Article

Stereological Quantification of Cerebellar Purkinje Cells

Literature Review and Description of a Variation of the Physical Disector Method Adapted to Confocal Laser Microscopy

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ABSTRACT

Estimation of cerebellar Purkinje cell number is an important evaluation parameter for the study of the development of the cerebellum as well as for investigating the changes occurring in various experimental and physiological conditions. The introduction of stereological techniques in biological and biomedical research has made quantification of Purkinje cells not only more reliable, because independent from morphology-related sources of bias, but also easier and less time consuming. In this paper, we will briefly overview the stereological techniques that have been used so far for estimation of Purkinje cell number in different animal species and we will describe a simple method (the *confocal physical disector*) for Purkinje cell quantification on confocal microscope optical slices, based on a variation of the physical disector technique that can be adopted without the need of any dedicated stereological workstation.

Key Words:cerebellum, Purkinje cells, design-based stereology, disector, laser confocal microscopy, Cavalieri method

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Introduction

Various cerebellar diseases involve the loss of Purkinje cells (PCs) and consequent severe cerebellar dysfunction. Today, the continuously increasing availability of transgenic mouse models of many clinical conditions (Tos *et al.*, 2009) provides the researchers with a powerful tool for investigating etio-pathogenesis and find out effective therapeutic strategies for many diseases, including those affecting the cerebellum (Savill *et al.*, 2005).

To reach this goal, researchers need to obtain reliable quantitative estimations of tissue changes occurring during disease progression and treatment and estimation of PC number is one of the most important, if not the single most important, predictor of disease and recovery for many cerebellar diseases. Yet, it also represents the key predictor of cerebellar development in physiological and pathological. Stereology has developed over the last decades as a group of investigation strategies which allow obtaining reliable quantitative information on the 3D structure of a biological structure (e.g. number and size of cells in a give organ or part of it) starting from quantitative information obtained on 2D images (Gundersen *et al.*, 1988). Neuromorphology is definitely one of most important fields of application of stereological tools (West, 1999) and, in this context, quantitative assessment of cerebellar PCs has received much attention from stereologists since the new methods have started to spread within the neuroscience community.

The aim of this paper is to briefly overview some of the main applications of stereological techniques for PC number estimation. Yet, the paper also aims to describe a simple method for PC quantification on confocal microscope optical slices based on a variation of the physical disector technique.

Quantitative estimation of Purkinje cell number: a stereological perspective

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Quantitative assessment of PCs is probably one of the earliest applications of the emerging stereological techniques to neuromorphology. It was along the last two decades of the 20th Century that the first papers describing the use of the unbiased stereology for estimating PC number in normal and experimental conditions in the rat were published (Bedi *et al.* 1980; Harvey and Napper, 1988; Korbo *et al.*, 1993). In 1989, Nairm *et al.* obtained estimates of PC number in twelve human brains using systematic random sampling and the fractionator technique followed by Andersen *et al.* (1992) who carried out stereological estimates of the numbers PCs in another five human brains by optical disector and Cavalieri methods.

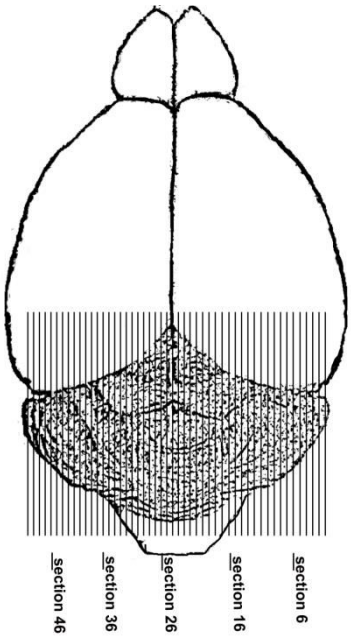

Figure 1.An example of systematic random sampling of cerebellar serial sections. After starting section is selected by chance (section n.6 in this example), the following sections that will be analyzed are selected by systematically jumping at a given distance (10 sections in this example) from the former section. The distance between sections is decided based on the amount of sampling (i.e. the total number of sections that are required by each study design).

