Tetraspanin-13 modulates voltage-gated CaV2.2 Ca\(^{2+}\) channels

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Integration of voltage-gated Ca\(^{2+}\) channels in a network of protein-interactions is a crucial requirement for proper regulation of channel activity. In this study, we took advantage of the specific properties of the yeast split-ubiquitin system to search for and characterize so far unknown interaction partners of CaV2.2 channels. We identified tetraspanin-13 (TSPAN-13) as an interaction partner of the \(\alpha_{1}\) subunit of N-type CaV2.2, but not of P/Q-type CaV2.1 or L- and T-type Ca\(^{2+}\) channels. Interaction could be located between domain IV of CaV2.2 and transmembrane segments S1 and S2 of TSPAN-13. Electrophysiological analysis revealed that TSPAN-13 specifically modulates the efficiency of coupling between voltage sensor activation and pore opening of the channel and accelerates the voltage-dependent activation and inactivation of the Ba\(^{2+}\) current through CaV2.2. These data indicate that TSPAN-13 might regulate CaV2.2 Ca\(^{2+}\) channel activity in defined synaptic membrane compartments and thereby influences transmitter release.

In the nervous system, presynaptic Ca\(^{2+}\) channels of the CaV2 family including CaV2.1 and CaV2.2 play pivotal roles in the Ca\(^{2+}\)-dependent neurotransmitter release\(^{1}\). Their activity is tightly regulated by a multitude of adjacent proteins forming defined interaction platforms. Innovative and comprehensive experimental approaches like the use of affinity purification and quantitative mass spectrometry have systematically characterized CaV2\(\alpha_{1}\) channel nanodomains\(^{2,3}\). Even though these and other studies shed light on the complex protein networks involved in the subtype-specific modulation of CaV2 family members, many aspects underlying their regulation remain unknown and could probably be accredited to yet unidentified interaction partners.

Different methodical approaches aiming at the identification of Ca\(^{2+}\) channel interaction partners might favour certain protein-protein interactions based on their biochemical character. Therefore the use of alternative methods to characterize the interactome of CaV2 channels is desirable.

Screening of cDNA libraries using specific bait proteins in yeast two-hybrid (YTH) assays has revealed so far unknown protein-protein interactions, which often are essential for complex regulatory mechanisms\(^{4}\). The use of CaV2.1 and CaV2.2 domains as bait proteins in a classical YTH system identified active zone RIM proteins as being presynaptic Ca\(^{2+}\) channel tethering adapter molecules that facilitate fast and synchronous neurotransmitter release\(^{5}\). However, due to intrinsic limitations of the classical YTH system, interactions between hydrophobic membrane proteins are not detected. A modified YTH system makes use of a split-ubiquitin which reconstitutes upon interaction of fused bait and prey proteins allowing for the detection of protein-protein interactions within or close to plasma membranes\(^{6}\). This experimental setup enables the identification and characterization of so far unknown interaction partners of CaV2 family members, which might comprise membrane proteins important for modulation of individual CaV2 functions.

**Results**

CaV2.2 \(\alpha_{1}\) subunit interacts with tetraspanin-13. In order to identify and characterize novel interaction partners of presynaptic Ca\(^{2+}\) channels, we constructed bait vectors encoding CaV2.1 and CaV2.2 Ca\(^{2+}\) channel \(\alpha_{1}\) subunit cDNA-fragments. Since our attempts to express full-length \(\tau_{2}\) subunit cDNA in yeast cells failed, we constructed bait-vectors encoding single domains and C-terminal fragments. We hypothesized that putative novel interaction partners might bind to the rather long intracellular C-terminus of CaV2 channels and selected domain IV as a kind of “membrane anchor” fused to the C-terminus. This construct also takes into account that putative interaction partners might bind not only to the C-terminus, but also to parts of the smaller intracellular loops within domain IV or even its transmembrane segments.
Amongst several candidate proteins TSPAN-13, a member of the tetraspanin family of membrane proteins demonstrated robust interaction with domain IV of the CaV2.2 α1 subunit. The usage of a series of truncated forms identified the entire domain IV of the CaV2.2 α1 subunit to be critical for the interaction with TSPAN-13 (Fig. S1a online). Interaction was confirmed by co-immunoprecipitation of CaV2.2-EGFP with TSPAN-13-Myc expressed in CHO cells (Fig. 1).

