

RAPID REPORT

EFFECTS OF STATUS EPILEPTICUS ON HIPPOCAMPAL GABA_A RECEPTORS ARE AGE-DEPENDENT

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Abstract—Long-term GABA_A receptor alterations occur in hippocampal dentate granule neurons of rats that develop epilepsy after status epilepticus in adulthood. Hippocampal GABA_A receptor expression undergoes marked reorganization during the postnatal period, however, and the effects of neonatal status epilepticus on subsequent GABA_A receptor development are unknown. In the current study, we utilize single cell electrophysiology and antisense mRNA amplification to determine the effect of status-epilepticus induced by lithium-pilocarpine in postnatal day 10 rat pups on GABA_A receptor subunit expression and function in hippocampal dentate granule neurons. We find that rats subjected to lithium-pilocarpine-induced status epilepticus at postnatal day 10 show long-term GABA_A receptor changes including a two-fold increase in $\alpha 1$ subunit expression (compared with lithium-injected controls) and enhanced type I benzodiazepine augmentation that are opposite of those seen after status epilepticus in adulthood and may serve to enhance dentate gyrus inhibition. Further, unlike adult rats, postnatal day 10 rats subjected to status epilepticus do not become epileptic. These findings suggest age-dependent differences in the effects of status epilepticus on hippocampal GABA_A receptors that could contribute to the selective resistance of the immature brain to epileptogenesis. © 2004 IBRO. Published by Elsevier Ltd. All rights reserved.

Key words: epilepsy, seizure, developing brain, dentate gyrus, benzodiazepine, mRNA amplification.

GABA_A receptors (GABARs) are heteromeric protein complexes that mediate most fast synaptic inhibition in forebrain. Many distinct subunit subtypes exist, and their distribution varies in a regional and cell type-specific manner (Wisden et al., 1992; Sperk et al., 1997; Barnard et al.,

1998). GABARs can be assembled in different subunit combinations to produce a variety of different receptor compositions, pharmacological profiles, and intrinsic receptor characteristics (Pritchett et al., 1989; Sieghart, 1995; Barnard et al., 1998). Adult rats that develop epilepsy following pilocarpine-induced status epilepticus (SE) and adult patients with refractory temporal lobe epilepsy demonstrate marked alterations in GABAR properties in hippocampal dentate granule neurons (DGNs; Buhl et al., 1996; Gibbs et al., 1997; Brooks-Kayal et al., 1998, 1999). GABAR subunit expression in DGNs vary during early postnatal development (Laurie et al., 1992; Fritschy et al., 1994; Brooks-Kayal et al., 2001), however, and the effects of neonatal SE on GABARs is not known. Here we examine the effect of SE in postnatal day 10 (P10) rat pups on subsequent GABAR development in DGN and find increased $\alpha 1$ subunit expression and type I benzodiazepine augmentation when the rats reach adulthood. These findings are opposite of those seen after SE in adulthood and could contribute to the selective resistance of the immature brain to epileptogenesis.

EXPERIMENTAL PROCEDURES

Lithium-pilocarpine injections

All studies were carried out in accordance with the National Institutes of Health Guide for the Care and Use of Laboratory Animals and with the approval of The Children's Hospital of Philadelphia Animal Care and Use Committee. Maximum care was taken to minimize the number of animals used and to minimize their suffering. At P9 Sprague–Dawley rat pups were injected i.p. with either lithium chloride (3 mEq/kg; P10-SE and lithium-control groups) or saline (saline controls). On P10, pups received i.p. injection of either 60 mg/kg pilocarpine (P10-SE group) or saline (lithium and saline control groups). Pups uniformly developed SE within 30 min of pilocarpine injection and seizures continued intermittently for 5–6 h. A separate set of pups (naive controls) received no injections and was not separated from mother until weaning at P21.

Video and EEG monitoring

As adults (P90 or older) the rats that had SE at P10 were video recorded for 24 h/day for 14 days ($n=6$) for any discrete alterations in behavior such as wild running, head bobbing, facial clonus, forelimb clonus, or tail stiffening that might indicate behavioral seizures. Three of the adult P10-SE rats that were video monitored were also implanted with electrodes in CA1 and frontal cortex and were video-EEG monitored using previously published methods (Raol et al., 2003; Zhang et al., 2004).