Since it is beyond the aims of this paper to describe the basic principles of stereological quantification on histological sections, the readers could refer to various extensive methodological papers (Gundersen *et al.*, 1988; West, 1999; Geuna, 2000) including those published in this issue (Geuna *et al.*, 2012; Kaplan *et al.*, 2012), we will briefly overview some of the main papers that have been published dealing with studies which investigated, using stereological methods, the quantitative changes occurring in PC number in various physiological, experimental and pathological conditions, contributing to significant scientific advancements in this field.

One of the main fields that received a significantly benefit from stereological quantification of PCs is neurotoxicology. In fact, various studies allowed disclosing the effects of different chemicals, of environmental (including drugs) and alimentary origin, on the cerebellum, especially when exposure to substances occurs during development. These included acrylamide (Larsen *et al.*, 1994; Sørensen *et al.*, 2000), methyl mercury (Larsen and Braendgaard, 1995), zinc (Gokce *et al.*, 2011; cocaine (Chen *et al.*, 1996); diclofenac sodium (Ragbetli *et al.*, 2007; Odaci *et al.*, 2010), and nicotine (Chen *et al.*, 2003). However, among potential toxic agents, the single substance that received the higher attention by researchers is definitely ethanol because of the high incidence and prevalence of alcoholism in many Countries. A number of stereology-based experimental studies allowed to demonstrate that exposure to ethanol during pre- and post-natal life causes a reduction in the total number of PCs (Napper and West, 1995; Pauli *et al.*, 1995; Goodlett and Eilers, 1997; Goodlett *et al.*, 1998; Thomas *et al.*, 1998; Miki *et al.*, 1999; Maier and West, 2001) thus emphasizing one of the most critical negative effects of alcohol consumption on the nervous system. Stereological quantitative data on PC loss after ethanol exposure were confirmed also in large animal models (West *et al.*, 2001; Ramadoss *et al.*, 2007) and in human patients (Baker *et al.*, 1999; Andersen, 2004).

Interestingly, stereological research, not only trough a light on the damage occurring to the cerebellum after ethanol exposure, but also allowed to investigate the effects of potential therapeutic strategies aimed at preventing the negative effects of alcohol, such as antioxidants (Grisel and Chen, 2005), zinc supplementation (Chen *et al.*, 2001), NGF (Heaton *et al.*, 2000), and motor training (Klintsova *et al.*, 1997; 2000; 2002)

Also the effects of the exposure various physical agents on PC number has been assessed by stereology, in various animal models. For instance, it has been recently shown, in rodents, that exposure to mobile phone electromagnetic fields (Rağbetli *et al.*,

2010; Sonmez *et al.*, 2010), X-irradiation (Li *et al.*, 2002) and altered gravity (Sajdel-Sulkowska *et al.*, 2005) induces a decrease in PC number. Stereological investigation also showed that that neonatal pinealectomy induces PC loss in the cerebellum of the chick (Tink *et al.*, 2006).

The increasing availability of mutants and genetically-modified mice has also opened new perspectives for the adoption of the stereological quantification of PC number for investigating the mechanisms of cerebellar function in normal and pathological conditions. Using genetically-modified mice, as an example, stereological analysis have shown that PCs undergo a period of naturally occurring cell death that is mediated by the cell death proteins Bcl-2 and Bax (Fan *et al.*, 2001). Yet the use of stereological methods in PC degeneration mutants as well as in transgenic mouse models of cerebellar ataxia have throw a light on the neurochemical changes occurring in cerebellar disease (Baurle *et al.*, 1997) and on the potential effectiveness of innovative treatment strategies based on the tissue engineering (stem cell transplantation) approach (Chintawar *et al.*, 2009).

