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Analysis of cerebellar Purkinje cells using EAAT4 glutamate transporter promoter reporter in mice generated via bacterial artificial chromosome-mediated transgenesis

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Abstract

The EAAT4 glutamate transporter helps regulate excitatory neurotransmission and prevents glutamate-mediated excitotoxicity in the cerebellum. Immunohistochemistry and *in situ* hybridization have previously defined a cerebellar cell population expressing this protein. These methods, however, are not well suited for evaluating the dynamic regulation of the transporter and its gene—especially in living tissues. To better study EAAT4 expression and regulation, we generated bacterial artificial chromosome (BAC) promoter eGFP reporter transgenic mice. Histological analysis of the transgenic mice revealed that the EAAT4 promoter is active predominantly in Purkinje cells, but can also be modestly detected in other neurons early postnatally. EAAT4 promoter activity was not present in non-neuronal cells. Cerebellar organotypic slice cultures prepared from BAC transgenic mice provided a unique reagent to study transporter and Purkinje cell expression and regulation in living tissue. The correlation of promoter activity to protein expression makes the EAAT4 BAC promoter reporter a valuable tool to study regulation of EAAT4 expression.

Keywords: EAAT4; Purkinje cells; Spinocerebellar degeneration; Radiation injury; Glutamate; Cell death; BAC; Transgenic; Promoter; Transport

Introduction

The glutamate transporter EAAT4 is the predominant transporter of the cerebellum (Danbolt, 2001). Both *in situ* hybridization (Yamada et al., 1997) and immunohistochemical (Furuta et al., 1997b) studies previously demonstrated that EAAT4 is mainly located in the membrane and cytoplasm of Purkinje neurons. EAAT4 transporters contribute to the removal of glutamate at climbing and parallel fiber synapses on Purkinje cells, and they limit activation of perisynaptic metabotropic glutamate receptors (mGluRs) (Huang et al., 2004). By shaping the activation of mGluRs, EAAT4 transporters can alter long-term depression at parallel fiber synapses (Wadiche and Jahr, 2005). Deficient transport, caused by a decreased number of transporters at the Purkinje cell membrane, is associated with several neurological disorders such as Canavan disease (Surendran et al., 2003), olivopontocerebellar atrophy (Dirson et al., 2002), global brain ischemia (Welsh et al., 2002), and spinocerebellar ataxia type 1 (Lin et al., 2000) and 5 (Ikeda et al., 2006), making it a protein whose regulation is of great interest.

Despite the demonstrated importance of EAAT4 in synaptic signaling and in protecting Purkinje neurons from ischemic damage, little is known about the mechanisms that regulate EAAT4 expression *in vivo*. Transcriptional regulation of protein expression is typically achieved through the promoter, usually upstream of the start codon, and can also involve 3′ untranslated regions as well. To better understand how EAAT4 expression is regulated, we created transgenic mice using a bacterial artificial

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chromosome (BAC) containing the EAAT4 gene plus 107 kb of DNA upstream of the first exon and 54 kb downstream of the last exon. EGFP cDNA was inserted in-frame into the first coding exon to drive expression of eGFP when the promoter is active. The ability to monitor EAAT4 promoter activity *in situ* provides a valuable tool for studying the regulation of EAAT4 expression and the role of this transporter in synaptic signaling and cerebellar disease.

Materials and methods

BAC constructs

Mouse BAC RPCI-23-202O10, obtained from BAC-PAC Resources (www.bacpac.chori.org), was used for the EAAT4 reporter mice. This BAC was modified using a double homologous combination approach with the SV-RecA shuttle vector as described (Yang et al., 1997) to insert cDNA for eGFP. The first homology region, termed the A-box, included the 401 bp between the sequence 5′-GACTAGCCTAACGGTGCCTT-3′ and the reverse complement sequence 5′-CAACGCGTCT-CTGCGGGGGA-3′ based on the published online mouse genome (http://www.ensembl.org/Mus_musculus/index.html). The EAAT4 start codon, in exon 2, was mutated to TTG, and the eGFP cDNA (Clontech) was inserted downstream, after the end of the A-box, within the same exon, as illustrated in Fig. 1A.

