Published in final edited form as:

Neuroscience. 2010 November 10; 170(4): 1328–1344. doi:10.1016/j.neuroscience.2010.07.059.

Inhibition of Cerebellar Granule Cell Turning by Alcohol

Tatsuro Kumada, Yutaro Komuro, Ying Li, Taofang Hu, Zhe Wang, Yoav Littner, and Hitoshi Komuro*

Department of Neurosciences, Lerner Research Institute, The Cleveland Clinic Foundation, Cleveland, Ohio 44195, USA

Abstract

Ectopic neurons are often found in the brains of fetal alcohol spectrum disorders (FASD) and fetal alcohol syndrome (FAS) patients, suggesting that alcohol exposure impairs neuronal cell migration. Although it has been reported that alcohol decreases the speed of neuronal cell migration, little is known about whether alcohol also affects the turning of neurons. Here we show that ethanol exposure inhibits the turning of cerebellar granule cells *in vivo* and *in vitro*. First, *in vivo* studies using P10 mice demonstrated that a single i.p. injection of ethanol not only reduces the number of turning granule cells but also alters the mode of turning at the EGL-ML border of the cerebellum. Second, *in vitro* analysis using microexplant cultures of P0-P3 mouse cerebella revealed that ethanol directly reduces the frequency of spontaneous granule cell turning in a dose-dependent manner. Third, the action of ethanol on the frequency of granule cell turning was significantly ameliorated by stimulating Ca^{2+} and cGMP signaling or by inhibiting cAMP signaling. Taken together, these results indicate that ethanol affects the frequency and mode of cerebellar granule cell turning through alteration of the Ca^{2+} and cyclic nucleotide signaling pathways, suggesting that the abnormal allocation of neurons found in the brains of FASD and FSA patients results, at least in part, from impaired turning of immature neurons by alcohol.

Keywords

neuronal cell migration; early postnatal mouse; fetal alcohol syndrome; second messenger signaling pathway; microexplant cultures; neuronal heterotopias

Introduction

Children of alcoholic mothers often suffer from many birth defects which are referred to as fetal alcohol spectrum disorders (FASD) of which fetal alcohol syndrome (FAS) is the most devastating case (Chiriboga, 2003; Lemoine et al., 2003; Sokol et al., 2003; Goodlett et al., 2005). One of the most serious features of FASD and FAS is abnormalities in the brain. Although several aspects of the developmental program are involved in the alcohol-induced malformation of the brain (Jones, 1988; Coulter et al., 1993; Guerri, 2002), the most striking abnormalities appear to involve the impairment of neuronal cell migration (Miller, 1986, 1993). For example, leptomeningeal neuroglial heterotopias that assume the form of a sheet of aberrant neuronal and glial cells covering portions of the cerebral, cerebellar and brain

To whom correspondence should be addressed. Dr. Hitoshi Komuro, Department of Neurosciences/NC30, Lerner Research Institute, Cleveland Clinic Foundation, 9500 Euclid Avenue, Cleveland, OH 44195, USA, Tel: +1 216-444-4497, Fax: +1 216-444-7927, komuroh@ccf.org.

Publisher's Disclaimer: This is a PDF file of an unedited manuscript that has been accepted for publication. As a service to our customers we are providing this early version of the manuscript. The manuscript will undergo copyediting, typesetting, and review of the resulting proof before it is published in its final citable form. Please note that during the production process errors may be discovered which could affect the content, and all legal disclaimers that apply to the journal pertain.

stem surfaces are often observed in the brains of FASD and FAS patients (Marcus, 1987; Clarren et al., 1978; Riley and McGee, 2005; Welch-Carre, 2005). Aberrations of brain stem and cerebellar development, in large part related to faulty cell migration, have also been especially frequent in the brains of FASD and FAS patients, along with the migrational disturbances of schizencephaly and polymicrogyria (Clarren et al., 1978; Peiffer et al., 1979).

Although it has been shown that alcohol exposure results in a significant reduction of the speed of neuronal cell migration (Kumada et al., 2006, 2007, 2010; Jiang et al., 2008), little is known about whether alcohol affects the turning of neurons. To attain their proper position, neurons are required to change their direction of movement at the right time and place during the journey towards their destination (Ramón y Cajal, 1929; Rakic, 1990; Yacubova and Komuro, 2003; Britanova et al., 2006; Kawauchi et al., 2006; Tanaka et al., 2006; Kumada et al., 2007; Nakajima, 2007; Cameron et al., 2007, 2009a; Kumada et al., 2009; Komuro et al., 2010). For example, in the developing cerebellum, postmitotic granule cells first migrate tangentially at the middle of the external granular layer (EGL) for 20–30 hours (Komuro et al., 2001; Komuro and Yacubova, 2003). Then, granule cells exhibit a right angle turn, changing the direction of migration from tangential to radial at the border of the EGL and the molecular layer (ML) (Rakic, 1971; Komuro et al., 2001; Komuro and Kumada, 2005). Thereafter, granule cells migrate radially through the ML and the Purkinje cell layer (PCL) to reach their final destination within the internal granular layer (IGL) (Komuro and Rakic, 1995, 1998a, 1998b; Rakic and Komuro, 1995; Cameron et al., 2009b). Therefore, the turning of granule cells at the EGL-ML border is essential for their proper allocation in the developing cerebellum (Yacubova and Komuro, 2003; Kumada et al., 2007).

In this study, we examined whether alcohol exposure affects neuronal cell turning in the developing brain. To address this question, we used the cerebellum as a model system because it has been reported that cerebellar abnormalities have been noted in autopsy and magnetic resonance imaging studies of FAS (Roebuck et al., 1998). In the developing human cerebellum, the most vulnerable period for alcohol exposure is during the third trimester (Clarren, 1986). The equivalent time of development in mice and rats is during the 1st and 2nd postnatal weeks (Dobbing and Sands, 1979; Kornguth et al., 1979). In fact, when alcohol exposure occurred during the early postnatal period, quantitative morphological changes were found in the rodent cerebellum (Bauer-Moffett and Altman, 1975, 1977; Kornguth et al., 1979; Shetty and Phillip, 1992; Sakata-Haga et al., 2001; Dikranian et al., 2005). In this series of studies, we determined whether alcohol exposure affects the turning of cerebellar granule cells. This is in part because it has been shown that alcohol exposure reduces the number and density of granule cells in the IGL (Anderson and Sides, 1979). Moreover, alcohol exposure resulted in an abnormal distribution of granule cells in the IGL (Anderson and Sides, 1979). Importantly, in an analysis of granule cell maturation in alcohol-exposed rats, Kornguth et al. (1979) found an appreciably thicker EGL, where granule cell precursors are actively proliferating and postmitotic granule cells remain before their radial migration towards the IGL, than in controls at P11 and P14 days, suggesting that the thickening of the EGL is a consequence of retarded migration from the EGL or diminished migration of granule cells out of the EGL. Based on these previous studies, we hypothesized that alcohol exposure affects the turning of granule cells in the developing cerebellum. To test this hypothesis, we first determined whether ethanol exposure affects the frequency and mode of granule cell turning at the EGL-ML border of the early postnatal mouse cerebellum. Second, to determine if ethanol directly affects granule cell turning, we examined the effects of ethanol exposure on the spontaneous turning of isolated granule cells, using microexplant cultures of early postnatal mouse cerebellum. Finally, we determined whether the action of ethanol on granule cell turning can be altered by

controlling second-messenger pathways, such as Ca^{2+} signaling and cyclic nucleotide signaling.

EXPERIMENTAL PROCEDURES

All animal procedures were approved by the Internal Animal Care and Use Committee of the Cleveland Clinic Foundation and conformed to the U.S. National Institutes of Health guidelines on the ethical use of animals. All efforts were made to minimize the number of animals used and their suffering.

Analysis of the effect of ethanol on granule cell migration *in vivo* using BrdU

Forty postnatal (P) 9-day-old mice (CD-1, both sexes) were injected intraperitoneally (i.p.) with 5-bromo-2'-deoxy-uridine (BrdU, 50 mg/kg body weight) (Komuro et al., 2001; Kumada et al., 2006). One day after BrdU injection (at P10), mice were injected i.p. with saline (100 μl , as a control) or one of three different doses of ethanol [1, 3, or 5 g/kg body weight (b.w.), 25%, v/v mixed in saline]. Two days after BrdU injection (at P11), all animals were transcardially perfused with 4% paraformaldehyde. Brains were post-fixed in 4% paraformaldehyde for 24 hours, stored in a 30% sucrose solution, and sectioned sagittally into 30 μm -thick slices on a cryostat. In each section, cells which had incorporated BrdU into DNA were detected by an anti-BrdU monoclonal antibody (BrdU labeling and Detection Kit I, Boehringer Mannheim) and fluorescein-conjugated secondary antibody (Komuro et al., 2001; Kumada et al., 2006). To examine the effects of ethanol in granule cell turning and migration, the positions of BrdU-labeled (fluorescein-positive) cells in the EGL, the ML, the PCL and the IGL of all lobules were detected by the use of a confocal microscope (TCS SP, Leica).

Determination of blood ethanol levels

Thirty P10 mice (CD-1, both sexes) were injected i.p. with one of three different doses of ethanol (1, 3, or 5 g/kg b.w.). At 1 hr after ethanol injection, blood samples were collected from the mice, and ethanol concentrations in blood were determined by the use of NAD-ADH Reagent Multiple Test Vial (Sigma) according to the manufacturers' instructions.

Examination of the effects of ethanol on granule cell turning *in vivo* using Golgi staining

Forty P10 mice (CD-1, both sexes) were injected i.p. with saline (100 μl , as a control) or one of three different doses of ethanol (0, 1, 3, or 5 g/kg b.w.). Six hours after injection, all animals were deeply anesthetized with ether and then euthanized by decapitation. Cerebella were quickly removed from the skull and frozen with isopentane precooled to -70°C with dry ice. Then, cerebella were sectioned transversely into 90- μm -thick sections on a cryostat. Golgi staining was performed by using an FD Rapid GolgiStain kit (FD NeuroTechnologies) according to the instructions of the manufacturer. After staining, the sections were examined with a bright field light microscope (DM 4000B, Leica), and photographed with $\times 63$ oil-immersion objective lens using digital camera (Xli, XL Imaging Ltd.). Images of the segments of Golgi-staining-positive granule cells of all lobules were obtained at different focal planes in order to have a clear definition of the whole cell morphology. The photomontage of Golgi-staining-positive granule cells was created from multiple images using Photoshop software (Adobe Systems).

