Long-lasting induction of Notch2 in the hippocampus of kainate-treated adult mice

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Notch proteins are involved in cell fate specification during development in tissues including brain. Little is known about their function in adulthood. Recently, Notch receptors have been hypothesized to play a role in neurodegeneration and in particular in Alzheimer's disease (Notch1) and CADASIL (Notch3). Here we show that another family member (Notch2) is constitutively expressed in adult mouse hippocampus in DG and not in CA1 and CA3 neurons. Treatment with kainic acid resulted in marked Notch2 induction in pyramidal neurons of CA1 and in a subpopulation of CA3 neurons surviving the lesion and protein expression was still detectable 6 weeks after drug treatment. These results suggest Notch2 involvement in the response of postmitotic neurons to excitotoxic stimuli. *NeuroReport* 14:917–921 © 2003 Lippincott Williams & Wilkins.

Key words: Alzheimer's disease; Development; Excitotoxicity; Hippocampus; Notch proteins

INTRODUCTION

The Notch gene family includes four members (Notch1, Notch2, Notch3, Notch4) encoding cell surface receptors involved in a wide variety of cellular interactions that specify cell fate during development [1]. The mechanism of Notch receptor activation involves cleavage and nuclear translocation of the intracellular domain which in turn activates transcription of specific target genes [2]. Recent studies have suggested a role of Notch in mature brain. In particular, in postmitotic neurons Notch1 may influence growth, branching and maintenance of dendrites [3–7]. A possible involvement of Notch1 and Notch3 in chronic neurodegeneration has been suggested by the following findings: (1) Notch1 and the amyloid precursor protein (APP) can both be cleaved by a presenilin-1-associated γ secretase activity [8,9]; (2) Notch1 protein expression is markedly increased in the hippocampal formation of Alzheimer's disease patients [10]; (3) Notch3 gene mutations lead to CADASIL, an adult-onset autosomal dominant neurological disease characterized by progressive subcortical infarcts and dementia [11].

We focused our interest on the family member referred to as Notch2 [12]. Previous data using the *in situ* hybridization technique have demonstrated that Notch2 mRNA is expressed in the embryonic ventricular zone, in the postnatal ependymal cells and in the choroid plexus throughout embryonic and postnatal development. Postnatal mouse brain strongly expresses Notch2 in the granular cell layer of the hippocampal dentate gyrus (DG), in the temporal cortex and in the cerebellum [13,14]. Although the functional role of Notch2 in specific cell populations of mature brain is not yet clearly established, it has been suggested that Notch2, like Notch1, may contribute to the stabilization of neuronal contacts and morphology [3,5]. To further elucidate the functional significance of Notch2 in adult brain, we evaluated (by immunohistochemistry and Western blot analysis) Notch2 protein expression in adult mouse after injection of kainic acid (KA), a well-established *in vivo* model of excitotoxicity. In rodents, systemic injection of KA results in cell death occurring in the hippocampal pyramidal neurons of the CA3 subfield [15,16]. We found that KA injection resulted in a sustained, time-dependent and cell-specific increase of Notch2 expression in the hippocampal neurons of the CA1 and CA3 fields. These results may suggest a novel role for Notch2 in postmitotic neurons.

MATERIALS AND METHODS

Experiments were carried out in accordance with the National Institute of Health guide for the care and use of laboratory animals and all efforts were made to minimize animal suffering. Twenty-five FVB/N mice (27–30 g body weight) received KA dissolved in saline in a single dose of 25 mg/kg by i.p. injection and were killed at 1, 3, 28, 42 days after the treatment. Mice were monitored after drug