The final modified BAC was transfected into HEK-293 cells using Fugene 6 Transfection Reagent (Roche) to validate reporter function, then linearized and injected into mouse pronuclei for generation of transgenic reporter mice. Founders

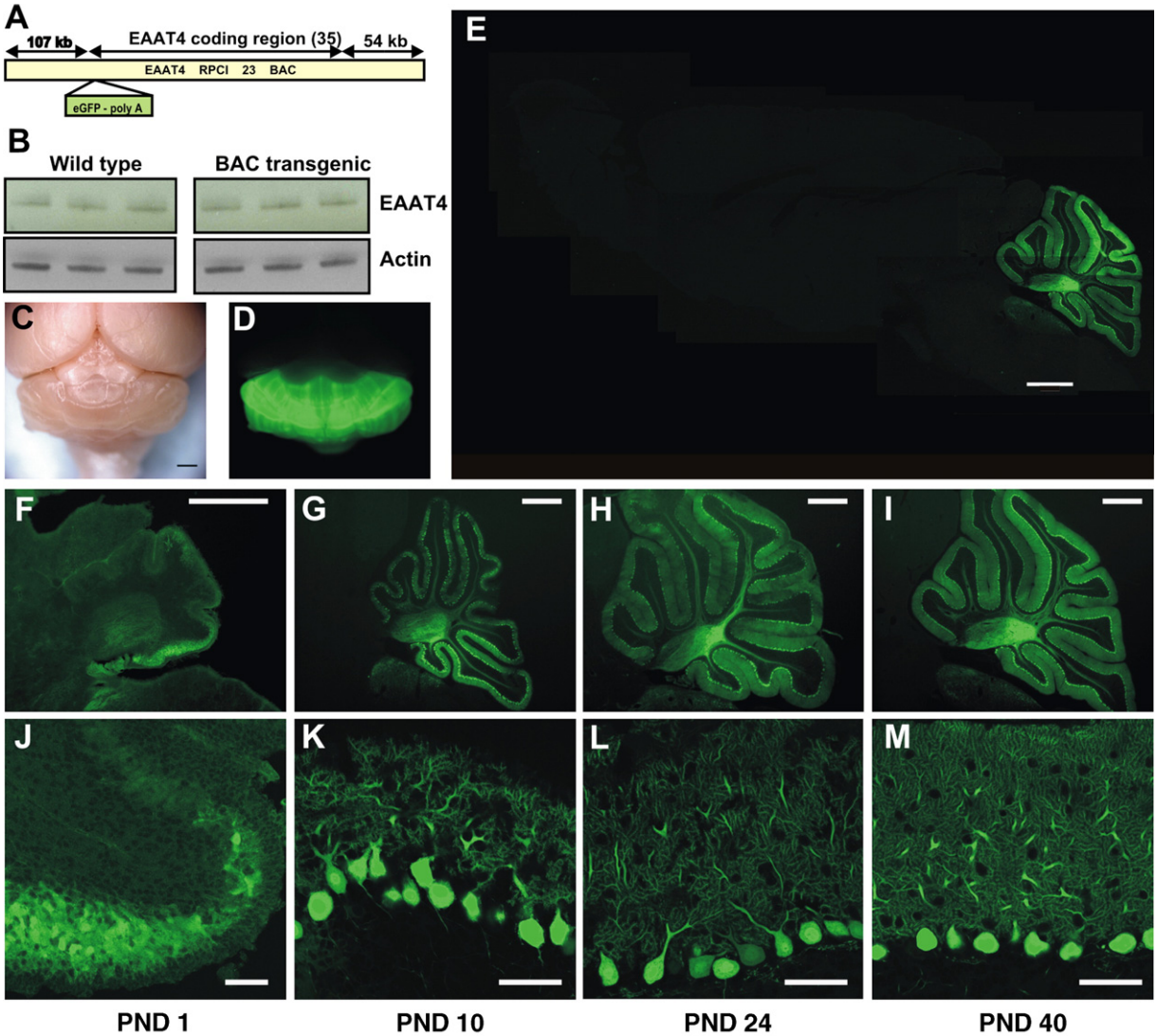

Fig. 1. EAAT4-BAC-eGFP promoter reporter mice and developmental changes in the cerebellum. (A) A schematic drawing of the BAC construct that was used to generate the EAAT4-BAC-eGFP transgenic mice. (B) Western blot of EAAT4 protein in three transgenic mice and three of their wild type littermates showing no difference in the expressed protein compared to actin. (C) Bright field and (D) eGFP fluorescence image of the brain from an adult (PND 40) EAAT4-BAC-eGFP mouse. The parasagittal banding patterns are clearly seen. (E) Sagittal image showing eGFP fluorescence in brain from a PND 40 EAAT4-BAC-eGFP mouse. (F–I) Low power and (J–M) high power images of eGFP expression in post natal developing cerebellum from EAAT4-BAC-eGFP mice. Scale bars: C, D, and E, 1 mm; F–I, 600 μm; J–M, 50 μm.

were identified via PCR to detect eGFP transcripts. These founders, of the B6SJL hybrid strain, were bred with C57Bl/6 wild type mice for more than eight generations to propagate the transgenic lines.