In this study, we examined whether ethanol affects the amount and mode of granule cell turning at the EGL-ML border. To this end, first, transverse sections of cerebella obtained from ethanol injected or saline injected mice were chosen according to the systematic random sampling scheme. The first section in the series to be analyzed was chosen randomly from the first 2–4 sections. This section and every 4th section thereafter were examined. All

analyses were conducted by observers blinded to treatment conditions. The EGL-ML border of all lobules was determined by cytoarchitectonic criteria including the relative density of granule cells, the position of the top end of Purkinje cell dendrites, and the upper location of parallel fibers. The length of EGL-ML border was measured by using ImageJ software. Thereafter, Golgi-staining-positive turning granule cells located within 10 μm from the EGL-ML border of all lobules were identified by using morphologic criteria: (1) the orientation, location, size and shape of the somata, (2) the length, number and orientation of the leading process and trailing process, (3) the direction of extension of the leading process, (4) the number of branches of the leading process. Subsequently, we measured the average number and mode of Golgi-staining-positive turning granule cells per 100 μm -length of the EGL-ML border in all lobules of cerebellar sections obtained from ethanol injected or saline injected mice. The average number and mode of granule cell turning per 100 μm -length of the EGL-ML border were determined in more than 500 different positions of all lobules.

In this study, to examine whether ethanol alters the number and mode of granule cell turning, we used Golgi-staining. Although it is known that the appearance of granule cells using Golgi-staining is sporadic (Ono et al., 1997; Kumada et al., 2009), we assumed that the frequency of Golgi-stained granule cells with a particular morphological feature among the total Golgi-stained granule cells reflected the proportion of granule cells with the same morphology in the early postnatal mouse cerebella. We also assumed that the application of ethanol does not affect the efficiency of the Golgi-staining.

Examination of the effects of ethanol on spontaneous granule cell turning *in vitro* using the microexplant culture of the early postnatal mouse cerebellum

Cerebella of P0-P3 mice (CD-1, both sexes) were quickly removed from the skull, placed in cold Hanks' balanced salt solution (Sigma) and freed from meninges and choroid plexus (Komuro and Rakic, 1999; Yacubova and Komuro, 2002a,b; Kumada et al., 2006, 2009; Cameron et al., 2007). Cerebellar slices were then made with a surgical blade from which white matter and deep cerebellar nuclei were removed. Rectangular pieces (50–100 μm) were dissected out from the remaining tissue, which mainly consisted of the cerebellar gray matter, using a surgical blade under a dissecting microscope. Such microexplants were rinsed with the culture medium and placed on 35 mm-glass-bottom microwell dishes (1 microexplant/dish, Mat-Tec Corporation) with 50 μl of the culture medium. The culture medium consisted of DMEM/F12 with N2 supplement, 90 U/ml penicillin and 90 $\mu\text{g}/\text{ml}$ streptomycin. Each dish was placed in a CO_2 incubator (37°C, 95% and 5% CO_2). The glass bottom microwell dishes were coated with poly-L-lysine (100 $\mu\text{g}/\text{ml}$)/laminin (20 $\mu\text{g}/\text{ml}$, Sigma) before use. Two hours after plating, 1.5 ml of the culture medium was added to each dish. In this culture, more than 95% of migrating cells were granule cells, which were easily distinguished from other neurons by the small size of their cell bodies (Komuro and Rakic, 1996; Yacubova and Komuro, 2002a; Kumada et al., 2009). Although granule cells were prepared from the EGL and the IGL of all lobules of the cerebellum, the vast majority of granule cells were derived from the EGL, since at the age of P0-P3 the IGL contains only very small numbers of postmigratory granule cells (Miale and Sidman, 1961; Fujita et al., 1966; Fujita, 1967; Altman, 1972). Therefore, the majority of granule cells were at the same developmental stage (Yacubova and Komuro, 2002a).

Twenty hours after plating, dishes were transferred into the chamber of a micro-incubator (PDMI-2, Harvard Apparatus) attached to the stage of a confocal microscope (TCS SP, Leica). The migratory behavior of granule cells is closely related to the temperature of the medium; lowering the medium temperature slows cell movement (Rakic and Komuro, 1995). Therefore, the chamber temperature was kept at $37.0 \pm 0.5^\circ\text{C}$ using a temperature controller (TC-202, Harvard Apparatus) during the observation of migration. The cells were provided with constant gas flow (95% air, 5% CO_2). A laser scanning confocal microscope

was used to visualize migrating granule cells in the microexplant cultures (Yacubova and Komuro, 2002a; Kumada et al., 2006, 2009). Granule cells were illuminated with a 488-nm wavelength light from an argon laser through an inverted microscope equipped with a 20× oil-immersion objective or a 40× oil-immersion objective, and the light transmitted through granule cells was detected by a photomultiplier (Yacubova and Komuro, 2002a; Kumada et al., 2009). To protect granule cells from cytotoxic effects of the laser beam, the light level was reduced by 99%. Images of granule cells in a single focal plane were collected with laser scans every 60 seconds for up to 10 hours. Ethanol is volatile. Therefore, in order to maintain ethanol concentrations in the medium, we continuously perfused the culture medium consisting of 0, 10, 25, 50, or 100 mM ethanol at a rate of 0.5 ml/min.

In this series of experiments, to examine how ethanol exposure alters granule cell turning, we used various pharmacological agents, which stimulate or inhibit the activity of Ca^{2+} signaling and cyclic nucleotide signaling. We chose dose levels for each agent which are able to alter the activity of the receptors, channels, and kinases but have less toxic side effects on granule cells (Komuro and Rakic, 1992, 1993, 1996, Yacubova and Komuro, 2002a,b; Kumada and Komuro 2004; Kumada et al., 2006; Cameron et al., 2007, 2009).

Statistical analysis

Statistical differences were determined using ANOVA. Statistical significance was defined at $P < 0.05$.

RESULTS

A single i.p. injection of ethanol into P10 mice reduces the number of granule cells migrating out from the EGL

To examine the effects of ethanol on granule cell turning, we determined whether ethanol exposure affects the translocation of granule cells from the EGL to the ML of the developing cerebellum. This is because as schematically represented in Fig. 1A, to enter the ML from the EGL, granule cells require a right angle turn at the EGL-ML border (Komuro et al., 2001; Yacubova and Komuro, 2003). In this study, we used three different doses of ethanol (1, 3 or 5 g/kg b.w.). First, we measured the elevation of blood ethanol levels one hour after i.p. injection of 1, 3 or 5 g/kg b.w. of ethanol. This is because our previous study indicated that blood ethanol levels reach their peak within 1 hour after i.p. injection of ethanol in early postnatal mice (Kumada et al., 2006). Analysis of blood ethanol levels revealed that one hour after i.p. injection, blood ethanol levels of P10 mice reached 15.2 ± 3.8 mM ($n=10$) in the case of 1 g/kg b.w. injection, 45.7 ± 5.8 mM ($n=10$) in the case of 3 g/kg b.w. injection, and 81.7 ± 7.3 mM ($n=10$) in the case of 5 g/kg b.w. injection. The injection of these amounts of ethanol made the pups intoxicated and decreased the activity of spontaneous movement for 1–7 hours, depending on the dosages used. However, these dose-levels of ethanol did not cause death.

Upon establishing the elevations of blood ethanol levels after i.p. injection of ethanol, we determined whether ethanol affects granule cell turning. To this end, we injected P9 mice i.p. with BrdU (50 mg/kg body weight), which is incorporated only into proliferating cells, such as granule cell precursors on the top of the EGL (Komuro et al., 2001; Kumada et al., 2006). One day after BrdU injection (at P10), mice were injected i.p. with one of three different doses of ethanol (1, 3 or 5 g/kg b.w.) or saline (100 μ l) as a control. Two days after BrdU injection (one day after ethanol injection, P11), the mice were euthanized and the cerebella were removed. Sections obtained from the cerebella were first stained with an anti-BrdU monoclonal antibody and then with fluorescein-conjugated secondary. The distribution of BrdU-labeled granule cells in the cortical layers of all lobules was examined

using a confocal microscope. As seen in Fig. 1B, in control mice (saline-injected mice), more than 50% of BrdU-labeled granule cells left the EGL and translocated their soma into the ML, the PCL, and the IGL within 2 days after BrdU injection (at P11). Because it has been reported that after final mitosis, postmitotic granule cells migrate tangentially at the middle and the bottom of the EGL (Komuro et al., 2001; Kumada et al., 2006), these results indicate that in the saline-injected animals (control animals), more than 50% of postmitotic granule cells undergo the right angle turn (tangential to radial) at the EGL-ML border and enter the ML within 48 hours after final mitosis. Interestingly, in the ethanol-injected animals, the number of the BrdU-labeled granule cells in the ML, PCL and IGL was significantly decreased (Fig. 1B and C). For example, the average number of the BrdU-labeled granule cells found in the ML, PCL and IGL was reduced to 89% (1 g/kg b.w. ethanol-injection), 62% (3 g/kg b.w. ethanol-injection) and 50% (5 g/kg b.w. ethanol-injection) of the control value (saline-injection), respectively. Furthermore, no difference was found in the effects of ethanol on the average number of the BrdU-labeled granule cells in the ML, PCL and IGL between different lobules (data not shown). Taken together, these results suggest that ethanol exposure prevents granule cells from turning at the EGL-ML border and entering the ML, the PCL and the IGL of the cerebellum.

Ethanol exposure decreases the number of turning granule cells at the EGL-ML border and alters the mode of turning

To further study the effects of ethanol exposure on granule cell turning *in vivo*, we examined whether ethanol exposure alters the number of granule cells that exhibit turning at the EGL-ML border and affects the mode of their turning. To this end, using Golgi-staining, we first determined the sequential changes in the morphology of granule cells in the P10 mouse cerebellum. At the top of the EGL, granule cell precursors had a polygonal cell body with several short processes (a1 in Fig. 2A). Beneath the layer of granule cell precursors, at the middle and bottom of the EGL, postmitotic granule cells had a horizontally-oriented soma with two horizontally-extending processes (a leading process and a trailing process) (a2-a6 in Fig. 2A). In the ML and the PCL, granule cells had a vertically-oriented soma with a voluminous leading process and a thin trailing process (a8 and a9 in Fig. 2A). In the IGL, post-migratory granule cells extended several short processes (future dendrites) from the soma (a11 and a12 in Fig. 2A). Importantly, at the EGL-ML border, granule cells exhibited characteristic features of their morphology indicating the process of their turning, which was classified into three distinct modes (T-, L-, and Y-shape turning; Fig. 2B). For example, T-shape turning was characterized by the extension of a single vertical process into the ML from the horizontally-oriented soma of granule cells, which had two horizontally-extended axon-like processes in the EGL (b1-b8 in Fig. 2B). L-shape turning was characterized by the turning of the tip of horizontally-extended leading processes towards the ML (b9-b23 in Fig. 2B). Y-shape turning was characterized by the bifurcation of the horizontally-oriented leading process (b24-b32 in Fig. 2B). One of the branches extended vertically and entered the ML, while the other extended horizontally in the EGL. There was a gradient in the occurrence of each mode of granule cell turning at the EGL-ML border of the cerebellum of control mice (saline-injected mice): L-shape turning ($41.5 \pm 4.3\%$) > T-shape turning ($32.9 \pm 3.6\%$) > Y-shape turning ($25.6 \pm 3.6\%$).