administration up to 6 h for evaluating the onset and extent of seizure and only mice reaching stage 4 of Racine's scale were included in the study ($n=5$, at day 1; $n=5$ at day 3; $n=4$ at day 28; $n=4$ at day 42). An additional five FVB/N mice were injected with saline and referred to as control mice. After killing, brains were collected and fixed in Carnoy for 24 h. Coronal sections from paraffin-embedded tissue were cut at 5 μm and used for haematoxylin–eosin staining. Haematoxylin–eosin-positive undamaged neurons were counted in CA1, CA2, CA3 and DG hippocampal subfields by estimating the mean profile number/mm^2, as described previously [17]. Some adjacent sections were incubated overnight with a polyclonal anti-Notch2 antiserum (1:30, Santa Cruz Biotechnology, CA, USA). Slides were then incubated with a biotinylated anti-rabbit IgG (1:300, Dako, Glostrup, Denmark), followed by incubation with avidin–biotin–horseradish peroxidase (HRP) complex (Dako, Glostrup, Denmark). Antigens were visualized by reaction with 3,3′-diaminobenzidine tetrahydrochloride (DAB, 0.05%) as chromogen in presence of H_2O_2 (0.003%).

TdT-mediated dUTP nick end labelling (TUNEL) was carried out using a commercially available kit (Roche Diagnostics, Mannheim, Germany). TUNEL-positive nuclei were visualized with 4-chloro-1-naphtol and H_2O_2. For double labelling, slides were then incubated with the anti-Notch2 antibody as described above and staining was visualized with DAB/H_2O_2. For double immunodetection of Notch2 and active caspase-3, immunohistochemical analysis with Notch2 antiserum was performed as described above. After DAB reaction, slides were incubated with a mouse monoclonal antibody raised against the activated form of caspase-3 (1:2; Ab 4, Calbiochem, Darmstadt, Germany); after rinses, a biotinylated anti-mouse IgG (1:300, Dako, Glostrup, Denmark) was added to the sections for 1 h at room temperature. Tissue sections were then incubated with avidin–biotin–HRP complex and immunodetection was performed in presence of DAB and nickel as chromogens, to obtain grey staining.

For Western blot analysis, additional FVB/N mice were treated with KA ($n=4$) or saline ($n=4$) as described previously and killed after 3 days. Protein extracts from hippocampi or frontal/parietal cortices (50 μg/lane) were electrophoresed onto 6% SDS-PAGE and transferred to nitrocellulose paper. Filters were incubated with an anti-Notch2 antiserum (1:200, Santa Cruz Biotechnology, CA, USA). An HRP-conjugated goat anti-rabbit secondary antibody (1:1500, Dako, Glostrup, Denmark) and a chemiluminescence blotting substrate kit (Amersham Biosciences UK, England) were used for immunodetection. Evaluation of immunoreactivity was performed on immunoblots by densitometric analysis using a LKB 2222-020 Ultra Scan XL laser densitometer.

Data were analysed by ANOVA with Student's t-test for simple comparisons.

RESULTS

KA treatment was chosen as an experimental model of excitotoxicity to explore the possible involvement of Notch2 in neuronal injury. As expected, at 3 days after the injection, KA produced selective hippocampal cell loss in the CA3 subfield [17] (data not shown). The percentage of surviving neurons, as estimated by computer-assisted measurement of profile counts as an index of cell number, was about 55–60% of the corresponding saline-treated animals. CA1 and DG subfields were less vulnerable to KA-induced neurodegeneration, with the number of surviving cells in the corresponding subregions not significantly different from that of saline-injected animals.

