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Research Report

Effect of chronic ethanol ingestion on Purkinje and Golgi cell firing in vivo and on motor coordination in mice

Laurent Servais[a,c,*], Bertrand Bearzatto[a], Vinciane Delvaux[d], Etienne Noël[d], Robert Leach[e], Michèle Brasseur[f], Serge N. Schiffmann[a], Cheron Guy[a,b,*]

[a]*Laboratory of Neurophysiology, Université Libre de Bruxelles (ULB), Campus Erasme 808 Route de Lennik, 1070 Brussels, Belgium*
[b]*Laboratory of Movement Biomechanics, Université Libre de Bruxelles (ULB), Campus Erasme 808 Route de Lennik, 1070 Brussels, Belgium*
[c]*Laboratory of Electrophysiology, Université Mons-Hainaut (UMH), Mons, Belgium*
[d]*Provincial Institute of Bacteriology and Hygiene, Mons, Belgium*
[e]*Department of Emergency Medicine, Centre Hospitalier Interuniversitaire Ambroise Paré, Mons, Belgium*
[f]*Laboratory of medical biology, Centre Hospitalier Interuniversitaire Ambroise Paré, Mons, Belgium*

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Abstract

As motor coordination impairment is a common symptom of acute and chronic alcohol intoxication, different studies have been conducted on cerebellar Purkinje cell sensitivity to ethanol since Purkinje cell firing constitutes the final integrative output of the cerebellar cortex. However, the effects of chronic ethanol ingestion on Purkinje firing and other cerebellar neurons such as Golgi cells remain unknown. Here, we studied the extracellular discharge of Purkinje and Golgi cells in four groups of non-anesthetized mice drinking ad libitum either 0%, 6%, 12% or 18% ethanol isocallorically compensated with sucrose 25% during a 3-month period. No difference in Golgi cell firing was found with respect to ethanol consumption. The only group that presented significant differences in Purkinje cell firing compared to the other groups was the 18% ethanol-drinking group. These mice presented decreased simple spike and complex spike firing and increased complex spike duration and pause. The 18% ethanol-drinking group was also the only one to present a slight but significant motor coordination impairment (evaluated by rotarod and runway) in naïve task. No motor coordination impairment was noticed in task learned before ethanol consumption. These results suggest that chronic high doses of ethanol are necessary to produce Purkinje cell firing alterations and measurable motor coordination impairment in naïve task. These alterations in Purkinje cell firing did not affect the ability to learn or to recall a motor coordination task.

Theme: Systems and sensorimotor integration
Topic: Cerebellum

Keywords: Ethanol; Purkinje cell; Golgi cell; Motor coordination

1. Introduction

Acute and chronic alcohol intoxication in humans affects different brain structures, including the cerebellum, producing ataxia [8]. As Purkinje cells constitute the single output of the cerebellar cortex to the deep cerebellar nuclei, the alteration of their firing by ethanol is crucial in the understanding of ataxia produced by alcohol consumption. Therefore, the effects of acute ethanol exposure on Purkinje cell firing has been described in cellular culture [19], in slice preparation [20,52], in anesthetized [46,55] and in alert animals [13]. However, there is to our knowledge no report about Purkinje cell firing during chronic (>1 month) ethanol exposure.

* Corresponding authors. L. Servais and C. Guy are to be contacted at Laboratory of Electrophysiology, Université de Mons Hainaut, Mons, Belgium. Fax: +32 065373566.
E-mail addresses: servais.laurent@ulb.ac.be (L. Servais), gcheron@ulb.ac.be (C. Guy).

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Most of the studies about acute ethanol exposure have demonstrated an inhibitory effect of ethanol on Purkinje cells [13,20,52], but it seems that lower dose of ethanol may promote an exciting effect [13,19,55,56]. Effect on the regularity of Purkinje cell simple spike firing has been demonstrated in the different paradigms [13,19,46,52,55], while the effect on the climbing fiber response, the so-called complex spike, differs widely from one study to another [13,46,55]. Plausible mechanisms involved in the action of ethanol on Purkinje cells includes direct effects on them, such as AMPA receptor inhibition [40] or GABA receptor potentiation [31] and indirect effects through other cerebellar neurons, such as granule cells [19] that express NMDA receptors, another well-known target of ethanol inhibition [45]. In vivo, the effect of ethanol on Purkinje cells may also result from the inhibition of other brain regions that project onto the cerebellum through excitatory mossy fibers, such as locus caeruleus [44] or raphe nuclei [14].

Golgi cells constitute another key element of the cerebellar cortex, since their selective ablation leads to severe ataxia [59]. Moreover, their firing also reflects the excitation of the cerebellar cortex by mossy fibers, since mossy fibers excite granule cells that in turn excite Golgi cells through the same type of AMPA receptors as Purkinje cells. However, only one study has to our knowledge been conducted on the effects of ethanol on Golgi cells, demonstrating the excitatory effect of ethanol on these neurons in slice preparation [20].

To better understand the effects of chronic ethanol on cerebellar cortex function, we studied motor coordination and spontaneous Purkinje and Golgi cell firing in non-anesthetized mice after a 3-month period of ethanol consumption.

2. Methods

2.1. Mice

Three- to four-month-old male NMRI mice (n = 74) were randomly divided into four groups. In addition to their normal dry diet, mice received ad libitum the following solutions as sole drinking fluids [30]:

Group Et0 (n = 17): Sucrose 25% (1000 kcal/l)
Group Et6 (n = 16): Ethanol 6% (V/V)/sucrose 16.7% (1000 kcal/l)
Group Et12 (n = 17): Ethanol 12% (V/V)/sucrose 8.4% (1000 kcal/l)
Group Et18 (n = 24): Ethanol 18% (V/V) (1000 kcal/l)

Mice of a same group were numbered and raised together. Each mouse was weighed before ethanol administration and their weight was monitored each month. Dry diet and fluid intake were also monitored each week for each group, in order to evaluate if the chronic ingestion of the different solutions would cause a difference in blood alcohol levels, individual blood alcohol level was measured in sera using the TDx/TDxFLx REA® Ethanol assay (Abbot Laboratories Diagnostics Division, USA) just before animal sacrifice. Osmolality was measured using the FISKE® 2400 Multi-Sample Osmometer (Fiske® Associates, Norwood, Massachusetts, USA).

The data from the mice that died during the 3-month period were excluded (n = 2, 1 and 6 in groups Group Et6, Et12 and Et18, respectively). All mice were tested for motor coordination. After the last test, six and five randomly selected mice from each group were used for electrophysiological and histochemical analysis, respectively.