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Abbreviations: DGN, dentate granule neuron; GABAR, GABA_A receptor; P10, postnatal day 10; SE, status epilepticus.

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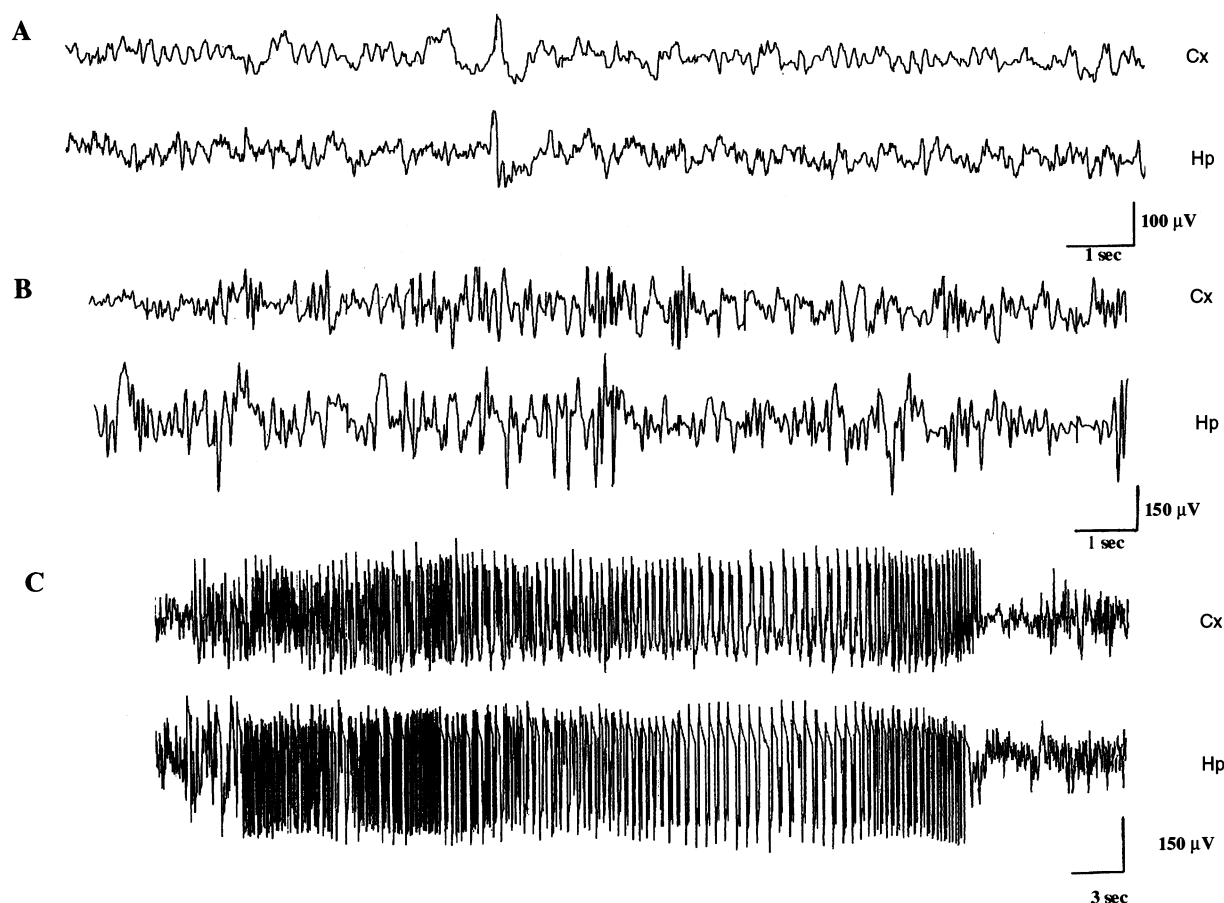


Fig. 1. Rats experiencing SE at P10 do not develop spontaneous seizures. (A) Representative hippocampal (Hp) and cortical (Cx) electroencephalogram tracing from an adult rat that had experienced SE 3 months earlier at P10. Note the absence of inter-ictal epileptiform discharges or electrographic seizures. (B, C) Representative Hp and Cx electroencephalogram tracing from a rat 1 month after experiencing SE in adulthood. Note the presence of inter-ictal epileptiform discharges (B) and an electrographic seizure (C).

