed the  
235 accepted lines were sampled, and those whose nucleolus came into focus  
236 within disector height were counted (Fig. 3).

237 The numerical density of Purkinje cells was calculated using the following  
238 formula (Kristiansen and Nyengaard, 2012):

$$239 \quad N_v (\text{Purkinje cells}) = [\Sigma Q^- / (a/f \times \Sigma P \times h)] \times t/BA$$

240 where  $\Sigma Q^-$  represents the total count of Purkinje cells,  $a/f$  ( $9895 \mu\text{m}^2$ ) is the  
241 area per frame,  $\Sigma P$  is the total number of frames,  $h$  ( $10 \mu\text{m}$ ) is the disector  
242 height,  $t$  is the sections mean thickness ( $18.5 \mu\text{m}$ ), measured for each  
243 microscopic field, and BA ( $25 \mu\text{m}$ ) is the block advance.

244 Finally, for the estimation of the total number of Purkinje cells, the  
245 numerical density was multiplied by the total volume of the cerebellum,  
246 estimated using the Cavalieri's principle.

### 247 **Estimation of mean Purkinje cellular and nuclear volumes**

248 To estimate the volumes of Purkinje cells and Purkinje cell nuclei,  
249 the nucleator technique was utilized (Gundersen *et al.*, 1988b). The volume  
250 of the sampled cells was measured by using the unbiased counting frame,

251 and following the formula (Gundersen *et al.*, 1988b):

$$252 \quad V_n = 4\pi/3 \cdot l_n^3$$

253 Where  $l_n$  refers to the intercept length from the nucleolus to the border of  
254 the cytoplasm (for cellular volume), or to the border of the nucleus (for  
255 nuclear volume) of Purkinje cells.

### 256 **Estimation of the coefficient of error (CE)**

257 The precision of the volume estimates, expressed in terms of CE, is  
258 related to the variability associated with systematic uniform random  
259 sampling (SURS) sampling and point counting of the estimator. The CE for  
260 the estimate of the volume (Gundersen and Jensen, 1987), surface area  
261 (Kroustrup and Gundersen, 1983) and Purkinje cell count (Braendgaard *et*  
262 *al.*, 1990) was calculated.

263

### 264 **Statistical analysis**

265 All data are expressed as mean  $\pm$  standard deviation (SD). As for  
266 right-skewed distributions, a logarithmic scale was used for individual  
267 estimates of Purkinje cellular and nuclear volumes (Weber *et al.*, 1997).

268

### 269 **Results**

270 All cerebella evaluated appeared normal both macroscopically and  
271 on histological examination, with all the microscopical structures being

272 distinctly identifiable and without any evidence of pathological processes.

273         The mean ( $\pm$ SD) weight of a guinea-pig cerebellar hemisphere was  
274  $0.285 \pm 0.028$  g. The mean volume of a guinea pig cerebellar hemisphere,  
275 calculated by dividing the cerebellar weight by its specific gravity, was  
276  $0.274 \pm 0.027$  cm<sup>3</sup>, while the value obtained by employing the Cavalieri's  
277 estimator, was  $0.110 \pm 0.015$  cm<sup>3</sup>. A  $61.34 \pm 5.39\%$  total cerebellar volume  
278 shrinkage, secondary to the process of paraffin embedding, was estimated.  
279 The relative volume fractions of the grey and white matters, expressed as a  
280 percentage of total cerebellar volume, were found to be  $78.06 \pm 2.66\%$  and  
281  $21.92 \pm 2.67\%$ , respectively. Their absolute volumes, on the other hand,  
282 were calculated to be  $0.21 \pm 0.02$  cm<sup>3</sup> for the grey matter, and  $0.060 \pm$   
283  $0.006$  cm<sup>3</sup> for the white matter. The separate and mean values for the  
284 above-mentioned parameters, are outlined in Table 1.

285         The surface area of the cerebellum measured  $611.4 \pm 96.8$  mm<sup>2</sup>. The  
286 volume of the molecular layer was estimated to be  $112.41 \pm 20.56$  mm<sup>3</sup>  
287 while that of the granular layer  $104.38 \pm 7.31$  mm<sup>3</sup>; the molecular and  
288 granular layers accounted for  $40.67 \pm 3.87\%$  and  $37.38 \pm 1.77\%$  of total  
289 cerebellar volume, respectively. The mean thickness of the molecular and  
290 granular layers was  $0.184 \pm 0.020$  mm and  $0.169 \pm 0.017$  mm, respectively.  
291 In Table 2 are shown the mean and individual data calculated for the above-  
292 mentioned criteria in the six guinea pigs.