Application of stereology to the study of the changes in PC number in the human cerebellum deserve particular mention. First, it has been shown that age-related selective loss of PCs does not occur (as it was previously thought) in the entire cerebellar cortex, but it occurs in the anterior lobe only (Andersen *et al.*, 2003). In addition, stereological research has also focused on the relation between PC loss and neuropsychiatric disorders showing, partially unexpectedly, that the number of PCs is reduced in some forms of autism (Andersen *et al.*, 2010) whereas in schizophrenia and Alzheimer's disease it does not change significantly (Andersen and Pakkenberg, 2003; Whitney *et al*, 2008).

Noteworthy, stereology recently led to a significant methodological improvement in the study of PCs, both in animal models and in human brain samples, namely the demonstration that traditional Nissl staining may not be a reliable marker for PCs since it does not label all cells, while calbindin-D28k is a better alternative (Whitney *et al.*, 2008b)

The confocal physical disector: an adaptation of the physical disector procedure to confocal optical slice galleries

Although the cost of stereological softwares and workstations is being progressively reduced over time, they are still not available in all laboratories thus partly limiting the spread of the use of stereological tools in quantitative neuromorphology. Recently, we have developed a simple method based on the employment of the physical disector principle on confocal laser microscopy optical slices that could be thus named the "*confocal physical disector*". The method, that has already been successfully applied to the quantification of PC number in SCA1-mutant mice, with or without neuron stem cells grafting (Chintawar *et al.*, 2009), is based on a three step procedure carried out on paraformaldehyde transcardially perfused mouse brains, embedded in ice, that were cryostat sliced at 18um and sections were immunostained by calbindinD-28 to reveal PCs soma and their dendrites in the molecular layer. The first two steps are aimed at respecting the equal opportunity rule (Geuna, 2005), i.e. that all histological sections of the entire cerebellum (*first step*) and then, in any selected section, all PCs (*second step*), have the same chance of being sampled. The *third step* is aimed at estimating the reference volume in order to eventually estimate the total PC number based on the PC density obtained in the sampled sections.

First stereological step: selection of sections by systematic random sampling

First, a systematic random sampling scheme is adopted to reach the goal all parts of the entire cerebellum are covered by sampling and have the same probability of being selected in the final sample. Systematic random sampling is carried out by the systematic selection of every n^{th} unit of the population from one randomly selected starting unit (Geuna, 2000). In the case of PC quantification, the units are represented by the serial sections of the cerebellum. In practical terms (fig. 1), all the serial sagittal sections including the whole cerebellar cortex are numbered and then, after *starting* section is selected by chance, the following sections that will be analyzed are selected by systematically jumping at a given distance from the former section. The distance between sections (10 sections in fig. 1) is decided based on the amount of sampling (i.e.

the total number of sections that are required by each study design). In our experience, a set of 6-8 sections is enough to get a sufficient sample of PCs in the mouse cerebellum.

Second stereological step: selection of PCs in each selected section by confocal physical disector probes

Once a sufficient number of sections have been sampled by systematic random sampling, the second stereological step is to define a sample of PCs in each section that is unbiased with respect to their size, shape and orientation. The definition of a set of inclusion/exclusion rules for clearly determining which PC profile fiber falls inside the sampling field is based on the axiom:

$$Nprofiles > Ncells \quad (1)$$

where *Nprofiles* is the total number of cell profiles on serial sections of an organ (or part of it) that is always higher than the true number of cells *Ncells* (since, independently of the size of the cells and the section thickness, at least some of the cells will be split into two sections, generating two profiles of one single cell). In 1984, a method named "disector" was described as a set of rules for obtaining unbiased estimates of the true number of cells on histological sections (Sterio, 1984). In its original version (named *physical disector*) method is based on the use of sets of pairs of parallel sections separated by a known distance and the counting of only those particles that appear in one of the two histological sections (named reference section) and not in the other (named look-up section). This goal is obtained by counting "tops", i.e. the first part of the object that encounters the progressing plane of observation. The tops are localized by identifying the last-first profile of an object seen only in the reference section (and not in the look-up section) of the disector pair of sections. In the *optical disector*, top's identification is carried out on single sections, not on section pairs, and the 3-D disector probe is determined by focusing on the z-axis for a given distance and the cells are counted when they first come into focus within the sampling volume (Gundersen *et al.*, 1988).

