EJN
European Journal of Neuroscience

European Journal of Neuroscience, Vol. 27, pp. 3118–3131, 2008 doi:10.1111/j.1460-9568.2008.06301.x

Cysteine string protein-α is essential for the high calcium sensitivity of exocytosis in a vertebrate synapse

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Keywords: calcium, mouse models, neurotransmission, synaptic proteins

Abstract

Cysteine string protein (CSPα) is a synaptic vesicle protein present in most central and peripheral nervous system synapses. Previous studies demonstrated that the deletion of CSPα results in postnatal sensorial and motor impairment and premature lethality. To understand the participation of CSPα in neural function in vertebrates, we have studied the properties of synaptic transmission of motor terminals in wild-type and CSPα knockout mice. Our results demonstrate that, in the absence of CSPα, fast Ca^{2+}-triggered release was not affected at postnatal day (P)14 but was dramatically reduced at P18 and P30 without a change in release kinetics. Although mutant terminals also exhibited a reduction in functional vesicle pool size by P30, further analysis showed that neurotransmission could be 'rescued' by high extracellular [Ca^{2+}] or by the presence of a phorbol ester, suggesting that an impairment in the fusion machinery, or in vesicle recycling, was not the primary cause of the dysfunction of this synapse. The specific shift to the right of the Ca^{2+} dependence of synchronous release, and the lineal dependence of secretion on extracellular [Ca^{2+}] in mutant terminals after P18, suggests that CSPα is indispensable for a normal Ca^{2+} sensitivity of exocytosis in vertebrate mature synapses.

Introduction

Cysteine string protein (CSP) is a highly conserved protein present in the central and peripheral nervous systems of invertebrates and vertebrates that is associated with synaptic vesicles and other secretory organelles (Buchner & Gundersen, 1997; Chamberlain & Burgoyne, 2000; Bronk *et al.*, 2001). CSP contains a DNA-J domain typical of co-chaperones of the Hsp40 family, and associates with the DNA-K domain heat-shock cognate protein (Hsc70) and the tetratricopeptide repeat protein SGT into a trimeric complex that exhibits an enzymatic chaperone activity *in vitro* (Tobaben *et al.*, 2001). The function(s) of CSP in a synapse is not completely understood but there is strong evidence that it participates in the maturation, maintenance and/or regulation of most synapses (Gundersen *et al.*, 1994; Fernandez-Chacon *et al.*, 2004). The deletion of *csp* in *Drosophila* is essentially lethal at the embryonic stage, with surviving individuals (4%) presenting severe synaptic defects (Gundersen *et al.*, 1994; Zinsmaier *et al.*, 1994). In mice, however, the deletion of the homologous gene, which expresses the predominant CSP in the nervous system of mammals (CSPα) (Braun & Scheller, 1995), does not affect survival of embryos and newborns. Nevertheless, the absence of CSPα in mice produces progressive and severe motor and sensorial impairment that begins by 2 weeks of age, when synapses are still undergoing significant developmental changes, which leads to muscular paralysis, blindness and premature death by postnatal day (P)40–60 (Fernandez-Chacon *et al.*, 2004; Schmitz *et al.*, 2006). Ultrastructural observation of neuromuscular junctions (NMJs) from CSPα knockout (KO) mice has confirmed an increasing number of abnormalities with age in the nerve terminals, such as small profiles, increased Schwann cell invasion and, finally, a decrease in the number of active zones (Fernandez-Chacon *et al.*, 2004). It has been proposed that the role of CSP in the synapse is to act as a co-chaperone through its interaction with heat-shock cognate protein (Hsc70) (Braun *et al.*, 1996; Chamberlain & Burgoyne, 1997; Dawson-Scully *et al.*, 2000; Tobaben *et al.*, 2003; Fernandez-Chacon *et al.*, 2004; Bronk *et al.*, 2005). In the absence of CSP, proteins denatured by use could not adequately be repaired, which leads to the progressive degeneration of the synapse. In addition to the co-chaperone function, which is mechanistically linked through the DNA-J domain of the protein, CSPα may participate in the regulation of other synaptic functions, such as synapse growth, thermal protection of evoked release and intraterminal Ca^{2+} sensitivity for neurotransmitter release, as suggested for *csp* in *Drosophila* (Bronk *et al.*, 2005).

To better understand the role of CSP in synaptic function in vertebrates, we have studied the synaptic properties of the NMJ in mutant mice that lack CSPα. The mouse NMJ provides a unique opportunity for performing a detailed electrophysiological analysis of the role of CSPα as it is accessible from postnatal ages to adulthood, which is more complicated for central synapse preparations. We have found that synaptic transmission in the mutants appear to be normal at P14 but, by P18, nerve stimulation-evoked secretion was much

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Received 3 January 2008, revised 8 April 2008, accepted 27 April 2008

reduced at physiological extracellular [Ca^{2+}] ([Ca^{2+}]$_e$), whereas the frequency of spontaneous quantal acetylcholine release was normal or even increased. We discovered that the decreased evoked postsynaptic responses could be rescued by increasing [Ca^{2+}]$_e$ beyond 2 mM or in the presence of a phorbol ester, which point to an apparent reduction in the Ca^{2+} sensitivity of the exocytic machinery in the absence of CSPα as the principal mechanism responsible for the reduced neurotransmission. The detailed characterization of these features might be useful to understand the role of this protein in neurotransmission and synapse maintenance.

Materials and methods

Identification of wild-type (WT) and homozygous CSPα KO mice was achieved through a DNA tail assay, as previously described (Fernandez-Chacon *et al.*, 2004). All WT mice used were age-matched littermates of mutants. Mice were anesthetized with tribromoethanol (2%, 0.15 mL/10 g body weight, i.p.) and killed by exangination while deeply anesthetized. All experiments were carried out according to the guidelines of the European Council Directive for the Care of Laboratory Animals.

Preparation

The levator auris longus muscles were dissected with facial nerve branches intact and pinned to the bottom of a 2 mL chamber, over a bed of cured silicone rubber (Sylgard, Dow Corning Corp., USA). Preparations were continuously perfused with a solution of the following composition (in mM): 125 NaCl, 5 KCl, 2 $CaCl_2$, 1 $MgCl_2$, 25 $NaHCO_3$ and 15 glucose. The solution was continuously gassed with 95% O_2 and 5% CO_2, which maintained the pH at 7.35. Recording was performed at room temperature (22-23°C).

Electrical stimulation

The nerve was stimulated supramaximally by means of a suction electrode. The stimulation consisted of square-wave pulses of 0.2 ms duration and 2–20 V amplitude, at variable frequencies (0.5–100 Hz).

Intracellular recording

A glass microelectrode (10–20 MΩ) filled with 3 M KCl was connected to an intracellular recording amplifier (Neuro Data IR283; Cygnus Technology, PA, USA). Evoked endplate potentials (EPPs) and miniature EPPs (mEPPs) were recorded from different NMJs within the muscle as described previously (Tabares *et al.*, 2007). The bath Ca^{2+} concentration varied from 0.1 to 5 mM as specified. Muscular contraction was prevented by including 1–4 μM μ-conotoxin (Alomone Laboratories, Israel) in the bath. 4-β-Phorbol-12,13-dibutyrate (Sigma-Aldrich, Spain) was applied at a concentration of 1 μM.

Perineural recording

Recordings of voltage changes in the perineural space were made using the perineural recording method (Mallart, 1985; Angaut-Petit *et al.*, 1987; Uchitel *et al.*, 1992). The perineural recording electrode was filled with normal Ringer solution and positioned under microscopic control inside the perineural space and near the ends of the myelin sheaths. Recordings were performed in the presence of 15 μM D-tubocurarine in bath solution (Sigma-Aldrich).

Data analysis

The mean amplitudes of the EPP and mEPPs recorded at each NMJ were linearly normalized to −70 mV resting membrane potential. EPP amplitudes were corrected for nonlinear summation (Martin, 1955)

$$\text{EPPc} = \text{Average Peak EPP}/1 - [\text{Average Peak EPP}/(V\text{m} - E\text{r})]$$

where Vm is the resting membrane potential and Er the reverse potential (assumed to be −5 mV for all of our experiments). Quantal content (QC) was estimated either by the direct method, which consists of recording mEPPs and EPPs (nerve stimulation 0.5 Hz) simultaneously and then calculating the ratio

$$\text{QC} = \text{Average Peak EPP/Average Peak mEPP}$$

or, when the [Ca^{2+}]$_e$ was low, by the number of failures (f) for a number (N) of stimuli (Del Castillo & Katz, 1954)

$$\text{QC} = \text{Ln}N/f$$

Latency was measured as the time from the positive peak of the artifact to the 10% onset of the postsynaptic event. All electrophysiological data are given as group mean values ± SEM unless otherwise stated, with n being the number of muscles fibers per group and N the number of mice per group. All experiments reported include the results of at least three animals per genotype. Statistical comparisons between mutant and control measures were made using Student's t-test (two-tails unless otherwise stated) when the distribution was normal and Mann–Whitney rank sum test when the distribution was not normal. Results were considered statistically different when the p value was < 0.05.

Endplate size measurements

At the end of a number of electrophysiological recordings, 2 μL of bungarotoxin-rhodamine (10 μg/mL; Sigma-Aldrich, Spain) was added to the chamber. After 10 min of incubation, the solution was exchanged with a solution without bungarotoxin-rhodamine and the endplates were visualized by epifluorescence with an Olympus BX50WI upright microscope fitted with a water immersion LUMPlanFI 60×/0.90 objective (Olympus). Images were acquired with an Orca ER or a 9100-02 camera (Hamamatsu, Japan). Offline analysis of the endplate area was performed with a macro of IGOR Pro 4.