293           The frequency distribution of the Purkinje cellular and nuclear  
294 volumes is plotted in Fig. 4. The Purkinje cell volumes were found to be  
295 ranging from 987 to 8246.8  $\mu\text{m}^3$ , of which cells with a volume of 3210.1  
296  $\mu\text{m}^3$  had a higher (13.71%) incidence of occurrence. The estimated volume  
297 of Purkinje nuclei was found to be ranging between <117 and 1623.4  $\mu\text{m}^3$ ,  
298 and nuclei with a volume of 470.9  $\mu\text{m}^3$  were the most frequently occurring  
299 ones (13.54%).

300           The mean total number of Purkinje cells for a cerebellar hemisphere  
301 was calculated to be  $253,090 \pm 34,754$  (Table 3).

302           The mean coefficient of error (CE) and coefficient of variation (CV),  
303 along with their ratio ( $\text{CE}^2/\text{CV}^2$ ), calculated for total cerebellar volume,  
304 grey and white matter volume fractions, granular and molecular layers  
305 volume fractions, cerebellar surface area, and total number of Purkinje cells  
306 are shown in Table 4.

307

## 308 **Discussion**

309           The mean total volume of a guinea-pig cerebellar hemisphere  
310 estimated in the present study is consistent with that calculated in a  
311 previous work, which investigated the brain morphology of domestic  
312 guinea pigs through quantitative cytoarchitectonic measurements (Kruska,  
313 2014). Cerebellar total volume has been previously assessed by



314 stereological techniques in other species such as humans, which exhibited a  
315 difference between sexes, with male cerebella measuring  $120.5 \pm 11.1 \text{ cm}^3$   
316 in volume, while females  $105.9 \pm 11.2 \text{ cm}^3$  (Taman *et al.*, 2020). Cerebellar  
317 volume has also been stereologically estimated in rabbits (Karabekir *et al.*,  
318 2014) and rats (Noorafshan *et al.*, 2018), presenting volumes of  $0.69 \pm 0.03$   
319  $\text{cm}^3$ , and  $0.080 \pm 0.004 \text{ cm}^3$  for each cerebellar hemisphere, respectively,  
320 but also in cats (Sadeghinezhad *et al.*, 2020), presenting a mean cerebellar  
321 hemisphere volume of  $2.06 \pm 0.29 \text{ cm}^3$ . When comparing total cerebellar  
322 volume (in  $\text{cm}^3$ ) in relation to body weight (in kg) in each species, it  
323 appears that the guinea pigs of the present study have a cerebellar volume  
324 to body weight ratio of 0.9, which is consistent with the 0.8 calculated for  
325 the rat (Noorafshan *et al.*, 2018), but greater than the 0.4 estimated for the  
326 rabbit (Karabekir *et al.*, 2014), and less than an approximate 1.7 for an  
327 adult individual of average weight (Taman *et al.*, 2020) and than the  
328 approximate 1.1 calculated for a medium-sized cat (Sadeghinezhad *et al.*,  
329 2020).

330         The cerebellar weight to body weight ratio was 0.1 in the guinea pig  
331 study population, which is in line with an approximate 0.13 calculated for a  
332 medium-sized cat (Sadeghinezhad *et al.*, 2020). Cerebellar volumetric  
333 modifications have been correlated with physiological factors such as age,  
334 gender (Raz *et al.*, 1998), cognitive capability, but also with several

335 pathological neurological conditions such as Alzheimer's disease,  
336 schizophrenia and epilepsy in humans (Bottmer *et al.*, 2005; Sato *et al.*,  
337 2007; Bas *et al.*, 2009; Andersen *et al.*, 2012). A study carried out on rats  
338 has also identified a correlation between maternal diabetes and a reduction  
339 of total cerebellar volume and thickness of all layers in the offspring (Hami  
340 *et al.*, 2016). Volumetric prediction of the cerebellum can therefore find a  
341 valuable use in further research on veterinary neurological disease affecting  
342 cognition.