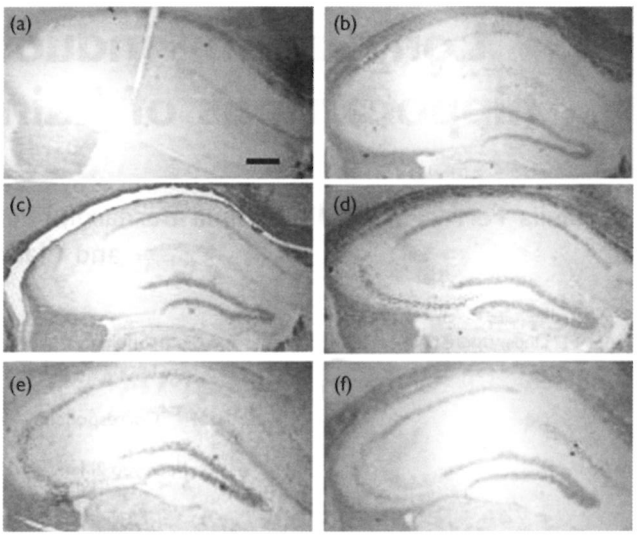

Fig. 1. Notch2 immunoreactivity in adult mouse hippocampus after KA treatment. Coronal sections of the hippocampus of saline- (**a**,**b**) and KA-treated mice (**c**–**f**). Animals were killed after 1 day (c), 3 days (d), 28 days (e), and 42 days (f) after the treatment. No signal was detectable when slices were pretreated with preimmune serum (a). Bar = 300 μm.

The effect of KA on the expression of Notch2 protein was evaluated at 1, 3, 28, and 42 days after the administration of the drug by immunohistochemical technique using a polyclonal antibody that recognizes the C-terminal portion of the protein. Figure 1 shows representative images of coronal hippocampal sections from KA- and saline-treated animals. In control mice, in agreement with previous studies [13,14], Notch2 immunoreactivity was detected predominantly in the DG subregion with few cells labelled in the CA1 subfield (about 4–5% of total) and with the CA3 region devoid of immunostaining (Fig. 1b). One day after KA treatment, the number of Notch2-immunoreactive cells in the hippocampus changed dramatically. An increased number of Notch2-positive cells was clearly detectable in the CA1 subfield, with about 80–85% of total CA1 neurons becoming immunolabelled (Fig. 1c). At that time point, no change in Notch2 expression levels was detected in the DG and CA3 hippocampal subfields, compared to control mice. Three days after the lesion, a time-point when excitotoxic damage becomes apparent in vulnerable pyramidal neurons of the CA3 subfield, about 40% of CA3 surviving cells were intensively immunolabelled (Fig. 1d). At that time point, neurons in the CA1 region were still markedly immunostained. Increased Notch2 immunoreactivity in the CA1 and CA3 regions represented a long-standing phenomenon, still detectable at 28 days (Fig. 1e) and 42 days (Fig. 1f) after KA injection. No significant change was observed in the DG and cortical regions at any evaluated time point. As shown in Fig. 2, the anatomical and temporal changes of Notch2 expression are better visualized at higher magnification in

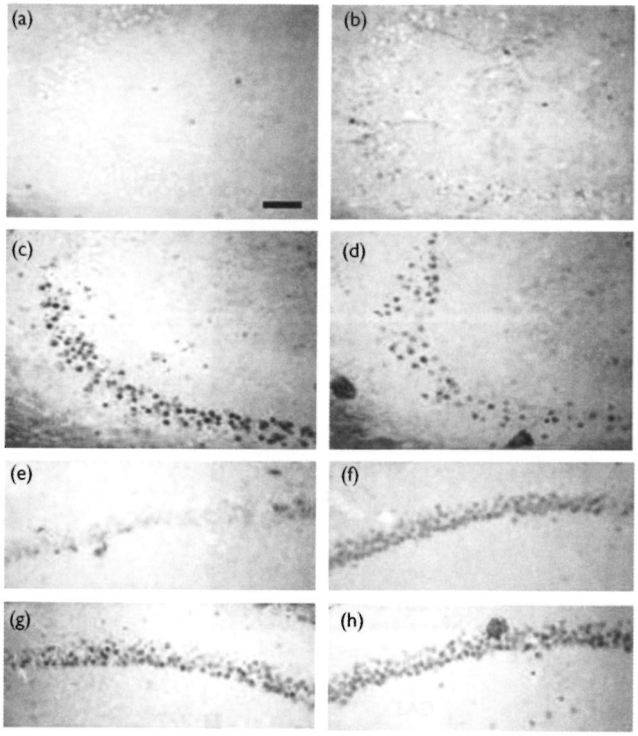

Fig. 2. Increased Notch2 immunoreactivity in CA3 and CA1 hippocampal subfields after KA treatment. Representative views of CA3 (**a–d**) and CA1 (**e–h**) regions of the hippocampus of saline- (a,e) and KA-treated mice 1 day (b,f), 3 days (c,g), and 42 days (d,h) after KA treatment. Notch2 overexpression was specifically restricted to pyramidal cells of CA3 and CA1 subfields. Bar = 40 μm.