2.2. Motor coordination tests

2.2.1. The accelerating rotarod

The rotarod apparatus (accelerating model Ugo Basile) consisted of a plastic roller (3 cm in diameter) with small grooves running along its turning axis. Mice received 4 trials per day. During each trial, animals were placed on the rod rotating at a constant speed (4 rpm), and as soon as all the animals were placed on the rod, the rod started to accelerate continuously from 4 to 40 rpm over 300 s. The latency to fall off the rotarod was recorded. Animals staying for 300 s were taken from the rotarod and recorded as 300 s.

2.2.2. The runway test

In this test, mice ran along an elevated runway with low obstacles intended to impede their progress. The runway was 100 cm long and 1.2 cm wide. Obstacles consisting of wood rods (1 cm diameter, 1.2 cm width) were disposed every 10 cm. The number of slips of the left hind leg was counted. Mice were placed on one brightly illuminated extremity of the runway and had to run to the other side to return to their cage.

In each group, mice were divided into a “rotarod” group that underwent a training session in the rotarod test before ethanol administration and into a “runway” group that underwent a similar training session in the runway test before ethanol administration. The training session lasted 5 days (4 trials/day). The task performed during this session will be further called the “learned task”. Ethanol administration began after the last day of the training session. After 1, 2 and 3 months, each mouse had to accomplish four consecutive trials of the learned task. The day after these last trials in the learned task, the mice that were trained with accelerating rotarod were tested with the runway (four consecutive trials), and the mice that were trained with the runway were tested with the accelerating rotarod (four consecutive trials). This task performed by the mice for the first time after 3 months of ethanol administration will be further called the “naïve task”.

2.3. Histochemistry

Under general anesthesia (Avertin 0.02 ml/g), mice were perfused with 20 ml PBS followed by 20 ml PAF. The

mice were then decapitated and the brain was removed and fixed for 24 h in 0.1 M phosphate-buffered 4% paraformaldehyde freshly prepared at 4 °C. Following a wash in 0.1 M PBS and consecutive 24-h incubations in 10%, 20% and 30% sucrose solutions, the brains were then embedded in Tissue-Tek OCT compound (Miles, Elkhart, IN), frozen in 2-methyl butane, cooled on dry ice and stored at −80 °C. Parasagittal sections, 15-μm-thick, were serially cut, mounted onto slides coated with poly-L-lysine. Mounted tissue sections were stored at −20 °C until use. Sections were labeled using a rabbit polyclonal anti-calbindin antiserum (1/5000; Swant). After quenching of the endogenous peroxidases, the sections were incubated for blocking 1 h in phosphate-buffered saline (PBS, pH 7.4) containing 10% swine normal horse serum (Hormonologie Laboratoire, Marloie, Belgium) and 0.1% Triton X-100. Sections were then incubated 36 h at 4 °C with the primary antibodies a polyclonal anti-calbindin antiserum (1/5000; Swant) diluted in PBS. Sections were then successively incubated with biotinylated donkey anti-rabbit IgG (H + L) (1/200; Jackson) and ABC complex (Elite ABC kit; Vector). The peroxidase activity was revealed by diaminobenzidine in the presence of hydrogen peroxide.

2.4. In vivo electrophysiology

2.4.1. Surgical preparation for recording

Under general anesthesia with xylido-dihydrothiazin (Rompun®, Bayer, 10 mg/kg) and ketamine (Ketalar®, Pfizer, 100 mg/kg), 24 mice (divided equally between the four groups) aged 7–9 months were prepared for chronic extracellular recording of neuronal activity in the cerebellum [11]. Anesthesia was monitored clinically and a supplemental dose of xylido-dihydrothiazin 3 mg/kg and ketamine 30 mg/kg was administered if the animal presented agitation or markedly increased respiration or heart rate during the procedure. In addition, local anesthesia with 0.5 ml Lidocaine 20 mg/ml + Adrenaline 1:80000 (Xylocaine®, Astra Zeneca) was administered subcutaneously during soft tissue removal. Two small bolts were cemented to the skull to immobilize the head during the experimental session. The surface of the cerebellum was exposed by reflecting the muscles overlying the cisterna magna, a craniotomy was performed above the cerebellum, and an acrylic recording chamber constructed around the craniotomy. Mice were allowed to recover from anesthesia for 24 h with their usual fluid to drink. The dura mater was removed locally above the vermis just prior to the recording session.

Single unit recordings were performed with glass micropipettes (1.5–5 MΩ impedance) in lobules IV–VIII. A signal was considered as originating from a Purkinje cell, if it presented two types of spiking activities: simple spikes characterized by single depolarization (300–800 μs) occurring at high frequencies (~50 Hz), and less frequent complex spikes (0.5–1 Hz) characterized by an initial fast depolarization (300–600 μs) followed by smaller components in a relatively consistent manner for the same Purkinje cell. Golgi cells were identified according to their firing properties [18,54], namely, a slow (4–10 Hz) and irregular discharge with interspike intervals always superior to 50 ms. After amplification (by a factor of 1000–2000) and band-pass filtering (10 Hz–10 kHz), unit activity was continuously stored on 4 mm digital audio tapes, transferred to a Pentium III personal computer with analog-to-digital converter boards (Power 1401, CED) and analyzed off-line with Spike 2 CED software. The recorded data were digitized continuously at 10 kHz.

For each Purkinje cell recording lasting more than 2 min, the simple spike autocorrelogram was constructed using time bins of 1 ms. The strength of the rhythmicity was quantified with a rhythm index [57]. The height and depth of all peaks and valleys that were significantly different from the baseline and that occurred at specific latencies with regard to the initial peak were summed. The sum was divided by the total number of spikes in the recording. In the autocorrelograms with no significant peaks and valleys, a value of zero was given to the rhythm index, and the activity was considered as non-rhythmic. The regularity of Purkinje and Golgi cells was measured using the coefficient of variation, defined as the standard deviation of the interspike intervals divided by their mean value. Golgi cell spike and Purkinje cell complex spike durations were measured on the averaged trace of their corresponding spikes.

Results are expressed and illustrated as mean ± SD. Groups were compared using two-way analysis of variance (ANOVA) and Bonferroni's post hoc test and were considered as significantly different for $P < 0.05$. In the analysis of behavioral data, to distinguish between motor coordination impairment and possible compensation by motor learning, we performed one-way ANOVA for each trial followed by two-way ANOVA for repeated measures and post hoc test on the complete set of values.