343         The cerebellar gray and white matter volumes have also been  
344 stereologically estimated in other species. The total gray matter volume of  
345 human cerebella has been calculated to be 88.5 cm<sup>3</sup>, while that of the white  
346 matter 22.5 cm<sup>3</sup> (Andersen *et al.*, 2012). Cerebellar grey and white matter  
347 volumes were estimated to be 1.46 ± 0.24 cm<sup>3</sup> and 0.60 ± 0.06 cm<sup>3</sup>,  
348 respectively, for the cat (Sadeghinezhad *et al.*, 2020). When compared to  
349 the guinea pig and cat, the proportionally more voluminous grey matter in  
350 humans can be likely ascribed to their increased development of motor  
351 control, coordination, as well as cognitive functions. Moreover, it was  
352 noted that, in the early domesticated mammals such as the guinea pig, a  
353 decrease in total brain size, which is proportional to the level of  
354 encephalization of the species, along with a decrease in total cortex and  
355 areas responsible for processing sensory information and motor control,

356 such as the grey matter, occurred as a consequence of the domestication  
357 process, with, however, the cognitive functions not being affected by this  
358 change (Kruska, 2005; Kaiser *et al.*, 2015; Welniak-Kaminska *et al.*, 2019).  
359 The volumes of the grey and white matter calculated in the present study  
360 are markedly greater than those reported by Mallard *et al.* (2000) for  
361 neonatal guinea pigs, which is likely due to the large age and body weight  
362 discrepancy. The influence of the physiological process of aging on  
363 volumetric changes in the cerebellar gray and especially the white matter  
364 has been assessed in several studies (Jernigan *et al.*, 2001; Walhovd *et al.*,  
365 2005).  
366 Several human neurological diseases affecting cognition have also been  
367 observed to cause volume losses of the cerebellar gray and white matters  
368 (Fennema-Notestine *et al.*, 2004; Anderson *et al.*, 2009), as evidence of the  
369 role that the cerebellum plays in cognition.

370         The mean volumes of the molecular and granular layers in the guinea  
371 pig cerebellum estimated in the present work are significantly greater than  
372 the corresponding values reported by Mallard *et al.* (2000) for neonatal  
373 guinea pigs, and, comparing the two studies, the volumes of the two layers  
374 are apparently not proportionally related to body weight. The mean  
375 corresponding volumes referring to humans are 54.4 cm<sup>3</sup> for the molecular  
376 layer, and 37.9 cm<sup>3</sup> for the granular layer (Andersen, 2004). The mean

377 volume of the molecular and granular layers of the cerebellum of normal  
378 rats was reported to be 0.035 cm<sup>3</sup> and 0.024 cm<sup>3</sup>, respectively (Dortaj *et al.*,  
379 2018). In cats' cerebella, the mean molecular layer volume had been  
380 reported to be 0.89 ± 0.16 cm<sup>3</sup>, while that of the granular layer 0.56 ± 0.10  
381 cm<sup>3</sup> (Sadeghinezhad *et al.*, 2020). The relative proportions of the molecular  
382 and granular layers of the cerebellum in the different species seem to be  
383 conserved, thanks to the similar cerebellar microscopical anatomy. As a  
384 matter of fact, the histological examination of the guinea pig cerebella  
385 permitted the clear identification of the molecular, Purkinje and granular  
386 layers with their characteristic cellular populations. The conserved  
387 volumetric trend seems to be therefore related to function.

388 A stereological study carried out on intrauterine growth-restricted guinea  
389 pigs secondary to placental insufficiency in the second half period of  
390 pregnancy, has been seen to cause a reduction in the volume of the  
391 molecular and granular layers, as well as in that of the white matter in  
392 prenatal guinea-pig cerebella, therefore causing cognitive, motor and  
393 behavioral deficits in the post-natal life (Mallard *et al.*, 2000).

394         When analyzing the distribution of the thickness of the molecular  
395 and granular layers in the different subjects comprising our study  
396 population, it appears that the measurements are quite consistent and

397 regular, in contrast with what Sultan and Braitenberg (1993) had reported  
398 for smaller mammalian species. Andersen (2004) calculated a mean  
399 thickness of the molecular layer of  $590.00 \pm 0.08 \mu\text{m}$  and  $410.00 \pm 0.15 \mu\text{m}$   
400 for the granular layer in human cerebella. Sadeghinezhad *et al.* (2020), on  
401 the other hand, calculated  $133.5 \pm 10.1 \mu\text{m}$  for the molecular layer and  $84.7$   
402  $\pm 17.3 \mu\text{m}$  for the granular layer in cats' cerebella. Consistently with human  
403 and cats' cerebella, the molecular layer appears thicker than the granular,  
404 although not in a statistically significant manner; however, it seems that the  
405 thickness in guinea pigs is more uniformly-distributed between the two  
406 layers when compared to cats and humans' data. This can be explained by  
407 different physiological factors such as age. Indeed, a study carried out on  
408 cats' cerebella showed that aging causes an increase in granular layer  
409 thickness at the expense of that of the molecular layer (Zhang *et al.*, 2006).

