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Critical role for TARPs in early development despite broad functional redundancy

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ABSTRACT

Transmembrane AMPA receptor regulatory proteins (TARPs), including γ-2, γ-3, γ-4, and γ-8, are auxiliary subunits for AMPA receptors. Based on studies in single knockout mice, it has been suggested that nearly all native AMPA receptors are associated with TARPs. To study the interplay between TARP family members and AMPA receptors *in vivo*, we generated mice lacking multiple TARPs. Triple knockout mice lacking γ-3, γ-4, and γ-8 are viable and fertile, and synaptic AMPA receptor activity is reduced to a level comparable to that seen in γ-8 single knockout mice. In contrast, triple knockout mice lacking γ-2, γ-3, and either γ-4 or γ-8 cannot survive *ex utero*. In particular, γ-2, γ-3, γ-4 triple knockout mice are born apneic and paralyzed, despite normal AMPA receptor function in cortical and spinal neurons. We found that γ-8 is expressed at low levels in early postnatal mice and regulates AMPA receptor levels at this developmental time period. Thus, the early expression of γ-8 may be responsible for maintaining AMPA receptor functions in neonatal neurons. Together, our data indicate that TARPs, in particular γ-2, are essential for early development, and that most neurons express multiple members of this functionally redundant protein family.

1. Introduction

The discovery that the *stargazer* strain of ataxic, epileptic mice lacked AMPA receptor expression in cerebellar granule cells revealed the critical role for stargazin, the absent protein, as an auxiliary AMPA receptor subunit (Chen et al., 2000; Hashimoto et al., 1999). Stargazin, also known as γ-2, is a member of a family of transmembrane AMPA receptor regulatory proteins (TARPs) (Nicoll et al., 2006; Ziff, 2007) that includes γ-3, γ-4, and γ-8 (Tomita et al., 2003), as well as γ-7, which exhibits some but not all TARP properties (Kato et al., 2007; Milstein et al., 2007). Like classical auxiliary subunits of voltage-gated channels, TARPs regulate many functional aspects of AMPA receptors; they augment AMPA receptor surface trafficking, enhance synaptic clustering, increase glutamate affinity, increase kainate efficacy, determine antagonist pharmacology, and slow channel deactivation and desensitization (Cho et al., 2007; Korber et al., 2007; Menuz et al., 2007; Milstein et al., 2007; Priel et al., 2005; Tomita et al., 2005; Turetsky et al., 2005; Yamazaki et al., 2004; Zhang et al., 2006). TARP regulation is emerging as a universal trait of native AMPA receptors, including those localized synaptically and extrasynaptically in both excitatory and inhibitory neurons (Chen et al., 1999, 2000; Hashimoto et al., 1999; Menuz et al., in press; Rouach et al., 2005). Although much is known about the distinct properties of individual TARP family members when expressed in heterologous systems, their individual roles *in vivo* remain elusive.

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γ-2 is the only TARP whose deletion results in a behavioral phenotype (Noebels et al., 1990), as mice lacking γ-3, γ-4 or γ-8 appear behaviorally indistinguishable from littermates (Kato et al., 2007; Letts et al., 2005; Menuz et al., in press; Rouach et al., 2005). Perhaps this reflects the widespread distribution of γ-2; whereas γ-2 is found in virtually all brain regions, expression of γ-3 is highest in the cortex, γ-4 in the olfactory bulb, striatum, and glia, and γ-8 in the hippocampus (Lein et al., 2007; Fukaya et al., 2005; Klugbauer et al., 2000; Tomita et al., 2003). However, significant overlap exists, with lower levels of γ-3, γ-4, and γ-8 also detectable in most other brain regions in adult mice (Lein et al., 2007; Fukaya et al., 2005). In the neonate, γ-4 was reported to be the sole TARP expressed (Tomita et al., 2003). However, its deletion does not preclude normal development (Kato et al., 2007; Letts et al., 2005), implying that TARPs may not be required at all developmental time periods, or that compensatory upregulation by other family members may occur.

We generated mice lacking multiple TARPs to determine the relationship between TARP expression and AMPA receptor function. We report for the first time the generation of γ-$3^{-/-}$;γ-$4^{-/-}$ (γ-3,4 KO) mice, as well as triple γ-3,4,8 KO and γ-2,3,4 KO strains. We found preserved AMPA receptor function in the hippocampus, cortex, and spinal cord, suggesting that a single remaining TARP is sufficient and thus highlighting TARP functional redundancy. Our data further suggest that γ-2 plays a special role in survival.