After examining the morphological features of granule cells at the EGL-ML border of the P10 mouse cerebellum, we i.p. injected one of three different doses of ethanol (1, 3 or 5 g/kg b.w.) or saline (100 μ l, as a control) into P10 mice. Six hours after injection of ethanol, all animals were euthanized, and cerebella were removed from the skull. After sectioning, with the use of Golgi-staining, we examined whether ethanol exposure alters the number of granule cells exhibiting turning at the EGL-ML border. Importantly, ethanol exposure significantly decreased the average number of granule cells exhibiting characteristic turning

morphologies (T-, Y-, and L-shape turning) at the EGL-ML border (Fig. 3). For example, at 6 hours after ethanol injection, the average number of turning granule cells (T-, Y-, and L-shape turning) at the EGL-ML border was reduced to 81% (1 g/kg b.w. ethanol injection), 61% (3 g/kg b.w. ethanol injection), and 44% (5 g/kg b.w. ethanol injection) of the control value (saline injection). Ethanol exposure also altered the ratio of T-, Y-, and L-shape turning of granule cells at the EGL-ML border (Fig. 4A and B). In the case of 1 g/kg b.w. of ethanol injection, the ratio of T-, Y-, and L-shape turning did not noticeably change, compared with the control value (saline injection). However, in the cases of 3 or 5 g/kg b.w. of ethanol injection, the ratio of T-shape turning significantly increased, while the ratios of L- and Y-shape turning decreased (Fig. 4B). Collectively, these results indicate that ethanol exposure inhibits granule cell turning at the EGL-ML border and alters the mode of turning in a dose-dependent manner, suggesting that ethanol exposure causes retention of postmitotic granule cells in the EGL, which leads to ectopic granule cells or granule cell death in the EGL.

Ethanol directly inhibits the occurrence of granule cell turning

Alcohol may alter granule cell turning directly or indirectly by modifying the surrounding environment. For example, it has been reported that ethanol affects the function of cell adhesion molecules expressed by neurons and glial cells (Baerer et al., 1999; Minana et al., 2000; Ozer et al., 2000; Guerri, 2002; Miller and Luo, 2002). It has been proposed that cell-cell contact between granule cells and Bergmann glial processes plays a role in the initiation of granule cell turning at the EGL-ML border (Rakic, 1990; Rakic et al., 1994). Therefore, ethanol may affect granule cell turning by altering cell-cell contact through the modification of the function of cell adhesion molecules. To determine whether ethanol directly affects granule cell turning, we used microexplant cultures of P0-P3 mouse cerebella (Komuro and Rakic, 1996; Yacubova and Komuro, 2002a, 2002b; Kumada et al., 2009). In this culture, isolated granule cells actively migrate in the absence of cell-cell contact (Yacubova, and Komuro, 2002a; Kumada et al., 2009). In this series of experiments, we started to monitor granule cell turning at 20 hours *in vitro* for up to 10 hours (until 30 hours *in vitro*), when the cells moved at the fastest rate (Yacubova and Komuro, 2002a). We selected granule cells that were located farthest from the cerebellar microexplant and were at least 300 μ m away from other cells and neuronal processes at the time of the start of the observation period. This selection allowed us to examine the turning of granule cells that had the least contact with other cells prior to observation. If granule cells came in contact with other cells or processes during the observation period, the data were excluded from this study. We first examined the turning of granule cells without cell-cell contact. Granule cells were considered to turn if the direction of their migration deviated more than 45° from the previous movement of their cell body within 30 consecutive minutes (schematically presented in Supplemental Fig. 1; Kumada et al., 2009). Fig. 5A represents a typical example showing granule cell turning over time: a granule cell exhibited turning six times during the period of observation from 20:00 *in vitro* to 23:25 *in vitro*. The 1st turning was observed at 20:25 *in vitro*: the granule cell turned towards the bottom of the photograph (Fig. 5A, B and C). The 2nd turning was observed at 21:15 *in vitro*: the cell turned towards the top of the photograph (Fig. 5A, C and D). The 3rd turning was observed at 21:35 *in vitro*: the cell turned towards the bottom-right of the photograph (Fig. 5A, D and E). The 4th turning was observed at 21:55 *in vitro*: the cell turned towards the bottom-left of the photograph (Fig. 5A, E and F). The 5th turning was observed at 23:10 *in vitro*: the cell turned towards the left of the photograph (Fig. 5A, F and G). The 6th turning was observed at 23:20 *in vitro*: the cell turned towards the bottom of the photograph (Fig. 5A, G and H). Furthermore, analysis of the trajectory of cell movement revealed that granule cells repeatedly undergo frequent and less frequent turning periods over time. Frequent turning periods were defined as the periods of when granule cells exhibited more than three turnings

within 60 consecutive minutes, whereas less frequent turning periods were defined as the periods of when the cells exhibited less than three turnings within 60 consecutive minutes (Kumada et al., 2009). Fig. 6 represents a typical example of the periodic turning of a granule cell over time: a granule cell exhibited the frequent turning periods (indicated by red circles and red lines) twice and the less frequent turning periods (indicated by black circles and black lines) twice during 7 hours of observation (from 20:00 after *in vitro* to 27:00 *in vitro*). The average cycle of the frequent and less frequent turning periods was 3.9 ± 0.7 hours ($n=52$). These results indicate that granule cells exhibit periodic turning without cell-cell contact.

After analyzing the characteristic features of granule cell turning *in vitro*, we determined whether ethanol directly affects granule cell turning. Because isolated granule cells exhibit the cycle of the frequent and less frequent turning periods every 3–5 hours, we averaged the data obtained from at least 200 isolated granule cells observed for 6–10 hours at each dose-level of ethanol exposure. Interestingly, the application of ethanol at concentrations ranging from 10 mM to 100 mM appreciably decreased the frequency of granule cell turning, which was defined as the change in the direction of their migration deviated more than 45° from the previous movement of their cell body within 30 consecutive minutes (Fig. 7A). For example, the average frequency of granule cell turning was reduced to 83% (10 mM ethanol), 60% (25 mM ethanol), 46% (50 mM ethanol), and 39% (100 mM ethanol) of the control value, respectively (Fig. 7A).

Ethanol may reduce the average frequency of granule cell turning by decreasing the turning angle of the cells. To test this possibility, we determined whether ethanol alters the frequency of low-angle turning of granule cells, which is defined as the change in the direction of their migration deviating between 30° and 45° from the previous movement of their cell body within 30 consecutive minutes. Interestingly, the application of ethanol at concentrations ranging from 10 mM to 100 mM significantly decreased the frequency of low-angle ($30\text{--}45^\circ$) turning of granule cells (Fig. 7B). For example, the average frequency of low-angle ($30\text{--}45^\circ$) turning of granule cells was reduced to 80% (10 mM ethanol), 65% (25 mM ethanol), 48% (50 mM ethanol), and 35% (100 mM ethanol) of the control value, respectively (Fig. 7B).

Taken together, these results indicated that ethanol directly reduces the occurrence of both high-angle ($>45^\circ$) turning and low-angle ($30\text{--}45^\circ$) turning of granule cells. Importantly, there is no significant difference in the reduction rate of the average frequency of granule cell turning by ethanol at a given concentration between high-angle ($>45^\circ$) turning and low-angle ($30\text{--}45^\circ$) turning, suggesting that ethanol directly inhibits granule cell turning without altering the turning angle. For the remaining experiments, we used high-angle ($>45^\circ$) turning as an indicator of granule cell turning. This was because ethanol induced similar effects on the frequency of high-angle ($>45^\circ$) turning and low-angle ($30\text{--}45^\circ$) turning of granule cells, and the frequency of high-angle ($>45^\circ$) turning is more accurately determined than that of low-angle ($30\text{--}45^\circ$) turning.

Reduction of the action of ethanol on granule cell turning by stimulating Ca^{2+} signaling

How does ethanol inhibit granule cell turning? Our working hypothesis is that ethanol affects granule cell turning by altering Ca^{2+} signaling pathways. This is because it has been reported that ethanol exposure inhibits spontaneous Ca^{2+} elevations in migrating granule cells (Kumada et al., 2006). Furthermore, granule cell migration is highly sensitive to changes in the Ca^{2+} signaling pathways, including changes in the frequency of spontaneous Ca^{2+} spikes (Komuro and Rakic, 1992, 1993, 1996, 1998b; Kumada and Komuro, 2004; Komuro and Kumada, 2005; Botia et al., 2007; Cameron et al., 2007). Moreover, it has been shown that modifying the Ca^{2+} signaling pathways alters the frequency of granule cell

turning *in vitro* (Kumada et al., 2009). Therefore, if ethanol affects granule cell turning by inhibiting Ca^{2+} signaling, stimulating Ca^{2+} release from internal Ca^{2+} stores or Ca^{2+} influxes across the plasma membrane may alter the action of ethanol on granule cell turning. To test this possibility, we utilized caffeine, which increases internal Ca^{2+} release through the ryanodine receptors, and N-methyl-D-aspartate (NMDA) and nicotine, which both induce Ca^{2+} influx through the NMDA type glutamate receptors and the nicotinic acetylcholine receptors. Intoxicating levels of ethanol have been reported to alter the activity of these receptors (Lovinger et al., 1989; Mezna et al., 1996; Narahashi et al., 1999). We added caffeine, NMDA and nicotine to the culture medium at dose levels which are able to activate their receptors and have less toxic side effects (Rossi and Slater, 1993; Narahashi et al., 2001; El Yacoubi et al., 2003; Connole et al., 2004; Dash et al., 2004; Chen and Harle, 2005; Riddoch et al., 2005). In the absence of ethanol, addition of nicotine (1 μM) to the culture medium did not appreciably change the frequency of granule cell turning, whereas addition of caffeine (1 mM) or NMDA (30 μM) increased the frequency of turning by 80% and 35%, respectively (Fig. 8). These results suggest that in the absence of ethanol, alterations of Ca^{2+} signaling affect the frequency of granule cell turning. Interestingly, the addition of NMDA (30 μM) to the culture medium increased the frequency of granule cell turning by 42% in the presence of 25 mM ethanol and by 52% in the presence of 100 mM ethanol (Fig. 8). The addition of caffeine (1 mM) did not appreciably change the frequency of turning in the presence of 25 mM ethanol, but increased the frequency by 16% in the presence of 100 mM ethanol (Fig. 8). In contrast, nicotine (1 μM) decreased the frequency of turning by 29% in the presence of 25 mM ethanol and by 19% in the presence of 100 mM ethanol (Fig. 8). Taken together, these results indicate that the effects of ethanol on granule cell turning are highly sensitive to changes in internal Ca^{2+} release and Ca^{2+} influx, suggesting that ethanol affects granule cell turning by altering multiple and distinct components of Ca^{2+} signaling.