410         With regard to the measurement of the cerebellar surface area, the  
411 ratio of cerebellar surface area to cerebellar weight in the different animals  
412 comprising the study population remains fairly constant, supporting the  
413 proportionality between cerebellar area and cerebellar weight hypothesized  
414 by Sultan and Braitenberg for larger mammals (1993), which is probably  
415 due, unlike other smaller mammalian species, to the equally constant  
416 distribution of grey matter thickness values in our guinea pig population.  
417 Further studies on larger population samples are needed to confirm this

418 finding. The average surface area of the human cerebellum has been  
419 previously estimated by different authors to be 550 cm<sup>2</sup> (Henery and  
420 Mayhew, 1989), 1027 cm<sup>2</sup> (Andersen *et al.*, 2012) and 1160 cm<sup>2</sup> (Andersen  
421 *et al.*, 1992). The human cerebellum, during evolution, underwent a  
422 significant expansion of its surface area both in absolute terms as well as in  
423 relation to the neocortex; this growth played a critical role in human  
424 cognitive development in comparison with other animals, given the role of  
425 the cerebellum in cognition (Barton and Venditti, 2014). In the animal  
426 kingdom, therefore, it is likely that the cerebellar surface area of highly  
427 encephalized species such as higher primates might show a greater  
428 development in comparison with mammals of a similar size. On the other  
429 hand, a mild but significant reduction in the total cerebellar area has been  
430 described in humans with advancing age, showing varying decline trends in  
431 the different vermian lobules (Raz *et al.*, 1998). A study carried out on  
432 experimentally vitamin C-deprived guinea pig fetuses has revealed a  
433 significant reduction in cerebellar surface area due to the obliteration of  
434 fissures and the fusion of opposing folia, resulting in a macroscopically-  
435 visible cerebellar dysplasia in terms of flattening of its surface, analogously  
436 to that observed in Lysencephaly Type 2 (Čapo *et al.*, 2015). The  
437 mentioned study is of clinical relevance in pet guinea pigs due to their  
438 natural incapability of endogenous vitamin C synthesis (Nishikimi *et al.*,

439 1992), analogously to humans, resulting in the necessity of its dietary  
440 supplementation, with the risk of developing vascular as well as  
441 neurological disease in case of deprivation.

442 Purkinje cells with a perikaryon volume of  $3210.1 \mu\text{m}^3$  and with a  
443 nuclear volume of  $470.9 \mu\text{m}^3$  were found to have the highest frequency of  
444 occurrence in the guinea pig cerebellum. Mean Purkinje cellular perikaryon  
445 volumes had been estimated to be  $12400 \mu\text{m}^3$  in humans (Korbo and  
446 Andersen, 1995),  $4900 \mu\text{m}^3$  (Korbo and Andersen, 1995) and  $5600 \mu\text{m}^3$   
447 (Sørensen *et al.*, 2000) in rats,  $17600 \mu\text{m}^3$  in adult minipigs (Jelsing *et al.*,  
448 2006),  $2207 \mu\text{m}^3$  in rabbits (Akosman *et al.*, 2011), and  $6994 \mu\text{m}^3$  in cats  
449 (Sadeghinezhad *et al.*, 2020). If considering a mean weight for an adult  
450 individual of each species, and calculating a ratio of Purkinje volume to  
451 body weight, these findings suggest a non-allometric correlation. Indeed,  
452 the mini-pig (Jelsing *et al.*, 2006) has a Purkinje volume to body weight  
453 ratio that is six times greater than that of humans (Korbo and Andersen,  
454 1995), whereas rodents such as the rat (Sørensen *et al.*, 2000) and the  
455 guinea pig have, respectively, ratios that are 40 and 180 times  
456 proportionally greater than that of humans. The variability encountered  
457 might be explained by the different degrees of tissue shrinkage (Andersen  
458 *et al.*, 1992), by the immersion time of the tissue in the fixative (Jelsing *et*  
459 *al.*, 2006), by the degree of postnatal development of Purkinje perikaryon

460 volume (Jelsing *et al.*, 2006), or by different degrees of significance of  
461 Purkinje cell roles in motor, sensory and cognitive functions among the  
462 different species.