2. Methods

2.1. Knockout mice

All experiments followed animal welfare guidelines established by the University of California, San Francisco I.A.C.U.C. Stargazer mice (γ-2 KO mice), γ-3 KO, γ-4 KO, and γ-8 KO mouse strains have been described previously (Letts et al., 1998; Menuz et al., in press; Milstein et al., 2007; Rouach et al., 2005). PCR genotyping of mouse tail DNA was performed with the following primers: γ-2: F-WT: CATTTGTT ATACATGCTCTAG, R-WT: ACTGTCACTCTATCTGGAATC, F-KO: GAGCAAGCAGGTTTCA GGC, R-KO: ACTGTCACTCTATCTGGAATC; γ-3: F-WT: AACTAGGTTCCCAGATAGCC, R-WT: GCTTCTAATGGGTTGCGCCC, F-KO: GGCTGCTCTTTGGTTAATCGG, R-KO: TACCCGGTAGAATTGA CCTGC; γ-4: F-WT: GGACTCCTGGGAGAGATGCC, R-WT: CGGCTGTAGATCCTCCCAGC, F-KO: GGTGATGGCGTTCAGTGCACGG R-KO: TACCCGGTAGAATTGACCTGC; γ-8: F-WT: TCGCGCTTTC CTCTCGTCCC, R-WT: GCTGCCACGAACAGGATCCC, F-KO: CGTTTAGGATCTACCCAGA TC, R-KO: TACCCGGTAGAATTGACCTGC.

As illustrated in Table 1, selective breeding generated a stable line of mice lacking both γ-3 and γ-4. Further crossbreeding generated γ-$3^{-/-}$;γ-$4^{-/-}$;γ-$8^{+/-}$ mice, who produced the γ-3,4 KO and γ-3,4,8 KO littermates used for experiments. The nonviable γ-2,3,4 KO mice that we studied resulted from breedings between viable γ-$2^{+/-}$;γ-$3^{-/-}$;γ-$4^{-/-}$ parents.

2.2. Electrophysiology in juvenile mice

Acute transverse hippocampal slices (300 μm) were prepared from postnatal day 15–22 animals using a cutting solution at 4–6 °C containing (in mM): 50 NaCl, 150 sucrose, 25 $NaHCO_3$, 1 NaH_2PO_4, 10 glucose, 7 $MgCl_2$, and 0.5 $CaCl_2$ bubbled continuously with 95% O_2/5% CO_2. Slices were submerged in artificial cerebrospinal fluid (ACSF) containing (in mM): 119 NaCl, 2.5 KCl, 26.3 $NaHCO_3$, 1 NaH_2PO_4, 11 glucose, 1.3 $MgSO_4$, and 2.5 $CaCl_2$, adjusted to osmolarity 290–300 mOsm and bubbled continuously as above. After incubation at 30–34 °C for 30 min, slices remained at room temperature for experiments that took place between 1 and 8 h after dissection. Slices were transferred to a recording chamber on the stage of an upright IR-DIC microscope (Olympus, Inc.) and perfused with ACSF containing picrotoxin (100 μM) (Sigma–Aldrich). Whole-cell recordings were obtained from visualized hippocampal CA1 pyramidal cells with borosilicate glass pipettes (2–6 MΩ) filled with a solution containing (in mM): 130 $CsCH_3SO_3$, 10 CsCl, 10 HEPES, 2 NaCl, 1 QX314-Cl, 5 TEA-Cl, 8 Na-phosphocreatine, 4 Na-ATP, 0.5 Na-GTP, 4 $MgCl_2$, 0.1 spermine, and 0.2 EGTA, adjusted to pH 7.3 and osmolarity 300 mOsm. Pipette capacitance was compensated, and series resistance, ranging 10–25 MΩ was monitored, uncompensated, throughout experiments. Cells or epochs were discarded when series resistance changed by more than 10–15%. Monosynaptic EPSCs were evoked by a monopolar stimulating electrode placed in the stratum radiatum. Data were amplified and filtered at 4 kHz with a MultiClamp 700B amplifier (Molecular Devices, Inc.), digitized at 10 kHz (National Instruments, Inc.), and analyzed with customized software for IgorPro (Wavemetrics, Inc.).