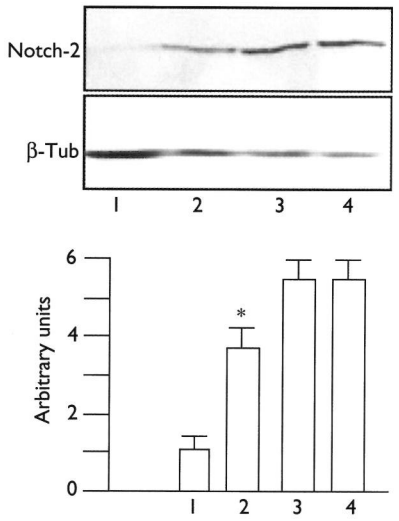

Fig. 3. Increase of Notch2 protein expression in CA3 subfields after KA treatment. Higher panel, representative Western blot analysis performed on protein extracts from hippocampus (1, 2) and cerebral cortex (3, 4) of mice treated with either saline (1, 3) or KA (2, 4) and killed 3 days after the treatment. Samples were immunoblotted with an anti-Notch2 or anti-ß tubulin (β-Tub) antibody, as indicated. Lower panel, semi-quantitative analysis of Notch2 protein levels from hippocampus (1, 2) and cerebral cortex (3, 4) of mice treated with either saline (1, 3) or KA (2, 4) and killed 3 days after the treatment. Values are expressed as densitometric arbitrary units with 1 corresponding to control and represent means ± s.e.m. of three different experiments. $^*p < 0.01$ *vs* the corresponding control values.

the CA3 (Fig. 2a–d) and CA1 (Fig. 2e–h) regions of the hippocampus of saline-treated and KA-treated mice at 1 day, 3 days and 42 days after KA treatment.

As shown in Fig. 3, Notch2 over-expression after KA injection was further supported by Western blot analysis of hippocampal protein extracts from mice at 3 days after KA treatment (lane 2) compared with mice injected with saline (lane 1). Densitometric analysis of three different blots demonstrated a 3- to 4-fold induction in Notch2 protein levels in drug-treated animals compared to control mice (Fig. 3, lower panel). Cortical Notch2 protein levels were also evaluated in KA- (lane 4) and saline-injected (lane 3) mice but no difference could be detected in the two animal groups. In all experimental samples, the antibody recognized a single band with an apparent mol. wt of about 115 kDa, consistent with the C-terminal fragment of Notch2.

To further investigate the functional significance of Notch2 up-regulation in selected hippocampal neuronal populations, we evaluated Notch2 expression and the spatio-temporal profile of apoptosis taking place in the hippocampus after KA treatment. To this aim, coronal brain sections from mice at 1, 3 and 28 days after drug injection were double-labelled for TUNEL staining and Notch2 immunoreactivity. Representative images are shown in Fig. 4. At 1 day after treatment, TUNEL-positive cells were detected in the CA1 (Fig. 4d) and CA3 (Fig. 4a) subfields but not in the DG (not shown). At 3 and 28 days after KA acid, no TUNEL-positive cells could be detected in both CA1 (not shown) and CA3 (Fig. 4b,c) regions. Semi-quantitative analysis showed (Fig. 4e) that at 1 day after KA in the CA3 subfield about 15–20% of the cells were TUNEL-positive while in the CA1 TUNEL-positive cells represented about 0.5–1% of the total number. None of the TUNEL-positive cells detected at 1 day in the CA1 was also immunoreactive to Notch2. We evaluated the correlation between Notch2 and another marker of apoptosis, i.e. the activated caspase-3 protease. The results were similar to those found for TUNEL/Notch2 double labelling experiments. Hippocampal pyramidal neurons expressing activated caspase-3 and representing about 15–20% of the total cell number, were identified in the CA3 subfield at 1 day after KA. At 3 and 28 days no caspase-3-positive cells could be demonstrated. In the CA1 subfield, we found few (< 1%) and scattered caspase-3-positive cells 1 day after the lesion and none at 3 or 28 days. None of the caspase-3-positive cells detected 1 day in the CA1 was also immunoreactive to Notch2. Based on these findings, it is reasonable to suggest that neuronal cells undergoing Notch2 induction and cells undergoing apoptosis in the CA1 and CA3 subfields after KA injection represent distinct populations.