463         The mean total number of Purkinje cells calculated in the present  
464 work is consistent with the value reported for the whole cerebellum in a  
465 previous work carried out on neonatal guinea pigs, that is in the order of  
466 500,000 (Mallard *et al.*, 2000). It has been demonstrated that the brain of  
467 newborn guinea pigs, species characterized by its precocity, presents a high  
468 degree of neurological maturity, and that postnatal cerebellar neurogenesis  
469 is minimal (Altman and Das, 1967). As a matter of fact, all cerebellar  
470 layers, including Purkinje cells, as well as white and gray matters, are well  
471 developed and differentiated as early as 45 days post conception (Silva *et al.*  
472 *al.*, 2016), and that all cerebellar cell proliferation events are entirely  
473 complete at birth in this species, unlike other similar rodents (Lossi *et al.*,  
474 1997). In the adult mini-pig cerebellum, on the other hand, the total number  
475 of Purkinje cells was in the order of 2.8 million (Jelsing *et al.*, 2006). The  
476 numerosity of the Purkinje cell count in the above-mentioned study was,  
477 indeed, partially explained by a significant postnatal development in total  
478 Purkinje cell number and perikaryon volume, as it had also been  
479 demonstrated in rats (Altman and Bayer, 1978), humans (Miyata *et al.*,  
480 1999), and cats (Vastagh *et al.*, 2005). The total number of Purkinje cells in



481 the whole adult rat cerebellum was estimated at around 320,000 cells  
482 (Sonmez *et al.*, 2010), which is markedly less than the value obtained for  
483 the guinea pig, and that could be explained by the complex heterogeneity of  
484 guinea pigs' Purkinje cells. It has been noted that Purkinje cells in the  
485 guinea pig cerebellum show a complex expression pattern of zebrin II, an  
486 immunohistochemical marker of cerebellar compartmental heterogeneity,  
487 showing three levels of zebrin II expression (Larouche *et al.*, 2003), as  
488 opposed to rats, where zebrin II expression only distinguishes two classes  
489 of Purkinje cells (Brochu *et al.*, 1990).

490 The hypothesis that less voluminous brains tend to have a higher cellular  
491 density than larger brains (Mwamengele *et al.*, 1993) does not seem to  
492 always be applicable, as is the case with the higher count of Purkinje cells  
493 in the guinea pigs comprising the present study when compared with the  
494 values reported for the rat (Sonmez *et al.*, 2010). Reports of acquired  
495 cerebellar degenerative disease in pet guinea pigs, mostly secondary to an  
496 infectious etiology, have been described in the literature, with ataxia and  
497 loss of voluntary motor control being common clinical signs, and  
498 meningoencephalitis and cerebellar cortical hypoplasia with necrosis of  
499 granule and Purkinje cells the principal histopathological findings (Monjan  
500 *et al.*, 1971; Van Andel *et al.*, 1995; Brabb *et al.*, 2012; Gentz and  
501 Carpenter, 2012; Hawkins and Bishop, 2012).

502           In conclusion, the present study represents the first detailed  
503 description of the morphometrical features of the guinea pig cerebellum  
504 using design-based stereological techniques. The reference morphometrical  
505 data provided for cerebellar structures might find a use as basic anatomical  
506 contribution to a greater understanding of neurological diseases when  
507 examining cerebellar pathology with relation to function in this exotic pet  
508 species of increasing veterinary interest. In addition, the present study  
509 might prove useful by providing a comparison with available data in  
510 humans and other mammals for future research investigating the basis of  
511 motor, cognitive and behavioral diseases in the different species.

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516   **Conflict of interests**

517   The authors have no conflict of interests to declare.

518   **Author contributions**

519   M.D.S.: acquisition of data, data analysis/interpretation, drafting of the

520 manuscript; J.S.: concept/design, acquisition of data, data  
521 analysis/interpretation, critical revision and approval of the manuscript;  
522 J.R.N.: data analysis/interpretation, critical revision of the manuscript and  
523 approval of the article; M.A.A.: data analysis/interpretation; A.S.: data  
524 analysis/interpretation; N.D.S.: acquisition of data; R.C.: concept/design;  
525 critical revision of the manuscript and approval of the article; A.G.:  
526 acquisition of data, critical revision of the manuscript and approval of the  
527 article.

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887 Table 1. Stereological data for total volume of cerebellar hemisphere and  
888 proportional volume of gray matter and white matter in six guinea pigs.