Histology

Mice were anesthetized and perfused with 4% paraformaldehyde via intra-ventricular cannulation, post-fixed in the same fixative overnight, cryoprotected with 20–30% glycerol in phosphate buffer for 24 h, and then frozen. Sections were cut at 40–45 μm and mounted on glass slides. Histological analysis was performed on parasagittal and/or coronal sections from two to three mice at each age. Digital images were taken from either a Nikon E800 microscope upright microscope or a Zeiss LSM 510 Meta confocal microscope using plan-neufluor 40×/1.3 oil, pinhole 66 μm and excitation wavelength 488 nm (3% intensity).

Immunohistochemistry and western blot

Cerebellum parasagittal sections were rinsed with phosphate-buffered saline (PBS), and blocked with 1% goat and 1% horse serum for 1 h at room temperature. Slices were then incubated overnight at 4°C with an antibody against Aldolase C (1:500). For fluorescence studies, slices were then washed with PBS and incubated with secondary antibodies conjugated to fluorescent AMCA (blue, Vector Labs, 1:200) for 1 h. Slices were then washed with PBS and mounted using aqueous mounting media (Gel/mount, Foster City, CA).

For Western blot analysis the right half of the cerebellum was dissected from PND 25 EAAT4-BAC-eGFP transgenic mice. The cerebellum was homogenized in 1× sucrose buffer with protease inhibitors. Following a protein assay, all samples were mixed with 2× Western buffer and 10 μg of protein was loaded per well on a 4–12% NuPAGE gradient gel (Invitrogen, Carlsbad CA). The gel was run for 1 h at 200 V in 1× MES buffer, transferred 1 h at 100 V, and blocked 1 h in 5% milk in TBS–Tween. The membrane was incubated O/N in 5% milk with anti-EAAT4 antibody (1:200 C-terminal EAAT4 aa 550–563) and anti-actin (1:50,000; Sigma). The following day, the membrane was washed 5× 5min in TBS–Tween and incubated 1 h in anti-rabbit HRP (1:5,000). HRP was detected with ECL reagents. Blot was exposed for 30 s, 1 min, and 2 min.

Organotypic slices

Organotypic cultures were prepared and maintained as described previously (Rothstein et al., 1993). Cerebella of 8-day-old EAAT4-BAC-eGFP pups were collected under sterile conditions and sectioned transversely into 350-μm slices using a Vibrotome vibrating microtome (Saint Louis, MO). Slices were cultured in Millicell CM semipermeable culture inserts at a density of 1–3 slices per well in an incubator at 37°C (5% CO_2, 95% humidity). Under these conditions, >95% of cultures retained cellular organization. Culture medium was changed twice weekly, propidium iodide (PI) was added (2 μg/ml) to the media to stain for dying cells using a rhodamine filter (510–560/590 nm) to visualize PI fluorescence emission. Analysis was performed as described previously (Vornov et al., 1991; Adamchik et al., 2000) Gains and black levels were standardized for each experiment. The fluorescence images were acquired and analyzed with the MetaMorph software package. Pixel intensity was measured either for the whole slice or in selected areas of the slice, using a standard size circle (20 pixels in diameter). For radiation induced damage, media was changed prior to radiation, cultures were irradiated with a single dose 3 gray using a cesium source (Gammacell 40, Atomic Energy). Organotypic cultures were then allowed to recover for 24 h and viewed live under an inverted microscope (Eclipse TE2000-U; Nikon, Japan). Slices exhibiting PI staining before radiation or those revealing any incomplete or absent cerebellum layer were excluded from the experiments.

Results

EAAT4-BAC-eGFP expression in adult mice is restricted to cerebellum

The EAAT4 BAC was modified as described in the Materials and methods and as shown in Fig. 1A, and transgenic mice, containing the BAC construct with the reporter eGFP cDNA under the control of the EAAT4 promoter, were generated. Five out of 12 pups were genotyped positive for the transgene by PCR, but only two lines expressed the reporter. Line 41 demonstrated a much stronger reporter expression phenotype than line 61, likely because of a greater number of head-to-tail inserted copies of the transgene. Adult and young animals from all the EAAT4-eGFP mouse lines were behaviorally normal, with no gross abnormalities in motor activity.