DISCUSSION AND CONCLUSION

Here we show that in adult mouse hippocampus Notch2 immunoreactivity is restricted to DG granule neurons with only few cells immunopositive in the CA1 subfield and the CA3 region devoid of immunostaining. These data are in agreement with *in situ* hybridization studies showing that, in the hippocampus of adult rat brain, Notch2 mRNA is

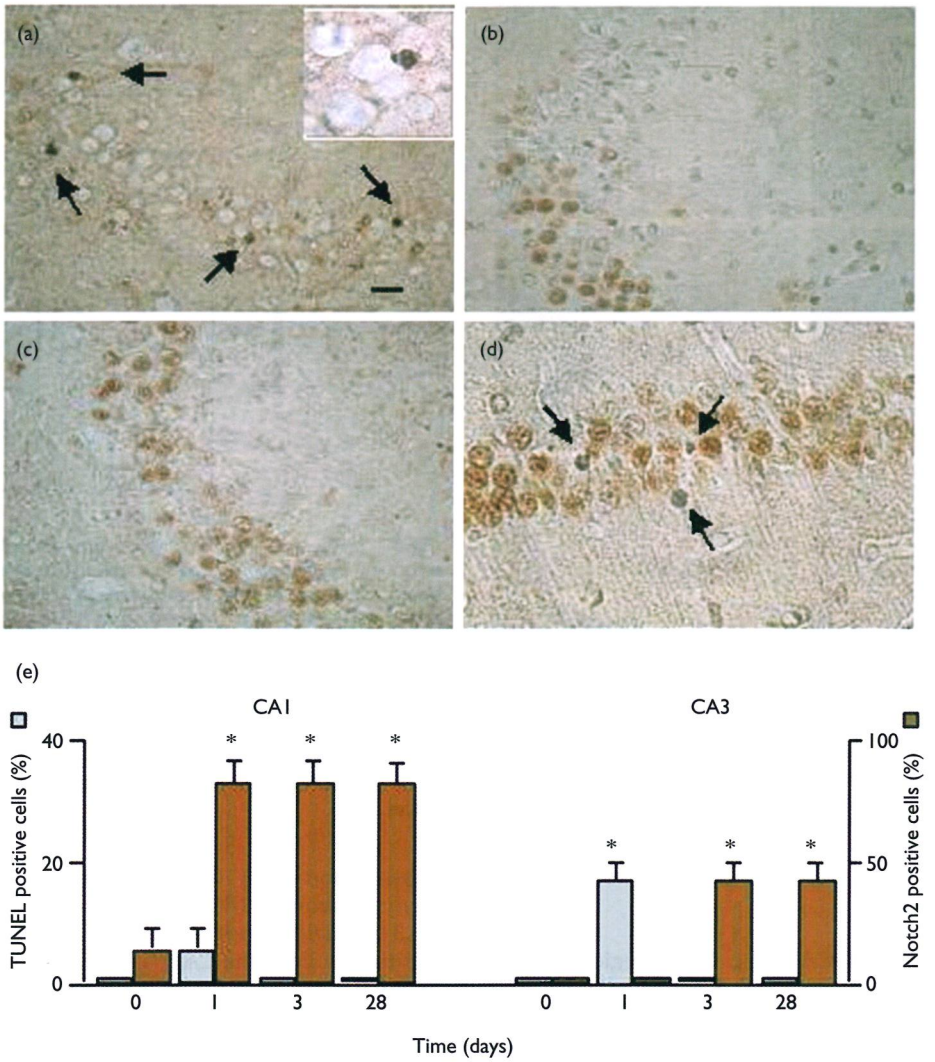

Fig. 4. Notch2 and TUNEL staining do not co-localize in hippocampal slices of KA-treated mice. Figure shows representative images from hippocampal CA3 (A, **a–c**) and CA1 region (**d**) double-labelled for TUNEL and Notch2 of mice killed 1 day (a,d), 3 days (b) and 28 days (c) after treatment with KA. TUNEL-positive cells are grey and are indicated by arrows. Notch2-immunopositive cells are brown. In (a) insert is a higher magnification view of a part of the figure. (**e**) Semi-quantitative evaluation of TUNEL and Notch2-positive cells. Data are expressed as percent of positive cells over total viable cells and represent means $\pm$ s.e.m. of $\geq$ 10 sections for each brain. $*p < 0.01$ *vs* the corresponding values at time 0. Bar = 10 μm.

expressed in the DG granule cells [14]. Western blot analysis further indicated that the 115 kDa intracellular fragment is the predominant form of Notch2 in adult mice hippocampi. This fragment represents the nuclear form of Notch2 which has been suggested to be generated by two sequential proteolytic cleavages of the 300 kDa precursor protein and to act as a transcription regulator [18]. Thus, our data suggest that Notch2 in DG granule cells is constitutively active. Function and target genes activated by Notch2 in DG are at the present unknown. Although a great deal of experimental work has raised attention on the involvement of Notch proteins in maturing and, more recently, in adult mammalian brain [3,10,14,19,20], very little information is available about the role of Notch2 in postmitotic neurons.

The selective expression of Notch2 in hippocampal subpopulations known to have different thresholds of vulnerability to neurotoxic stimuli prompted us to investigate Notch2 expression in an experimental model of neuronal injury, namely KA injection. It is well established that pyramidal neurons in the hippocampal CA3 region are particularly vulnerable while DG and CA1 neurons are more resistant to systemic injection of the toxin. Although the precise mechanism is not completely