Isolation of neurons

DGNs were acutely isolated from hippocampal slices using our previously published protocol (Hsu et al., 2003; Zhang et al., 2004). DGNs were identified based on their size (approximately 10 μ m) and characteristic morphology (oval cell body with a single neuronal process).

Electrophysiology

Recordings were made using the whole cell patch clamp technique as described previously (Hsu et al., 2003). GABA-elicited current was recorded at room temperature. Neurons were voltage-clamped at -20 mV and signals were amplified and saved at 10 kHz using pClamp 8.01 software (Axon Instruments, Foster City, CA, USA) for off-line analysis. Curves were fitted using the Marquardt-Levenberg nonlinear least-squares algorithm.

RNA measurement

Relative expression of GABAR mRNAs within individual acutely isolated DGNs were measured using antisense RNA amplification and reverse northern blotting as previously described in detail (Brooks-Kayal et al., 1998, 1999). The relative abundance of total GABAR subunit mRNA was calculated as the sum of the hybridization signal for all subunit cDNAs on the blot for each cell divided by the hybridization signal for β -actin

cDNA on that blot. The effect of group on the various outcomes as measured repeatedly across different cells was analyzed using a longitudinal mixed effects model (SAS Proc Mixed models; SAS Institute Inc., Cary, NC, USA) which accounts for potential within-subject correlation associated with making repeated measures from individual animals.

Timm's and Cresyl Violet staining

The staining was performed using previously published methods (Sloviter, 1982; Raol et al., 2003).

RESULTS

Rats do not become epileptic after SE at postnatal day 10 (P10-SE)

No evidence of behavioral or electrographic seizures was seen during prolonged video and EEG monitoring of adult animals previously subjected to P10-SE (Fig. 1A). Further no interictal epileptiform abnormalities were found in any of the P10-SE animals. This contrasted markedly to the uniform development of spontaneous seizures in adult rats subjected to SE (Fig. 1B, C; see also Gibbs et al., 1997; Brooks-Kayal et al., 1998). No

hippocampal cell loss or mossy fiber sprouting was visualized using light microscopy in any of the P10-SE rats ($n=6$) or controls ($n=6$).

GABAR properties after P10-SE

Expression of 16 GABAR subunits ($\alpha 1$ – 6 , $\beta 1$ – 3 , $\gamma 1$ – 3 , δ , ϵ , π , θ) mRNAs was examined in each DGN isolated from the four groups (Fig. 2A). GABAR expression was similar in the lithium- and saline-control groups, and only the former is presented. Overall GABAR mRNA expression (total GABAR) relative to β -actin expression was increased in DGNs from P10-SE rats compared with DGNs from lithium- or naive-controls (Fig. 2B; $P=0.04$).

Mean relative expression (as a percent of total GABAR) of $\alpha 1$ subunit mRNA in DGNs from P10-SE rats was also significantly increased compared with either control group (Fig. 2C; $P=0.001$). Of additional note, the levels of $\alpha 1$ subunit mRNA were lower in the lithium-control group compared with naive-controls (Fig. 2C; $P=0.05$), consistent with an effect of neonatal handling on $\alpha 1$ subunit expression as has been previously reported (Hsu et al., 2003). No other significant change was found in expression of any of the other 15 subunits examined.