Gross promoter activation in the entire cerebellum can be seen even at the whole brain level, as shown in Figs. 1C and D, and a lateral banding pattern of expression across the cerebellum is clearly seen. A low power magnification of sagittal brain sections (Fig. 1E) also reveals that, in the adult mouse, promoter expression is largely restricted to cerebellum. The was no evidence of abnormality in neural development associated with the EAAT4-eGFP BAC transgene with normal appearing CNS morphology in neonatal and adult brain.

Although the entire EAAT4 gene is present in the BAC construct, no EAAT4 mRNA or protein is expressed by these transgenes because the first coding exon (exon 2) is interrupted by the reporter cDNA followed by a poly-A tail sequence. In addition, the respective transporter protein levels are unchanged in the BAC reporter transgenic mice as compared to wild type mice littermates. We thought it possible that extra copies of the EAAT4 promoter might serve as a sink for transcription elements, thus actually reducing the specific transporter protein levels in the transgenic mice. This would, in turn, make the mice less useful for studies of up- or down-regulation of the transporters. However, this was not the case. The Western

blots in Fig. 1B show that EAAT4 protein expression in cerebellum is identical between BAC transgenic mice and wild type littermates. For all blots, actin served as a control to validate equal protein loading among lanes (Fig. 1B).

Developmental changes in EAAT4 promoter activity in the cerebellum

In order to characterize the change in EAAT4 promoter activity during development, we inspected EAAT4 promoter activation at postnatal days 1, 10, 25, and 40 (Figs. 1F–M). As shown in these panels, the posterior and inferior lobes of the cerebellar cortex mature earlier than the anterior lobes. As a result, EAAT4 promoter activity is first observed in these regions (Figs. 1F, G and J, K). The early development of the posterior lobe in the cerebellum has also been seen in organotypic slice culture (unpublished data). This developmental profile is comparable to previous reports of EAAT4 protein expression using immunohistochemistry (Furuta et al., 1997a) and other cerebellar proteins (Armstrong et al., 2001; Kadowaki et al., 2004). EAAT4 promoter activity increases as the external granule cell layer dissipates after PND 10 (Figs. 1H, I and L, M). By PND 10, eGFP can be observed in the maturing dendrites and dendritic spines of Purkinje cells.

EAAT4 promoter activity is observed in rare non-cerebellar neurons

In order to study EAAT4 promoter activity in tissue other then cerebellum we examined sagittal sections of whole brain at multiple postnatal time points. Although the majority of the brain was almost completely eGFP negative, EAAT4 promoter activity was observed in other brain regions. In particular, eGFP reporter activity was detected in rare neurons in the thalamus and spinal cord, up to PND 10, and in hippocampal CA1 neurons up to PND 40 (Fig. 2). Thus, the eGFP reporter enables us to predict expression of EAAT4 in cells where the level of expression is too low to be detected by antibodies or where the expression lasted for only a short period of time.

EAAT4 promoter activity predicts protein expression

Two complementary approaches to expression analysis were performed to further investigate whether EAAT4 promoter activity accurately reflects transporter protein expression patterns. First, we looked for co-localization of eGFP fluorescence with the Aldolase C (Zebrin II) protein. Co-localization of EAAT4 and Aldolase C protein has been previously described (Dehnes et al., 1998). The parasagittal banding of Aldolase C completely co-localized with the EAAT4 promoter activity (Figs. 3A–C). EAAT4 immunoreactivity in cerebellum was identical to the eGFP reporter activity as well (not shown).

In the second experiment we compared the distribution of the neuronal EAAT4 promoter to that of the glial glutamate transporter GLAST promoter in mouse cerebellum. GLAST is highly expressed in the cerebellar molecular layer (Rothstein et al., 1994). We mated the EAAT4-BAC-eGFP promoter reporter mouse with a GLAST-BAC-DsRed promoter reporter mouse (Regan et al., in preparation) and examined the fluorescent protein distribution. As shown (Fig. 3D), both glial and neuronal glutamate transporter reporters were enriched in cerebellar cortex in PND 10 mice. Promoter activation for glial glutamate transporter GLAST was localized to Bergmann glia surrounding the Purkinje neurons while EAAT4 promoter activation was confined to the Purkinje cells. Both experiments indicate that the promoter reporter can accurately reveal the protein expression pattern.