clarified, it is proposed that in the hippocampus KA triggers a chain of time- and space-dependent events characterized by neuronal death, including apoptosis and necrosis, extensive gliosis, and dramatic synaptic reorganization [16,21,22]. These changes lead to increased seizure susceptibility, as a result of the progressive formation of an excitatory network between granule cells and CA3 pyramidal neurons [23,24]. Interestingly, we found that KA injection caused a marked induction of Notch2 in the hippocampus with a peculiar temporal and anatomical pattern. Notch2 protein levels were dramatically elevated in CA1 neurons at 1 day, and in

CA3 neurons at 3 days. These changes were long lasting since Notch2 immunolabelled neurons in both CA1 and CA3 of KA-treated animals were still detectable up to 6 weeks after the injury.

Several genes either up-regulated or down-regulated after systemic administration of KA have been implicated in the selective vulnerability of specific neuronal phenotypes to this type of damage and in the prevention or promotion of excitotoxicity. It is intriguing that neurons less vulnerable to excitotoxicity constitutively express (DG granule cells) or rapidly up-regulate (CA1 pyramidal neurons) Notch2 1 day after drug administration. Conversely, in the CA3 vulnerable subregion, Notch2 cannot be detected at 1 day. At 3 days, when about 40–45% neurons have died, a subpopulation of surviving neurons markedly expresses Notch2 protein.

An intriguing possibility to be explored is that Notch2 induction may represent a distinctive feature of a neuronal population resistant to excitotoxicity. To better understand the functional significance of this phenomenon, we asked the question whether cells expressing Notch2 could at some time express apoptotic markers. In CA3 both TUNEL and activated caspase-3-positive cells could be detected at 1 day (when Notch2 is not expressed) and not at 3 and 28 days (when Notch2 is up-regulated) after KA. Thus, our data suggest that apoptosis and Notch2 expression in KA vulnerable regions are two temporally distinct phenomena involving different cell populations. In CA1, few and scattered TUNEL-positive cells were detected at 1 day and none at 3 and 28 days. None of the cells which were TUNEL positive (in CA1 at 1 day) was double labelled with Notch2, thus further supporting the view that neurons undergoing apoptosis and neurons expressing Notch2 are distinct cell populations.