The *confocal physical disector* that we describe here has already been used for PC number estimation in mice (Chintawar *et al.*, 2009) and is a method which combines the physical and optical disector procedures taking advantage of the possibility offered by laser confocal microscopy to obtain a set of serial optical slices inside the thickness of a histological section. Knowing the thickness of each optical slice (that can be set up by the investigator) a gallery of images can be used to define a *optical reference section* and an *optical look-up section* that can eventually be used to sample PCs according to the principles of the physical disector (i.e. counting of only those PCs that appear in the reference section and not in the look-up section). Figure 2 illustrates how this is done in practical terms.

The number of PCs (*N*) which respects the inclusion rules inside each disector probe volume (*V*) is then used to calculate PC density (*Nv*) according to the formula:

$$Nv = N/V \quad (2)$$

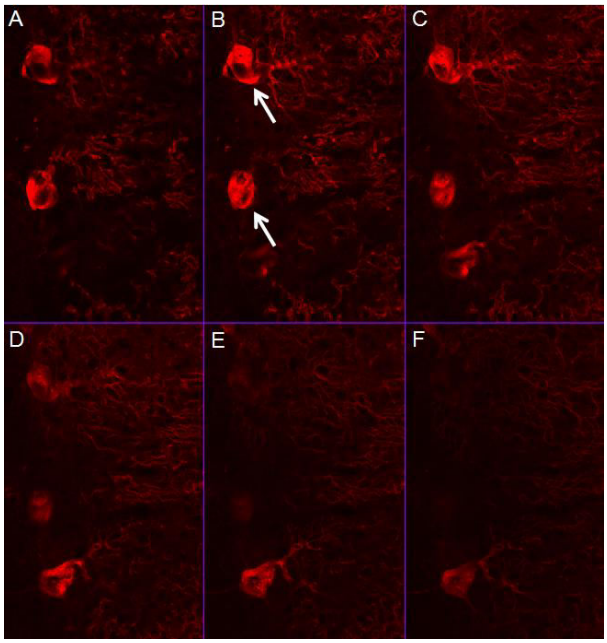

Figure 2. An example of confocal physical disector PC counting. A gallery of images (confocal optical sections taken inside the histological section's thickness) is created. Then, an *optical reference section* (panel B in this example) and an *optical look-up section* (panel E in this example) can be used to sample PCs according to the principles of the physical disector: namely, counting of only those PCs (arrows) that appear in the *reference section* and not in the *look-up section*.

Third stereological step: estimation of the reference volume in order to estimate the final total number of PCs

In order to obtain the final estimates of the total PC number from the average PC density assessed in the sampled sections, it is necessary to first estimate the reference

volume (*Vref*) of the structure under analysis. Provided that all PCs are included, the size of the reference volume could vary depending on the investigator choice: of course if a large reference volume (e.g. the entire cerebellum) is selected the average PC density will be lower, and vice versa, and thus size of this parameter is not expected to influence the final total number estimates. Therefore, selection of the reference volume should be guided by easiness in recognizing its borders on the histological sections in order to facilitate and speed up its measurement. In our experience, the best *Vref* for PC number estimation is the whole volume occupied by PC layer and molecular layer together (Fig. 2). In practical terms, *Vref* is estimated using the Cavalieri principle as follows: first the area (*A*) occupied by the PC and molecular layers is measured in each sections. Then, knowing the distance between sampled sections (calculated by multiplying the number of interval sections for the mean section thickness) the total reference volume is calculated according to the formula:

$$Vref = \Sigma A \times T \quad (3)$$

where ΣA is the sum of areas occupied by the PC and molecular layers and T is the sum of all distances between sampled sections.