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Animals	Cerebellum weight (g)	Total volume of cerebellum (weight/specific gravity) (cm <sup>3</sup> )	Total volume of cerebellum (Cavalieri estimator) (cm <sup>3</sup> )	Shrinkage (%)	Gray matter		White matter	
					Volume fraction (%)	Volume (cm <sup>3</sup> )	Volume fraction (%)	Volume (cm <sup>3</sup> )
1	0.257	0.247	0.117	54.47	75.94	0.1875	24.05	0.0594
2	0.282	0.271	0.118	58.15	80.42	0.2179	19.57	0.0530
3	0.298	0.286	0.117	60.73	77.60	0.2219	22.39	0.0640
4	0.263	0.252	0.079	69.96	78.64	0.1981	21.35	0.0538
5	0.280	0.269	0.112	60	74.37	0.2000	25.62	0.0689
6	0.335	0.322	0.118	64.77	81.42	0.2621	18.57	0.0597
Mean±SD	0.285±0.028	0.274±0.027	0.110±0.015	61.34±5.39	78.06±2.66	0.2145±0.0266	21.92±2.66	0.0598±0.0060

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916 Table 2. Stereological data for surface area, volume and thickness of  
917 molecular and granular layers in cerebellar hemisphere in six guinea pigs.

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Animals	1	2	3	4	5	6	Mean±SD
Surface area (mm <sup>2</sup> )	486.066	555.984	630.003	627.302	592.925	776.140	611.40±96.8
Volume fraction of molecular layer (%)	40.28	42.24	39.3	41.63	34.42	46.19	40.67±3.87
Volume of molecular layer (mm <sup>3</sup> )	99.4	114.4	112.3	104.9	92.5	151.0	112.41±20.56
Volume fraction of granular layer (%)	35.65	38.18	38.29	37.01	39.94	35.23	37.38±1.77
Volume of granular layer (mm <sup>3</sup> )	88.0	103.4	109.5	93.2	107.4	113.4	104.38±7.31
Thickness of molecular layer (mm)	0.204	0.205	0.178	0.167	0.156	0.194	0.184±0.020
Thickness of granular layer (mm)	0.181	0.185	0.173	0.148	0.181	0.146	0.169±0.017

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Table 3. Stereological data for numeral density and total number of Purkinje cells in cerebellar hemisphere in six guinea pigs.

Animals	1	2	3	4	5	6	Mean±SD
Numeral density (cells/mm <sup>3</sup> )	2532	2413	2215	2546	2197	1986	2314.833 ± 220.099
Total number	296010	284380	258570	200660	245280	233640	253090 ± 34754

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961 Table 4. The mean coefficient of error (CE) and coefficient of variation

	Total volume	Grey matter volume	White matter volume	Granular layer volume	Molecular layer volume	Surface area	Total number of Purkinje cells
CE	0.016	0.080	0.050	0.033	0.031	0.0162	0.080
CV	0.140	0.123	0.101	0.096	0.182	0.158	0.137
$CE^2/CV^2$	0.013	0.423	0.252	0.121	0.029	0.479	0.346

962 (CV) of stereological analysis of guinea pig cerebellum (n=6)

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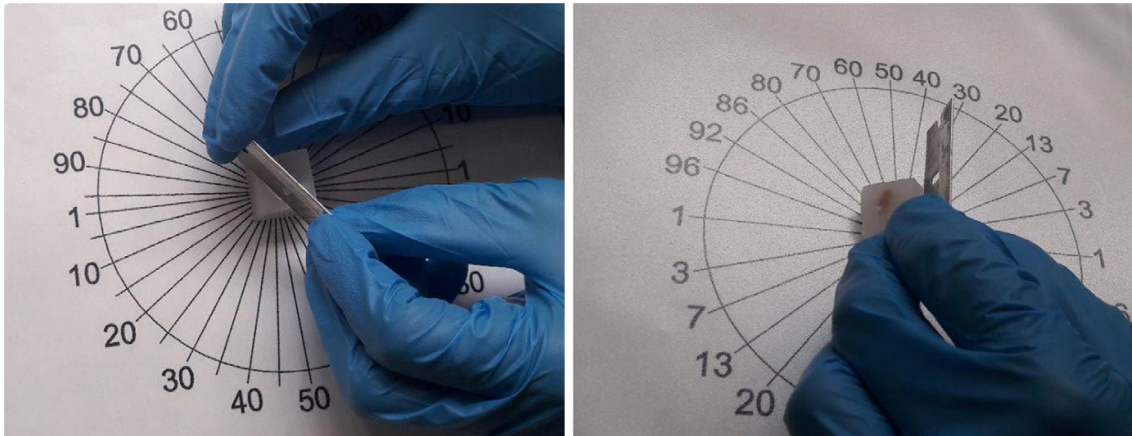
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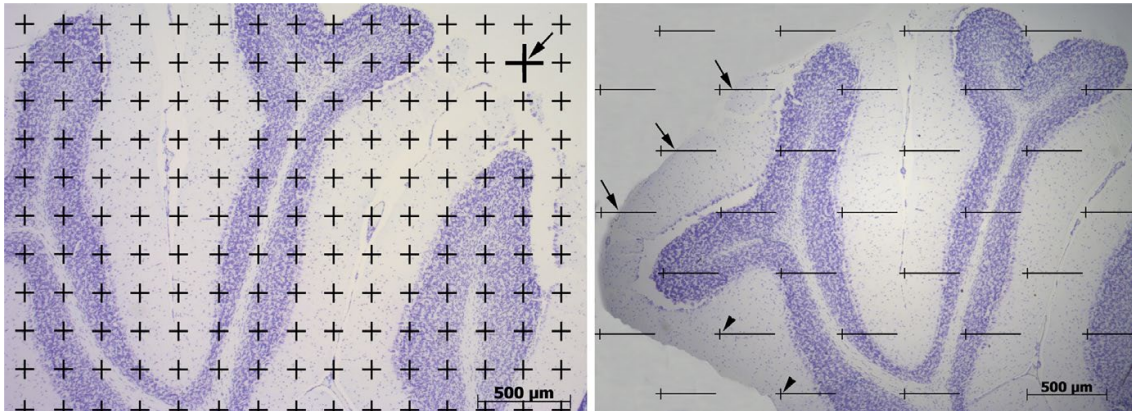