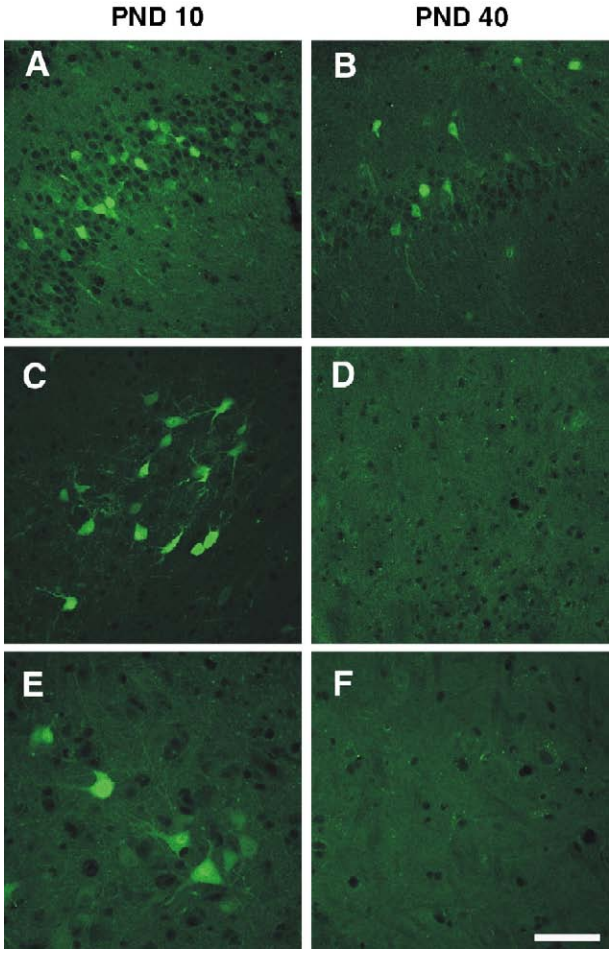

Fig. 2. eGFP fluorescence shows EAAT4 promoter activity in non-cerebellar neurons in early post natal developing mice. (A, B) Hippocampus (CA1 area); (C, D) thalamus and (E, F) Spinal cord (ventral gray). Scale bar, 50 μm.

Cerebellar organotypic slice cultures from EAAT4-BAC-eGFP mice

One advantage of a naturally fluorescent promoter reporter mouse line is the ability to perform experiments with live tissue. Whether following EAAT4 promoter activity, Purkinje neuron development, or tissue viability, the EAAT4 promoter reporter mice enable us to study tissue without fixing or staining, and therefore, to measure changes over time. We have used the EAAT4-BAC-eGFP promoter reporter mice to indicate the