3. Results

3.1. Mice

Fluid intake decreased significantly according to ethanol concentration (Table 1). However, the daily ethanol intake and the mean blood alcohol level at the end of the study remained significantly proportional to ethanol concentration (Table 1). Weight gain during the test period was inversely proportional to ethanol concentration in the fluids (Table 1). To determine whether this difference in weight gain was due to malnutrition induced by ethanol or to the sucrose consumption in Et0, the daily dry diet intake of each group was compared. A positive correlation between ethanol concentration in the fluids and dry diet intake was found (Table 1). To further assess the cause of

Table 1
Nutritional data of the experimental groups

	Et0	Et6	Et12	Et18	Controls
Daily fluid intake (ml/day/mouse)	15.7 ± 1.9	14.4 ± 2.2	9.4 ± 1.0	8.0 ± 1.2	11.6 ± 1.5
Daily ethanol intake (ml/day/mouse)	0	0.86 ± 0.13	1.12 ± 0.11	1.45 ± 0.21	
Mean blood alcohol level (mg/dl)	2 ± 2	13 ± 14	33 ± 57	87 ± 64	
Monthly weight gain (g/mouse/month)	0.83 ± 0.49	0.40 ± 0.45	0.07 ± 0.67	−1.8 ± 0.8	0.80 ± 0.30
Daily dry diet intake (g/day/mouse)	4.2 ± 0.5	4.5 ± 0.7	4.6 ± 0.8	4.7 ± 0.7	7.2 ± 0.9
Osmolality (mM/l)	340 ± 9	361 ± 12	349 ± 18	363 ± 13	322 ± 8

the difference in weight gain between groups, we measured the weight gain, the fluid and the dry diet intake of an age-matched NMRI mice group (n = 7) drinking water ad libitum. These mice demonstrated a weight gain similar to the one observed in Et0, but their dry food intake was significantly higher than the Et0 group, whereas their fluid intake was lower. To rule out that the high blood alcohol level in Et_{18} was overestimated by dehydration, we determined osmolality in the blood of the 4 experimental groups and of the water-drinking group (Table 1). When compared with the water-drinking group, all experimental groups presented a significantly increased osmolality, as did the Et_{18} when compared with the Et_0. However, there was no significant difference between the different ethanol-drinking groups (Et_6, Et_{12} and Et_{18}), even when pooling the Et_6 and Et_{12} mice. This indicates that the higher level of ethanol in Et_{18} is not due to an increased level of dehydration in these mice.

3.2. Motor coordination

Before ethanol consumption, 40 mice were trained in the runway test (Et0 (n = 11), Et6 (n = 8), Et12 (n = 10) and Et18 (n = 11); Fig. 1A, left) and 25 were trained in the rotarod (Et0 (n = 6), Et6 (n = 6), Et12 (n = 6) and Et18 (n = 7); Fig. 1B, left). After 1, 2 and 3 months of ethanol consumption, mice were re-tested in their learned task. No differences could be pointed out between the different groups whether in runway (Fig. 1A, center) or in rotarod. (Fig. 1B, center). This demonstrates that 3 months of ethanol consumption did not

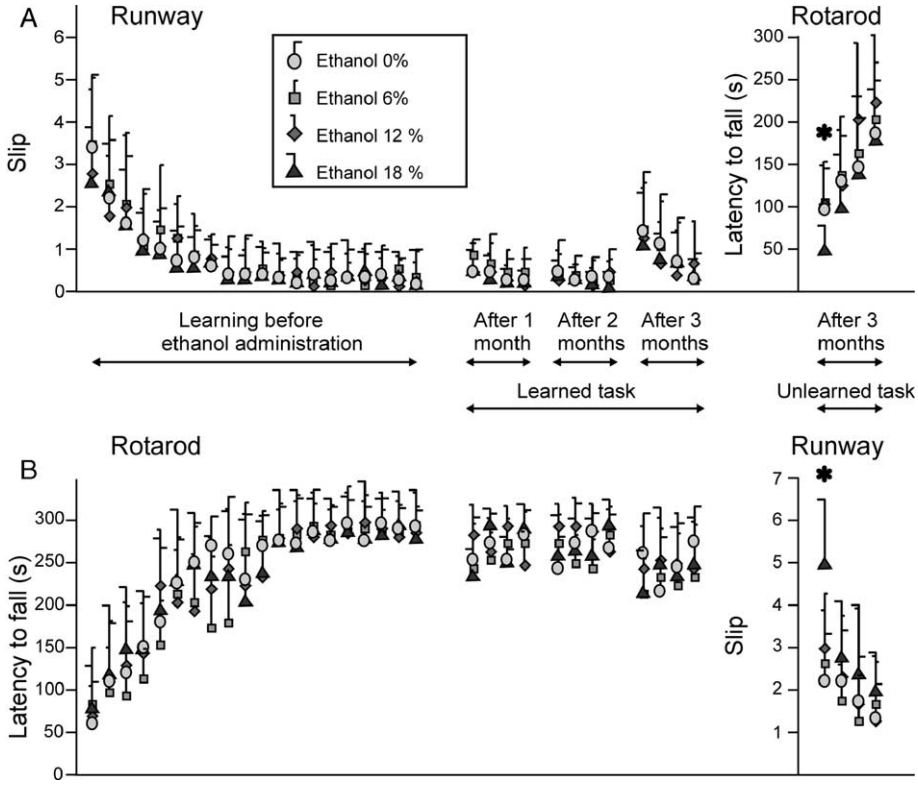

Fig. 1. Chronic ingestion of high doses of ethanol produces motor coordination impairment in naïve, but not in learned tasks. (A) Learning curves of mice before ethanol ingestion in the runway test (left). Monthly trials in the runway test of the same mice after 1, 2 and 3 months of 0% (n = 11), 6% (n = 8), 12% (n = 10) or 18% (n = 11) ethanol ingestion. (Center) Rotarod performance in these same mice after 3 months of ethanol ingestion. Asterisk indicates significant difference with the control group ($P < 0.05$). (B) Learning curves of mice before ethanol ingestion in the rotarod test (left). Monthly trials in the rotarod test in these same mice after 1, 2 and 3 months of 0% (n = 6), 6% (n = 6), 12% (n = 6) or 18% (n = 7) ethanol ingestion. (Center) Runway performance of these same mice after 3 months of ethanol ingestion.

produce a measurable motor coordination impairment in the execution of a task learned before alcohol consumption. Then in order to test the motor coordination in a naïve task, mice trained with the runway were tested with the rotarod (Fig. 1A, right) and those trained with the rotarod were tested with the runway (Fig. 1B, right). During the first trial, mice of Et18 demonstrated a significant impairment in comparison with all the other groups whatever the naïve task, while the other ethanol-consuming groups did not demonstrate any significant impairment. The second and subsequent trials of the naïve task did not demonstrate a significant difference between Et18 and the other groups because of rapid motor learning in all groups. Two-way ANOVA for repeated measures did not show any significant differences between groups.