Results

As CSPα is proposed to play a role in synapse maintenance with use, we examined neuromuscular transmission at three postnatal ages: P14, where no major *in vivo* phenotype was apparent, P18 and P > 30, where there was an altered phenotype *in vivo* as assessed by EMG recordings and motor tests (Fernandez-Chacon *et al.*, 2004). In the *ex vivo* neuromuscular preparation, action potentials in CSPα KO muscle fibers had similar characteristics as in control fibers and were efficiently blocked by the same amount of μ-conotoxin (1–4 μM) as in controls. At P > 30, the resting membrane potential was less negative in CSPα KO (−49.9 ± 1.4 mV; n, N = 100, 17) than in WT (−56.4 ± 1.6 mV; n, N = 62, 13) ($P < 0.002$) animals.

Loss of CSPα altered the frequency and magnitude of spontaneous release

In order to investigate the effect of the absence of CSPα on the probability of spontaneous neurotransmitter release, we compared the

occurrence of spontaneous mEPPs in control and CSPα KO terminals. The frequency of mEPPs varied greatly between terminals in mutant mice at all ages examined (Fig. 1A and B; Table 1); e.g. at P30–40 mEPP frequencies ranged from normal values of ∼1 event/s (47% of fibers) to high frequencies of up to 51.3 events/s (11%) (variation coefficient was 2.17 vs. 0.84 in control mice). The rest

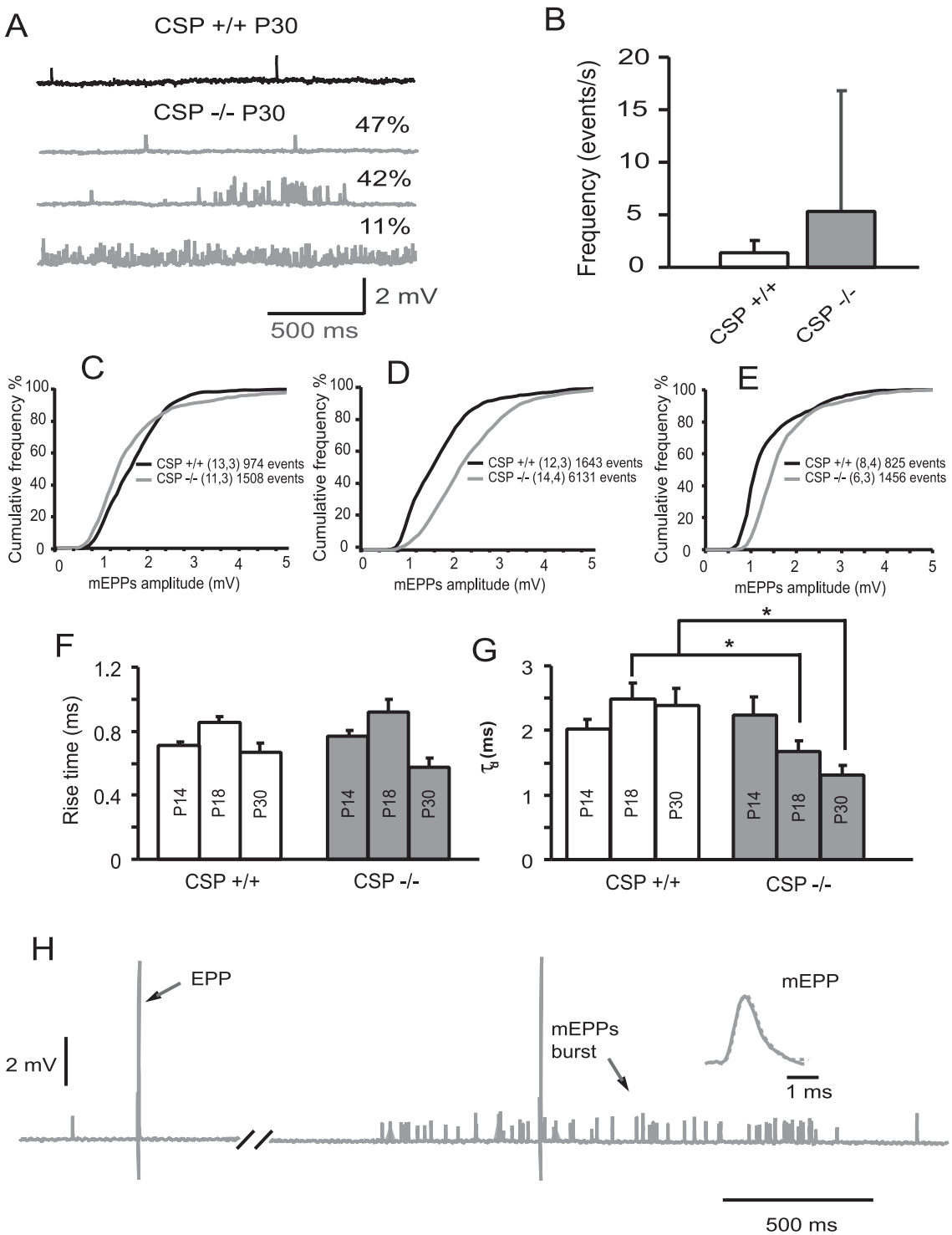

FIG. 1. Spontaneous neurotransmitter release in CSPα KO terminals. (A) Representative examples of mEPP frequency in control and CSPα mutants at P30 showing the great variability found in mutants. (B) Mean ± SD of mEPP frequency in control (n, N = 15, 4) and mutant (n, N = 19, 9) mouse terminals. (C–E) Cumulative frequency of mEPP amplitudes in control and mutants at P14 (C), P18 (D) and P > 30 (E). (F and G) Kinetics of mEPPs (rise and decay times) in controls and mutants at P14, P18 and P > 30; rise times were measured between 10 and 90% on the leading edge of the peak, and decay times from 50% of the peak to the baseline. Decay time constants were significantly faster in mutants at P18 ($P < 0.01$) and P > 30 ($P < 0.01$). (H) Example of a spontaneous burst of mEPPs after a period of normal frequency during evoked release (EPP) at 0.5 Hz. The inset shows the kinetics of the two mEPPs (one outside and the other inside the burst) *$P < 0.08$.

TABLE 1. mEPP frequencies at P14, P18 and P > 30 in control (CSP+/+) and mutant (CSP−/−) fibers

	Bursting fibers %		High-frequency fibers %	
Age	CSP+/+	CSP−/−	CSP+/+	CSP−/−
P14	0 (11, 3)	25 (16, 3)	0 (11, 3)	12.5 (16, 3)
P18	0 (19, 4)	53.8 (26, 5)	5.3 (19, 4)	3.8 (26, 5)
P > 30	0 (29, 7)	42 (25, 6)	3.4 (29, 7)	11 (25, 6)

Data in parentheses (*n*, *N*): *n*, the number of muscles fibers per group; *N*, number of mice per group.

(42%) of the mutant fibers at this age displayed bursts of high-frequency spontaneous release intermixed with normal frequency periods, a feature never seen in control littermate terminals at any explored age (Table 1). The spontaneous release bursts lasted between 0.2 and 7 s, and were also recorded in the presence of tetrodotoxin (8 μM), with low external Ca^{2+} (0.1 mM), or in the presence of ω-agatoxin (200 nM) (data not shown), suggesting that they were not induced by nerve activity. The mEPP bursts might be due to transient intraterminal Ca^{2+} increases, similar to the spontaneous Ca^{2+} 'flashes' that occasionally occur in *csp* null NMJs in *Drosophila* (Dawson-Scully *et al.*, 2000). In addition, the relatively high percentage of fibers with almost continuous high frequency on mEPPs could be related to long-lasting elevations of intraterminal Ca^{2+}, either a cause or consequence of the degeneration process. From a different point of view, the presence of a continuous high frequency of mEPPs also revealed that these mutant terminals were not depleted of fusion-competent vesicles.

We next compared the size and shape of mEPPs in mutant and WT fibers. No size differences were found at P14 (Fig. 1C; $P > 0.59$) but at P18 and at $P > 30$ the mean mEPP amplitude in mutant fibers was significantly greater than in controls ($P < 0.001$), as assessed by the nonparametric Mann–Whitney rank sum test (Fig. 1D and E), which seems to exclude the possibly of a reduced sensitivity of the postsynaptic muscle to neurotransmitter or a decrease in the amount of transmitter contained in synaptic vesicles. Interestingly, vesicle sizes in presynaptic photoreceptor ribbon terminals, quantified by electron microscopy, demonstrated that vesicles are more heterogeneous and larger in CSPα KO mice than in control littermates (Schmitz *et al.*, 2006). Similarly, in the calyx of Held, the miniature excitatory postsynaptic current (mEPSC) amplitude in CSPα KO mice is significantly increased at P20–23 (Fernandez-Chacon *et al.*, 2004).

The mean mEPP rise time was not significantly different in CSPα KO and control terminals at any examined age (Fig. 1F), suggesting that spontaneous vesicle release occurs at the right sites facing the postsynaptic membrane. The decay phase of mEPPs was not significantly different in CSPα KO and control terminals at P14 but was faster in mutant terminals at P18 and P30 (mutants, 1.3 ± 0.15 ms; controls, 2.4 ± 0.26 ms; $P < 0.008$, *t*-test) (Fig. 1G). This may reflect the fact that muscle fibers were smaller in mutants than in controls, which could also contribute to the increase in mEPP amplitude seen at these ages (see Fig. 1D and E). The increase in the mEPP amplitude will be traduced in an apparent larger QC, which is the opposite of what we have found in mutants at P18 and P30 (see below), meaning that the real decrease in the QC in the mutant may even be underestimated.