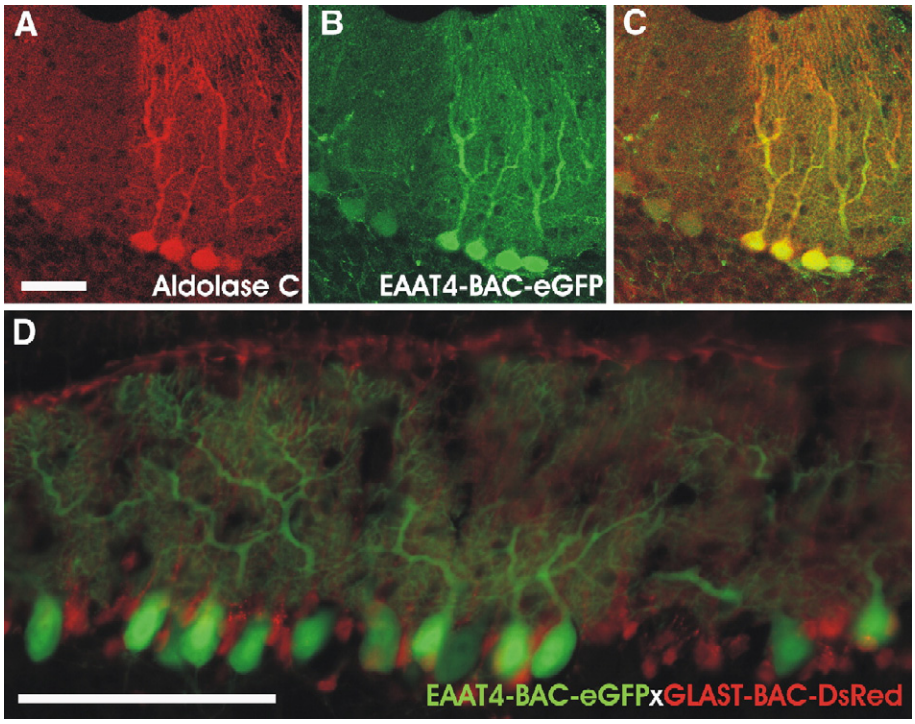

Fig. 3. Co-localization of EAAT4 promoter activity with Aldolase C but not with the glial glutamate transporter GLAST. Parasagittal from sections of EAAT4-BAC-eGFP mice slices were incubated with Aldolase C antibody (A) and co-localized with the EAAT4-BAC-eGFP fluorescence (B). The merge is presented in panel C. In panel D, GLAST promoter activity (red) is restricted to the Bergmann glial cells surrounding Purkinje neurons while EAAT4 promoter activity (green) is seen in Purkinje cell bodies and dendrites and does not co-localize with GLAST. Scale bar in panel A–C, 25 μm and 50 μm in panel D.

vitality of organotypic slices following damage induced by treatments and have found that slices lose their eGFP fluorescence before they show any other sign of cell death and long before the tissue loses its structure.

Fig. 4 shows an example of a cerebellum organotypic slice culture from an EAAT4-BAC-eGFP PND 8 mouse used for studying survival following radiation induced damage of the slices and the survival of Purkinje neurons over time. While the tissue viewed with light microscopy looks intact up to 21 days in culture (not shown), the Purkinje neurons start losing their fluorescence over time, suggesting that the cells are injured or dying and, therefore, that other proteins may also be dysregulated. When we quantified the intensity of eGFP-expressing cells and compared it to the fluorescence intensity from propidium iodide staining, we found that the higher the intensity of the eGFP from the Purkinje neurons, the healthier the tissue was, as determined by staining with propidium iodide (Fig 4). Also, whereas the propidium iodide staining was not always consistent and gave high standard errors, eGFP fluorescence was much more consistent (Fig. 4B). We also quantified the eGFP (EAAT4 promoter activity) and propidium iodide intensity in different areas of the same slices and found that there were differences between eGFP and propidium iodide staining (Table 1). Propidium iodide staining was not always consistent with eGFP fluorescence in the different areas. While the propidium iodide staining served as an indicator of cell death for all the cells in the slice, the loss of eGFP was more localized and indicated early damage specifically to Purkinje neurons.

Following 3 gray of gamma radiation to induce damage, we measured up to 62.8% more eGFP fluorescence (promoter activity) in some areas of the slices than in other areas, indicating that the damage to Purkinje neurons is uneven while the cell death in those areas indicated by propidium iodide staining was about the same, only up to a 2% difference in most cases (Table 1).

Discussion

In this report, we describe transgenic BAC promoter reporter mice in which the fluorescent reporter eGFP, incorporated into the first coding exon for EAAT4, is expressed under the control of full-length promoter and regulatory elements. These transgenic mice provide the opportunity to easily view promoter regulation as well as a tool to follow Purkinje cell function and survival.

EAAT4 promoter activity

EAAT4 promoter activity in the adult mouse is highly restricted to cerebellar Purkinje neurons. Previous reports have documented the cellular localization of EAAT4 protein in humans and rodents; however, we gained several new observations through analysis of the EAAT4 BAC promoter reporter mice. They provide insight into the dynamic developmental profile of gene expression in the cerebellum and revealed a regional distribution to EAAT4 gene activation. The gene appears to be first activated in the posterior and inferior neurons of the cerebellum, followed by a gradual