No significant relationship was found between alcohol level measured before animal sacrifice and motor performances.

3.3. Electrophysiology

A total of 90 Purkinje cells were recorded (n = 24,18, 24 and 24 in Et0, Et6, Et12, and Et18, respectively). A typical extracellular recording is illustrated in Fig. 2A. Simple (center) and complex (right) spikes may easily be discriminated and averaged.

Complex spike firing rate was decreased in Et18 in comparison with all groups, whereas no significant differences could be pointed out between Et6, Et12 and Et0 (Fig. 2B). The complex spike duration (Fig. 2C) and pause (Fig. 2D) of Purkinje cells recorded in Et18 were longer than in Et0. Again, no significant differences were found between the other groups.

The alterations in simple spike firing in ethanol-consuming groups were also restricted to Et18. Indeed, these mice demonstrated a decreased simple spike firing rate (Fig. 2E) and regularity (Fig. 2G) in comparison with all the other groups, whereas the other ethanol-consuming groups did not show any significant differences with Et0. Rhythmicity of simple spike firing did not differ between groups (Fig. 2F). We did not find a significant relationship between alcohol level measured before animal sacrifice and Purkinje cell firing alterations.

The Golgi cell is the other cerebellar neuron that can be easily identified on the basis of its spontaneous firing in vivo [18,54]. A typical recording is illustrated in Fig. 2H (left). Spikes may be easily discriminated and averaged (center). To determine if ethanol consumption would inhibit Golgi cells as it inhibits Purkinje and olivary neurons, we compared Golgi cell firing rate (Fig. 2I), duration (Fig. 2J) and regularity (Fig. 2K) in Et0 (n = 8), Et6 (n = 5), Et12 (n = 5) and in Et18 (n = 6). No significant differences could be pointed out between these groups.

3.4. Histochemistry

Granule cells are more sensitive than Purkinje cells to chronic ethanol consumption, so that the granular layer is

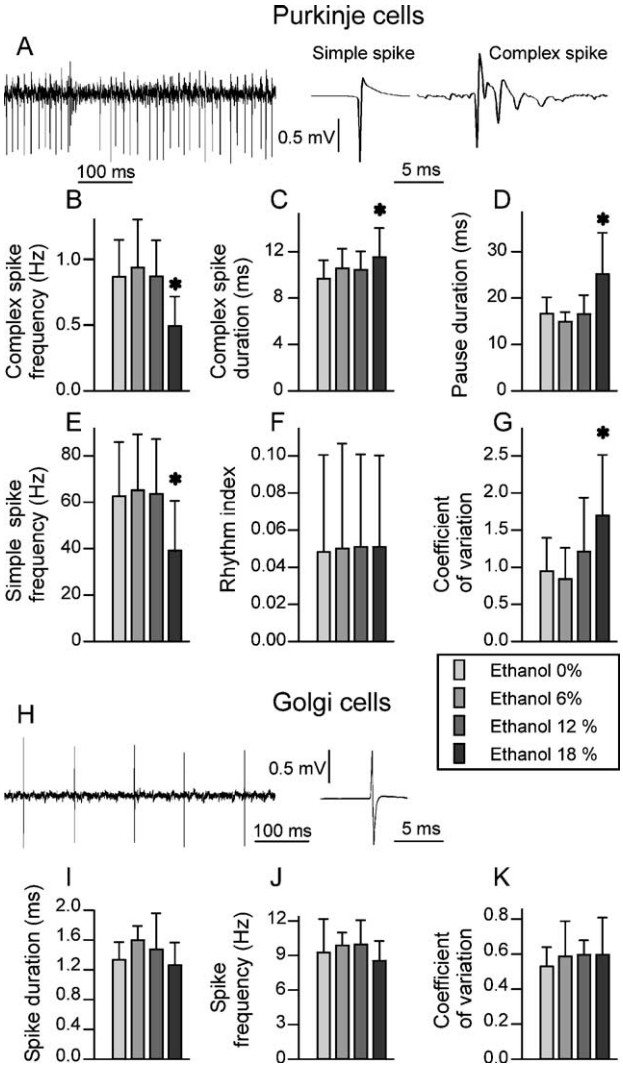

Fig. 2. Chronic ingestion of high doses of ethanol produces alteration in Purkinje, but not in Golgi cell firing. (A). Recording of a Purkinje cell in a control mouse (left). Averaged trace of the simple (center) and complex (right) spike. (B–D) Complex spike frequency (B), duration (C) and pause (D) in 0% (n = 24), 6% (n = 18), 12% (n = 24) and 18% (n = 24) ethanol-drinking mice after 3 months ethanol consumption. Asterisks indicate significant difference with the control group ($P < 0.05$). (E–G) Simple spike frequency (E), rhythmicity (F) and regularity (G), same cells. (H) Golgi cell recording in a control mouse (left) and averaged trace of its spike (right). (I–K) Golgi cells spikes frequency (I), duration (J) and regularity (K), in the same mice as in panels B–G (n = 8, 5, 5 and 6 in Et_0, Et_6, Et_{12} and Et_{18}, respectively.

significantly thinned before any Purkinje cell loss during long-term ethanol ingestion [58]. To determine if this could be a plausible hypothesis to explain depressed Purkinje cell firing in the present model, we measured granular layer and counted Purkinje cells in the lobule IV from lateral and median vermis of 3 Et0 and 3 Et18 mice. Purkinje cells soma and dendrites labeling by calbindin was more pronounced in Et0, but mean Purkinje cells number was not different in Et18 in comparison with Et0 (Fig. 3) (42.9 ± 12.6 (n = 16) vs. 40.8 ± 7.7 (n = 12). Mean granular layer thickness measured in ten randomly selected areas of lobule IV on the same slice was not