Controlling cyclic nucleotide pathways alters the action of ethanol on granule cell turning

It has been shown that ethanol exposure increases cyclic AMP (cAMP) levels, but decreases cyclic GMP (cGMP) levels in cerebellar cells (Kumada et al., 2006). This is intriguing because in the absence of ethanol, altering the cAMP and cGMP signaling pathways affects the frequency and mode of granule cell turning (Kumada et al., 2009). Moreover, cyclic nucleotide signaling interacts with Ca^{2+} signaling: stimulating the cAMP signaling pathways decreases the frequency of spontaneous Ca^{2+} spikes in migrating granule cells, while inhibiting the cAMP signaling pathways increases the frequency (Kumada et al., 2006). Taken together, these previous studies suggest that ethanol affects granule cell turning by altering cAMP and cGMP signaling pathways. To test this possibility, we examined whether the manipulations of the cAMP signaling pathways alter the action of ethanol on the frequency of granule cell turning. In the absence of ethanol, application of 100 μM Rp-cAMPS (a competitive cAMP antagonist), 5 μM PKI (inhibitor of protein kinase A), or 30 μM 9CP-Ade (inhibitor of adenylyl cyclase) increased the frequency of turning by 35%, 99% and 27%, respectively (Fig. 9). In contrast, without ethanol, application of 20 μM Sp-cAMPS (a competitive cAMP agonist) or 30 μM forskolin (stimulator of adenylyl cyclase) did not appreciably change the frequency of granule cell turning (Fig. 9). These results suggest that in the absence of ethanol, inhibition of cAMP signaling increases the frequency of granule cell turning, while stimulation of cAMP signaling decreases the frequency of turning. Interestingly, inhibiting cAMP signaling with 100 μM Rp-cAMPS increased the frequency of granule cell turning by 59% in the presence of 25 mM ethanol and by 65% in the presence of 100 mM ethanol (Fig. 9). Inhibiting protein kinase A with 5 μM PKI increased the frequency of turning by 103% in the presence of 25 mM ethanol and by 75% in the presence of 100 mM ethanol (Fig. 9). Likewise, inhibiting adenylyl cyclase with 30 μM 9CP-Ade increased the frequency of turning by 22% in the presence of 25 mM ethanol

and by 27% in the presence of 100 mM ethanol (Fig. 9). In contrast, stimulating cAMP signaling with 20 μ M Sp-cAMPS decreased the frequency of turning by 33% in the presence of 25 mM ethanol, but did not appreciably change the frequency in the presence of 100 mM ethanol (Fig. 9). Stimulating adenylyl cyclase with 30 μ M forskolin decreased the frequency of turning by 31% in the presence of 25 mM ethanol and by 64% in the presence of 100 mM ethanol (Fig. 9). Collectively, these results indicate that stimulating cAMP signaling amplifies the effects of ethanol on granule cell turning, whereas inhibiting cAMP signaling ameliorates the effects. Because ethanol increases cAMP levels (Kumada et al., 2006), these results suggest that ethanol decreases the frequency of granule cell turning by stimulating the cAMP signaling pathways.

Next, we examined whether the cGMP signaling pathways also are involved in the action of ethanol on granule cell turning. In the absence of ethanol, application of 100 μ M Br-cGMP (a cGMP analogue), 5 μ M Rp-8-pCPT-cGMPS (a cGMP antagonist), or 1.5 μ M ODQ (a guanylyl cyclase inhibitor) did not appreciably change the frequency of granule cell turning (Fig. 10). These results suggest that in the absence of ethanol, the activity of cGMP signaling is not involved in controlling the frequency of granule cell turning. Interestingly, stimulating cGMP signaling with 100 μ M Br-cGMP increased the frequency of granule cell turning by 103% in the presence of 25 mM ethanol and by 50% in the presence of 100 mM ethanol (Fig. 10). In contrast, inhibiting cGMP signaling with 5 μ M Rp-8-pCPT-cGMPS or 1.5 μ M ODQ did not appreciably change the frequency of turning in the presence of either 25 mM or 100 mM ethanol (Fig. 10). These results indicate that stimulating cGMP signaling decreases the effects of ethanol on granule cell turning, but inhibiting cGMP signaling does not alter the effects. These results suggest that ethanol alters granule cell turning by inhibiting cGMP signaling because ethanol decreases cGMP levels (Kumada et al., 2006).

Inhibiting cyclic nucleotide phosphodiesterase reduces the action of ethanol on granule cell turning

To further examine the role of cyclic nucleotide signaling pathways in ethanol-induced inhibition of granule cell turning, we determined whether altering cyclic nucleotide phosphodiesterases (PDE), which control the intracellular levels of cAMP and/or cGMP by catalyzing the hydrolysis of cAMP and/or cGMP, affects the action of ethanol on granule cell turning. In the absence of ethanol, application of 30 μ M IBMX (a broad spectrum PDE inhibitor), 20 μ M 8-MM-IBMX [a PDE1 inhibitor that blocks the Ca^{2+} /calmodulin-dependent cleavage of cAMP and cGMP (Juilfs et al., 1999)], or 10 μ M EHNA [a PDE2 inhibitor that blocks the cGMP-dependent cleavage of cAMP and cGMP (Juilfs et al., 1999)] did not appreciably change the frequency of granule cell turning (Fig. 11). These results suggest that in the absence of ethanol, the activity of PDE family does not play a key role in controlling the frequency of granule cell turning. Interestingly, the application of IBMX (30 μ M) increased the frequency of granule cell turning by 68% in the presence of 25 mM and by 31% in the presence of 100 mM ethanol (Fig. 11). Similarly, the application of EHNA (10 μ M) increased the frequency of turning by 23% in the presence of 25 mM and by 17% in the presence of 100 mM ethanol (Fig. 11). In contrast, the application of 8-MM-IBMX (20 μ M) did not alter the frequency of turning in the presence of either 25 mM or 100 mM ethanol (Fig. 11). Taken together, these results suggest that ethanol inhibits granule cell turning by altering the amplitude and duration of cyclic nucleotide signals by modifying the activity of a specific PDE family, such as PDE2.

DISCUSSION

Multiple aspects of central nervous system development can be affected by alcohol, but the most striking abnormalities appear to involve neuronal cell migration (Jones, 1975; Clarren, 1977). Many ectopic neurons are found in the brain of FAS and FASD patients (Clarren, and

Smith, 1978; Clarren et al., 1978; Peiffer et al., 1979; Konovalov et al., 1997), but little is known about how alcohol exposure results in ectopic neurons. In this study, we hypothesized that ethanol exposure affects neuronal cell turning, leading to the abnormal allocation of neurons. To test this hypothesis, we examined whether ethanol exposure affects the turning of cerebellar granule cells. This is because the effect of ethanol on brain growth is especially marked in the cerebellum (McCaffery et al., 2004; Gonzalez-Burgos and Alexandre-Gomez, 2005; Ohrtman et al., 2006), and ethanol exposure reduces the number of granule cells in their final destination (the IGL) of the cerebellum (Anderson and Sides, 1979; Borges and Lewis, 1983; Yanai and Waknin, 1985). The present results revealed that a single i.p. injection of ethanol reduces the number of turning granule cells and alters the mode of turning at the EGL-ML border of the early postnatal mouse cerebellum. Furthermore, ethanol exposure directly reduced the frequency of spontaneous granule cell turning *in vitro*. Moreover, the effects of ethanol on the frequency of spontaneous granule cell turning were ameliorated by altering the Ca^{2+} and cyclic nucleotide signaling pathways. Collectively, the present results demonstrate that ethanol exposure inhibits granule cell turning by altering the Ca^{2+} and cyclic nucleotide signaling pathways. The present results also suggest that the ectopic neurons found in the brains of FAS and FASD patients are due, at least in part, to ethanol-induced impairment of neuronal cell turning.

The present results indicated that a single i.p. injection of ethanol into P9 mice results in the significant reduction of BrdU-labeled granule cells in the ML, PCL and IGL at P10 (Fig. 1B and C), suggesting that ethanol exposure reduces the number of granule cells migrating out from the EGL. Furthermore, the use of Golgi staining indicated that ethanol exposure significantly reduces the number of granule cells exhibiting the turning phenotype at the EGL-ML border (Fig. 3). However, we cannot rule out the possibility that ethanol exposure induces the selective cell death of granule cells engaging in turning. This is because it has been shown that single ethanol exposure increases the cell death of granule cell precursors and postmitotic granule cells in the EGL of P10 mice (Kumada et al., 2006) and significantly decreases the total number of BrdU-positive granule cells in the ML, PCL and IGL (Fig. 1C). The question of whether ethanol exposure causes the selective cell death of granule cells engaging in turning is still open.

Although ethanol is known to affect the function of a wide variety of molecules and signaling pathways (McCaffery et al., 2004; Ohrtman et al., 2006; Kumada et al., 2007; Jiang et al., 2008), the present results indicate that Ca^{2+} and cyclic nucleotide signaling pathways play a key role in the ethanol-induced impairment of granule cell turning. The question of how ethanol inhibits granule cell turning remains to be answered, but previous studies that examined the cellular mechanisms underlying growth cone turning provide us some clues (Gomez et al., 2001; Gorbunova and Spitzer, 2002; Conklin et al., 2005; Spitzer, 2006). First, Ca^{2+} signaling plays a role in organizing the assembly and disassembly of cytoskeletal components, which are required for growth cones turning (Henley and Poo, 2004). Therefore, changes in Ca^{2+} signaling by ethanol may alter the initiation of granule cell turning by modifying the organization of cytoskeletal components. Second, transient elevations of intracellular Ca^{2+} levels are essential for the formation of new focal adhesion sites, which are required for changing the direction of growth cone migration (Conklin et al., 2005). Because ethanol reduces the frequency of spontaneous Ca^{2+} transients in migrating granule cells (Kumada et al., 2006), ethanol may affect granule cell turning by altering the formation of new focal adhesion sites. Third, Ca^{2+} signals localized to one side of the neuronal growth cone can cause asymmetric activation of effector enzymes to steer the growth cone (Petersen and Cancela, 2000; Henley and Poo, 2004). Therefore, alterations by ethanol to the distribution of Ca^{2+} signals in the leading process may affect granule cell turning. Fourth, changes in the ratio of cAMP/cGMP or cytoplasmic cAMP gradients affect growth cone turning (Song et al., 1998; Nishiyama et al., 2003; Munck et al., 2004),

suggesting that alterations in the cAMP and cGMP signaling by ethanol affect granule cell turning by modifying the turning of the leading process.