GABAR pharmacology is largely determined by receptor subunit composition. GABARs containing an $\alpha 1$ subunit

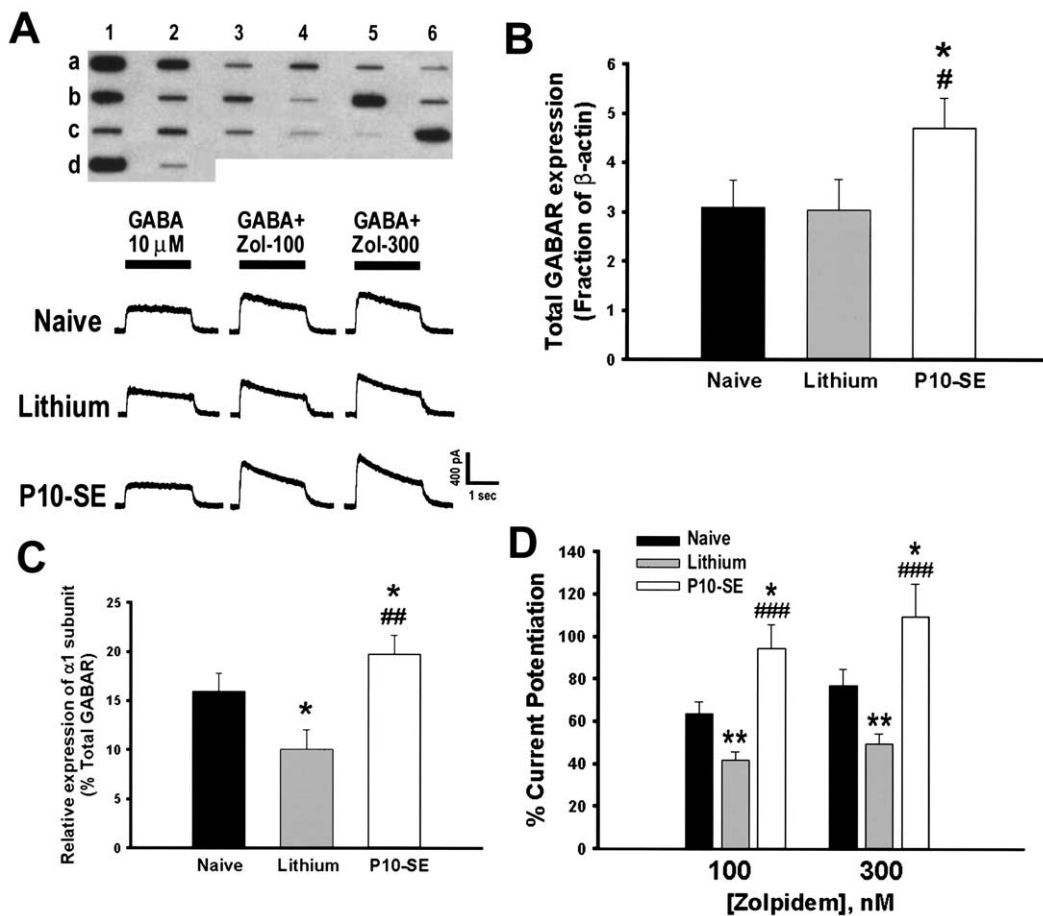


Fig. 2. P10-SE altered GABAR subunit expression and pharmacology. (A) Top panel: Representative reverse northern blot from a single DGN isolated from a P10-SE rat. The slot-blot contains cDNAs encoding GABAR subunit $\alpha 1$ – $\alpha 6$ (a1–6), $\beta 1$ – $\beta 3$ (b1–3), $\gamma 1$ – $\gamma 3$ (b4–6), δ , ϵ , π and θ (c1–4), glial fibrillary acidic protein (GFAP; c5), neurofilament-L (NF-L, c6), β -actin (d1), pBluescript (d2). Bottom panels: Representative responses to 10 μ M GABA application alone and co-application with zolpidem (100 and 300 nM) in DGNs isolated from naive control, lithium control and P10-SE rats. (B) Total GABAR mRNA expression was increased in P10-SE rats compared with controls (effect of group: $F=3.11$; $P=0.04$; pair-wise comparison to naive * $P<0.05$, lithium # $P<0.05$, SAS Proc Mixed models; $n=21$ cells total from nine rats for P10-SE [1–4 cells/rat]; 24 cells total from eight rats for naive-controls [1–6 cells/rat]; 18 cells total from eight rats for lithium-controls [1–4 cells/rat]). (C) Relative expression of $\alpha 1$ subunit mRNA was increased in DGNs from P10-SE rats compared with controls (effect of group: $F=7.28$; $P=0.001$; pair-wise comparison to naive * $P<0.05$, lithium ### $P<0.01$, SAS Proc Mixed models). (D) GABA current potentiation by zolpidem, a BDZ₁ receptor specific positive modulator, was significantly increased in DGNs from P10-SE rats compared with DGNs from naive or lithium control rats. Each cell was treated with 100 nM and 300 nM zolpidem which was pre-applied alone for 30 s before co-applying drug with 10 μ M GABA for 2 s. Cells were washed with drug-free recording solution for 2 min between applications to reduce the effects of current run-down and/or desensitization (* $P<0.05$, ** $P<0.01$ compared with naive; ### $P<0.001$ compared with lithium; one-way ANOVA with Tukey-Kramer test for pair-wise post hoc comparison; $n=18$ cells from four rats for P10-SE, 31 cells from seven rats for lithium-controls and 15 cells from three rats for naives). Note also handling-induced differences in $\alpha 1$ mRNA expression and zolpidem potentiation between lithium- and naive-control groups as has been previously reported (Hsu et al., 2003).