The amplitude and kinetics of mEPPs within the spontaneous release bursts appeared similar to those outside the burst (Fig. 1H, inset).

Evoked neurotransmitter release was reduced in CSPα KO terminals

We next examined whether the deficiency in CSPα affects nerve-evoked neurotransmission release by studying the mean size of the EPPs in response to 0.5 Hz stimulation. At P14, neither the EPP amplitude (Fig. 2A, upper traces) nor the mean QC (number of quanta released per stimulus) (Fig. 2B) was statistically different between control and mutant terminals (QC in KO: 15.9 ± 3.0; *n*, *N* = 11, 3; QC in WT: 16.3 ± 1.4; *n*, *N* = 13, 3). However, at P18, the mean QC in mutant terminals was decreased to 56.7% of controls (KO: 10.1 ± 1.1; *n*, *N* = 14, 4; WT: 17.8 ± 2.4; *n*, *N* = 12, 3) ($P < 0.012$, *t*-test) (Fig. 2B). Later, at P30–40, this difference became even greater as WT terminals further increased the amplitude of their EPPs and their QC (33.5 ± 3.8; *n*, *N* = 14, 5), whereas mutant terminals did not (12.6 ± 2.9; *n*, *N* = 10, 6) ($P < 0.0002$, *t*-test) (Fig. 2A, lower traces, and 2B). QC mean values in mutants were not different in fibers with and without a burst of mEPPs (data not shown).

Interestingly, the postsynaptic area, estimated by labeling the nicotinic receptors with bungarotoxin-rhodamine (Fig. 2C), was similar in mutants to the controls at P14 but was ∼16% smaller than in the controls at P18 (KO: 212.3 ± 12.4 μm^2; *n*, *N* = 36, 3; WT: 251.8 ± 8.87 μm^2; *n*, *N* = 54, 3) ($P < 0.012$, *t*-test) and ∼37% smaller at P30–40 (KO: 206.4 ± 13.7 μm^2; *n*, *N* = 22, 3; WT: 328.4 ± 21.5 μm^2; *n*, *N* = 21, 3) ($P < 0.00003$, *t*-test) (Fig. 2D). No apparent differences in the overall intensities of receptor clusters between mutant and WT terminals were, however, observed at any age.

Repetitive stimulation increases neurotransmitter release in CSPα KO terminals

Among the possible explanations for the reduction in quantal release observed on or after P18 in CSPα KO terminals are an impairment in vesicle recycling and a reduction in the size of the readily releasable pool, which become apparent in the inability of the terminal to sustain normal release during repetitive stimulation (Liu & Tsien, 1995). To explore these possibilities we compared the EPP amplitudes during a train of 10 stimuli applied at 100 Hz. The resulting short-term facilitation or depression curves were then normalized in order to average all age-matched WT and CSPα KO fibers. At P14, no differences were found between mutant and control terminals (Fig. 3A), again suggesting a normal development and function of the synapse in the absence of CSPα up to this age.

At P18, however, mutant terminals facilitated more strongly than controls due to their smaller response to the first stimulus (Fig. 3B), consistent with the low quantal release observed during 0.5 Hz stimulation (Fig. 2B). Nevertheless, the increase in the amplitude of the subsequent responses, probably in response to the accumulation of residual Ca^{2+}, demonstrates the capability of mutant terminals to increase the amount of secretion during repetitive stimulation and suggests that a defect in vesicle recycling, or a reduction in pool size, was not the main reason for the decreased QC found at this age.

Impaired calcium dependency of neurotransmitter release in CSPα KO terminals

To test the calcium dependence of QC in mutant and WT terminals ($P \geq 30$), we performed experiments at different $[Ca^{2+}]_e$ (0.1–5 mM) while the extracellular $[Mg^{2+}]$ was fixed at 1 mM. At low $[Ca^{2+}]_e$, QC values were calculated either through direct or failure methods (see Materials and methods), with the exception of some fibers on which

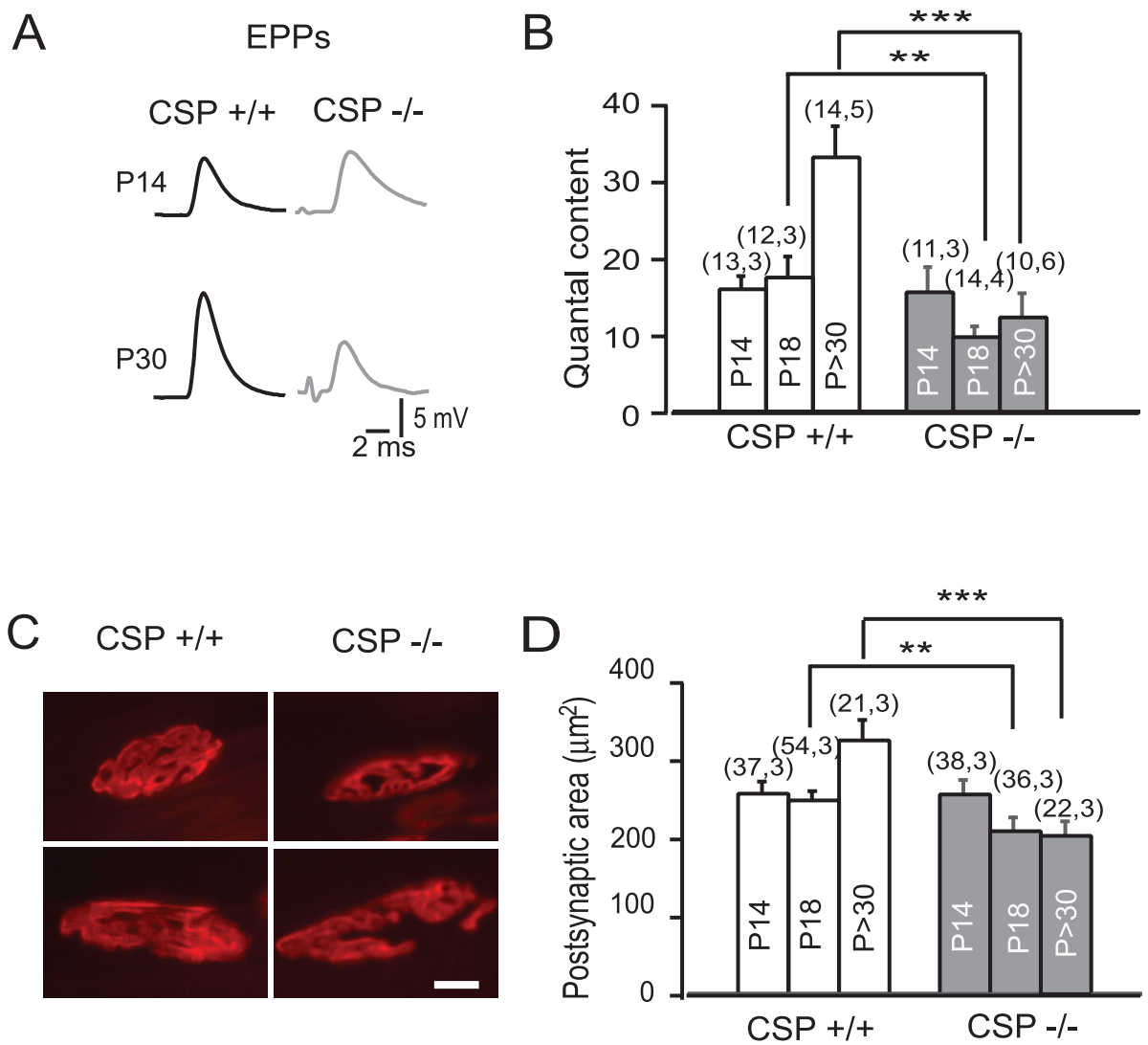

FIG. 2. Evoked release in CSPα KO mice. (A) Representative EPP traces at P14 and P30 in CSPα KO and WT mice. (B) Mean QC in WT and CSPα KO at P14, P18 and P > 30 in 2 mM $[Ca^{2+}]_e$. EPPs were sequentially recorded at 0.5 Hz, a frequency at which the EPP amplitudes did not progressively diminish. QC was significantly lower in CSPα KO than control at P18 (**$P < 0.006$) and P30 (***$P < 0.0002$). (C) Representative examples of end-plates labeled with bungarotoxin-rhodamine from P18 control and CSPα KO mice. Note the smaller area and anomalous 'open' appearance of end-plates in mutants. Calibration bar, 10 μm. (D) Mean postsynaptic area in CSPα WT and KO labeled terminal, as in C (**$P < 0.005$; ***$P < 0.00002$).

both methods were used in order to validate the results. Fig. 4B shows that the values obtained from the two methods are in reasonable agreement ($r^2 = 0.91$). In control terminals, the amplitude of the EPPs increased with external Ca^{2+}, as illustrated in the example in Fig. 4A (upper traces) where Ca^{2+} was changed from 0.5 to 1 mM. Figure 4C shows the mean calcium dependence of the QC in both controls and mutants at P30. In controls, quantal release increased steeply from 0.25 to 0.75 mM Ca^{2+}, demonstrating signs of saturation at higher $[Ca^{2+}]$ (see Fig. 4C, empty circles); the data points fitted well with a Hill function

$$QC = QC\ \max\{[Ca^{2+}]^n/(k^n + [Ca^{2+}]^n)\}$$

where k is the $[Ca^{2+}]$ that produced half-maximum QC and n is the Hill coefficient representing the apparent Ca^{2+} cooperativity. The fit curve showed a maximum QC value of 33.78 ± 2.25, an n value of 5.03 ± 1.63 and a k value of 0.58 ± 0.05 mM. An apparent cooperativity of ∼5 has also been observed in frog NMJs (Shahrezaei *et al.*, 2006) and in the calyx of the Held synapse (Bollmann *et al.*, 2000; Schneggenburger & Neher, 2000).