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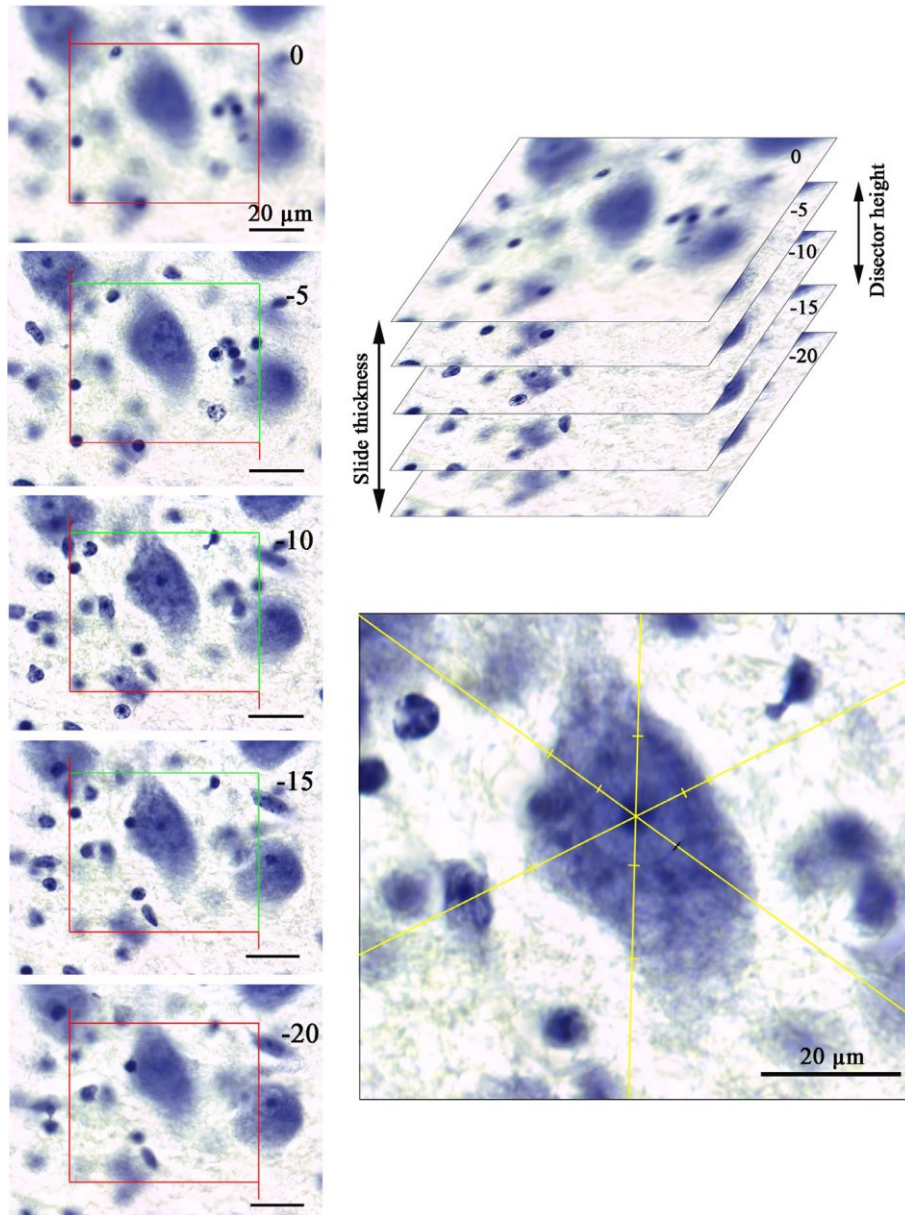
980 **Figure 1.** Isotropic, uniform random sections of the guinea-pig cerebellar  
981 hemispheres were obtained by applying the orientator method. (a): A  
982 randomly chosen cerebellar hemisphere for each animal was embedded in a  
983 paraffin block and placed at the centre of a circle with 90 equidistant  
984 divisions along the perimeter. A random number between 0 and 90 was  
985 looked up and the paraffin medium was cut along a line parallel to the  
986 direction of the selected number (here, 75). (b): The block was placed on its  
987 cut surface at the center of a second circle, with 96 nonequidistant divisions  
988 along its perimeter. The paraffin was cut along a line parallel to the  
989 direction of a random number ranging from 0 to 96 (here, 50), and the  
990 block was finally re-embedded in paraffin while placed on its cut surface,  
991 and consecutive 25  $\mu\text{m}$ -thick sections were cut with a microtome.