Table 1. Consequences of pilocarpine-induced SE

	P10-SE	Adult-SE
Behavior		
Spontaneous seizure	No	Yes ^{2,12}
Anatomy		
Hippocampal cell loss	No	CA1, CA3, hilar DG ¹²
Mossy fiber sprouting	No	Yes ¹²
DGN GABAR function		
Current density	No change	Increased ²
Zolpidem augmentation	Increased	Decreased ²
DGN subunit mRNA expression		
Total GABAR mRNA	Increased	Increased ²
GABAR α 1-subunit	Increased	Decreased ²
GABAR α 4-subunit	No change	Increased ²
GABAR δ -subunit	No change	Increased ²

are more highly augmented by type I benzodiazepine agonists such as zolpidem than GABARs containing any other α -subtype (Pritchett et al., 1989; Barnard et al., 1998), although GABARs containing other α -subunits can respond to zolpidem at high concentrations (Goldstein et al., 2003). There was significantly more augmentation of GABA-induced currents by 100 nM zolpidem in DGNs from P10-SE rats ($94.6 \pm 11.4\%$) compared with DGNs from either lithium-control rats ($41.8 \pm 4.2\%$) or naive-control rats ($63.9 \pm 5.3\%$; Fig. 2D). As α 1 was the only subunit to show a significant increase in relative expression after P10-SE, the greater zolpidem augmentation of GABA currents in DGNs from P10-SE rats most likely reflects an increased number of α 1 containing GABARs on the membrane of these cells. The GABA concentration-response curve demonstrated no difference in the GABA EC_{50} or maximum GABA current density between DGNs isolated from pilocarpine-treated animals (P10-SE; EC_{50} 29.55 ± 4.82 μ M), lithium-controls (EC_{50} 33.37 ± 7.17 μ M) or naive-controls (27.92 ± 6.68 μ M).

DISCUSSION

The current study demonstrates that rats experiencing pilocarpine-induced SE at P10 respond differently than those subjected to SE in adulthood (Table 1). Unlike rats experiencing SE as adults, P10-SE rats do not become epileptic and the alterations in hippocampal GABARs in P10-SE rats are of lesser magnitude and in the opposite direction from those seen in adult SE rats. DGNs from P10-SE rats show increased expression of GABAR α 1 subunit mRNA and enhanced zolpidem augmentation of GABA-mediated currents compared with controls. In contrast, GABAR α 1 subunit expression in DGNs is decreased after SE in adulthood and zolpidem potentiation of GABA-mediated currents is reduced (Brooks-Kayal et al., 1998). Further, the α 4, β 3 and δ subunits are all up-regulated and the β 1 subunit is down-regulated in adult SE rats, while none of these subunits are changed following P10 SE. Taken together these findings demonstrate that the effects of SE on GABARs are highly age-dependent and suggest that the immature dentate may have a selective resistance to GABAR changes after SE compared with the adult.

