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Dynamic expression of *SynDIG1* mRNA in cerebellar Purkinje neurons

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Abstract

During development of the central nervous system, successive intrinsic and extrinsic programs control the specification, proliferation, and migration of individual neuronal cell types that ultimately result in the formation of precise synaptic connections. In previous research, expression profiling of the developing mouse cerebellum identified genes expressed during specific phases of neuronal differentiation. Based on this study, a novel type II transmembrane protein (SynDIG1) that regulates AMPA receptor content at developing synapses was discovered. Here I provide a detailed analysis of *SynDIG1* mRNA expression in the developing mouse cerebellum. *SynDIG1* mRNA is expressed exclusively in Purkinje neurons as demonstrated by analysis of *Lurcher* and *weaver* mutant mice in which Purkinje neurons or granule neurons degenerate, respectively. *SynDIG1* mRNA expression is most prominent in mouse cerebellum at postnatal day 14, the peak of synaptogenesis in rodents. Interestingly, *SynDIG1* mRNA appears to localize to Purkinje neuron dendrites, suggesting the intriguing possibility that *SynDIG1* mRNA is subject to local protein synthesis at synapses. Taken together, these results demonstrate that *SynDIG1* mRNA expression is highly dynamic with restricted expression to Purkinje neurons in the developing mouse cerebellum.

Introduction

The cerebellar cortex is a popular model system for the analysis of neuronal differentiation due to the simplicity of its cellular architecture. The cerebellum is composed of a small number of morphologically and molecularly distinct neuronal cell types (see [1] for review). Mature granule neurons extend multiple short dendrites that receive synaptic input from mossy fiber afferents and Golgi neurons, whereas granule neuron axons (the parallel fibers of the cerebellum) synapse on distal dendritic spines of Purkinje neurons. The proximal dendrites of Purkinje cells are innervated by multiple synapses from a single climbing fiber originating from the inferior olive.

During development of the cerebellum, Purkinje neurons arise from the ventricular zone along the lining of the dorsal aspect of the fourth ventricle (see [2] for review). Purkinje neurons become postmitotic during embryonic development and migrate along radial glia fibers to form a multi-cell layer below the external granular layer (EGL). Beginning at birth, Purkinje neuron dendrites undergo dynamic remodeling based on intrinsic and extrinsic cues [3]. The Purkinje neuron dendritic arbor is mature by postnatal day 13 (P13); however, Purkinje neuron dendrites undergo dynamic synapse remodeling events until P30. For example, at birth, each Purkinje neuron is innervated by multiple climbing fibers of the inferior olive nuclei; however, these surplus climbing fibers are eliminated during postnatal development, such that by P20 mono-innervation is attained.

An attractive aspect of the cerebellum as a model system is the large number of mouse mutants that affect selectively Purkinje neuron or granule neuron development. For example, in heterozygous *Lurcher* (*Lc*) mice, Purkinje neurons degenerate in the second postnatal week of development due to a point mutation in the δ2 glutamate receptor [4], which is selectively expressed in cerebellar Purkinje neurons [5]. Subsequently, a dramatic loss of granule neurons is observed [6]. In *weaver* (*wv*) mice, granule neurons die in the EGL due to expression of a mutant potassium channel in granule neurons [7], resulting in greatly reduced numbers of mature granule neurons in the internal granule layer (IGL) [8, 9]. This in turn results in defects in Purkinje neuron development and aberrant morphologies of Purkinje neuron dendrites [10, 11].