If Notch2 induction may represent an epiphenomenon, a sign of suffering or may actively contribute to neuroprotection would need to be further evaluated. In this regard, preliminary *in vitro* studies using DNA microarray technology indicated that exposure of primary neurons to submaximal concentrations of glutamate resulted in a 3-fold increase of Notch2 mRNA levels (data not shown). *In vitro* systems are, therefore, suitable for studies aimed at investigating molecular mechanisms underlying Notch2 induction in neuron.

It is notable that Notch2 induction represents a long-lasting event, being still detectable up to several weeks after neurodegeneration occurs. In adult brain, constitutively active Notch2 has been proposed to play a role in neurite stability and connections. An additional line of investigation to be pursued is that Notch2 expression may contribute to KA-induced neuronal circuit re-organization.

REFERENCES

1. Artavanis-Tsakonas S, Rand MD and Lake RJ. *Science* **284**, 770–776 (1999).
2. Shimizu K, Chiba S, Saito T *et al. Biochem Biophys Res Commun* **291**, 775–779 (2002).
3. Sestan N, Artavanis-Tsakonas S and Rakic P. *Science* **286**, 741–746 (1999).
4. Redmond L, Oh SR, Hicks C *et al. Nature Neurosci* **3**, 30–40 (2000).
5. Whitford KL, Dijkhuizen P, Polleux F and Ghosh A. *Annu Rev Neurosci* **25**, 127–149 (2002).
6. El Hachimi KH and Foncin JF. *CR Acad Sci* **311**, 397–402 (1990).
7. McNeill TH, Brown SA, Rafols JA and Shoulson I. *Brain Res* **455**, 148–152 (1988).
8. Song W, Nadeau P, Yuan M *et al. Proc Natl Acad Sci USA* **96**, 6959–6963 (1999).
9. Kopan R and Goate A. *Genes Dev* **14**, 2799–2806 (2000).
10. Berezovska O, Xia MQ and Hyman BT. *J Neuropathol Exp Neurol* **57**, 738–795 (1998).
11. Joutel A, Corpechot C, Ducros A *et al. Ann NY Acad Sci* **826**, 213–217 (1997).
12. Weinmaster G, Roberts VJ and Lemke G. *Development* **116**, 931–941 (1992).
13. Higuchi M, Kiyama H, Hayakawa T *et al. Mol Brain Res* **29**, 263–272 (1995).
14. Irvin D, Zurcher SD, Nguyen T *et al. J Comp Neurol* **436**, 167–181 (2001).
15. Pollard H, Charraut-Marlangue C, Cantagrel S *et al. Neuroscience* **63**, 7–18 (1994).
16. Kaminska B, Filipkowski RK, Biedermann IW *et al. Acta Biochem Pol* **44**, 781–789 (1997).
17. Grilli M, Diodato E, Lozza G *et al. Proc Natl Acad Sci USA* **97**, 12822–12827 (2000).
18. Shimizu K, Chiba S, Hosoya N *et al. Mol Cell Biol* **20**, 6913–6922 (2000).
19. Tanaka M, Kadokawa Y, Hamada Y and Marunouchi T. *J Neurobiol* **41**, 524–539 (1999).
20. Solecki DJ, Liu XL, Tomoda T *et al. Neuron* **31**, 557–568 (2001).
21. Suzuki F, Junier M, Guilhem D *et al. Neuroscience* **64**, 665–674 (1995).
22. Zagulska-Szymczak S, Filipkowski RK and Kaczmarek L. *Neurochem Int* **38**, 485–501 (2001).
23. Ben-Ari Y. *Epilepsia* **42**, 5–7 (2001).
24. Wuarin JP and Dudek FE. *J Neurophysiol* **85**, 1067–1077 (2001).

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