Rats do not develop epilepsy after pilocarpine-induced SE at P10 (Fig. 1) in contrast to the nearly uniform development of spontaneous recurrent seizures in rats experiencing SE as adults (Gibbs et al., 1997; Brooks-Kayal et al., 1998; Shumate et al., 1998). This relative protection from development of epilepsy after P10 SE could be related, in part, to the increased GABAR α 1 subunit expression. Dentate gyrus serves as a gate in the hippocampal circuit (Heinemann et al., 1992), and the increased GABAR expression in DGN following P10 SE could augment dentate gyrus inhibition and prevent excessive excitation from propagating into hippocampus. In addition, the up-regulation of α 4 and δ subunits and the associated increase in zinc inhibition of GABA-currents that are hypothesized to result in a “failure of inhibition” in dentate after adult SE (Buhl et al., 1996; Brooks-Kayal et al., 1998) did not occur after P10 SE. Thus, it is possible that age-dependent differences in SE-induced GABAR effects could contribute to the selective resistance of the immature brain to epileptogenesis. As both anatomical and molecular consequences of SE vary based on the age at which it occurs (Table 1), however, further studies will be needed to determine whether differential effects on GABAR properties contribute to this difference in the rate of later epilepsy development.

The mechanisms regulating GABAR expression are not well understood. GABAR α 1 subunit expression in DGNs increases markedly during the first postnatal month (Laurie et al., 1992; Brooks-Kayal et al., 2001). Several lines of evidence suggest that neuronal activity (Meinecke and Rakic, 1990; Huntsman et al., 1994; Holopainen and Lauren, 2003), specifically synaptic signaling through the NMDA selective glutamate receptor (Memo et al., 1991; Harris et al., 1995; Zhu et al., 1995) may be critical for stimulating GABAR α 1 receptor subunit expression. Thus the increased α 1 subunit expression after P10 SE could be an “over-maturation” of DGN GABARs due to the excessive neuronal activity associated with SE. On the contrary, the diminished α 1 expression after SE in adulthood suggests a regression of mature DGN to a more immature state. Determining whether this difference is due to a derangement of “normal” developmental cues for GABAR expression or due to signaling mechanisms that are unique to SE will require additional investigation. These findings reinforce the need for better understanding of age-related differences in the consequences of SE and the mechanisms underlying these differences in order to develop optimal therapeutic approaches for the treatment of epilepsy throughout the lifespan.

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REFERENCES

- Barnard EA, Skolnick P, Olsen RW, Mohler H, Sieghart W, Biggio G, Braestrup C, Bateson AN, Langer SZ (1998) International Union of Pharmacology: XV. Subtypes of gamma-aminobutyric acid A