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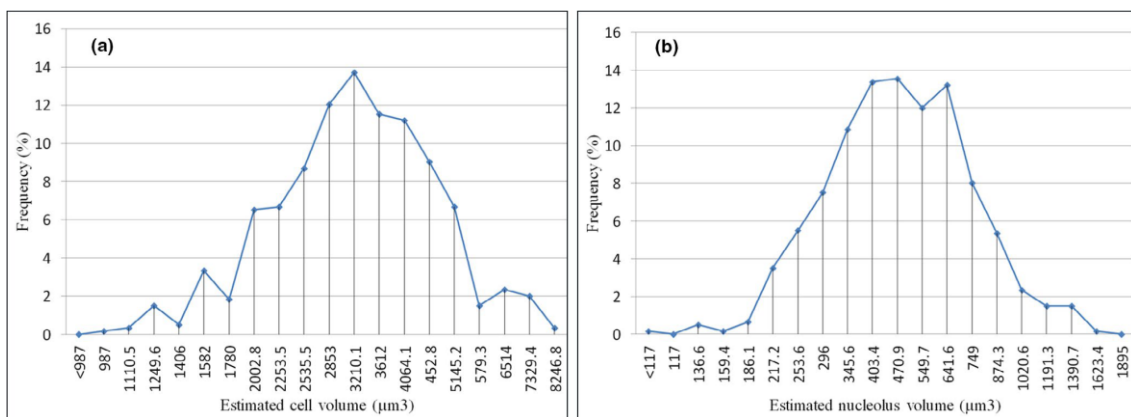
994 **Figure 2.** Estimation of cerebellar volume and surface area by employing  
995 the point-counting and the test-lines systems. (a): The volume of the  
996 cerebellar structures was estimated by randomly superimposing a point-  
997 counting probe onto each section. The upper right corner of each point  
998 (arrow) was taken as a reference for the count of the number of points  
999 hitting the region of interest. (b): The surface area of the cerebellum was  
1000 estimated by superimposing test-lines onto each section. The arrowheads  
1001 show two points hitting the molecular layer, whereas the arrows indicate  
1002 the intersection between test lines with the outer cerebellar surface.



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1004 **Figure 3.** Use of the optical disector technique for the Purkinje cell count  
 1005 and of the nucleator technique for the estimation of the Purkinje cellular  
 1006 and nuclear volumes. (a): The microscopic fields were selected by moving  
 1007 the microscope stage in the x and y directions for a constant distance. Then,  
 1008 the stage of microscope moved in z-axis and the consecutive focal planes  
 1009 were evaluated within optical disector height (10 μm from -5 to -15 μm).

1010 (b-f): The unbiased counting frame principle was applied for the Purkinje  
 1011 cell count. The cells whose nucleolus was located inside the counting frame  
 1012 or crossed the accepted lines were sampled, and those whose nucleolus  
 1013 came into focus within disector height were counted. (g): The intercept  
 1014 length from the nucleolus to the border of the cytoplasm, or to the border of  
 1015 the nucleus, was measured for the estimation of Purkinje cellular and  
 1016 nuclear volumes, respectively.  
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 1019 **Figure 4.** Graphs showing the frequency distribution of the Purkinje  
 1020 cellular (a) and nuclear (b) volumes in the guinea-pig cerebellum.

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