In mutants (P > 30), the amplitudes of the evoked potentials were smaller than in controls at all $[Ca^{2+}]_e$ tested, as seen in the example of Fig. 4A (lower traces), as were the QCs (see Fig. 4C, filled circles). In addition, neurotransmitter release in mutant terminals depended less strongly on the $[Ca^{2+}]_e$ than in controls; due to the apparent nonsaturation of the QC values, the calcium dependence in mutants was best fit with a power function of the form

$$QC = \alpha\,[Ca^{2+}]^m$$

with $m = 0.95 \pm 0.46$ and $\alpha = 5.29 \pm 4.47$. These results demonstrate that the calcium dependence of release had a large shift to the right in CSPα KO terminals, with near restoration of normal maximal release by raising $[Ca^{2+}]_e$ above physiological concentrations.

We also studied the Ca^{2+} dependence of release at P18. At this age, mutant terminals showed the same impairment in Ca^{2+} dependence

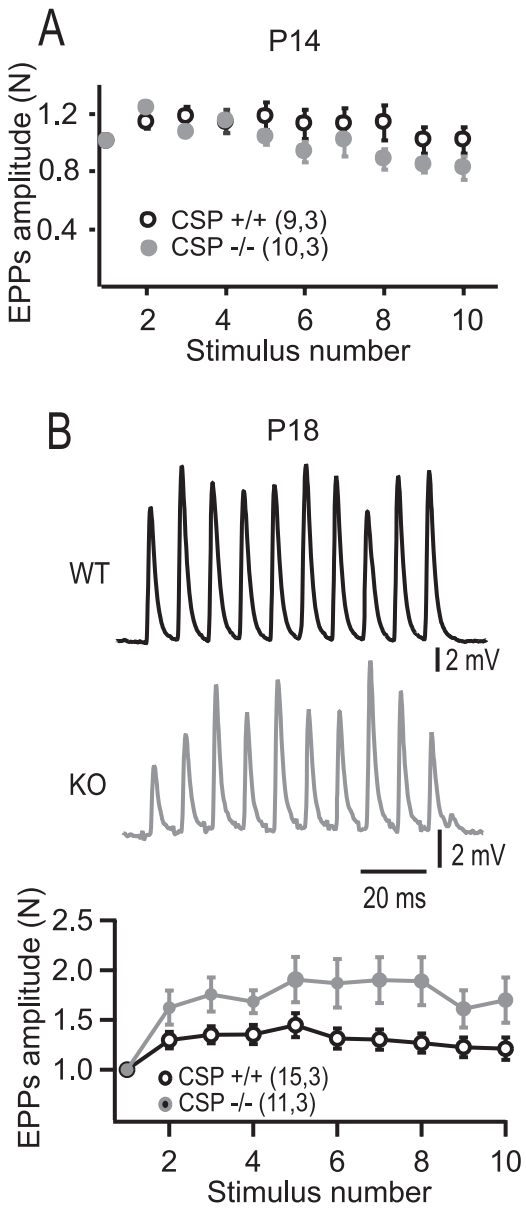

FIG. 3. Repetitive stimulation increases neurotransmitter release in CSPα KO mice. (A and B) EPP amplitudes during trains of stimuli at 100 Hz in WT and KO terminals with physiological $[Ca^{2+}]_e$. At P14 (A) mutant responses did not differ from controls but at P18 (B), CSPα KO fibers facilitated more strongly than did WT fibers due to the small first response in the train. Stimulation frequency was 100 Hz.

(Fig. 4D, $m = 0.91 \pm 0.13$; $\alpha = 6.21 \pm 1.43$) as found at P30 (Fig. 4C). Control terminal values at P18 were, however, fitted well with a Hill function (maximum QC value, 18.17 ± 0.24, $n = 3.9 \pm 0.18$; $k = 0.71 \pm 0.01$ mM). These last results suggest that the alteration in the Ca^{2+} dependence of release in CSPα mutants was not a secondary consequence of the degeneration process itself but a consequence of the absence of CSPα from the terminal. At P14, however, neurotransmitter release in CSPα KO terminals was not different from controls (Fig. 2B), suggesting that CSPα is not critical for a normal Ca^{2+} sensitivity and potentially Ca^{2+} cooperativity of transmitter release as such (it only becomes critical for this later on).

Together these results suggest that the loss of CSPα reduced, after P18, both the Ca^{2+} sensitivity of evoked release and the apparent number of cooperative Ca^{2+}-binding sites needed to release a quantum.

Ca^{2+} channel subtype responsible for neurotransmitter release in CSPα KO terminals

As in other mammal synapses, expression of Ca^{2+} channel subtypes in the NMJ is developmentally regulated (Rosato Siri & Uchitel, 1999), with N-type Ca^{2+} channels having a certain relevance early in development, whereas P/Q channels are mainly responsible for regulating neurotransmitter release in normal adult terminals (Uchitel *et al.*, 1992; Protti & Uchitel, 1993; Protti *et al.*, 1993; Westenbroek *et al.*, 1995; Day *et al.*, 1997). We checked whether P/Q Ca^{2+} channels played a main role in neurotransmitter secretion in CSPα mutant mice, given the 'apparent' arrest in the development of the NMJ in CSPα KO mice (Fig. 2B and D). Using specific subtype blockers, evoked release was equally inhibited (>90%) by ω-agatoxin IVA (200 nM), a P/Q-type voltage-dependent calcium channels blocker, in normal and CSPα mutants at $P > 30$ (Fig. 5A–C), whereas the N-type VDCC antagonist ω-conotoxin GVIA (1 and 5 μM) did not significantly affect release in either WTs or mutants (data not shown), indicating that P/Q Ca^{2+} channels were mainly mediating neurotransmitter secretion in CSPα mutant mice and that the altered Ca^{2+} dependence of release was not due to the coupling of the secretory machinery to a different Ca^{2+} channel subtype.

We next checked whether there was an impairment in the release time course in CSPα KO motor terminals by comparing the rise time (10–90%) of their EPPs with those of controls. We found no statistical difference between WT and KO (WT: 0.76 ± 0.03 ms n, $N = 5, 3$; KO: 0.76 ± 0.02 ms, n, $N = 10, 3$) (Fig. 5D), which indicates that in mutant terminals the Ca^{2+} increment due to the activation of P/Q Ca^{2+} channels occurred in a temporal and spatial window that allowed fast and synchronized evoked neurotransmitter release.

Phorbol ester increased neurotransmission in CSPα KO terminals

In addition to changing the $[Ca^{2+}]_e$ to study the Ca^{2+} dependence of release in CSPα KO terminals, we have explored the sensitivity of release to phorbol ester, a well-known positive regulator of neurotransmission (Malenka *et al.*, 1986; Shapira *et al.*, 1987). Phorbol esters have been shown to potentiate neurotransmission by increasing the size of the readily releasable pool (Stevens & Sullivan, 1998; Virmani *et al.*, 2005) and the Ca^{2+} sensitivity of the fusion machinery (Yawo, 1999; Wu & Wu, 2001; Lou *et al.*, 2005), either by direct protein kinase C (Shapira *et al.*, 1987; Stevens & Sullivan, 1998; Waters & Smith, 2000) or Munc-13 activation (Betz *et al.*, 1998; Rhee *et al.*, 2002). As the effect of phorbol esters depends on the maturation state of the synapses (Virmani *et al.*, 2005), we performed these experiments at P30, an age at which it is considered that the synapse is already mature but at which synaptic transmission in CSPα KO mice was already much impaired. Surprisingly, at this age, CSPα KO synapses responded equally well to the bath application of 4-β-phorbol-12,13-dibutyrate (1 μM) as controls, i.e. with a large increase in EPP amplitude (Fig. 6A–C); however, no changes in mean mEPP amplitudes were observed (Fig. 6D) and the amount of fused vesicles per action potential (calculated as the ratio of the mean EPP size to the mean size of the mEPPs) was equally potentiated (about 1.5-fold) by the phorbol ester in control and mutant terminals (Fig. 6E). In addition, 4-β-phorbol-12,13-dibutyrate increased the frequency of spontaneous neurotransmission in both control and