Finally the total number (*Ntot*) of PCs is estimated, based on the values obtained as described in the formulas (2) and (3), according to the formula:

$$Ntot = Vref \cdot Nv \quad (4)$$

Conclusions

The spread among neuroscientists of the use of stereological techniques has made estimation of PC number, in various physiological and pathological conditions, not only independent from morphological bias and thus much more reliable than in the past, but also much easier and faster since the progress of stereological workstations have made them user-friendly and relatively simple to be used (Geuna, 2005). Yet, quantitative data from different studies can be more easily compared when unbiased stereological counting techniques are adopted.

Literature overview shows that the employment of stereological techniques to the study of PCs in various animals' species, including humans, has contributed to significant advancements in scientific knowledge about cerebellar changes in various physiological and pathological conditions.

Therefore, always keeping in mind that there is no absolutely correct method for solving any problem that involves inductive reasoning (Smith, 1994; Geuna, 2005) and that any experimental approach should be always critically assessed, it can be concluded that, today, the adoption of traditional assumption-based morphometrical methods for PC number estimation, especially those based on simple profile counting, is no longer justifiable. Researchers in the field should thus primarily consider the adoption of design-based stereological approaches for quantitative morphology of the cerebellum, and especially of PCs.

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References

Agashiwala RM, Louis ED, Hof PR, Perl DP. A novel approach to non-biased systematic random sampling: a stereologic estimate of Purkinje cells in the human cerebellum. Brain Res 2008; 1236:73-78.

Andersen BB, Korbo L, Pakkenberg B. A quantitative study of the human cerebellum with unbiased stereological techniques. J Comp Neurol 1992; 326(4):549-560.

Andersen BB, Gundersen HJ, Pakkenberg B. Aging of the human cerebellum: a stereological study. J Comp Neurol 2003; 466:356-365.

Andersen BB, Pakkenberg B. Stereological quantitation in cerebella from people with schizophrenia. Br J Psychiatry 2003; 182:354-361.

Andersen BB. Reduction of Purkinje cell volume in cerebellum of alcoholics. Brain Res 2004; 1007:10-18.

Baker KG, Harding AJ, Halliday GM, Kril JJ, Harper CG. Neuronal loss in functional zones of the cerebellum of chronic alcoholics with and without Wernicke's encephalopathy. Neuroscience. 1999;91:429-438.

Bäurle J, Grüsser-Cornehls U. Differential number of glycine- and GABA-immunopositive neurons and terminals in the deep cerebellar nuclei of normal and Purkinje cell degeneration mutant mice. J Comp Neurol 1997; 382:443-458.

Bedi KS, Hall R, Davies CA, Dobbing J. A stereological analysis of the cerebellar granule and Purkinje cells of 30-day-old and adult rats undernourished during early postnatal life. J Comp Neurol 1980; 193:863-870.

Chen WJ, Berryhill EC, West JR. Zinc supplementation does not attenuate alcohol-induced cerebellar Purkinje cell loss during the brain growth spurt period. Alcohol Clin Exp Res 2001; 25:600-605.

Chen WJ, Edwards RB, Romero RD, Parnell SE, Monk RJ. Long-term nicotine exposure reduces Purkinje

cell number in the adult rat cerebellar vermis. Neurotoxicol Teratol 2003; 25:329-334.

Chen WJ, McAlhany RE Jr, Maier SE, West JR. Cocaine exposure during the brain growth spurt failed to produce cerebellar Purkinje cell loss in rat pups. Teratology 1996; 53:145-151

Chintawar S, Hourez R, Ravella A, Gall D, Orduz D, Rai M, Bishop DP, Geuna S, Schiffmann SN, Pandolfo M. Grafting neural precursor cells promotes functional recovery in an SCA1 mouse model. J Neurosci 2009; 29:13126-13135.

Fan H, Favero M, Vogel MW. Elimination of Bax expression in mice increases cerebellar Purkinje cell numbers but not the number of granule cells. J Comp Neurol 2001; 436:82-91.

Geuna S, Gigo-Benato D, de Castro Rodrigues A. 2004. On sampling and sampling errors in histomorphometry of peripheral nerve fibers. Microsurgery 24:72-76.

Geuna S. 2000. Appreciating the difference between design-based and model-based sampling strategies in quantitative morphology of the nervous system. J Comp Neurol 427:333-339.