The present results demonstrated that ethanol directly inhibits granule cell turning *in vivo* and *in vitro*, but we cannot rule out the possibility that ethanol may also indirectly affect granule cell turning *in vivo*. It has been shown that ethanol exposure alters the expression and the function of cell adhesion molecules (Bearer et al., 1999; Minana et al., 2000; Ozer et al., 2000; Guerri, 2002; Miller and Luo, 2002), and that cell adhesion molecules play a key role in glia-associated granule cell migration in the ML (Rakic, 1971; Hatten, 1990; Cameron and Rakic, 1994; Komuro and Rakic, 1995, 1998a, 1998b). Although the role of cell adhesion molecules in granule cell turning has never been examined, it is possible that ethanol indirectly affects the initiation of granule cell turning at the EGL-ML border by altering the expression and function of cell adhesion molecules on the Bergmann glial process and granule cells. Furthermore, ethanol may also affect granule cell turning indirectly by altering the role of extracellular guidance molecules. It has been reported that the Ca^{2+} and cyclic nucleotide signaling pathways play a role in converting extracellular guidance signals to intracellular signals for controlling cell migration (Bix and Clark, 1998; Zou et al., 1998; Yuen and Mobley, 1999; Klein et al., 2001; Borghesani et al., 2002; Du et al., 2006; Gudz et al., 2006; Botia et al., 2007; Cameron et al., 2007; Jiang et al., 2008). In addition, it has been shown that the response of growing axons to external guidance cues is altered by the basal activity of the Ca^{2+} and cyclic nucleotide signaling pathways (Ming et al., 2001; Nishiyama et al., 2003; Wen et al., 2004; Jin et al., 2005). Therefore, ethanol may affect the response of granule cells to potential external guidance molecules by modifying the Ca^{2+} and cyclic nucleotide signaling pathways, leading to alterations of the turning *in vivo*.

Although, to date, little is known about the signals that initiate the turning (from tangential to radial) of granule cells at the EGL-ML border, recent studies suggest possible involvement of stromal cell-derived factor 1 α (SDF-1 α) and its receptor CXCR4, Ephrin-B2 and its receptor EphB2. SDF-1 α expression is present in the pia membrane which is adjacent to the EGL, while granule cell precursors express its receptor CXCR4 (Zou et al., 1998; McGrath et al., 1999). In the SDF-1 α or CXCR4-deficient mice, granule cell precursors prematurely migrate away from the EGL and locate ectopically outside the EGL (Zou et al., 1998; Ma et al., 1998). SDF-1 α induces chemotactic responses in granule cell precursors (Klein et al., 2001). Importantly, the chemoattractant effect of SDF-1 α to granule cells is selectively inhibited by soluble EphB2 receptor through reverse signaling of ephrin-B2 (Lu et al., 2001). Collectively, these results suggest that SDF-1 α and CXCR4 play a crucial role in retaining granule cell precursors in the EGL by chemoattracting toward the pia mater, and inhibit the initiation of radial migration of granule cells towards the ML. The loss of responsiveness to SDF-1 α induced by Ephrin-B2 and its receptor, EphB2, is possibly initiation signals, which allow granule cells to exhibit their turning (in other words, change the direction of migration from tangential to radial) at the EGL-ML border. The question of whether ethanol affects the roles of SDF-1 α , CXCR4, Ephrin-B2, and EphB2 in controlling the initiation of granule cell turning at the EGL-ML border remains to be determined.

The present results indicated that granule cells exhibit the characteristic behavior of migration *in vitro* without cell-cell interaction and in the absence of potential guidance cues (Figs. 5 and 6). Although the question of whether granule cell migration *in vitro* can compare to that observed *in vivo* has not yet been answered, there are some hints. It has been shown that in microexplant cultures, isolated granule cells sequentially go through three distinct phases of migration without cell-cell contact (Yacubova and Komuro, 2002a). The comparison between migration *in vivo* and *in vitro* suggests possible roles of intrinsic signals and external guidance cues in granule cell migration *in vivo*. For example, during the

early periods of migration *in vitro*, isolated granule cells most frequently turn left or right, while granule cells in the EGL *in vivo* migrate tangentially and do not alter the direction of cell movement until 20 hours after the initiation of migration (Komuro et al., 2001; Yacubova and Komuro, 2002a). This difference of turning among granule cells *in vitro* and *in vivo* suggests that localized external cues or cell-cell contacts suppress the intrinsic turning activity of granule cells in the EGL. During the middle periods of migration *in vitro*, isolated granule cells have two long processes and move at the fastest rate, while in the ML *in vivo* (20–30 hours after the initiation of migration) granule cells have a long leading process and a trailing process and move radially at an increased rate (Komuro and Rakic, 1998a; Yacubova and Komuro, 2002a). The similarity suggests that the alteration of granule cell migration observed in the ML may be regulated, at least in part, by intrinsic programs. During the late periods of migration *in vitro*, isolated granule cells terminate their migration without cell-cell contact and start to express the $\alpha 6$ subunit of GABA_A receptors, which are expressed only when the cells arrive in the IGL *in vivo* (Yacubova and Komuro, 2001), suggesting that granule cells in PIII may be in a similar stage of differentiation with those in the IGL. Collectively, the comparison of granule cell migration *in vitro* and *in vivo* suggests that ethanol may alter the turning of granule cells *in vivo* by affecting the intrinsic program for cell movement and by modifying the role of external guidance cues for cell migration.

The present results demonstrate that ethanol inhibits granule cell turning, and our previous study demonstrated that ethanol decreases the speed of granule cell migration (Kumada et al., 2006). Is there any relation between the changes in the speed of granule cell migration and the frequency of turning? The combination of present and previous studies (Kumada et al., 2006, 2009) indicates that in the presence of ethanol, the relation between the changes in the speed of granule cell migration and the frequency of turning can be divided into five categories (as summarized in Fig. 12). The 1st category is defined as when the speed of migration and the frequency of turning change in the same direction. The 2nd category is defined as when the frequency of turning alters, but the speed of migration does not change. The 3rd category is defined as when the speed of migration alters, but the frequency of turning does not change. The 4th category is defined as when the turning and speed change in the opposite directions. The 5th category is defined as when both the turning and speed do not change. The 1st and 4th categories indicate that the changes in the speed of granule cell migration by ethanol positively or negatively affect the changes in the frequency of turning. In contrast, the 2nd and 3rd categories indicate that there is no direct link between the changes in the speed of migration and the frequency of turning by ethanol. These results suggest that ethanol affects granule cell turning by cell motility-dependent and -independent manners.

Depending on the dosage of ethanol, the application of a single reagent often resulted in different effects on the action of ethanol in granule cell turning (shown in Fig 12). For example, caffeine did not alter the effects of 25 mM ethanol on the frequency of granule cell turning, but significantly reduced the effects of 100 mM ethanol on the frequency. In contrast, Sp-cAMPS significantly amplified the effects of 25 mM ethanol on the frequency of turning, but did not alter the effects of 100 mM ethanol on the frequency. These results suggest that cellular mechanisms by which ethanol affects granule cell turning vary as the dosage of ethanol increases.

The use of Golgi-staining demonstrated that at the EGL-ML border, granule cells exhibit three distinct modes (T-, L-, and Y-shape turning) of turning (Fig. 2B). In the absence of ethanol, there is a preference in the occurrence of each mode of granule cell turning at the EGL-ML border: L-shape turning > T-shape turning > Y-shape turning mode. Interestingly, ethanol exposure not only inhibited granule cell turning at the EGL-ML border, but changed the preference in the occurrence of each mode of granule cell turning: T-shape turning > L-

shape turning > Y-shape turning (Fig. 4). These results are very intriguing. This is because these results suggest that normal stimulus can induce changes in the three different regions (the soma for T-shape turning, the dendritic tip for L-shape turning, the intervening dendritic process length for Y-shape turning) of migrating granule cells, and alters their mode of turning. The question of how a single extracellular guidance molecule controls the mode of granule cell turning by altering the three different regions of the cells remains to be determined.

The present study demonstrates that even a single exposure of a high level of ethanol causes serious effects on granule cell turning in the developing cerebellum. Also, the present study indicates that the effects of ethanol on the frequency of granule cell turning are ameliorated by caffeine, NMDA, Rp-cAMPS, or Br-cGMP. Taken together, these results suggest that controlling the Ca^{2+} and cyclic nucleotide signaling pathways are possible new targets for preventing the abnormal allocation of granule cells in the brains of the offspring of alcoholic mothers. However, the questions of whether and how chronic ethanol exposure affects granule cell turning remain to be answered. Moreover, the question of whether ethanol exposure impairs the turning of neurons in other parts of the developing brain, especially the cerebral cortex, remains open.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

We thank C. Nelson for critically reading the manuscript. This work was supported by a grant (ES015612) from National Institute of Environmental Health Sciences.

Abbreviations

AC	adenylate cyclase
b.w	body weight
cAMP	cyclic AMP
cGMP	cyclic GMP
EGL	external granular layer
ML	molecular layer, IGL, internal granular layer
i.p	intraperitoneally
PCL	Purkinje cell layer
PKA	protein kinase A
PKC	protein kinase C
PKI	PKA inhibitor fragment 14–22 myristoylated trifluoroacetate salt

References

- Altman J. Postnatal development of the cerebellar cortex in the rat. I. The external germinal layer and the transitional molecular layer. *J Comp Neurol.* 1972; 145:353–398. [PubMed: 4113154]
- Anderson WJ, Sides GR. Alcohol induced defects in cerebellar development in the rat. *Curr Alcohol.* 1979; 5:135–153. [PubMed: 755617]