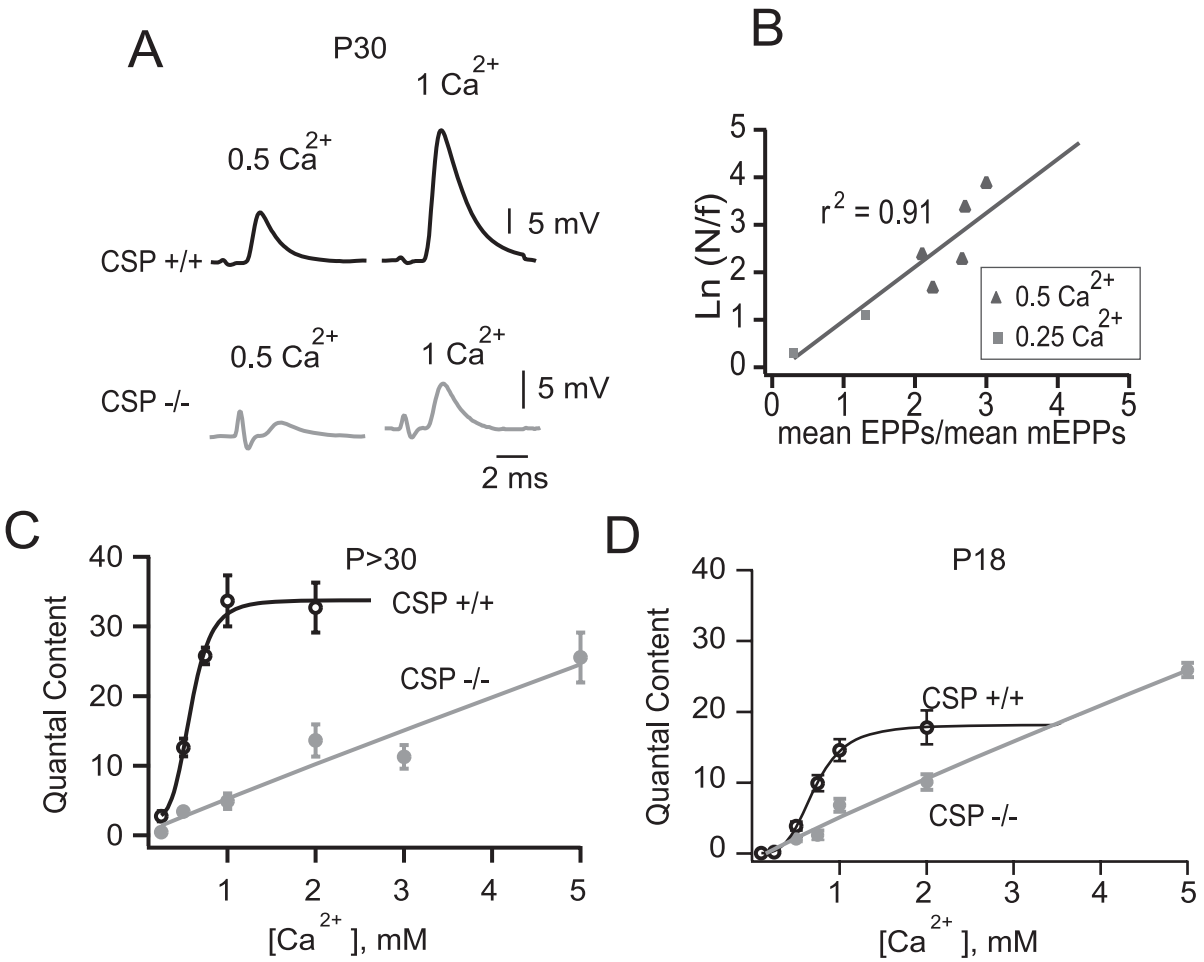

FIG. 4. Calcium dependency of neurotransmitter release in CSPα KO terminals. (A) Representative WT and CSPα KO EPP traces at different $[Ca^{2+}]_e$ from P30 mice. (B) Correlation between direct and indirect methods of measuring the QC, as measured in seven mutant fibers. (C and D) WT and CSPα KO calcium dependence of the QC from P > 30 (C) and P18 (D) mice. Data were fit with a Hill function in controls and with a power function in mutants.

mutant terminals (Fig. 6F), except in a mutant fiber that already had a high spontaneous frequency of mEPPs. The application of α-phorbol (10 μM), a nonactive homologue of 4-β-phorbol-12,13-dibutyrate, was ineffective (data not shown). All of these results together demonstrate that neurotransmission in CSPα KO terminals could still be positively modulated at an age when synaptic function appeared to be much impaired.

Synaptic efficacy to maintain evoked release was altered in CSPα KO mice after P30

At P30–40, we found a strong variability in short-term plasticity among mutant mice terminals when stimulated with trains of pulses at different frequencies. At 100 Hz stimulation, over 26% of mutant fibers (four out of 15) still demonstrated short-term facilitation, although with marked cyclic changes in the amplitude of the EPPs during the train (Fig. 7A, middle trace), probably resulting from the combined effect of Ca^{2+} accumulation and partial vesicle depletion; the remaining mutant fibers (74%) displayed a significantly larger synaptic depression than WT controls (depression at the end of the train in KO: 44 ± 3.1% of their initial value; *n*, *N* = 11, 6; WT: 19.7 ± 6%; *n*, *N* = 5, 3; $P < 0.01$, *t*-test) (Fig. 7B). A test of normality confirmed that the mutant fiber population did not follow a normal distribution, whereas control fibers did ($P = 0.017$ and $P > 0.2$, respectively, Kolmogorov–Smirnov test). In mutants, the short-term depression demonstrated frequency dependence (see Fig. 7C), such that the steady-state EPP amplitude was smaller at higher stimulation frequencies and always significantly smaller than in controls at all explored frequencies (1–100 Hz). However, when we lowered the $[Ca^{2+}]_e$ to 0.5 mM to decrease the probability of release and the likelihood of a premature depletion of vesicles, fibers showed facilitation instead of depression and no differences were observed between WT and KO (Fig. 7D). Together, these results demonstrate the inability of most CSPα KO terminals to maintain the amount of neurotransmitter released with repetitive stimulation at P30, suggesting that at this age they start to suffer from a reduction in the size of the readily releasable vesicle pool or from a defect in the recycling of vesicles.

Evoked release synchrony and latency in CSPα KO terminals

In mutants at P ≥ 30 the rise time of the EPPs within the train did not increase significantly from EPP_1 (0.76 ± 0.02 ms) to EPP_{10} (0.78 ± 0.01 ms) (*n*, *N* = 10, 3; $P > 0.36$), suggesting that, in CSPα KO motor terminals, the release kinetics did not become desynchronized with repetitive stimulations. However, when we compared the delay from the first peak of the stimulation artifact with the initial 10% of the first evoked response we discovered that it was slightly larger in

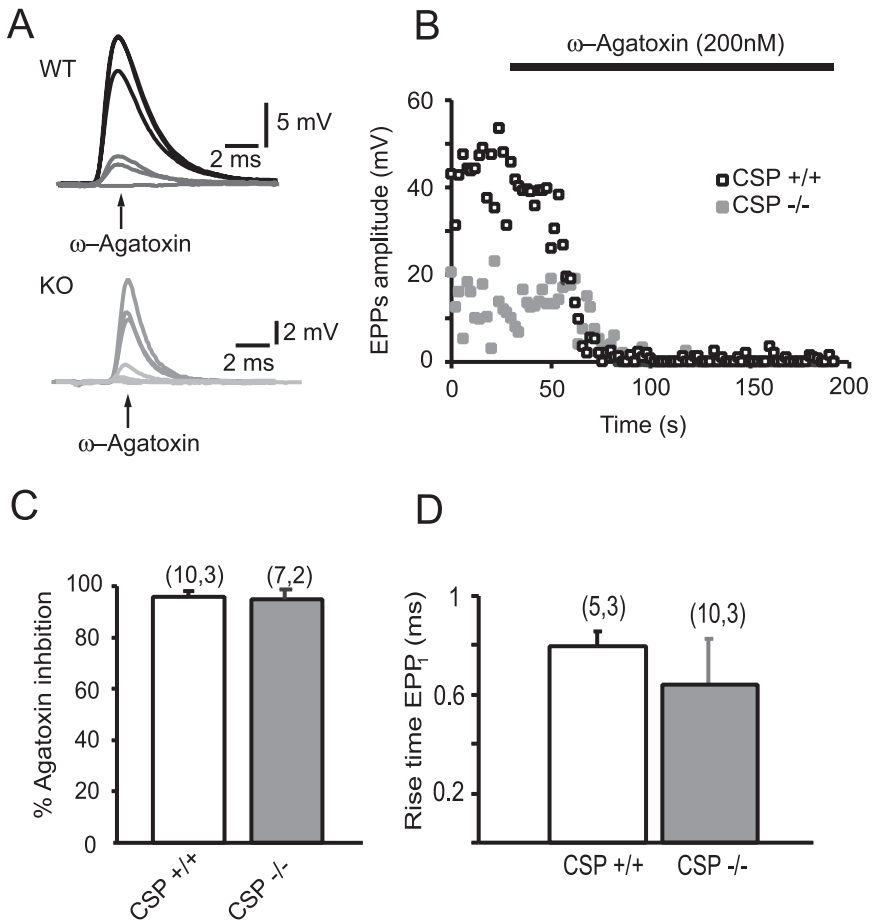

FIG. 5. Effect of ω-agatoxin (Aga) (200 nM) on EPP amplitude. (A) Sample traces of the EPP before and after Aga application in a WT and a KO terminal. (B) Examples of the Aga blocking effect as assessed by the progressive reduction of the EPPs evoked by single nerve stimuli given at 0.5 Hz in a WT and a mutant fiber. (C) Mean Aga inhibition in CSPα KO and WT terminals. Evoked responses were recorded first in a number of fibers in the absence of the toxin; Aga was added to the chamber later and kept for the remainder of the experiment, with recording starting after 30 min of incubation with Aga. (D) EPP rise time (10–90%) in control and mutant terminals showed no differences. All data are from P > 30 mice.

CSPα KO than in littermate controls at P > 30 (KO: 2.05 ± 0.16 ms, *n*, *N*, 12, 3; WT: 1.73 ± 0.07 ms, *n*, *N*, 10, 3) ($P < 0.05$, *t*-test, one tail). Furthermore, during the stimulus train, there was progressive prolongation of this latency (delta delay) with the stimulus number. Figure 8A shows eight representative superimposed responses from a control (upper panel) and a mutant (lower panel) fiber during a 100-Hz stimulus train in 1 mM $[Ca^{2+}]_e$. Figure 8B shows the mean delta delay values as a function of the pulse number in control and mutant fibers. In both groups, the latency increased with each stimulus but, in mutants, the average mean latency increment by EPP_{10} was almost three times longer than in control terminals (KO: 0.35 ± 0.08 ms, *n*, $N = 12, 3$; WT: 0.13 ± 0.04 ms, *n*, $N = 10, 3$; $P < 0.01$, *t*-test).