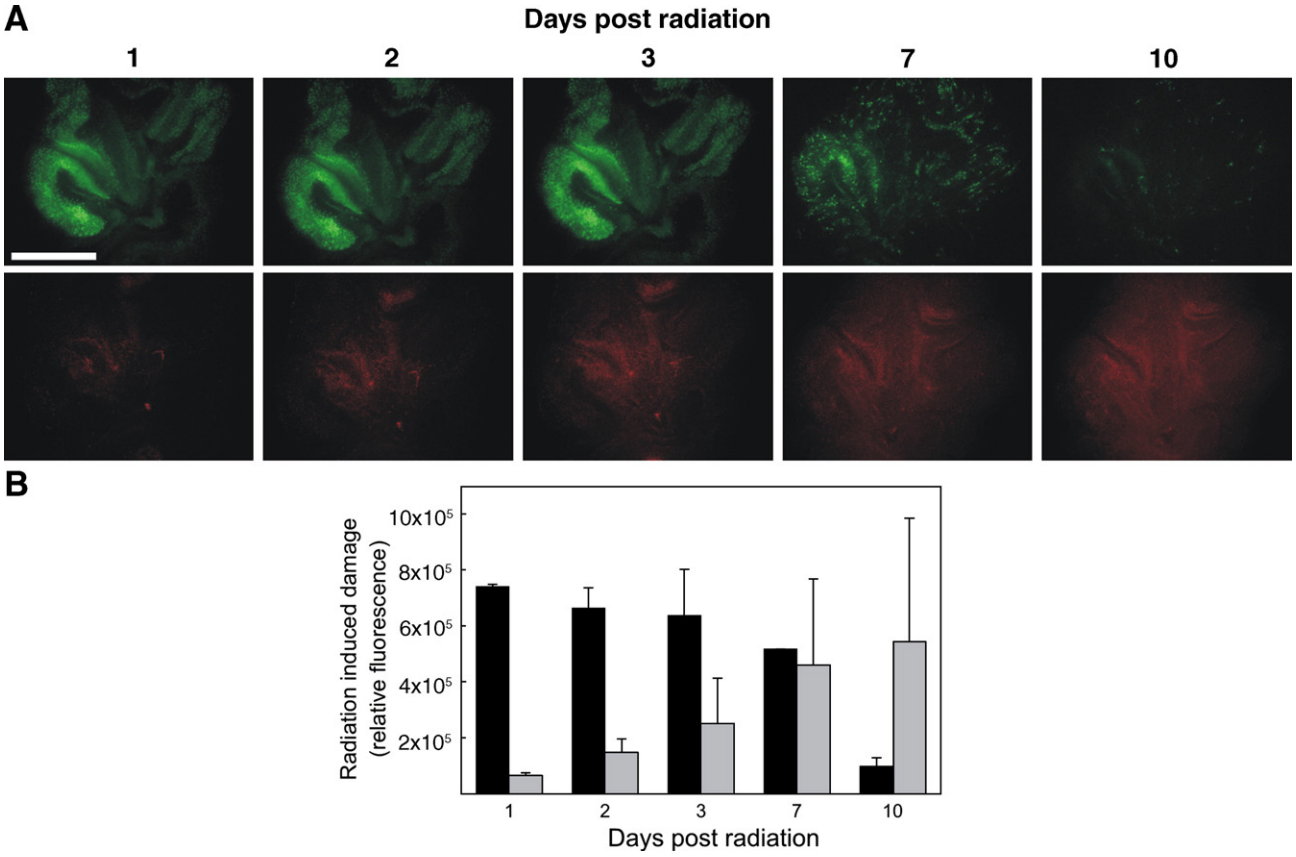

Fig. 4. Organotypic slices from EAAT4-BAC-eGFP. Postnatal day 8 cerebellum sagittal organotypic slices (350 μm) were plated and 24 h later exposed to 3 gray radiation. (A) Fluorescence of eGFP (upper) and propidium iodide (PI) (lower) was viewed and measured under the same exposure conditions 1, 2, 3, 7 and 10 days post radiation. Cell death is evident with time. Scale bar, 600 μm. (B) Average cell death ($n=3$) in injured cerebellum slices after radiation induced damage. The loss of fluorescence emission of eGFP (black) and increased propidium iodide emission (gray) was measured for the whole slice using a preset threshold.

advancement towards the anterior superior cerebellar folia and neurons. This pattern follows the developmental maturation of the cerebellum (Vastagh et al., 2005).

Unexpectedly, we observed that rare neurons, in various forebrain regions from young animals, expressed the EAAT4 promoter reporter. This had not been previously reported with antibody-mediated techniques. At the level of protein expression, our laboratory and others have demonstrated that forebrain expression of EAAT4 protein is more than 100-fold lower than in cerebellar tissue (Furuta et al., 1997b). To date there has been no physiological evidence of EAAT4 transporter current in forebrain neurons. However, most studies of forebrain glutamate transport have been restricted to hippocampus. In addition, the ability to identify individual EAAT4 expressing neurons in living cells had not been previously possible. Thus, these BAC promoter reporter mice can aid in examining the individual EAAT4 reporting neurons to assess their transporter biology. Whether or not neurons that express EAAT4 are in some way different physiologically, can withstand neural injury, or have unique developmental properties, is not yet clear.