Table 1
Survival of mice lacking multiple TARPs

Colony	Breeding scheme	Age[a]	+/+	+/−	−/−	Litters	Chi-square
γ-2,3	γ-$2^{+/-}$;γ-$3^{-/-}$ X γ-$2^{+/-}$;γ-$3^{-/-}$	P0	59 (28%)	111 (53%)	41 (19%)	37	$P = 0.16$
		P14	62 (35%)	106 (61%)	7 (4%)	46	$P < 0.0001$
		Died <P14	5 (9%)	23 (42%)	27 (49%)	30	$P < 0.0001$
γ-2,4	γ-$2^{+/-}$;γ-$4^{-/-}$ X γ-$2^{+/-}$;γ-$4^{-/-}$	[b]	13 (23%)	32 (57%)	11 (20%)	23	$P = 0.52$
γ-2,8	γ-$2^{+/-}$;γ-$8^{-/-}$ X γ-$2^{+/-}$;γ-$8^{+/-}$		[c]				
γ-2,3,4	γ-$2^{+/-}$;γ-$3^{-/-}$;γ-$4^{-/-}$ X γ-$2^{+/-}$;γ-$3^{-/-}$;γ-$4^{-/-}$	E18.5	15 (27%)	36 (64%)	5 (9%)	10	$P < 0.05$
γ-2,3,8	γ-$2^{+/-}$;γ-$3^{-/-}$;γ-$8^{-/-}$ X γ-$2^{+/-}$;γ-$3^{-/-}$;γ-$8^{+/-}$		[d]				
γ-3,4	γ-$3^{-/-}$;γ-$4^{-/-}$ X γ-$3^{-/-}$;γ-$4^{-/-}$	[b]	–	–	(100%)		[e]
γ-3,4,8	γ-$3^{-/-}$;γ-$4^{-/-}$;γ-$8^{+/-}$ X γ-$3^{-/-}$;γ-$4^{-/-}$;γ-$8^{+/-}$	[b]	38 (28%)	74 (54%)	25 (18%)	17	$P = 0.27$

[a] Genotypes of offspring are tabulated by postnatal (P) or embryonic (E) age in days.
[b] Age did not influence the proportions of offspring in these crossings, in which no early deaths were observed.
[c] In our limited experience with this breeding, we did successfully produce γ-2,8 KO offspring, some of whom died soon after birth and some who lived long enough for acute slice experiments (Rouach et al., 2005).
[d] In our limited attempts to breed these mice, no living γ-2,3,8 KO pups resulted (12.5% predicted); of the dead pups genotyped, approximately half were γ-2,3,8 KOs.
[e] These homozygous breeding pairs produced normal litters of healthy offspring. During creation of this line, γ-3,4 KO mice and their heterozygous siblings appeared in normal Mendelian ratios ($P = 0.34$).

$$\mathrm{RI} = \frac{\text{EPSC amplitude at } +40\text{ mV}}{\text{PSC amplitude at } -60\text{ mV}} \times \frac{60 + X_0}{40 - X_0}$$

Rectification index (RI) was calculated by obtaining AMPA-only EPSC amplitudes at −60, 0, and +40 mV. X_0 is the voltage at which current is 0, extrapolated from EPSC amplitudes at −60 and 0 mV (typically 0–5 mV). An RI value of 0 corresponds to complete inward rectification, whereas 1 corresponds to a purely linear response.

2.3. Preparation of acute slices from perinatal mice

Transverse hippocampal slices (300 μm) from P0–P1 mice were cut in cold (4–6 °C) cutting solution containing (in mM): 125 NaCl, 2.5 KCl, 26 $NaHCO_3$, 1.25 NaH_2PO_4, 25 glucose, 4 $MgCl_2$, and 1 $CaCl_2$ saturated with 95% O_2/5% CO_2. Hippocampal slices were incubated at 30–34 °C for 30 min, and then maintained at room temperature. For cortical and spinal cord slice preparations, timed-pregnant γ-$2^{+/-}$;γ-$3^{-/-}$;γ-$4^{-/-}$ mother mice were sacrificed by cervical dislocation, and E16.5–E18.5 embryos were removed. As γ-2,3,4 KO fetuses are motionless, one motionless pup and one normal-appearing littermate were selected for an experiment, and the genotypes were confirmed later. For recording cortical neurons, coronal brain slices (300 μm) were prepared in the same manner as hippocampal slices. For spinal cord slices, the meninges surrounding the spinal cord were removed and the spinal cord was set into an agarose block for cutting slices (300–400 μm). The cutting solution was the same as described above, but at a temperature of 0–4 °C. Spinal cord slices were incubated at 37 °C and then left at room temperature prior to recordings.

2.4. Preparation of dissociated neuron cultures

Dissociated neuronal cultures were prepared from the hippocampus of E16–E19 wild type and γ-8 KO mice, spinal cord of E13–E16 wild type and γ-2,3,4 KO mice, and cerebral cortex of E14–E18 γ-2,3,4 KO mice and their littermates. The tissues were dissociated by papain digestion and brief mechanical trituration. Cells were plated on poly-D-lysine-coated glass coverslips (10–12 mm). Hippocampal and cortical cultures were plated in Neurobasal media (Gibco) with B-27 and 5% FBS (Invitrogen) (Craven et al., 1999). After 3 days, the media were replaced by serum free media. Spinal cultures were plated and maintained in L15 media with FBS. After 3 days in culture, cytosine arabinoside was added. Recordings were performed after 10–19 days *in vitro* (DIV).

2.5. Electrophysiology in perinatal slices or cultured neurons

Slices or coverslips were transferred to a chamber on the stage of an upright IR-DIC microscope (Olympus, Inc.) and perfused with ACSF containing tetrodotoxin