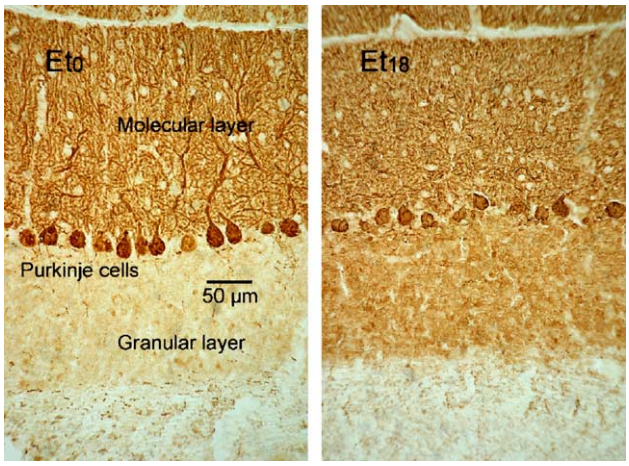

Fig. 3. Immunohistology of cerebellar cortex in Et0 (left) and Et18 (right), labeling by anticalbindin antibody. Note that despite a lesser labeling in the soma, and even more so in the dendrites of Purkinje cells in Et18, Purkinje cell density and granular layer thickness are similar in both groups.

significantly different in Et0 when compared with Et18 (84.7 ± 25.9 vs. 85.9 ± 24.7 µm).

4. Discussion

The present results demonstrate that long-term ingestion of high doses of ethanol causes motor coordination impairment in naïve tasks and a decrease in spontaneous firing of the Purkinje cell simple and complex spikes.

Since Purkinje cells constitute the sole output of cerebellar cortex, the regulation of their firing is crucial for cerebellar function [1,28,36]. As motor coordination impairment is a common symptom of acute, chronic and even fetal ethanol intoxication in humans [6,8,9,35], the effect of pre- and postnatal exposure to ethanol on Purkinje cell morphology has been extensively studied for more than 20 years [16,32,38,43,58]. However, the effects of chronic ethanol administration (>1 month) on Purkinje cell function, namely, their in vivo firing, have to our knowledge not yet been reported. Rogers and colleagues did not point out any significant change in Purkinje cell firing after 11–14 days ethanol treatment leading to a blood alcohol level around 150 mg/dl [46]. This could be due either to the short period of ethanol administration, either to the paradigm of this study realized on halothane-anesthetized rats. Indeed, this study also failed to detect any significant change in simple spike firing following acute ethanol administration, which contrasts with all other reports [13,55].

Spontaneous simple spike firing reflects Purkinje cell intrinsic excitability and the integration of inhibition by the molecular interneurons and of the excitation by parallel fiber and climbing fiber input [17,48,51]. Complex spikes directly reflect the firing activity of olivary neurons because every discharge of an olivary neuron produces a complex spike in the targeted Purkinje cells [29]. The present results mainly demonstrate the depressing effect of high doses ethanol consumption on Purkinje cell simple spike and complex spike firing. The decrease in complex spike activity in Et18 may not explain the decrease in the simple spike firing rate observed in these mice, as decreased climbing fiber activity normally tends to increase Purkinje cell simple spike firing [5,39].

In contrast with the clear effects of long-term high concentration ethanol consumption on Purkinje cell firing, Golgi cell firing was not inhibited by chronic ethanol consumption. This demonstrates the different sensitivity of cerebellar neurons to chronic ethanol administration. The depressing effect of chronic ethanol consumption on Purkinje cell firing might be related to the inhibiting effects of acute ethanol administration that has been characterized by in vitro [20,52] and in vivo [13] studies. Different molecular targets mediate this inhibition, namely, GABA receptor activation [24,31,62] or glutamate receptor inhibition [23,40,61]. In addition, the sensitivity of other cerebellar cells such as granule cells [19,24] may also mediate a network effect.

In chronic administration, one may not only consider the direct excitatory or inhibitory effect of ethanol on the different receptors or channels, but also the alterations in their function, their structure and their expression [15]. For instance, chronic ethanol exposure increases both NMDA and AMPA receptor subunit expression in different brain regions [42] and Ca^{+} entry following NMDA [27] or AMPA [41] application. Chronic ethanol exposure also alters GABAa receptor gene expression and function [47]. In addition, chronic ethanol exposure also causes different structural and ultrastructural changes [16,32,43], biochemical induction [26] and death of certain cells [58]. About this later point, our findings are in line with the report of Tavares and colleagues who did not find any Purkinje cell death after 3 months of ethanol administration [58]. However, we did not find any reduction of the granular layer in Et18 mice. Despite the similarity in Purkinje cells number and granular layer thickness between chronic ethanol-consuming mice and controls, the immunolabeling of Purkinje cells dendritic tree by anti-calbindin-antibody appeared stronger in the latter, even this feature is difficult to quantify. This is in line with the findings of Pentney and Dlugos [43], who demonstrated that long-term ethanol ingestion in rats results in longer terminal dendritic segments. These authors have proposed that such alterations could be the result of smooth endoplasmic reticulum dilatation [32] that is known to be associated with imbalances in the intracellular calcium concentration [21]. Since calbindin immunoreactivity depends on the calcium concentration [50], this could explain the lesser labeling in Et18 mice.

Do these different structural, ultrastructural or molecular effects of chronic ethanol ingestion on the cerebellar cortex result in a net inhibition or excitation of its sole output, namely, the Purkinje cells? The present results demonstrate that high dose ethanol in chronic consumption are required to

elicit a net inhibition of Purkinje cells. In addition to the different mechanisms directly induced by ethanol, malnutrition may also enhance direct ethanol action on Purkinje cells. Indeed, malnutrition by itself induces cytoplasmic and synaptic structural change in Purkinje cells [10] and decreases Purkinje cell excitability [53]. To distinguish between the direct effects of ethanol and more complex malnutrition-induced deficit remain challenging, even when administrating isocaloric fluids to controls. Indeed, malnutrition in chronic alcoholism results not only from decreased food intake, but also from more complex factors such as malabsorption or catabolism induction [33]. For instance, long-term ethanol ingestion in humans results in cytochrome P450 induction, which decreases the half-life of different substrates. This is well illustrated in the present model, since mice of all ethanol-consuming groups presented decreased weight gain when compared with controls, despite increased dry diet intake. However, as only Et18 present Purkinje cell firing alteration and motor coordination impairment, malnutrition may not be considered as the sole factor that mediates cerebellar dysfunction. Ethanol-consuming mice may be mildly dehydrated, as suggested by their increased osmolality when compared to controls. However, the osmolality was increased in all mice groups, including the Et0, when compared to water-drinking mice. Moreover, osmolality was not significantly different in Et18 when compared with other ethanol-consuming group. This not only indicates that blood alcohol level in Et18 is not largely overestimated by dehydration, but also that this condition does not account for the specific behavioral impairment and electrophysiological alterations of this group.