Previously, a DNA microarray approach was applied to expression profile the cerebellum in wild type and mutant mouse lines with defects in neuronal differentiation [12]. This approach identified a novel transmembrane protein (SynDIG1) that regulates excitatory synapse development via interaction with AMPA receptors [13]. In wild type cerebellum, *SynDIG1* mRNA is upregulated during postnatal development; in contrast, *SynDIG1* upregulation is defective in *Lc*

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cerebellum [12]. *In situ* hybridization with SynDIG1 digoxigenin-labeled riboprobes in mouse brain sections demonstrated that *SynDIG1* mRNA is expressed in Purkinje neurons in cerebellum as well as throughout the hippocampus [13]. Here I provide a detailed analysis of *SynDIG1* mRNA expression in the developing mouse cerebellum using the more sensitive radioactive *in situ* hybridization method. Consistent with the microarray expression profiling study [12], *SynDIG1* mRNA is expressed exclusively in Purkinje neurons as demonstrated by analysis of *Lc* and *wv* mutant mice in which Purkinje neurons or granule neurons degenerate, respectively. *SynDIG1* mRNA is expressed in immature Purkinje neurons; however expression is most prominent at P14, during the peak of synapse development in rodents. Interestingly, *SynDIG1* mRNA appears to localize to both Purkinje neuron cell bodies and dendrites, suggesting the intriguing possibility that *SynDIG1* mRNA is subject to local protein synthesis at synapses. Taken together, these results demonstrate that *SynDIG1* mRNA expression is highly dynamic with restricted expression to Purkinje neurons in the developing mouse cerebellum.

Materials and Methods

Animals

CD-1 mice were purchased from Charles River. Breeder pairs for mutant mouse lines were purchased from the Jackson Laboratory. All mice were bred and maintained in the animal facility at UC Davis. *Weaver homozygotes (wv/wv), heterozygotes (wv/+)* and wild-type littermates were generated by mating B6CBACa-*A* $^{w\text{-}J}$*/A –Kcnj6* ^{wv} mice. Genotypes were determined by a restriction site-generating PCR protocol [18]. *Lurcher heterozygotes (Lc/+)* and wild-type littermates were generated by mating B6CBACa-*A* $^{w\text{-}J}$*/A –Grid2* ^{Lc} male mice and wild-type females; genotypes were determined by restriction fragment length polymorphism analysis [4] and/or behavior for adult animals. The use and maintenance of animals were according to the guidelines set forth by UC Davis, NIH, and AALAC.

In situ hybridization

In situ hybridization on fresh frozen sections was performed as described [19]. Briefly, SynDIG1 cDNA sequence (Riken AV149920) was used as template for *in vitro* transcription to produce anti-sense ^{35}S-UTP labeled probes. Probes were purified on S-200 micro-spin columns (Amersham) and diluted to $8x10^8$ cpm/ml in Wilkinson's hybridization buffer [20]. Fresh frozen CD-1 brains were cut into 20 µm sections on a cryostat. Sections were fixed in 4% paraformaldehyde / 1X phosphate buffered saline (PBS) for 10 min, washed in PBS, acetylated for 10 min, washed in PBS, and incubated with Wilkinson's hybridization buffer for 4 hr. RNA probes were applied evenly by "painting" with parafilm. Slides were coverslipped and incubated in a humidified chamber overnight at 65°C. The next day, slides were washed in 0.2 X SSC, 10 mM DTT at 72°C for 4 x 30 min (100 mM DTT in the first wash). Slides were cooled in RNase buffer (0.5 M NaCl, 10 mM Tris pH 7.5, 5 mM EDTA), incubated with 5 µg/ml RNaseA for 30 min at 37°C, washed 2 x 30 min with RNase buffer, 4 x 15 min with 0.2 X SSC at 72°C, and dehydrated through an ethanol series with 0.3 M NH_4OAc. Slides were coated with NTB2 emulsion (Kodak) and exposed for 10-14 days. Slides were developed with D19 developer (Kodak), rinsed with water and fixed at 15°C. Slides were dehydrated through an ethanol series, incubated with xylenes and mounted. Images were taken with a digital camera mounted on a Zeiss Axioplan2 upright microscope under dark-field conditions.