Geuna S. 2005. The revolution of counting "tops": two decades of the disector principle in morphological research. Microsc Res Tech 66:270-274.

Gökçe MF, Kaplan S, Türkkani A, Kozan R, Ayyildiz M, Emirzeoglu M, Aslan H, Marangoz C. 2011. Nitric oxide synthase inhibitors protect cerebellar Purkinje cells from zinc-induced cell loss in adult rat. J Chem Neuroanat 41:25-31.

Goodlett CR, Eilers AT. Alcohol-induced Purkinje cell loss with a single binge exposure in neonatal rats: a stereological study of temporal windows of vulnerability.Alcohol Clin Exp Res 1997; 21:738-744.

Goodlett CR, Pearlman AD, Lundahl KR. Binge neonatal alcohol intubations induce dose-dependent loss of Purkinje cells. Neurotoxicol Teratol 1998; 20:285-292.

Grisel JJ, Chen WJ. Antioxidant pretreatment does not ameliorate alcohol-induced Purkinje cell loss in the developing rat cerebellum. Alcohol Clin Exp Res 2005; 29:1223-1229.

Gundersen HJ, Bagger P, Bendtsen TF, Evans SM, Korbo L, Marcussen N, Møller A, Nielsen K, Nyengaard JR, Pakkenberg B. The new stereological tools: disector, fractionator, nucleator and point sampled intercepts and their use in pathological research and diagnosis. APMIS 1988; 96:857-881.

Harvey RJ, Napper RM. Quantitative study of granule and Purkinje cells in the cerebellar cortex of the rat. J Comp Neurol 1988; 274:151-157.

Heaton MB, Mitchell JJ, Paiva M. Overexpression of NGF ameliorates ethanol neurotoxicity in the developing cerebellum. J Neurobiol 2000; 45:95-104.

Jelsing J, Gundersen HJ, Nielsen R, Hemmingsen R, Pakkenberg B. The postnatal development of cerebellar Purkinje cells in the Göttingen minipig estimated with a new stereological sampling technique--the vertical bar fractionator. J Anat 2006; 209: 321-331.

Klintsova AY, Goodlett CR, Greenough WT. Therapeutic motor training ameliorates cerebellar effects of postnatal binge alcohol. Neurotoxicol Teratol 2000; 22:125-132.

Klintsova AY, Matthews JT, Goodlett CR, Napper RM, Greenough WT. Therapeutic motor training increases parallel fiber synapse number per Purkinje neuron in cerebellar cortex of rats given postnatal binge alcohol exposure: preliminary report. Alcohol Clin Exp Res 1997; 21:1257-1263.

Klintsova AY, Scamra C, Hoffman M, Napper RM, Goodlett CR, Greenough WT.Therapeutic effects of complex motor training on motor performance deficits induced by neonatal binge-like alcohol exposure in rats: II. A quantitative stereological study of synaptic plasticity in female rat cerebellum. Brain Res 2002; 937:83-93.

Korbo L, Andersen BB, Ladefoged O, Møller A. Total numbers of various cell types in rat cerebellar cortex estimated using an unbiased stereological method. Brain Res 1993; 609:262-268.

Larsen JO, Braendgaard H. Structural preservation of cerebellar granule cells following neurointoxication with methyl mercury: a stereological study of the rat cerebellum. Acta Neuropathol 1995; 90:251-256.

Li HP, Miki T, Gu H, Satriotomo I, Mastumoto Y, Kuma H, Gonzalez D, Bedi KS, Suwaki H, Takeuchi Y. 2002. The effect of the timing of prenatal X-irradiation on Purkinje cell numbers in rat cerebellum. Brain Res Dev Brain Res 139:159-166.

Maier SE, West JR. Regional differences in cell loss associated with binge-like alcohol exposure during the first two trimesters equivalent in the rat. Alcohol 2001; 23:49-57.