The variation of mean alcohol blood level among a same group of mice is similar to that observed in previous studies concerning chronic ethanol ingestion [37,60]. This variation not only reflects the difference in ethanol metabolization in the different animals, but also the time differences between the blood dosage sampling and the last spontaneous fluid intake by the animal. Therefore, the blood alcohol dosage was realized rather to demonstrate that the mean blood alcohol level was proportional to the concentration in the consumed fluid rather than to correlate the performance of each animal with its alcohol blood level.

Rotarod and runway tests are frequently used to evaluate motor coordination impairment in mice with cerebellar diseases, including ethanol intoxication [24]. Despite their rather different design, these two tests have been demonstrated to provide similar motor coordination evaluation, when they are correctly adapted to the performances expected from the tested mice [3]. In the present protocol, it cannot be ruled out that mice presented cross-learning between the two tasks, since all groups of mice performed better in the rotarod when this test was the "naïve task", rather than when this was the first task learned. However, this difference may also be explained by the age difference between mice that performed rotarod as a naive task (~7 months old) and mice that performed rotarod as a learned task (~4 months old). Indeed motor performance of mice in the different motor coordination tests is highly age dependant [3]. Rotarod performances also depend on animal weight that is positively correlated with age [7]. Moreover, we did not observe similar differences in runway performance, which demonstrates that the so-called naïve tasks may really be considered as naïve.

In the present study, the Et18 group, which was the only one to present significant electrophysiological differences, also demonstrated motor coordination impairment in naïve tasks, whereas the other groups did not. This strongly suggests that alterations in Purkinje cell firing regulation in mice chronically drinking high doses of ethanol could cause motor coordination impairment when mice are challenged in a task that they do not know, while their ability to learn or to recall remains intact. The fact that the difference between Et18 and other mice was limited to the first trial of the naïve task and disappeared with motor learning is not surprising, since this statistically significant difference was minor. In different transgenic mice models [50] or genetic background [3,4] that present mild (that requires specific motor test to be pointed out) motor coordination impairment when compared with controls or with other backgrounds, it is common to observe the disappearance of motor coordination impairment through trials because of motor learning. In contrast, severe impairment that may be noticed without any motor task generally remains throughout the trials [49].

The normal performances of the same mice in learned tasks strongly suggest that the impairment observed in naïve tasks does not result from chronic neuropathy [30] or ethanol-induced myopathy, but from central nervous system dysfunction.

Purkinje cells firing rate at rest is around 50 Hz in mammals [12,22,25,34,50]. Different cerebellum-targeted mutant models have already demonstrated that altered Purkinje cell simple spike and complex spike firing are associated with motor coordination impairment despite a normal ability to improve motor task tests through trials [2,4]. However, the pattern of Purkinje cell alteration was completely different in these mice. Indeed, they present shorter complex spike pause and duration and increased simple spike firing rate, rhythmicity and synchronicity, giving rise to fast local field potential oscillation. In contrast, the present toxic model shows depressed Purkinje cell activity, no oscillation and a slight motor coordination impairment rapidly compensated by motor learning. This suggests that precise Purkinje cell firing level at rest is necessary for the cerebellar cortex to optimally integrate sensory input and provide rapid motor adaptation to new situations. In addition to the differences observed in simple and complex spike firing rate, Et18 mice present an increased coefficient of variation, indicating simple spike irregularity. Increased simple spike irregularity has also been recently demonstrated in absence of any change in simple and complex spikes firing in another model of ataxic mice lacking P/Q-type voltage-gated calcium channels [25].

In conclusion, the present model demonstrates that in mice high doses of ethanol are required in chronic ingestion to cause functional Purkinje cell alterations and motor coordination impairment in the execution of naïve tasks.