Results and Discussion

SynDIG1 mRNA is expressed in Purkinje neuron cell bodies and dendrites

To examine the distribution of *SynDIG1* mRNA in finer detail, *in situ* hybridization with ^{35}S-UTP-labeled riboprobes was performed with CD-1 mouse brain sections at different ages (Figure 1). As previously demonstrated with digoxigenin-labeled riboprobes, *SynDIG1* mRNA is expressed in Purkinje neurons in cerebellum [13]. *SynDIG1* mRNA expression is detected as early as P6 in immature Purkinje neurons throughout the cerebellar cortex (Figure 1A). At P6, immature Purkinje neurons are practically devoid of dendrites [see [3] for review]. Thus, as expected, examination of higher magnification images revealed that *SynDIG1* mRNA expression is restricted to Purkinje neuron cell bodies (Figure 1B).

SynDIG1 mRNA expression is most prominent at P14 in mouse cerebellum (Figure 1C), consistent with its peak of protein expression in whole brain extracts [13]. Strikingly, examination of higher magnification images revealed that *SynDIG1* transcripts are detectable in Purkinje neuron cell bodies and dendrites within the molecular layer (Figure 1D). By P14, the dendrites of young Purkinje neurons exhibit all features of adult Purkinje neurons [see [3] for review]; however, Purkinje neuron dendrites undergo dynamic synapse remodeling events until P30. At P22, *SynDIG1* transcripts are still present in Purkinje neuron cell bodies (Figure 1E); however, examination of higher magnification images revealed that *SynDIG1* mRNA expression in dendrites was significantly reduced compared with P14 cerebellum

(compare Figures 1F and 1D). Thus, while *SynDIG1* mRNA expression is maintained in Purkinje neuron cell bodies throughout development, *SynDIG1* mRNA expression in dendrites
peaks during the period of Purkinje neuron synaptogenesis.

The observation that *SynDIG1* mRNA expression is most prominent during the peak of synaptogenesis in rodents in consistent with the previous demonstration that SynDIG1 functions to regulate AMPA receptor content at developing synapses [13]. SynDIG1 protein is present in adult brain but at a reduced level compared with P14 brain [13].

Thus, *SynDIG1* mRNA abundance dictates SynDIG1 protein level. Intriguingly, SynDIG1 transcripts are detected in the molecular layer of the cerebellum, the location of Purkinje cell dendrites, suggesting that *SynDIG1* mRNA might be subject to local mRNA translation at synapses. Indeed, local protein synthesis is thought to underlie aspects of synaptic plasticity and higher order cognitive function (see [14] for review). However, *SynDIG1* mRNA expression in dendrites is enriched in P14 brain, suggesting that local translation of *SynDIG1* mRNA might occur only at nascent synapses.

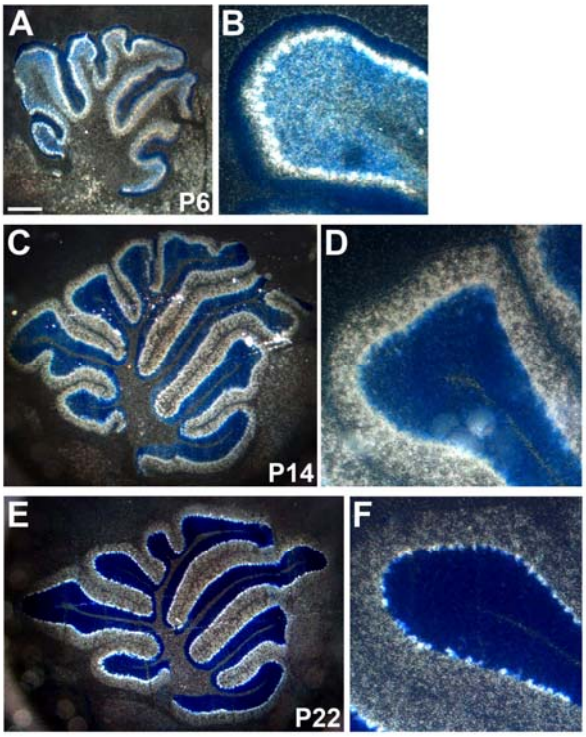

Figure 1. SynDIG1 mRNA expression in the developing mouse cerebellum.
Sagittal sections of mouse cerebellum at postnatal day 6 (A, B), postnatal day 14 (C, D), and postnatal day 22 (E, F) were hybridized in situ with antisense ^{35} S-UTP labeled probe for SynDIG1. Note that SynDIG1 mRNA expression is most prominent at P14 in Purkinje neuron cell bodies and dendrites.
Scale bar, 400 µm (A, C, E), 100 µm (B, D, E).