- Bauer-Moffet C, Altman J. Ethanol-induced reductions in cerebellar growth of infant rats. *Expl Neurol.* 1975; 48:378–382.
- Bauer-Moffet C, Altman J. The effect of ethanol chronically administered to preweanling rats on cerebellar development: a morphological study. *Brain Res.* 1977; 119:249–268. [PubMed: 830387]
- Bearer CF, Swick AR, O’Riordan MA, Cheng G. Ethanol inhibits L1-mediated neurite outgrowth in postnatal rat cerebellar granule cells. *J Biol Chem.* 1999; 274:13264–13270. [PubMed: 10224086]
- Bix GJ, Clark GD. Platelet-activating factor receptor stimulation disrupts neuronal migration in vitro. *J Neurosci.* 1998; 18:307–318. [PubMed: 9412509]
- Borges S, Lewis PD. Effects of ethanol on postnatal cell acquisition in the rat cerebellum. *Brain Res.* 1983; 271:388–391. [PubMed: 6616187]
- Borghesani PR, Peyrin JM, Klein R, Rubin J, Carter AR, Schwartz PM, Luster A, Corfas G, Segal RA. BDNF stimulates migration of cerebellar granule cells. *Development.* 2002; 129:1435–1442. [PubMed: 11880352]
- Botia B, Basille M, Allais A, Raoult E, Falluel-Morel A, Galas L, Jolivel V, Wurtz O, Komuro H, Fournier A, Vaudry H, Burel D, Gonzalez B, Vaudry D. Neurotrophic effects of PACAP in the cerebellar cortex. *Peptides.* 2007; 28:1746–1752. [PubMed: 17544170]
- Britanova O, Alifragis P, Junek S, Jones K, Gruss P, Tarabykin V. A novel mode of tangential migration of cortical projection neurons. *Dev Biol.* 2006; 298:299–311. [PubMed: 16901480]
- Cameron RS, Rakic P. Polypeptides that comprise the plasmalemmal microdomain between migrating neuronal and glial cells. *J Neurosci.* 1994; 14:3139–3155. [PubMed: 8182462]
- Cameron DB, Galas L, Jiang Y, Raoult E, Vaudry D, Komuro H. Cerebellar cortical-layer-specific control of neuronal migration by pituitary adenylate cyclase-activating polypeptide. *Neuroscience.* 2007; 146:697–712. [PubMed: 17383102]
- Cameron DB, Kasai K, Jiang Y, Hu T, Saeki Y, Komuro H. Four distinct phases of basket/stellate cell migration after entering their final destination (the molecular layer) in the developing cerebellum. *Dev Biol.* 2009a; 332:309–324. [PubMed: 19500566]
- Cameron DB, Raoult E, Galas L, Jiang Y, Lee K, Hu T, Vaudry D, Komuro H. Role of PACAP in controlling granule cell migration. *Cerebellum.* 2009b; 8:433–440. [PubMed: 19548046]
- Chen WJ, Harle LK. Interactive effect of alcohol and nicotine on developing cerebellum: an investigation of the temporal pattern of alcohol and nicotine administration. *Alcohol Clin Exp Res.* 2005; 29:427–442.
- Clarren SK. Central nervous system malformations in two offspring of alcoholic women. *Birth Def.* 1977; 7:131–132.
- Clarren, SK. Neuropathology in fetal alcohol syndrome. In: West, JR., editor. *Alcohol and Brain Development.* New York: Oxford UP; 1986. p. 158
- Clarren SK, Smith DW. The fetal alcohol syndrome. *N Engl J Med.* 1978; 298:64–67.
- Clarren SK, Alvord EC, Sumi SM. Brain malformations related to prenatal exposure to ethanol. *J Pediatr.* 1978; 92:64. [PubMed: 619080]
- Clarren SK, Alvord EC Jr, Sumi SM, Streissguth AP, Smith DW. Brain malformations related to prenatal exposure to ethanol. *J Pediatr.* 1978; 92:1063–1067.
- Chiriboga CA. Fetal alcohol and drug effects. *Neurologist.* 2003; 9:267–279. [PubMed: 14629781]
- Connole L, Harkin A, Maginn M. Adenosine A1 receptor blockade mimics caffeine’s attenuation of ethanol-induced motor incoordination. *Basic Clin Pharmacol Toxicol.* 2004; 95:299–304. [PubMed: 15569276]
- Conklin MW, Lin MS, Spitzer NC. Local calcium transients contribute to disappearance of pFAK, focal complex removal and deadehesion of neuronal growth cones and fibroblasts. *Dev Biol.* 2005; 287:201–212. [PubMed: 16202989]
- Coulter CL, Leech RW, Schaefer GB, Scheithauer BW, Brumback RA. Midline cerebral dysgenesis, dysfunction of the hypothalamic-pituitary axis, and fetal alcohol effects. *Arch Neurol.* 1993; 50:771–775. [PubMed: 8323485]
- Dash PK, Moore AN, Moody MR, Treadwell R, Felix JL, Clifton GL. Post-trauma administration of caffeine plus ethanol reduces contusion volume and improves working memory in rats. *J Neurotrauma.* 2004; 21:1573–1583. [PubMed: 15684650]

- Dikranian K, Qin YQ, Labruyere J, Nemmers B, Olney JW. Ethanol-induced neuroapoptosis in the developing rodent cerebellum and related brain stem structures. *Dev Brain Res.* 2005; 155:1–13. [PubMed: 15763270]
- Dobbing J, Sands J. Comparative aspects of the brain growth spurt. *Early Hum Dev.* 1979; 3:79–83. [PubMed: 118862]
- Du Y, Lercher LD, Zhou R, Dreyfus CF. Mitogen-activated protein kinase pathway mediates effects of brain-derived neurotrophic factor on differentiation of basal forebrain oligodendrocytes. *J Neurosci Res.* 2006; 84:1692–1702. [PubMed: 17044032]
- El Yacoubi M, Ledent C, Parmentier M, Costentin J, Vaugeois JM. Caffeine reduces hypnotic effects of alcohol through adenosine A2A receptor blockade. *Neuropharmacology.* 2003; 45:977–985. [PubMed: 14573390]
- Fujita S. Quantitative analysis of cell proliferation and differentiation in the cortex of the postnatal mouse cerebellum. *J Cell Biol.* 1967; 32:277–287. [PubMed: 10976221]
- Fujita S, Shimada M, Nakamura T. H³-thymidine autoradiographic studies on the cell proliferation and differentiation in the external and the internal granular layers of the mouse cerebellum. *J Comp Neurol.* 1966; 128:191–208. [PubMed: 5970298]
- Gomez TM, Robles E, Poo MM, Spitzer NC. Filopodial calcium transients promote substrate-dependent growth cone turning. *Science.* 2001; 291:1983–1987. [PubMed: 11239161]
- Gorbunova YV, Spitzer NC. Dynamic interactions of cyclic AMP transients and spontaneous Ca²⁺ spikes. *Nature.* 2002; 418:93–96. [PubMed: 12097913]
- Gonzalez-Burgos I, Alejandre-Gomez M. Cerebellar granule cell and Bergmann glial cell maturation in the rat is disrupted by pre- and post-natal exposure to moderate levels of ethanol. *Int J Devl Neuroscience.* 2005; 23:383–388.
- Goodlett CR, Horn KH, Zhou FC. Alcohol teratogenesis: mechanisms of damage and strategies for intervention. *Exp Biol Med.* 2005; 230:394–406.
- Gudz TI, Komuro H, Macklin WB. Glutamate stimulates oligodendrocyte progenitor migration via an α_v -integrin/myelin proteolipid protein complex. *J Neurosci.* 2006; 26:2458–2466. [PubMed: 16510724]
- Guerri C. Mechanisms involved in central nervous system dysfunctions induced by prenatal ethanol exposure. *Neurotox Res.* 2002; 4:327–335. [PubMed: 12829422]
- Hatten ME. Riding the glial monorail: A common mechanism for glial-guided migration in different regions of the developing brain. *Trend Neurosci.* 1990; 13:179–184. [PubMed: 1693236]
- Henley J, Poo MM. Guiding neuronal growth cones using Ca²⁺ signals. *Trends Cell Biol.* 2004; 14:320–330. [PubMed: 15183189]
- Jiang Y, Kumada T, Cameron DB, Komuro H. Cerebellar granule cell migration and the effects of alcohol. *Dev Neurosci.* 2008; 30:7–23. [PubMed: 18075250]
- Jin M, Guan CB, Jiang YA, Chen G, Zhao CT, Cui K, Song YQ, Wu CP, Poo MM, Yuan XB. Ca²⁺-dependent regulation of Rho GTPases triggers turning of nerve growth cones. *J Neurosci.* 2005; 25:2338–2347. [PubMed: 15745960]
- Jones KL. Aberrant neuronal migration in the fetal alcohol syndrome. *Birth Def.* 1975; 7:131–132.
- Jones DG. Influence of ethanol on neuronal and synaptic maturation in the central nervous system-morphological investigations. *Prog Neurobiol.* 1988; 31:171–197. [PubMed: 3047814]
- Juilfs DM, Soderling S, Burns F, Beavo JA. Cyclic GMP as substrate and regulator of cyclic nucleotide phosphodiesterases (PDEs). *Rev Physiol Biochem Pharmacol.* 1999; 135:67–104. [PubMed: 9932481]
- Kawauchi D, Taniguchi H, Watanabe H, Saito T, Murakami F. Direct visualization of nucleogenesis by precerebellar neurons: involvement of ventricle-directed, radial fiber-associated migration. *Development.* 2006; 133:1113–1123. [PubMed: 16501169]
- Klein RS, Rubin JB, Gibson HD, DeHaan EN, Alvarez-Hernandez X, Segal RA, Luster AD. SDF-1 α induces chemotaxis and enhances Sonic hedgehog-induced proliferation of cerebellar granule cells. *Development.* 2001; 128:1971–1981. [PubMed: 11493520]
- Komuro H, Kumada T. Ca²⁺ transients control CNS neuronal migration. *Cell Calcium.* 2005; 37:387–393. [PubMed: 15820385]