Table 1
Stability of EAAT4 eGFP promoter reporter versus variability of propidium iodide in cerebellar organotypic slice cultures following radiation induced acute damage

Days after induced injury	eGFP fluorescence		PI fluorescence	
	Area 1	Area 2	Area 1	Area 2
1	146.0±14.2	60.3±4.4	73.1±7.5	73.6±12.9
2	139.9±14.3	58.6±2.9	63.1±7.8	64.4±2.1
3	145.6±26.9	54.2±0.9	57.7±4.6	80.7±35.2
7	77.3±3.9	65.4±3.9	60.3±6.3	58.5±3.6
10	73.9±3.3	55.5±0.8	61.1±15.1	60.4±9.1

EAAT4-BAC-eGFP organotypic slices where exposed to 3 Gy radiation, and fluorescence from promoter activity of EAAT4 (eGFP) and from propidium iodide staining was measured in two independent non-overlapping areas of the same slices. Each measurement represents fluorescence intensity±standard error in a 20-pixel circle as indicated in the Materials and methods section and measured by image analysis software.

Promoter activity of EAAT4 correlates with protein expression

Several studies have been done on the localization of EAAT4 using immunohistochemistry (Furuta et al., 1997b; Furuta et al., 1997a), electron microscopy (Lin et al., 1998) and *in situ* hybridization (Yamada et al., 1997; Dehnes et al., 1998). All of these studies demonstrate that EAAT4 protein in mice is localized to cerebellar cortex and in some cases very faintly to

other areas of the brain. The EAAT4 promoter reporter expression observed in the BAC reporter mice appears to be coincident with protein expression. Furthermore, the parallel localization of the EAAT4 promoter to Purkinje neurons, and the zebrin banding seen with the Aldolase C immunostains (Dehnes et al., 1998) confirm that we can consider the promoter activity to be a good indicator of EAAT4 protein expression. Thus the dynamic regulation of the EAAT4 gene and its relationship to Aldolase C expression can be studied in these mice. The specific localization of EAAT4 promoter reporter fluorescence to Purkinje cells and the lack of co-localization with the GLAST promoter reporter confirm the independent gene expression of these related glutamate transporters. The findings also provide the opportunity to study the cellular basis of transporter gene regulation among neurons and glia.

EAAT4 is located at high density on the membranes of postsynaptic spines surrounding the synaptic cleft (Danbolt, 2001) and is responsible for the vast majority of Purkinje cell uptake at climbing fiber synapses (Huang et al., 2004). Monitoring EAAT4 promoter activity can serve as a tool to investigate cerebellar tissue or Purkinje cell survival *in situ*. Organotypic slice cultures can be reliably prepared from neonatal rodents and maintained chronically *in vitro* for weeks, similar to spinal cord slice cultures (Rothstein et al., 1993). These preparations offer the ability to examine local synaptic circuitry, viability of Purkinje cells, and gene expression regulation over periods of time when exposed to certain toxins and mutant proteins. Our studies provide new data on the utility of cerebellar slice cultures and the ability to investigate Purkinje cell biology. Although propidium iodide staining provides some indication of the viability of slices, it was not consistent over all slices perhaps due to variable diffusion through the thickness of the organotypic slices (350 mm), and thus gave higher standard errors between slices (Fig. 4). We also could not see any difference within different areas in the same slice with the propidium iodide staining, while eGFP-mediated fluorescence could distinguish between them (Table 1). Thus, the use of EAAT4-BAC-eGFP mice not only reveals EAAT4 gene expression but can also be used to indicate early Purkinje neuron damage and neurodegeneration *in vitro*, in organotypic slices, and *in vivo*.

In conclusion, we generated transgenic BAC promoter reporter mice for the glutamate transporter EAAT4 in which EAAT4 promoter activity can be detected throughout the life of the animals. We show that EAAT4 promoter activity typically occurs in Purkinje neurons but is also seen infrequently in other neurons in CNS regions during early postnatal development. The EAAT4-BAC-eGFP mice are useful for analyzing transporter and Purkinje neuron function in primary cell culture, living organotypic slices, and *in vivo*, and to assess up- and down-regulation of transporter promoter activation in various pathological, physiological, and pharmaceutical conditions.

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