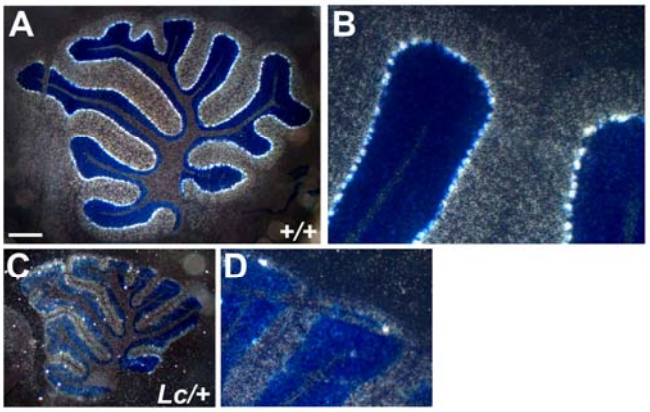

Figure 2. SynDIG1 mRNA is expressed selectively in cerebellar Purkinje neurons.
Sagittal sections of cerebellum at postnatal day 28 from wild type mice (A, B) and Lurcher heterozygous littermates (C, D) were hybridized in situ with antisense ^{35} S-UTP labeled probe for SynDIG1. Note that SynDIG1 mRNA expression is absent in Lc/+ cerebellum compared with wild type littermates.
Scale bar, 400 µm (A, C, E), 100 µm (B, D, E).

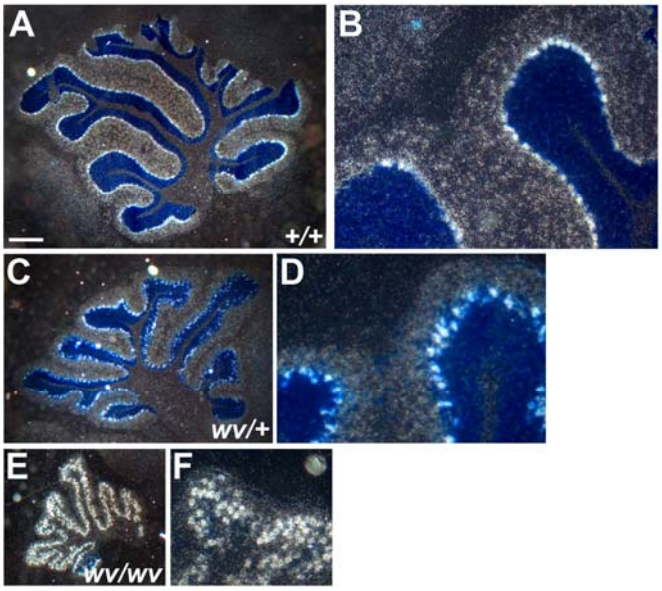

Figure 3. SynDIG1 expression in Purkinje neurons does not require synaptic input from granule neurons
Sagittal sections of cerebellum at postnatal day 25 from wild type mice (A, B), weaver heterozygous littermates (C, D), and weaver homozygous littermates (E, F) were hybridized in situ with antisense ^{35} S-UTP labeled probe for SynDIG1. Note that SynDIG1 mRNA expression in dendrites but not cell bodies is dependent on the presence of granule neurons.
Scale bar, 400 µm (A, C, E), 100 µm (B, D, E).