- Komuro H, Rakic P. Selective role of N-type calcium channels in neuronal migration. *Science*. 1992; 257:806–809. [PubMed: 1323145]
- Komuro H, Rakic P. Modulation of neuronal migration by NMDA receptors. *Science*. 1993; 260:95–97. [PubMed: 8096653]
- Komuro H, Rakic P. Dynamics of granule cell migration: a confocal microscopic study in acute cerebellar slice preparations. *J Neurosci*. 1995; 15:1110–1120. [PubMed: 7869087]
- Komuro H, Rakic P. Intracellular Ca^{2+} fluctuations modulate the rate of neuronal migration. *Neuron*. 1996; 17:275–285. [PubMed: 8780651]
- Komuro H, Rakic P. Distinct modes of neuronal migration in different domains of developing cerebellar cortex. *J Neurosci*. 1998a; 18:1478–1490. [PubMed: 9454856]
- Komuro H, Rakic P. Orchestration of neuronal migration by activity of ion channels, neurotransmitter receptors, and intracellular Ca^{2+} fluctuations. *J Neurobiol*. 1998b; 37:110–130. [PubMed: 9777736]
- Komuro, H.; Rakic, P. *In vitro* analysis of signal mechanisms involved in neuronal migration. In: Haynes, LW., editor. *The neuron in tissue culture*. New York: John Wiley & Sons; 1999. p. 57–69.
- Komuro H, Yacubova E, Yacubova E, Rakic P. Mode and tempo of tangential cell migration in the cerebellar external granular layer. *J Neurosci*. 2001; 21:527–40. [PubMed: 11160432]
- Komuro H, Yacubova E. Recent advances in cerebellar granule cell migration. *Cell Mol Life Sci*. 2003; 60:1084–1098. [PubMed: 12861377]
- Komuro, Y.; Kumada, T.; Ohno, N.; Foote, KD.; Komuro, H. Migration in the cerebellum. In: Rubenstein, J.; Rakic, P., editors. *Comprehensive Developmental Neuroscience*. Oxford: Elsevier; 2010.
- Konovalov HV, Kovetsky NS, Bobryshev YV, Ashwell KW. Disorders of brain development in the progeny of mothers who used alcohol during pregnancy. *Early Hum Dev*. 1997; 48:153–166. [PubMed: 9131316]
- Kornguth SE, Rutledge JJ, Sunderland E, Siegal F, Carlson I, Smollens J, Juhl U, Young B. Impeded cerebellar development and reduced serum thyroxine levels associated with fetal alcohol intoxication. *Brain Res*. 1979; 177:347–360. [PubMed: 497836]
- Kumada T, Komuro H. Completion of neuronal migration regulated by loss of Ca^{2+} transients. *Proc Natl Acad Sci USA*. 2004; 101:8479–8484. [PubMed: 15150416]
- Kumada T, Lakshmana MK, Komuro H. Reversal of neuronal migration in a mouse model of fetal alcohol syndrome by controlling second-messenger signalings. *J Neurosci*. 2006; 26:742–756. [PubMed: 16421294]
- Kumada T, Jiang Y, Cameron DB, Komuro H. How does alcohol impair neuronal migration? *J Neurosci Res*. 2007; 85:465–470. [PubMed: 17139684]
- Kumada T, Jiang Y, Kawanami A, Cameron DB, Komuro H. Autonomous turning of cerebellar granule cells *in vitro* by intrinsic programs. *Dev Biol*. 2009; 326:237–249. [PubMed: 19063877]
- Kumada, T.; Komuro, Y.; Li, Y.; Littner, Y.; Komuro, H.; Preedy, VR.; Watson, RR.; Martin, CR. *International Handbook of Behavior, Diet and Nutrition*. New York: Springer; 2010. Neuronal cell migration in fetal alcohol syndrome.
- Lemoine P, Harousseau H, Borteyru JP, Menuet JC. Children of alcoholic parents-observed anomalies: discussion of 127 cases. *Ther Drug Monit*. 2003; 25:132–136. [PubMed: 12657907]
- Lovinger DM, White G, Weight FF. Ethanol inhibits NMDA-activated ion current in hippocampal neurons. *Science*. 1989; 243:1721–1724. [PubMed: 2467382]
- Lu Q, Sun EE, Klein RS, Flanagan JG. Ephrin-B reverse signaling is mediated by a novel PDZ-RGS protein and selectively inhibits G protein-coupled chemoattraction. *Cell*. 2001; 105:69–79. [PubMed: 11301003]
- Ma Q, Jones D, Borghesani PR, Segal RA, Nagasawa T, Kishimoto T, Bronson RT, Springer TA. Impaired B-lymphopoiesis, myelopoiesis, and derailed cerebellar neuron migration in CXCR4- and SDF-1-deficient mice. *Proc Natl Acad Sci USA*. 1998; 95:9448–9453. [PubMed: 9689100]
- Marcus JC. Neurological findings in the fetal alcohol syndrome. *Neuropediatrics*. 1987; 18:158–160. [PubMed: 3683756]

- McCaffery P, Omanands Koul, Smith D, Napoli JL, Chen N, Ullman MD. Ethanol increases retinoic acid production in cerebellar astrocytes and in cerebellum. *Dev Brain Res.* 2004; 153:233–241. [PubMed: 15527891]
- McGrath K, Koniski AD, Maltby KM, McGann JK, Palis J. Embryonic expression and function of the chemokine SDF-1 and its receptor, CXCR4. *Dev Biol.* 1999; 213:442–456. [PubMed: 10479460]
- Mezna M, Patchick T, Tovey S, Michelangeli F. Inhibition of the cerebellar inositol 1,4,5-trisphosphate-sensitive Ca^{2+} channel by ethanol and other aliphatic alcohols. *Biochem J.* 1996; 314:175–179. [PubMed: 8660280]
- Miale LL, Sidman RL. An autoradiographic analysis of histogenesis in the mouse cerebellum. *Exp Neurol.* 1961; 4:277–296. [PubMed: 14473282]
- Miller MW. Effects of alcohol on the generation and migration of cerebral cortical neurons. *Science.* 1986; 233:1308–1311. [PubMed: 3749878]
- Miller MW. Migration of cortical neurons is altered by gestational exposure to ethanol. *Alcohol Clin Exp Res.* 1993; 17:304–314. [PubMed: 8488973]
- Miller MW, Luo J. Effects of ethanol and transforming growth factor beta (TGF beta) on neuronal proliferation and nCAM expression. *Alcohol Clin Exp Res.* 2002; 26:1281–1285. [PubMed: 12198406]
- Ming G, Henley J, Tessier-Lavigne M, Song H, Poo MM. Electrical activity modulates growth cone guidance by diffusible factors. *Neuron.* 2001; 29:441–452. [PubMed: 11239434]
- Minana R, Climent E, Barettino D, Segui JM, Renau-Piqueras J, Guerri C. Alcohol exposure alters the expression pattern of neural cell adhesion molecules during brain development. *J Neurochem.* 2000; 75:954–964. [PubMed: 10936176]
- Munck S, Bedner P, Bottaro T, Harz H. Spatiotemporal properties of cytoplasmic cyclic AMP gradients can alter the turning behavior of neuronal growth cones. *Eur J Neurosci.* 2004; 19:791–797. [PubMed: 15009126]
- Nakajima K. Control of tangential/non-radial migration of neurons in the developing cerebral cortex. *Neurochem Int.* 2007; 51:121–131. [PubMed: 17588709]
- Narahashi T, Aistrup GL, Marszalec, Nagata K. Neuronal nicotinic acetylcholine receptors: a new target site of ethanol. *Neurochem Int.* 1999; 35:131–141. [PubMed: 10405997]
- Narahashi T, Soderpalm B, Ericson M, Olausson P, Engel JA, Zhang X, Nordberg A, Marszalec W, Aistrup GL, Schmidt LG, Kalouti U, Smolka M, Hedlund L. Mechanisms of alcohol-nicotine interactions: alcoholics versus smokers. *Alcohol Clin Exp Res.* 2001; 25:152S–156S. [PubMed: 11391065]
- Nishiyama M, Hoshino A, Tsai L, Henley JR, Goshima Y, Tessier-Lavigne M, Poo MM, Hong K. Cyclic AMP/GMP-dependent modulation of Ca^{2+} channels sets the polarity of nerve growth-cone turning. *Nature.* 2003; 423:990–995. [PubMed: 12827203]
- Ohrtmann JD, Stancik EK, Lovinger DM, Davis MI. Ethanol inhibits brain-derived neurotrophic factor stimulation of extracellular signal-regulated/mitogen-activated protein kinase in cerebellar granule cells. *Alcohol.* 2006; 39:29–37. [PubMed: 16938627]
- Ono K, Shokunbi T, Nagata I, Tokunaga A, Yasui Y, Nakatsuji N. Filopodia and growth cones in the vertically migrating granule cells of the postnatal mouse cerebellum. *Exp Brain Res.* 1997; 117:17–29. [PubMed: 9386001]
- Ozer E, Sarioglu S, Gure A. Effects of prenatal ethanol exposure on neuronal migration, neurogenesis and brain myelination in the mice brain. *Clin Neuropathol.* 2000; 19:21–25. [PubMed: 10774947]
- Peiffer J, Majewski F, Fischbach H, Bierich JR, Volk B. Alcohol embryo- and fetopathy. Neuropathology of 3 children and 3 fetuses. *J Neurosci Sci.* 1979; 41:125–137.
- Petersen OH, Cancela JM. Attraction or repulsion by local Ca^{2+} signals. *Curr Biol.* 2000; 10:R311–R314. [PubMed: 10801404]
- Rakic P. Neuron-glia relationship during granule cell migration in developing cerebellar cortex. A golgi and electron microscopic study in *Macacus rhesus*. *J Comp Neurol.* 1971; 141:283–312. [PubMed: 4101340]
- Rakic P. Principles of neuronal cell migration. *Experientia.* 1990; 46:882–891. [PubMed: 2209797]
- Rakic P, Komuro H. The role of receptor/channel activity in neuronal cell migration. *J Neurobiol.* 1995; 26:299–315. [PubMed: 7775964]

- Rakic P, Cameron SR, Komuro H. Recognition, adhesion, transmembrane signaling and cell motility in guided neuronal migration. *Curr Opin Neurobiol.* 1994; 4:63–69. [PubMed: 8173327]
- Ramón, Y.; Cajal, S. *Études sur la Neurogenèse de Quelques Vertébrés.* Madrid: Tipografía Artística; 1929.
- Riddoch FC, Rowbotham SE, Brown AM, Redfern CP, Cheek TR. Release and sequestration of Ca^{2+} by a caffeine- and ryanodine-sensitive store in a sub-population of human SH-SY5Y neuroblastoma cells. *Cell Calcium.* 2005; 38:111–120. [PubMed: 16095688]
- Riley EP, McGee CL. Fetal alcohol spectrum disorders: an overview with emphasis on changes in brain and behavior. *Exp Biol Med.* 2005; 230:357–365.
- Roebuck TM, Mattson SN, Riley EP. A review of the neuroanatomical findings in children with fetal alcohol syndrome or prenatal exposure to alcohol. *Alcohol Clin Exp res.* 1998; 22:339–344. [PubMed: 9581638]
- Rossi D, Slater TN. The developmental onset of NMDA receptor channel activity during neuronal migration. *Neuropharmacology.* 1993; 32:1239–1248. [PubMed: 7509049]
- Sakata-Haga H, Sawada K, Hisano S, Fukui Y. Abnormalities of cerebellar foliation in rats prenatally exposed to ethanol. *Acta Neuropathol.* 2001; 102:36–40. [PubMed: 11547949]
- Shetty AK, Phillips DE. Effects of prenatal ethanol exposure on the development of Bergmann glia and astrocytes in the rat cerebellum: An immunohistochemical study. *J Comp Neurol.* 1992; 321:19–32.
- Sokol RJ, Delaney-Black V, Nordstrom B. Fetal alcohol spectrum disorder. *JAMA.* 2003; 290:2996–2999. [PubMed: 14665662]
- Song H, Ming G, He Z, Lehmann M, McKerracher L, Tessier-Lavigne M, Poo MM. Conversion of neuronal growth cone responses from repulsion to attraction by cyclic nucleotides. *Science.* 1998; 281:1515–1518. [PubMed: 9727979]
- Spitzer NC. Electrical activity in early neuronal development. *Nature.* 2006; 444:707–712. [PubMed: 17151658]
- Tanaka DH, Maekawa K, Yanagawa Y, Obata K, Murakami F. Multidirectional and multizonal tangential migration of GABAergic interneurons in the developing cerebral cortex. *Development.* 2006; 133:2167–2176. [PubMed: 16672340]
- Welch-Carre E. the neurodevelopmental consequences of prenatal alcohol exposure. *Adv Neonatal Care.* 2005; 5:2117–229.
- Wen Z, Guirland C, Ming G, Zheng JA. CaMKII/Calcineurin switch controls the direction of Ca^{2+} -dependent growth cone guidance. *Neuron.* 2004; 43:835–846. [PubMed: 15363394]
- Yacubova E, Komuro H. Intrinsic program for migration of cerebellar granule cells in vitro. *J Neurosci.* 2002a; 22:5966–5981. [PubMed: 12122059]
- Yacubova E, Komuro H. Stage-specific control of neuronal migration by somatostatin. *Nature.* 2002b; 415:77–81. [PubMed: 11780120]
- Yacubova E, Komuro H. Cellular and molecular mechanisms of cerebellar granule cell migration. *Cell Biochem Biophys.* 2003; 37:213–234. [PubMed: 12625628]
- Yanai J, Waknin S. Comparison of the effects of barbiturate and ethanol given to neonates on the cerebellar morphology. *Acta Ana.* 1985; 123:145–147.
- Yuen EC, Mobley WC. Early BDNF, NT-3, and NT-4 signaling events. *Exp Neurol.* 1999; 159:297–308. [PubMed: 10486198]
- Zou YR, Kottmann AH, Kuroda M, Taniuchi I, Littman DR. Function of the chemokine receptor CXCR4 in haematopoiesis and in cerebellar development. *Nature.* 1998; 393:595–599. [PubMed: 9634238]

Fig. 1.