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References

[1] J.S. Albus, A theory of cerebellar function, Math. Biosci. 10 (1971) 25–26.
[2] J.J. Barski, J. Hartmann, C.R. Rose, F. Hoebeek, K. Morl, M. Noll-Hussong, C.I. De Zeeuw, A. Konnerth, M. Meyer, Calbindin in cerebellar Purkinje cells is a critical determinant of the precision of motor coordination, J. Neurosci. 23 (2003) 3469–3477.
[3] B. Bearzatto, L. Servais, G. Cheron, S.N. Schiffmann, Age-dependence of strain determinant on mice motor coordination, Brain Res. 1039 (2005) 37–42.
[4] B. Bearzatto, L. Servais, D. Gall, C. Roussel, F. Baba-Aïssa, A. De Kerchove d'Exaerde, S. Schurmans, G. Cheron, S.N. Schiffmann, Selective expression of calretinin into cerebellar granule cells is essential for granule cell excitability, Purkinje cells firing, and motor coordination, Soc. Neurosci (2004) Abstract No. 827.9.
[5] F. Bengtsson, P. Svensson, G. Hesslow, Feedback control of Purkinje cell activity by the cerebello-olivary pathway, Eur. J. Neurosci. 20 (2004) 2999–3005.
[6] B.A. Blansjaar, G.J. Vielvoye, J.G. van Dijk, R.J. Rijnders, Similar brain lesions in alcoholics and Korsakoff patients: MRI, psychometric and clinical findings, Clin. Neurol. Neurosurg. 94 (1992) 197–203.
[7] R.E. Brown, L. Stanford, D. Houghton, M. Williamson, L. Currie, K. Luedemann, C. Hawken, Body Weight as a Confound in Rotarod Studies of Motor Learning and Coordination in Rats and Mice, Society for Neuroscience, Orlando, FL, 2002, (Abstract No. 267.12).
[8] J.C. Brust, Neurologic complications of substance abuse, J. Acquired Immune Defic. Syndr. 31 (2002) S29–S34.
[9] R.F. Butterworth, Pathophysiology of cerebellar dysfunction in the Wernicke–Korsakoff syndrome, Can. J. Neurol. Sci. 3 (1993) S123–S126.
[10] S. Chen, D.E. Hillman, Giant spines and enlarged synapses induced in Purkinje cells by malnutrition, Brain Res. 187 (1980) 487–493.
[11] G. Cheron, D. Gall, L. Servais, B. Dan, R. Maex, S.N. Schiffmann, Inactivation of calcium-binding protein genes induces 160 Hz oscillations in the cerebellar cortex of alert mice, J. Neurosci. 24 (2004) 434–441.
[12] G. Cheron, L. Servais, J. Wagstaff, B. Dan, Fast cerebellar oscillation associated with ataxia in a mouse model of Angelman syndrome, Neuroscience 130 (2005) 631–637.
[13] N.S. Chu, Effects of ethanol on rat cerebellar Purkinje cells, Int. J. Neurosci. 21 (1983) 265–277.
[14] N.S. Chu, Responses of midbrain raphe neurons to ethanol, Brain Res. 311 (1984) 348–352.
[15] F.T. Crews, A.L. Morrow, H. Criswell, G. Breese, Effects of ethanol on ion channels, Int. Rev. Neurobiol. 39 (1996) 283–367.
[16] C.A. Dlugos, R.J. Pentney, Effects of chronic ethanol consumption on SER of Purkinje neurons in old F344 rats, Alcohol 20 (2000) 125–132.
[17] J.C. Eccles, M. Ito, J. Szenthagothai, The Cerebellum as a Neuronal Machine, Springer-Verlag, Berlin, 1967.
[18] S.A. Edgley, M. Lidierth, The discharges of cerebellar Golgi cells during locomotion in the cat, J. Physiol. (London) 392 (1987) 315–332.
[19] C.L. Franklin, D.L. Gruol, Acute ethanol alters the firing pattern and glutamate response of cerebellar Purkinje neurons in culture, Brain Res. 416 (1987) 205–218.
[20] R.K. Freund, Y. Wang, M.R. Palmer, Differential effects of ethanol on the firing rates of Golgi-like neurons and Purkinje neurons in cerebellar slices in vitro, Neurosci. Lett. 164 (1993) 9–12.
[21] G. Garthwaite, F. Hojes, J. Garthwaite, Morphological response of endoplasmic reticulum in cerebellar Purkinje cells to calcium deprivation, Neuroscience 48 (1992) 681–688.
[22] B. Ghelarducci, M. Ito, N. Yagi, Impulses discharges from flocculus Purkinje cells of alert rabbits during visual stimulation combined with horizontal head rotation, Brain Res. 87 (1975) 66–72.
[23] D.L. Gruol, K.L. Parsons, N. DiJulio, Acute ethanol alters calcium signals elicited by glutamate receptor agonists and K+ depolarisation in cultured cerebellar Purkinje neurons, Brain Res. 773 (1997) 82–89.
[24] H.J. Hanchar, P.D. Dodson, R.W. Olsen, T.S. Otis, M. Wallner, Alcohol-induced motor impairment caused by increased extrasynaptic GABA(A) receptor activity, Nat. Neurosci. 8 (2005) 339–345.
[25] F.E. Hoebeek, J.S. Stahl, A.M. van Alphen, M. Schonewille, C. Luo, M. Rutteman, A.M. van den Maagdenberg, P.C. Molenaar, H.H. Goossens, M.A. Frens, C.I. De Zeeuw, Increased noise level of Purkinje cell activities minimizes impact of their modulation during sensorimotor control, Neuron 45 (2005) 953–965.
[26] L.A. Howard, S. Miksys, E. Hoffmann, D. Mash, R.F. Tyndale, Brain CYP2E1 is induced by nicotine and ethanol in rat and is higher in smokers and alcoholics, Br. J. Pharmacol. 138 (2003) 1376–1386.
[27] K.R. Iorio, L. Reinlib, B. Tabakoff, P.L. Hoffman, Chronic exposure of cerebellar granule cells to ethanol results in increased *N*-methyl-D-aspartate receptor function, Mol. Pharmacol. 41 (1992) 1142–1148.
[28] M. Ito, The Cerebellum and Neural Control, Raven Press, New York, 1984.
[29] M. Ito, J.I. Simpson, Discharges in Purkinje cell axons during climbing fibre activation, Brain Res. 31 (1971) 215–219.
[30] J. Juntunen, T. Widenius, T.E. Reed, R. Ylikahri, E. Matikainen, M. Sarviharju, H. Teravainen, Tolerance for the decrease in nerve conduction velocity and for the motor impairment produced by ethanol in mice: differential development during chronic ethanol consumption, Neurobehav. Toxicol. Teratol. 6 (1984) 245–248.
[31] R.S. Lee, S.S. Smith, J.K. Chapin, B.D. Waterhouse, N. Shimizu, B.N. Maddux, D.J. Woodward, Effects of systemic and local ethanol on responses of rat cerebellar Purkinje neurons to iontophoretically applied gamma-aminobutyric acid, Brain Res. 687 (1995) 1–11.
[32] E. Lewandowska, M. Kujawa, A. Jedrzejewska, Ethanol-induced changes in Purkinje cells of rat cerebellum: II. The ultrastructural changes after chronic ethanol intoxication (Morphometric evaluation), Folia Neuropathol. 32 (1994) 61–64.
[33] C.S. Lieber, Relationships between nutrition, alcohol use, and liver disease, Alcohol Res. Health 27 (2003) 220–231.
[34] S.G. Lisberger, A.F. Fuchs, Response of flocculus Purkinje cells to adequate vestibular stimulation in the alert monkey: fixation vs compensatory eye movement, Brain Res. 69 (1974) 347–353.
[35] J.C. Marcus, Neurologic findings in the fetal alcohol syndrome, Neuropediatrics 18 (1987) 158–160.
[36] D. Marr, A theory of cerebellar cortex, J. Physiol. (London) 202 (1969) 437–470.