- receptors: classification on the basis of subunit structure and receptor function. *Pharmacol Rev* 50:291–313.
- Brooks-Kayal AR, Shumate MD, Jin H, Rikhter TY, Coulter DA (1998) Selective changes in single cell GABA(A) receptor subunit expression and function in temporal lobe epilepsy. *Nat Med* 4:1166–1172.
- Brooks-Kayal AR, Shumate MD, Jin H, Lin DD, Rikhter TY, Holloway KL, Coulter DA (1999) Human neuronal gamma-aminobutyric acid(A) receptors: coordinated subunit mRNA expression and functional correlates in individual dentate granule cells. *J Neurosci* 19:8312–8318.
- Brooks-Kayal AR, Shumate MD, Jin H, Rikhter TY, Kelly ME, Coulter DA (2001) gamma aminobutyric acid (A) receptor subunit expression predicts functional changes in hippocampal dentate granule cells during postnatal development. *J Neurochem* 77:1266–1278.
- Buhl E, Otis T, Mody I (1996) Zinc-induced collapse of augmented inhibition by GABA in a temporal lobe epilepsy model. *Science* 271:369–373.
- Fritschy JM, Paysan J, Enna A, Möhler H (1994) Switch in the expression of rat GABA_A-receptor subtypes during postnatal development: an immunohistochemical study. *J Neurosci* 14:5302–5324.
- Gibbs JW 3rd, Shumate MD, Coulter DA (1997) Differential epilepsy-associated alterations in postsynaptic GABA(A) receptor function in dentate granule and CA1 neurons. *J Neurophysiol* 77:1924–1938.
- Goldstein PA, Elsen PE, Ying S-W, Ferguson C, Homanics GE, Harrison NL (2003) Prolongation of hippocampal miniature inhibitory postsynaptic currents in mice lacking the GABA(A) receptor $\alpha 1$ subunit. *J Neurophysiol* 88:3208–3217.
- Harris B, Costa E, Grayson D (1995) Exposure of neuronal cultures to K⁺ depolarization or to N-methyl-D-aspartate increases the transcription of genes encoding the $\alpha 1$ and $\alpha 5$ GABA(A) receptor subunits. *Mol Brain Res* 28:338–342.
- Heinemann U, Beck H, Dreier JP, Ficker E, Stabel J, Zhang CL (1992) The dentate gyrus as a regulated gate for the propagation of epileptiform activity. In: *The dentate gyrus and role in seizures* (Ribak CE, Gall CM, Mody I, eds), pp 273–280. Amsterdam: Elsevier.
- Holopainen IE, Lauren HB (2003) Neuronal activity regulates GABA_A receptor subunit expression in organotypic hippocampal slice cultures. *Neuroscience* 118(4):967–974.
- Hsu FC, Zhang GJ, Raol Y, Valentino RJ, Coulter DA, Brooks-Kayal AR (2003) Repeated neonatal handling with maternal separation permanently alters hippocampal GABA(A) receptors and behavioral stress responses. *Proc Natl Acad Sci USA* 100:12213–12218.
- Huntsman M, Isackson P, Jones E (1994) Lamina-specific expression and activity-dependant regulation of seven GABA(A) receptor subunit mRNAs in monkey visual cortex. *J Neurosci* 14:2236–2259.
- Laurie DJ, Wisden W, Seeburg PH (1992) The distribution of 13 GABA(A) receptor subunits in the rat brain: III. Embryonic and postnatal development. *J Neurosci* 12:4151–4172.
- Meinecke D, Rakic P (1990) Developmental expression of GABA and the subunits of the GABA(A) receptor complex in an inhibitory synaptic circuit in the rat cerebellum. *Dev Brain Res* 55:73–86.
- Memo M, Bovolin P, Costa E, Grayson D (1991) Regulation of GABA(A) receptor subunit expression by activation of NMDA-selective glutamate receptors. *Mol Pharmacol* 39:599–603.
- Pritchett DB, Luddens H, Seeburg PH (1989) Type I and type II GABA(A)-benzodiazepine receptors produced in transfected cells. *Science* 245:1389–1392.
- Raol Y, Budreck EC, Brooks-Kayal AR (2003) Epilepsy after early life seizures can be independent of structural hippocampal injury. *Ann Neurol* 53:503–511.
- Sloviter RS (1982) A simplified Timm stain procedure compatible with formaldehyde fixation and routine paraffin embedding of rat brain. *Brain Res Bull* 8:771–774.
- Shumate MD, Lin DD, Gibbs JW 3rd, Holloway KL, Coulter DA (1998) GABA(A) receptor function in epileptic human dentate granule cells: comparison to epileptic and control rat. *Epilepsy Res* 32:114–128.
- Sieghart W (1995) Structure and pharmacology of gamma-aminobutyric acid-A receptor subtypes. *Pharmacol Rev* 47:181–234.
- Sperk G, Schwarzer C, Tsunashima K, Fuchs K, Sieghart W (1997) GABA_A receptor subunits in the rat hippocampus: I. Immunocytochemical distribution of 13 subunits. *Neuroscience* 80:987–1000.
- Wisden W, Laurie D, Monyer M, Seeburg P (1992) The distribution of 13 GABA_A receptor subunit mRNAs in the rat brain. I. Telencephalon, diencephalon, mesencephalon. *J. Neurosci* 12:1040–1062.
- Zhang G, Raol Y, Hsu F, Brooks-Kayal AR (2004) Selective alterations in glutamate receptors and transporters in hippocampal dentate granule neurons after seizures in the developing brain. *J Neurochem* 88:91–101.
- Zhu W, Vicini S, Harris B, Grayson D (1995) NMDA-mediated modulation of γ -aminobutyric acid type A receptor function in cerebellar granule neurons. *J Neurosci* 15:7692–7701.

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