Napper RM, West JR. Permanent neuronal cell loss in the cerebellum of rats exposed to continuous low blood alcohol levels during the brain growth spurt: a stereological investigation. J Comp Neurol 1995; 362:283-292.

Odaci E, Cihan OF, Aslan H, Ragbetli MC, Kaplan S. Prenatal diclofenac sodium administration increases the number of Purkinje cells in female rats: a stereological study. Int J Dev Neurosci 2010; 28:145-151.

Overgaard Larsen J, Tandrup T, Braendgaard H. The volume of Purkinje cells decreases in the cerebellum of acrylamide-intoxicated rats, but no cells are lost. Acta Neuropathol 1994; 88:307-312

Pauli J, Wilce P, Bedi KS. Acute exposure to alcohol during early postnatal life causes a deficit in the total number of cerebellar Purkinje cells in the rat. J Comp Neurol 1995; 360:506-512.

Rağbetli MC, Aydinlioğlu A, Koyun N, Rağbetli C, Bektas S, Ozdemir S. The effect of mobile phone on the number of Purkinje cells: a stereological study. Int J Radiat Biol 2010; 86:548-554.

Ragbetli MC, Ozyurt B, Aslan H, Odaci E, Gokcimen A, Sahin B, Kaplan S. Effect of prenatal exposure to diclofenac sodium on Purkinje cell numbers in rat cerebellum: a stereological study. Brain Res 2007; 1174:130-135.

Ramadoss J, Lunde ER, Chen WJ, West JR, Cudd TA. Temporal vulnerability of fetal cerebellar Purkinje cells to chronic binge alcohol exposure: ovine model. Alcohol Clin Exp Res 2007; 31:1738-1745.

Sajdel-Sulkowska EM, Nguon K, Sulkowski ZL, Rosen GD, Baxter MG Purkinje cell loss accompanies motor impairment in rats developing at altered gravity. Neuroreport 2005; 16:2037-2040.

Savill RM, Scotting PJ, Coyle B. Strategies to investigate gene expression and function in granule cells. Cerebellum 2005; 4:271-278

Sonmez OF, Odaci E, Bas O, Kaplan S. Purkinje cell number decreases in the adult female rat cerebellum following exposure to 900 MHz electromagnetic field. Brain Res 2010; 1356:95-101.

Sørensen FW, Larsen JO, Eide R, Schiønning JD. Neuron loss in cerebellar cortex of rats exposed to mercury vapor: a stereological study. Acta Neuropathol 2000; 100:95-100.

Thomas JD, Goodlett CR, West JR. Alcohol-induced Purkinje cell loss depends on developmental timing of alcohol exposure and correlates with motor performance. Dev Brain Res 1998; 105:159-166.

Tos P, Ronchi G, Papalia I, Sallen V, Legagneux J, Geuna S, Giacobini-Robecchi MG.Methods and protocols in peripheral nerve regeneration experimental research: part I-experimental models. Int Rev Neurobiol 2009; 87:47-79.

Tunç AT, Turgut M, Aslan H, Sahin B, Yurtseven ME, Kaplan S. Neonatal pinealectomy induces Purkinje cell loss in the cerebellum of the chick: a stereological study. Brain Res 2006; 1067:95-102.

West JR, Parnell SE, Chen WJ, Cudd TA. Alcohol-mediated Purkinje cell loss in the absence of hypoxemia during the third trimester in an ovine model system. Alcohol Clin Exp Res 2001; 25:1051-1057.

West MJ. Stereological methods for estimating the total number of neurons and synapses: issues of precision and bias. Trends Neurosci 1999; 22:51-61.

Whitney ER, Kemper TL, Bauman ML, Rosene DL, Blatt GJ. Cerebellar Purkinje cells are reduced in a subpopulation of autistic brains: a stereological experiment using calbindin-D28k. Cerebellum 2008b; 7:406-416.

Whitney ER, Kemper TL, Rosene DL, Bauman ML, Blatt GJ. Calbindin-D28k is a more reliable marker of human Purkinje cells than standard Nissl stains: a stereological experiment. J Neurosci Methods 2008a; 168:42-47.