Mutant mice also presented a significant larger delta delay by EPP_{10} (0.6 ± 0.1 ms, *n*, $N = 13$, 3) at an earlier age (P18) in comparison with littermate controls (0.28 ± 0.02 ms, *n*, $N = 13$, 3; $P < 0.012$, *t*-test), in the range of that observed at P > 30. No differences in delta delay were found at P14, demonstrating that it is not the lack of CSPα by itself that produces the increased delay. A possible reason for an ongoing increment in the delay in the EPPs during the train is the progressive alteration of the presynaptic action potentials during successive stimulations (Datyner & Gage, 1980; Sabatini & Regehr, 1996). To test this possibility we performed perineural recordings near the endplate areas, which reflect the membrane permeability changes associated with the action potential (Mallart, 1985; Angaut-Petit *et al.*, 1987; Uchitel *et al.*, 1992). The analysis of these recordings showed that the absolute value of the delay between the stimulation artifact and the negative peak of the perineural waveform was not different in controls (*n*, $N = 16$, 3) and mutants (*n*, $N = 22$, 3); furthermore, during trains of 10 stimuli at 100 Hz, the delta delays were not different between WT (67.2 ± 12.6 μs) and KO (57.3 ± 9.2 μs) ($P > 0.05$) terminals (Fig. 8E and F). In addition, the width of the recorded responses also remained constant during the train in both groups (data not shown). These data suggest that the increment in the delay was not due to a defect in the conduction of the action potential along the axon but, most probably, to a defect in the intraterminal axonal branches.

Finally, we found no correlation between latency increments and short-term changes in plasticity; e.g. Fig. 8C shows a mutant fiber with cyclic changes in the amplitude of the EPPs during a train in which the delay progressively increased. The corresponding plot representing the amount of delta delay vs. the EPP amplitude for this fiber is illustrated in Fig. 8D. The data show that no good correlation existed between these two parameters ($r^2 = 0.17$). Similarly, no correlation was found in the fibers where EPP amplitudes either depressed or facilitated monotonically during the train (data not shown), suggesting that the origin of the delay increment was not related with the availability of vesicles for release.

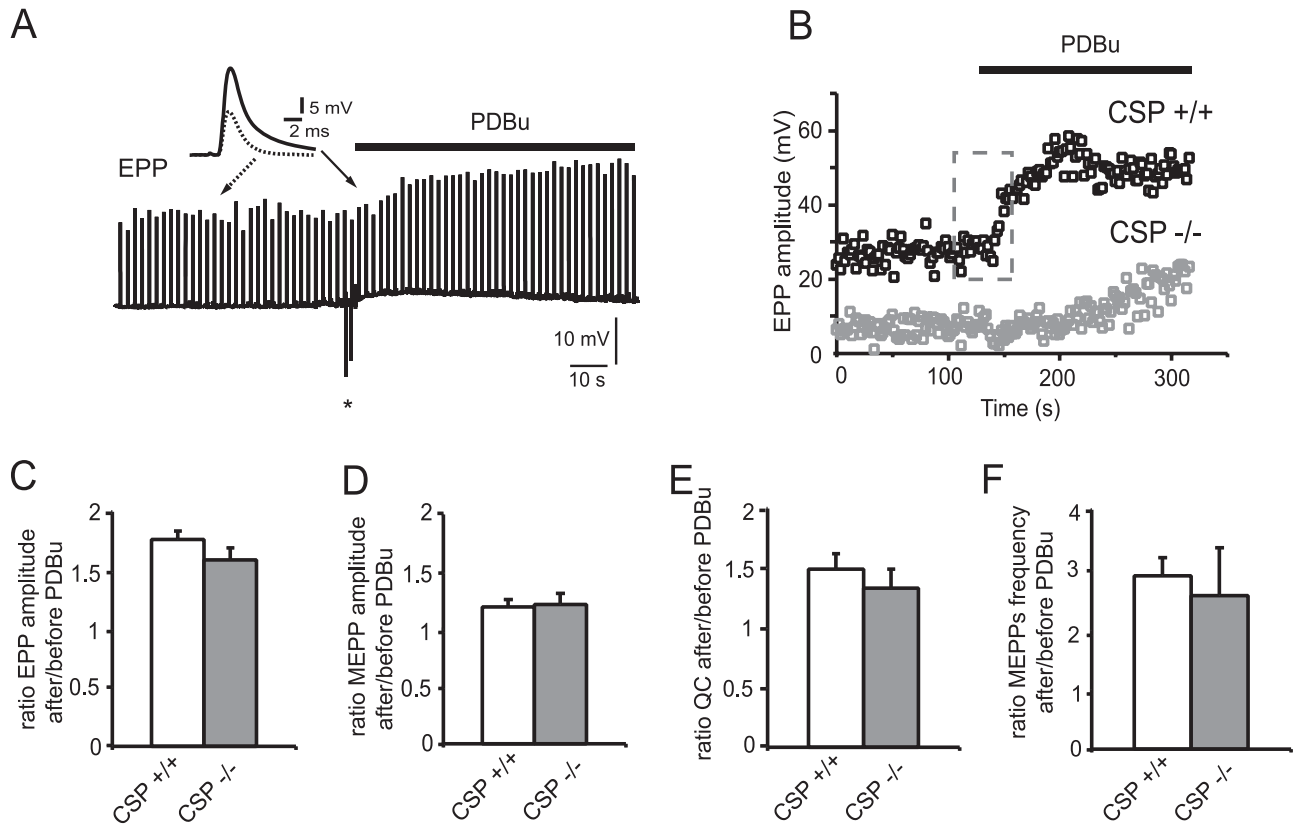

FIG. 6. Phorbol ester potentiation of evoked and spontaneous transmitter release in CSPα KO terminals. (A) EPP traces recorded (0.5 Hz stimulation) from a WT terminal before and during 4-β-phorbol-12,13-dibutyrate (PDBu) bath application (1 μM). Inset: two superimposed EPPs on an expanded time scale; asterisk indicates the artifact of adding the drug. (B) Time course of the change in amplitude of the evoked responses in a WT and a CSPα KO terminal induced by PDBu. Mean ratio (after and before PDBu) of EPP amplitude (C), mEPP amplitude (D), QC (E) and mEPP frequency (F) measured in WT (n = 3) and KO (n = 3) terminals from littermate mice (P30).

CSPα-deficient NMJs had a greater probability of neurotransmission failure at physiological external Ca^{2+} concentrations

In low $[Ca^{2+}]_e$, the number of vesicle fusions per nerve impulse is reduced and a fraction of the stimuli fail to evoke a response (probability of neurotransmission failure). We have compared the number of failures in control and CSPα mutants with 0.25 mM $[Ca^{2+}]_e$ and found that, in P30–40 mutants, the fraction of stimuli failing to evoke transmitter release during a 10-stimuli train was approx. threefold higher than in WT littermates at 100 Hz stimulation (Fig. 9A and B). To examine whether the greater number of failures in the mutants could be explained by the smaller size of their evoked responses, we calculated the predicted probability of failures ($P_f = e^{-QC}$) for each given QC. Figure 9B shows that the means of the expected (dashed lines) and measured (bars) values were similar in both groups of mice, indicating that the excessive number of failures observed in mutants was due to 'true' stochastic failures of synaptic transmission. Surprisingly, however, with 2 mM $[Ca^{2+}]_e$, terminals from CSPα KO mice presented intermittent failures (Fig. 9C and D) with a probability much higher than that expected (dashed line in Fig. 9D) by their relatively low QC. No failures were observed in control terminals, as was expected with this $[Ca^{2+}]_e$. The occurrence of failures in physiological Ca^{2+} was not significantly different at P30–40 than at P18 but this trait was not observed in mutant fibers at P14 (n, N = 10, 3, data not shown). The probability of failures in the mutants increased exponentially to an asymptote with the stimulation frequency (Fig. 9E) but was not related to the latency of the evoked responses or the presence of bursts of mEPPs (data not shown). Although one possible explanation for the transmission failures in CSPα KO terminals would be a block in the spread of the nerve axonal currents, no failures were found in perineural recordings in CSPα mutants (n, N = 22, 3), similar to that found in neural cell adhesion molecule (NCAM)-mutant mice, which also present failures without any alteration in nerve terminal currents (Polo-Parada *et al.*, 2004), suggesting that the defect occurs at the nerve terminal itself.

Discussion

In CSPα KO mice, the neuromuscular synapse is apparently formed normally, indicating that CSPα is not essential for synaptogenesis. However, our data demonstrate that, although synaptic transmission appeared to be normal during the first 2 weeks of life with the exception of the appearance of sporadic bursts of mEPPs in 25% of fibers (Table 1), evoked synaptic neurotransmission became increasingly impaired during the third week of life. In addition, we found that the Ca^{2+} sensitivity of release was much decreased in CSPα KO terminals and that, unexpectedly, with high $[Ca^{2+}]_e$, the amplitudes of the postsynaptic responses approached those of control littermates in physiological $[Ca^{2+}]$.