[37] R.P. Messner, N.L. Meryhew, E.G. DeMaster, Effect of ethanol on immune clearance in mice: biphasic alteration of complement-mediated clearance with chronic ethanol ingestion, J. Lab. Clin. Med. 122 (1993) 506–517.
[38] T. Miki, S. Harris, P. Wilce, Y. Takeuchi, K.S. Bedi, The effect of the timing of ethanol exposure during early postnatal life on total number of Purkinje cells in rat cerebellum, J. Anat. 194 (1999) 423–431.
[39] P.G. Montarolo, M. Palestini, P. Strata, The inhibitory effect of the olivocerebellar input on the cerebellar Purkinje cells in the rat, J. Physiol. (London) 332 (1982) 187–202.
[40] T. Moykkynen, E.R. Korpi, D.M. Lovinger, Ethanol inhibits alpha-amino-3-hydyroxy-5-methyl-4-isoxazolepropionic acid (AMPA) receptor function in central nervous system neurons by stabilizing desensitization, J. Pharmacol. Exp. Ther. 306 (2003) 546–555.
[41] J.G. Netzeband, C. Trotter, J.N. Caguioa, D.L. Gruol, Chronic ethanol exposure enhances AMPA-elicited Ca2+ signals in the somatic and dendritic regions of cerebellar Purkinje neurons, Neurochem. Int. 35 (1999) 163–174.
[42] J. Ortiz, L.W. Fitzgerald, M. Charlton, S. Lane, L. Trevisan, X. Guitart, W. Shoemaker, R.S. Duman, E.J. Nestler, Biochemical actions of chronic ethanol exposure in the mesolimbic dopamine system, Synapse 21 (1995) 289–298.
[43] R.J. Pentney, C.A. Dlugos, Cerebellar Purkinje neurons with altered terminal dendritic segments are present in all lobules of the cerebellar vermis of ageing, ethanol-treated F344 rats, Alcohol Alcohol. 35 (2000) 35–43.
[44] L.A. Pohorecky, J. Brick, Activity in neurons in the locus coeruleus of the rat: inhibition by ethanol, Brain Res. 131 (1977) 174–179.
[45] M. Roberto, P. Schweitzer, S.G. Madamba, D.G. Stouffer, L.H. Parsons, G.R. Siggins, Acute and chronic ethanol alter glutamatergic transmission in rat central amygdala: an in vitro and in vivo analysis, J. Neurosci. 24 (2004) 1594–1603.
[46] J. Rogers, G.R. Siggins, J.A. Schulman, F.E. Bloom, Physiological correlates of ethanol intoxication tolerance, and dependence in rat cerebellar Purkinje cells, Brain Res. 196 (1980) 183–198.
[47] E. Sanna, M.C. Mostallino, F. Busonero, G. Talani, S. Tranquilli, M. Mameli, S. Spiga, P. Follesa, G. Biggio, Changes in GABA(A) receptor gene expression associated with selective alterations in receptor function and pharmacology after ethanol withdrawal, J. Neurosci. 23 (2003) 11711–11724.
[48] Y. Sato, A. Miura, H. Fushiki, T. Kawasaki, Short-term modulation of cerebellar Purkinje cell activity after spontaneous climbing fiber input, J. Neurophysiol. 68 (1992) 2051–2062.
[49] M. Sausbier, H. Hu, C. Arntz, S. Feil, S. Kamm, H. Adelsberger, U. Sausbier, C.A. Sailer, R. Feil, F. Hofmann, M. Korth, M.J. Shipston, H.G. Knaus, D.P. Wolfer, C.M. Pedroarena, J.F. Storm, P. Ruth, Cerebellar ataxia and Purkinje cell dysfunction caused by Ca2+-activated K+ channel deficiency, Proc. Natl. Acad. Sci. 101 (1994) 9474–9478.
[50] S.N. Schiffmann, G. Cheron, A. Lohof, P. d'Alcantara, M. Meyer, M. Parmentier, S. Schurmans, Impaired motor coordination and Purkinje cell excitability in mice lacking calretinin, Proc. Natl. Acad. Sci. U. S. A. 96 (1999) 5257–5262.
[51] C. Schwarz, J.P. Welsh, Dynamic modulation of mossy fiber system throughput by inferior olive synchrony: a multielectrode study of cerebellar cortex activated by motor cortex, J. Neurophysiol. 86 (2001) 2489–2504.
[52] W.S. Seo, C.K. Suh, Acute effect of ethanol on firing patterns of Purkinje cells in the rat cerebellar slice preparation, Yonsei Med. J. 42 (2001) 384–389.
[53] S.K. Sharma, U. Nayar, M.C. Maheshwari, B. Singh, Purkinje cell evoked unit activity in developing normal and undernourished rats, J. Neurol. Sci. 116 (1993) 212–219.
[54] J.I. Simpson, H.C. Hulscher, E. Sabel-Goedknegt, T.J. Ruigrok, Between in and out: linking morphology and physiology of cerebellar cortical interneurons, Prog. Brain Res. 148 (2004) 329–340.
[55] J.G. Sinclair, G.F. Lo, Ethanol effects on rat cerebellar Purkinje cells, Gen. Pharmacol. 13 (1982) 449–451.
[56] S. Sorensen, D. Carter, J. Marwaha, R. Baker, R. Freedman, Disinhibition of rat cerebellar Purkinje neurons from noradrenergic inhibition during rising blood ethanol, J. Stud. Alcohol 42 (1981) 908–917.
[57] I. Sugihara, E.J. Lang, R. Llinás, Serotonin modulation of inferior olivary oscillations and synchronicity: a multiple-electrode study in the rat cerebellum, Eur. J. Neurosci. 7 (1995) 521–534.
[58] M.A. Tavares, M.M. Paula-Barbosa, A. Cadete-Leite, Chronic alcohol consumption reduces the cortical layer volumes and the number of neurons of the rat cerebellar cortex, Alcohol., Clin. Exp. Res. 11 (1987) 315–319.
[59] D. Watanabe, H. Inokawa, K. Hashimoto, N. Suzuki, M. Kano, R. Shigemoto, T. Hirano, K. Toyama, S. Kaneko, M. Yokoi, K. Moriyoshi, M. Suzuki, K. Kobayashi, T. Nagatsu, R.J. Kreitman, I. Pastan, S. Nakanishi, Ablation of cerebellar Golgi cells disrupts synaptic integration involving GABA inhibition and NMDA receptor activation in motor coordination, Cell 95 (1998) 17–27.
[60] B.R. Willis, R.A. Anderson, C. Oswald, L.J. Zaneveld, Ethanol-induced male reproductive tract pathology as a function of ethanol dose and duration of exposure, J. Pharmacol. Exp. Ther. 225 (1983) 470–478.
[61] J.J. Woodward, Ionotropic glutamate receptors as site of action for ethanol in the brain, Neurochem. Int. 35 (1999) 107–113.
[62] X. Yang, H.E. Criswell, G.R. Breese, Ethanol modulation of gamma-aminobutyric acid (GABA)-mediated inhibition of cerebellar Purkinje neurons: relationship to GABAb receptor input, Alcohol., Clin. Exp. Res. 24 (2000) 682–690.