Ethanol exposure reduces the number of granule cells that entered the ML of the early postnatal mouse cerebellum. (A) The three-dimensional representation of granule cell migration from the EGL to the IGL in the developing mouse cerebellum. 1, Extension of two uneven horizontal processes near the top of the EGL; 2, Tangential migration in the middle of the EGL; 3, Development of vertical process near the EGL-ML border; 4, initiation of turning at the EGL-ML border; 5, Bergmann glia-associated radial migration in the ML; 6, Stationary state in the PCL; 7, Glia-independent radial migration in the IGL; 8, Completion of migration in the middle or the bottom of the IGL. Abbreviations: P, Purkinje cell; B, Bergmann glia; G, Golgi cell; g, postmigratory granule cell; cf, climbing fiber; mft, mossy fiber terminal. (B) The effects of ethanol on the translocation of BrdU-labeled granule cells from the EGL to the ML in the postnatal mouse cerebellum. P9 mice were injected i.p. with BrdU (50 mg/kg b.w.). One day after BrdU injection (at P10), mice were injected i.p. with saline (100 μl) or one of three different doses of ethanol (1, 3, or 5 g/kg b.w.). Two days after BrdU injection (at P11), all mice were transcardially perfused with 4% paraformaldehyde. The distribution of BrdU-labeled granule cells was examined by the use of an anti-BrdU monoclonal antibody and fluorescein-conjugated secondary antibody. (C) The effects of ethanol on the number of BrdU-labeled granule cells in the ML, PCL and IGL of the cerebellum. Each column represents the average number obtained from more than 500

positions in all lobules. Each bar is S.D. Single ($p < 0.05$) and double ($p < 0.01$) asterisks indicate statistical significance.

Fig. 2.

Morphological changes of granule cells in the early postnatal mouse cerebellum revealed by Golgi-staining. (A) Micrographs showing the migration and differentiation of granule cells in the P10 mouse cerebellum. a1, granule cell precursors (marked by the white circles) at the top of the EGL. a2-a6, tangentially oriented granule cells (marked by the white asterisks) at the middle and bottom of the EGL. a7, Bergmann glial cell. a8-a10, radially oriented granule cells (marked by the white asterisks) in the ML and PCL. a11-a12, postmigratory granule cells (marked by the white squares) in the IGL. Bar: 15 μ m in a1-a11. (B) Micrographs showing three different modes of granule cell turning at the EGL-ML border of the P10 mouse cerebellum. b1-b8, T-shape turning of granule cells. Micrographs showing the extension of a single vertical process into the ML from the granule cell soma. The cells also had two horizontally-extended axon-like processes (future parallel fibers). b9-b23, L-shape turning of granule cells. Micrographs showing the turning of the tip of the horizontally-oriented leading process of granule cells towards the ML. b24-b32, Y-shape turning of granule cells. Micrographs showing the bifurcation of the horizontally-oriented leading process of granule cells. One of the branches extended vertically and entered the ML, while the other extended horizontally in the EGL. In b1-b32, white asterisks mark the somata of granule cells. Bar: 15 μ m in b1-b32.

Fig. 3.

Reduction of the average number of turning granule cells at the EGL-ML border of the P10 mouse cerebella after the injection of one of three different doses of ethanol (0, 1, 3, or 5 g/kg b.w.). Each column represents the average number of turning granule cells at the 100 μm-length EGL-ML border obtained from at least 500 positions of all lobules. Single ($p < 0.05$) and double ($p < 0.01$) asterisks indicate statistical significance. Bar: S.D.

Fig. 4. Alterations of the ratio of T-, L-, and Y-shape granule cell turning at the EGL-ML border of the P10 mouse cerebella by ethanol exposure. (A) Schematic representation of T-, L-, and Y-shape turning of granule cells at the EGL-ML border. (B) Histograms showing the effects of the i.p. injection of one of three different doses of ethanol (0, 1, 3, or 5 g/kg b.w.) on the ratio of the T-, L-, and Y-shape turning of granule cells at the EGL-ML border. Single ($p < 0.05$) and double ($p < 0.01$) asterisks indicate statistical significance. Bar: S.D.

Fig. 5.

Spontaneous turning of granule cells in microexplant cultures of the early postnatal mouse cerebellum. (A) Time-lapse series of images showing the turning of a granule cell in microexplant cultures of P2 mouse cerebella over time. Elapsed time *in vitro* is indicated on the top of each photograph. Arrows and asterisks indicate the processes and the soma of a granule cell, respectively. Bar: 15 μ m. (B) Migration of the granule cell (shown in A) towards the right side of the photograph. Five pseudocolor images of the cell taken every 5 minutes during the period of 20:00–20:20 *in vitro* are superimposed. (C) Migration of the granule cell (shown in A) towards the bottom of the photograph. Ten pseudocolor images of the cell taken every 5 minutes during the period of 20:25–21:10 *in vitro* are superimposed. (D) Migration of the granule cell (shown in A) towards the top of the photograph. Four pseudocolor images of the cell taken every 5 minutes during the period of 21:15–21:30 *in vitro* are superimposed. (E) Migration of the granule cell (shown in A) towards the right-bottom of the photograph. Four pseudocolor images of the cell taken every 5 minutes during the period of 21:35–21:50 *in vitro* are superimposed. (F) Migration of the granule cell (shown in A) towards the left-bottom of the photograph. Fifteen pseudocolor images of the cell taken every 5 minutes during the period of 21:55–23:05 *in vitro* are superimposed. (G) Migration of the granule cell (shown in A) towards the left of the photograph. Two pseudocolor images of the cell taken every 5 minutes during the period of 23:10–23:15 *in vitro* are superimposed. (H) Migration of the granule cell (shown in A) towards the bottom of the photograph. Two pseudocolor images of the cell taken every 5 minutes during the period of 23:20–23:25 *in vitro* are superimposed. Bar: 15 μ m in B-H.

Fig. 6.

Fluctuations of the frequency of granule cell turning over time. Line drawing represents the trajectory of the migration of a granule cell in the microexplant cultures of P2 mouse cerebella over time. Black open circles represent the position of a granule cell at the beginning of observation (at 20:00 *in vitro*). The interval between large filled black and red circles represents 30 minutes. The interval between small filled black and red circles represents 3 minutes. The numbers in the figure represent the elapsed time after *in vitro*. Red circles and red lines indicated the frequent turning periods, while black circles and black lines indicated the less frequent turning periods. Bar: 15 μ m.

Fig. 7.

Reduction of the frequency of granule cell turning in microexplant cultures of P2-P3 mouse cerebella by ethanol (0–100 mM). (A) The dose-dependent effects of ethanol on the number of high-angle ($>45^\circ$) turning of granule cells, which is defined as the change in the direction of their migration deviating more than 45° from the previous movement of their cell body within 30 consecutive minutes. (B) The dose-dependent effects of ethanol on the number of low-angle ($30\text{--}45^\circ$) turning of granule cells, which is defined as the change in the direction of their migration deviating between 30° and 45° from the previous movement of their cell body within 30 consecutive minutes. In A and B, each column represents the average frequency of granule cell turning obtained from at least 200 cells during a 6–10 hour-period of observation starting at 20 hours *in vitro*. Double ($p < 0.01$) asterisks indicate statistical significance. Bar: S.D.

Fig. 8.

Alterations of the effects of ethanol on the frequency of granule cell turning by stimulating the Ca^{2+} signaling pathway. Each column represents the average frequency of granule cell turning obtained from at least 100 cells during a 6–10 hour-period of observation starting at 20 hours after *in vitro*. Each reagent was added to the culture medium at 20 hours after *in vitro*. Single ($p < 0.05$) and double ($p < 0.01$) asterisks indicate statistical significance. Bar: S.D.

Fig. 9.

Amelioration of the effects of ethanol on the frequency of granule cell turning by altering the cAMP signaling pathway. Each column represents the average frequency of granule cell turning obtained from at least 100 cells during a 6–10 hour-period of observation starting at 20 hours after *in vitro*. Each reagent was added to the culture medium at 20 hours after *in vitro*. Single ($p < 0.05$) and double ($p < 0.01$) asterisks indicate statistical significance. Bar: S.D.

Fig. 10.

Changes in the effects of ethanol on the frequency of granule cell turning by altering the cGMP signaling pathway. Each column represents the average frequency of granule cell turning obtained from at least 100 cells during a 6–10 hour-period of observation starting at 20 hours after *in vitro*. Each reagent was added to the culture medium at 20 hours after *in vitro*. Double (p < 0.01) asterisks indicate statistical significance. Bar: S.D.

Fig. 11.

Reduction of the effects of ethanol on the frequency of granule cell turning by altering the activity of PDE. Each column represents the average frequency of granule cell turning obtained from at least 100 cells during a 6–10 hour-period of observation starting at 20 hours after *in vitro*. Each reagent was added to the culture medium at 20 hours after *in vitro*. Single ($p < 0.05$) and double ($p < 0.01$) asterisks indicate statistical significance. Bar: S.D.

	0 mM EtOH		25 mM EtOH		100 mM EtOH	
	Turning	Speed	Turning	Speed	Turning	Speed
NMDA	↑	↑	↑	↑	↑	↑
PKI	↑	↑	↑	↑	↑	—
caffeine	↑	—	—	↑	↑	↑
9CP-Ado	↑	—	↓	—	↓	↑
Rp-cAMPS	↑	—	↑	↑	↑	↑
forskolin	—	↓	↓	↓	↓	—
Sp-cAMPS	—	↓	↓	—	—	—

Category 1	Turning & speed changed in the same direction.
Category 2	Turning changed, but speed did not change.
Category 3	Turning did not change, but speed changed.
Category 4	Turning & speed changed in the opposite direction.
Category 5	Turning & speed did not change.

Fig. 12.
The relationship between the changes in the speed of granule cell migration and the frequency of turning in the presence and absence of ethanol.