At P18, with the exception of an increase in short-term synaptic depression that appeared by P30, the CSPα KO phenotype was fully developed, i.e. decreased EPP amplitudes and QCs, increased delay in evoked responses, failures of transmission at physiological $[Ca^{2+}]_e$ and occurrence of high-frequency bursts of spontaneous release. Remark-

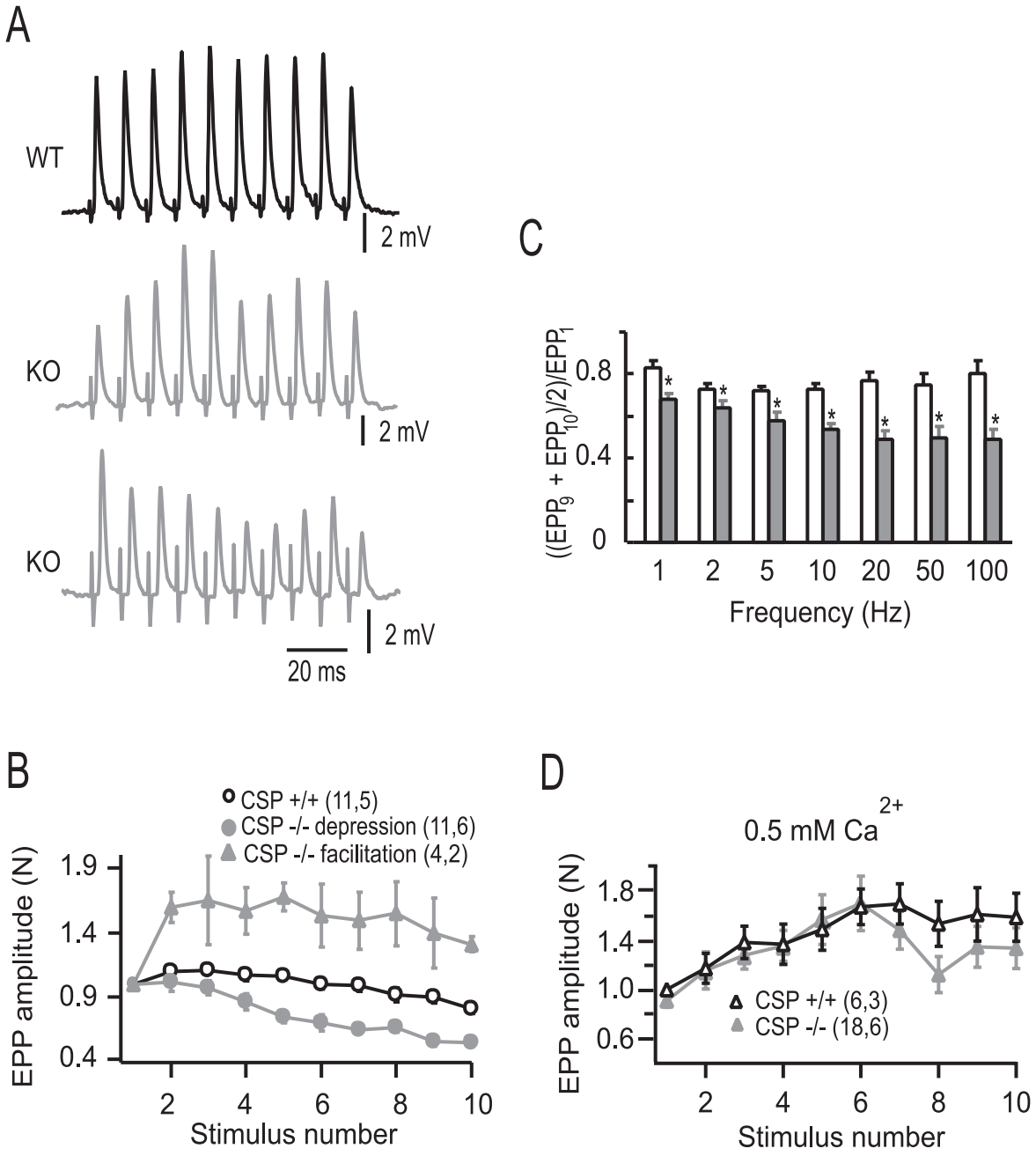

FIG. 7. Short-term plasticity in CSPα KO mice at P > 30. (A) At this age, fiber responses in mutants were much scattered, with some fibers showing facilitation (middle trace) and others strong depression (lower trace). (B) For each fiber, we constructed the mean curve observed for three sequential stimulus trains, separated by 20 s intervals. (C) Normalized depression at the end of a 10-stimuli train at different frequencies (1–100 Hz) in CSPα KO (grey bars) and control (white bars) terminals in normal Ca^{2+}. (D) Mean EPP amplitudes during 10 stimuli at 100 Hz in CSPα KO and WT terminals (P30) in 0.5 mM $[Ca^{2+}]_e$ (fibers with transmission failures have been excluded). *$P < 0.003$.

ably, in spite of all of these abnormalities, evoked postsynaptic responses were synchronized, different from what has been described in the calyx of Held in the same mouse model (Fernandez-Chacon *et al.*, 2004) or in *Drosophila* NMJs (Heckmann *et al.*, 1997 but see Umbach & Gundersen, 1997; Dawson-Scully *et al.*, 2000, 2007).

The big reduction (62%) of the QC in CSPα KO NMJ terminals by P30 was quantitatively similar to the diminishment of evoked release described in *Drosophila csp* null NMJs (Umbach *et al.*, 1994; Zinsmaier *et al.*, 1994; Dawson-Scully *et al.*, 2007), in frog motoneurons after the injection of CSP antibodies (Poage *et al.*, 1999) and in mouse CSPα KO calyx of Held synapses at P20–23 (Fernandez-Chacon *et al.*, 2004). We found, nevertheless, that with high $[Ca^{2+}]_e$, repetitive stimulation or in the presence of a phorbol ester the amplitudes of the postsynaptic responses approached those of control littermates in physiological conditions (Figs 3, 4 and 6). Remarkably, a similar 'rescue' of neurotransmission by high Ca^{2+} has also been reported in *Drosophila csp* null NMJ terminals (Dawson-Scully *et al.*, 2000). There are several alternative explanations that can be considered for these results: there could be a reduction in stimulus-evoked Ca^{2+} entry, a diminution in the ability of Ca^{2+} to trigger exocytosis, or release in the KO could be due to nonsynaptic vesicle pools that are less sensitive to Ca^{2+} and that may remain after partial

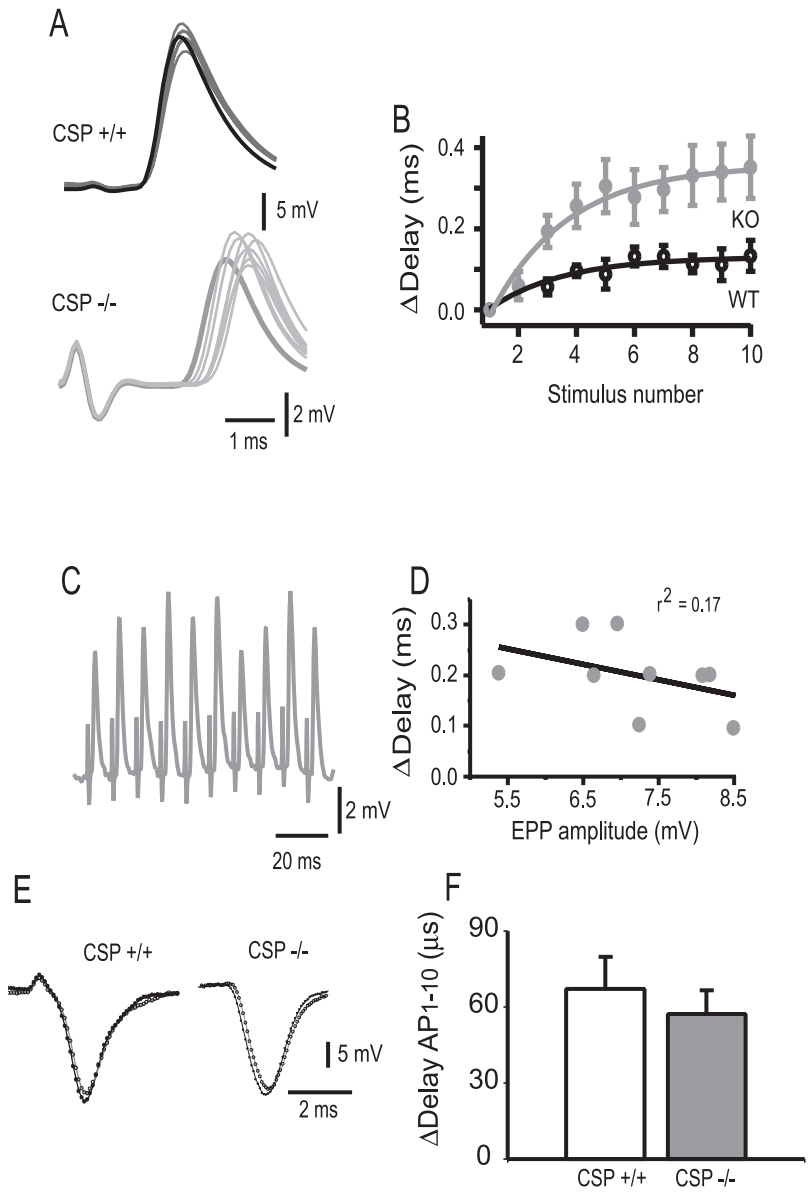

FIG. 8. CSPα KO demonstrates a progressive increment in synaptic delay. (A) Representative recordings of WT (upper traces) and CSPα KO (lower traces) EPPs during a stimulation train at 100 Hz. The continuous dark line represents the first EPP in the train. (B) Average delay increment as a function of the successive stimuli in CSPα KO (12 fibers, three mice) and WT (10 fibers, three mice) in 1 mM Ca^{2+}. (C) Representative recording from a CSPα KO muscle fiber showing cyclic variation in the amplitude of the evoked responses. (D) Plot of EPP amplitude vs. delta delay from the recording shown in C. (E) Representative examples of perineural recordings during a train of 10 stimuli at 100 Hz from a WT and a CSPα KO fiber; for clarity only the first (continuous line) and last (discontinuous line) perineural waveforms of the train are shown superimposed. (F) Mean delta delay between the 10th and first response from perineural recordings in WT (15, 3) and CSPα KO (15, 3) fibers; these recordings were performed in the presence of 15 μM D-tubocurarine in bath solution.

neurodegeneration in the CSPα-deficient terminals, resulting in an overall shift of the apparent Ca^{2+} sensitivity of release.