SynDIG1 mRNA expression is restricted to Purkinje neurons in cerebellum

To conclusively prove that SynDIG1 is expressed in Purkinje neurons and not radial glial cells that are also present in the Purkinje cell layer [1], mouse cerebellum sections from *Lc*/+mice and wild type littermates were examined with *in situ* hybridization (Figure 2). In *Lc*/+ mice, there is massive Purkinje neuron death during the second postnatal week of development due to a point mutation in the δ2 glutamate receptor [4], which is selectively expressed in cerebellar Purkinje neurons [5]. As expected, *SynDIG1* transcripts are present in Purkinje neuron cell bodies in cerebellar sections from wild type mice (Figures 2A-2B). In contrast, *SynDIG1* transcripts are absent in cerebellar sections derived from *Lc/+* littermates (Figure 2C). Indeed, examination of higher magnification images of *Lc/+* cerebellum did not reveal any significant labeling above background levels (Figure 2D). Thus, *SynDIG1* mRNA is expressed selectively in Purkinje neurons in the mouse cerebellum.

SynDIG1 mRNA expression does not require granule neuron synaptic input

The development of Purkinje neurons is affected by bi-directional cell-cell interactions with granule neurons [15]. To test if *SynDIG1* expression in Purkinje neurons is dependent on the presence of granule neurons, cerebellar sections from *wv* mutant mice were examined with *in situ* hybridization (Figure 3). In homozygous *wv/wv* mice, granule neuron precursors are correctly specified and proliferate in the EGL; however, the post-mitotic cells die before they migrate to the IGL [10, 16].

As expected, *SynDIG1* transcripts are present in Purkinje neuron cell bodies in cerebellar sections from wild type mice (Figures 3A, 3B). In addition, *SynDIG1* transcripts are present in cerebellar sections derived from *wv/+* littermates (Figures 3C, 3D) and *wv/wv* littermates (Figures 3E, 3F). Indeed, examination of higher magnification images of *wv/+* and *wv/wv* cerebellum revealed that although Purkinje neurons are disordered in these mutant mice, *SynDIG1* mRNA

expression is detectable in Purkinje neuron cell bodies (Figures 3D, 3F). In contrast, SynDIG1 transcripts are absent from Purkinje neuron dendrites in *wv/wv* mice (Figure 3F). Thus, while *SynDIG1* expression in Purkinje neuron cell bodies does not require synaptic input from granule neurons, *SynDIG1* expression in Purkinje neuron dendrites is dependent on the presence of granule neurons.

The observation that *SynDIG1* mRNA expression in Purkinje neuron dendrites but not cell bodies requires synaptic input from granule neurons suggests that *SynDIG1* mRNA is transported to developing synapses via retrograde signaling from presynaptic parallel fibers of the granule neurons. Early events in synapse development include clustering of synaptic vesicles to the presynaptic active zone, and NMDA receptors to the postsynaptic density while later events include clustering of AMPA receptors and synaptic activity directs whether synapses will be stabilized, eliminated or strengthened (see [17] for review). SynDIG1 regulates the content of AMPA receptors but not NMDA receptors at developing excitatory synapses [13], suggesting that SynDIG1 mRNA translation at nascent synapse might provide an immediate signal to trigger synapse stabilization via the incorporation of synaptic AMPA receptors. Further experiments will be necessary to test this interesting possibility.

In summary, *SynDIG1* mRNA is expressed exclusively in Purkinje neurons and is most prominent at P14, the peak of synaptogenesis in rodents. Interestingly, *SynDIG1* mRNA appears to localize to Purkinje neuron dendrites, suggesting the intriguing possibility that *SynDIG1* mRNA is subject to local protein synthesis at synapses. Furthermore, SynDIG1 mRNA localization to Purkinje neuron dendrites but not cell bodies is dependent on the presence of granule neurons. Taken together, these results demonstrate that *SynDIG1* mRNA expression is highly dynamic with restricted expression to Purkinje neuron cell bodies and dendrites in the developing mouse cerebellum.

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