In the first case, the data obtained in CSP-deficient mutants in different laboratories are contradictory; in some cases, a reduction in Ca^{2+} entry has been postulated (Gundersen & Umbach, 1992; Umbach *et al.*, 1998; Chen *et al.*, 2002), whereas in others, the size of the Ca^{2+} currents under voltage clamp has not been found to be diminished (Morales *et al.*, 1999; Fernandez-Chacon *et al.*, 2004). Furthermore, it has also been reported that nerve electrical stimulation produces a larger increase in intracellular [Ca^{2+}] on *csp* null NMJ *Drosophila* terminals in comparison to controls (Dawson-Scully *et al.*, 2000). In addition to a change in the number of Ca^{2+} channels, a variation of the type of Ca^{2+} channel coupled to release could also account for the altered Ca^{2+} responses in CSP mutants, e.g. in the calyx of Held, Ca^{2+} influx through the P/Q-type channels triggers release more effectively than Ca^{2+} influx through N- or R-type channels (Wu *et al.*, 1999). In mammalian NMJs, Ca^{2+} channels implicated in evoked neurotransmitter release undergo developmental changes during the early postnatal period (Rosato Siri & Uchitel, 1999) (Santafe *et al.*, 2001; Rosato-Siri *et al.*, 2002). As the absence of *csp* in *Drosophila* NMJs retards the development of this synapse (Bronk *et al.*, 2005; Dawson-Scully *et al.*, 2007), we tested the possibility that the reduced Ca^{2+} sensitivity for release in mice could be due to a delay in the expression of the mature Ca^{2+} channel subtype. Nevertheless, our experiments

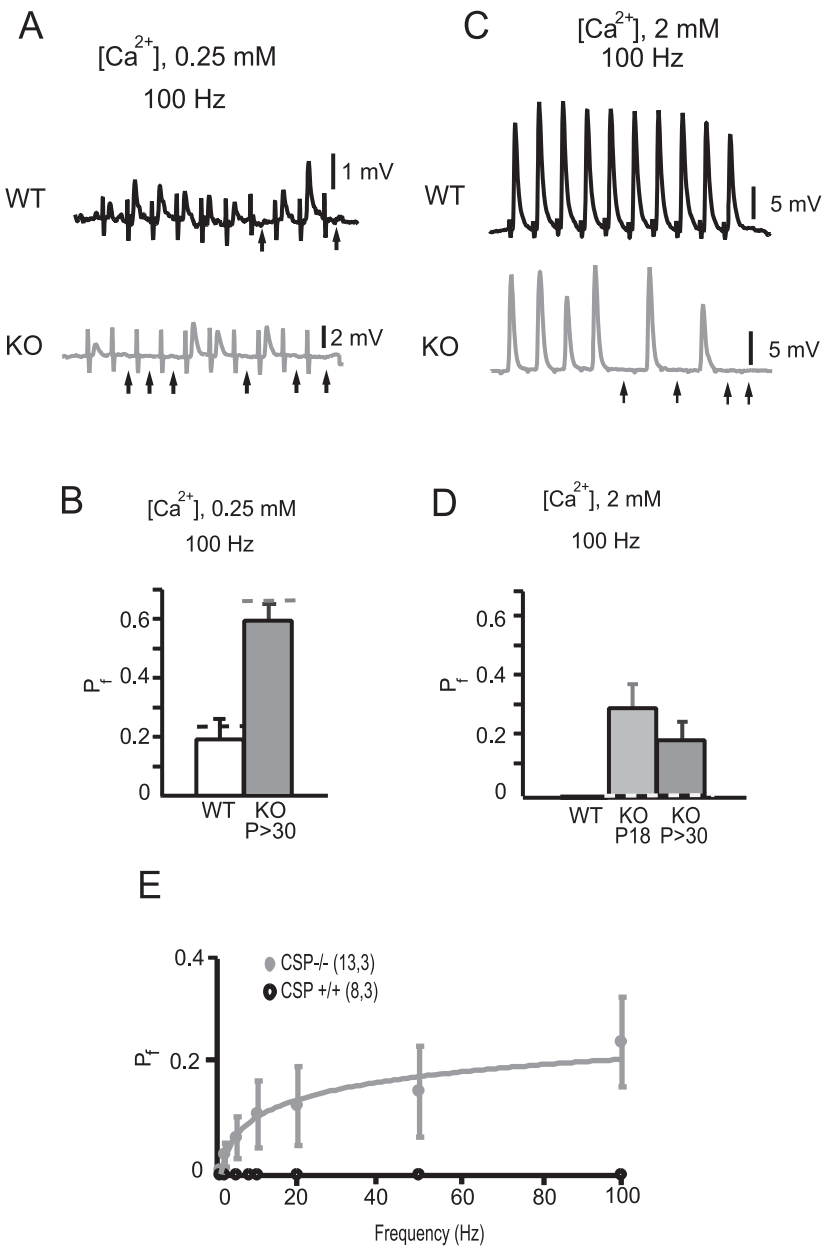

FIG. 9. Failures in synaptic transmission with repetitive stimulation in CSPα mice. (A–D) Representative traces of evoked responses at low $[Ca^{2+}]_e$ (A) and physiological $[Ca^{2+}]$ (C) in WT and CSPα KO at P30. The mean probability of failures (P_f) measured in WT and mutants (bars) was similar to the theoretical calculated value (dashed lines) in low Ca^{2+} (B) but, at physiological $[Ca^{2+}]$, P_f was greater than expected in mutants at both P18 and P > 30 (D). (E) The P_f in CSPα KO increased with the frequency of stimulation.

discard this possibility as, in mutants as well as WTs, evoked release was strongly dependent on the activation of P/Q-type Ca^{2+} channels (see Fig. 5A–C).

The second possibility, a reduction of Ca^{2+} sensitivity of transmitter release downstream of Ca^{2+} entry, has also been demonstrated, not only in *Drosophila* null *csp* NMJs (Dawson-Scully *et al.*, 2000) but also in a null mutant of the chaperone protein that interacts with *csp* [heat-shock cognate protein (Hsc70–4)] (Bronk *et al.*, 2001). In these two invertebrate models, mutants demonstrate a shift to the right of the calcium dependence of neurotransmitter release so that high Ca^{2+} is able to 'rescue' the mutant defect in neurotransmission. Many scenarios are possible to explain these and our results, ranging from the diminished activity of an essential protein for Ca^{2+} sensitivity due to the lost co-chaperone action of CSP (Tobaben *et al.*, 2001, 2003; Zinsmaier & Bronk, 2001; Evans *et al.*, 2003) to the lack of a direct effect of CSP with the secretory machinery, i.e. the soluble NSF-attachment protein receptor (SNARE) complex (Nie *et al.*, 1999; Evans & Morgan, 2002), Ca^{2+} channels (Leveque *et al.*, 1998) or G protein subunits regulating Ca^{2+} channels (Magga *et al.*, 2000). As the P/Q Ca^{2+} channel α_{1A} subunit associates with the SNARE core complex via a synaptic protein interaction 'synprint' site (Catterall, 1998), the diminished assembly of SNARE complex in the absence of CSP (Chandra *et al.*, 2005) may reduce the interaction between presynaptic Ca^{2+} channels and the SNARE complex, necessary for releasing the tonic inhibition of the Ca^{2+} channels and eliciting efficient neurotransmitter release (Zhong *et al.*, 1999).

The third possibility, finally, is consistent with all available data but is difficult to prove because the notion would be that vesicles are

resistant to the normal synaptic SNARE-dependent triggering pathway that is the target of CSPα function but are still sensitive to a parallel pathway that is not normally used.

In summary, neurotransmission in the NMJ of CSPα KO mice is not significantly impaired during early postnatal development but becomes greatly impaired after 2 weeks of age. These results demonstrate that CSPα KO presynaptic terminals have enough vesicles to secrete neurotransmitter at a normal level, vesicles can recycle in the terminal and release neurotransmitter in a synchronic way, and secretion can still be regulated by increasing the extra- or intracellular Ca^{2+} concentration, the effective Ca^{2+} sensitivity of vesicle fusion or the pool of vesicles available for release. Our data, therefore, suggest that the absence of CSPα produced an impairment of neurotransmitter secretion downstream of calcium entry through P/Q calcium channels but upstream of the fusion process itself, the calcium sensitivity for release between these two processes being the main mechanism impaired in the synaptic terminal. Viewed together, the severe functional and structural changes that take place in the absence of CSP, both in invertebrates and vertebrates, point to the importance of the distinct roles of this protein in the synapse, which may range from repairing other proteins, participating in the assembly and dissociation of multi-protein complexes, and regulating preterminal Ca^{2+} sensitivity for release.

Acknowledgements

We are very grateful to Dr R. Fernández-Chacón for fruitful discussion and for mouse genotyping (C. de Cires Segura and Dr G. Cantero) and to I. Benito for excellent mouse husbandry. Supported by a Grant from the Spanish Ministry of Education and Science (BFU2004-00350 and BES-2005-6739).

Abbreviations

$[Ca^{2+}]_e$, extracellular $[Ca^{2+}]$; CSP, cysteine string protein; EPP, evoked endplate potential; KO, knockout; mEPP, miniature evoked endplate potential; NMJ, neuromuscular junction; P, postnatal day; QC, quantal content; SNARE, soluble NSF-attachment protein receptor; WT, wild-type.

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