Tetraspans, as implicated by its names, consist of four transmembrane segments and are characterized by a long extracellular loop (LEL) containing a conserved CCMG motif

Using a yeast pellet-β-galactosidase assay we analysed the interaction between different TSPAN-13 deletion mutants with domain IV of the CaV2.2 α1 subunit. Remarkably, the interaction between TSPAN-13 and CaV2.2 was not affected by deletion of transmembrane segment S3 and S4, the LEL and the C-terminus of TSPAN-13 in the mutant TSPAN-13 ΔS3-S4 and domain IV of CaV2.2 was confirmed by a yeast β-galactosidase filter-lift assay. Expression of TSPAN-13 in the murine brain was demonstrated by an RT-PCR analysis (Fig. S2 online). We identified TSPAN-13 mRNA at about equal levels in the hippocampus, neocortex and cerebellum.

TSPAN-13 affects Ca\textsuperscript{2+}-currents in NG108-15 cells. Since CaV channel activity depends on the integrity of complex protein networks, which contain a multitude of CaV channel interaction partners, we analysed whether TSPAN-13 affects channel properties of its putative interaction partner CaV2.2 using differentiated NG108-15 cells. Upon cultivation in the presence of cAMP, these cells express representatives of high-voltage-activated L-type and non-L-type (predominantly N-type) and low-voltage-activated T-type CaV channels

Amplitudes of low-voltage-activated CaV \textsuperscript{2+} currents activated by depolarization up to \(-20\) mV were not affected by TSPAN-13 (Fig. 2a and b). In contrast, high-voltage-activated Ba\textsuperscript{2+} current amplitude activated by depolarization to 0 mV and above was significantly decreased (unpaired two-tail t-test; 0 mV: \(p = 0.0167; +10\) mV: \(p = 0.0270; +20\) mV: \(p = 0.0177; +30\) mV: \(p = 0.0177\)). High-voltage-activated Ca\textsuperscript{2+} channels were pharmacologically classified. P/Q type (CaV1.2) are abundant in NG108-15 cells, in agreement with observations by Liu and colleagues

Figure 1 | Co-immunoprecipitation analysis using solubilisates of membrane-fractions obtained from CHO cells expressing CaV2.2-EGFP and transiently transfected with TSPAN-13-Myc. Eluates from co-immunoprecipitations with either anti-Myc (left) or IgG-control antibodies (middle) and the input controls (right) were separated on 7% SDS-polyacrylamide gels, respectively. Shown is a representative example of three independent co-immunoprecipitation experiments. The figure shows the relevant area of the antibody-staining.

Effects of TSPAN-13 on recombinant CaV1.2, CaV2.2 and CaV3.1 Ca\textsuperscript{2+}-channels. Next, we confirmed these results using cell lines expressing recombinant CaV \textsuperscript{2+} \textsuperscript{2+} channels co-transfected either with a control EGFP-vector or with a vector encoding TSPAN-13-EGFP. TSPAN-13 did not affect recombinant CaV1.2 and CaV3.1 Ca\textsuperscript{2+} current (Fig. 2c and d) and also did not significantly suppress current amplitude through recombinant CaV2.2 (Fig. 2e). Neither in NG108-15 nor in CHO cells the inhibition of the N-type Ca\textsuperscript{2+} current was accompanied by a shift of the I-V relationship. In contrast to CHO cells, NG108-15 cells do express a considerable outwards K\textsuperscript{+} current. This current was probably responsible for an apparent shift of the reversal potential which was observed in NG108-15 cells (Fig. 2b), but was much less pronounced in CHO cells (Figure 2e).

This result points out that TSPAN-13 might have a specific functional impact on CaV2.2 Ca\textsuperscript{2+} channels. TSPAN-13 suppressed the amplitude of current through CaV2.2 channels in NG108-15 cells and also in a cell line expressing CaV2.2 (Fig. 2e), though, the latter was statistically not significant. We optimized the voltage protocols for the analysis of possible effects of TSPAN-13 on current kinetics (Fig. 3). TSPAN-13 significantly accelerated the inactivation of Ba\textsuperscript{2+} current activation (Fig. 3a) when time constants at individual depolarisations were compared. This acceleration was due to a shift of the voltage dependence of the activation time constant towards less depolarized membrane potentials (Table S1 online). Further, TSPAN-13 significantly accelerated the inactivation of Ba\textsuperscript{2+} current to an enhanced proportion of the current inactivating with a fast time constant (Fig. 3b and Table 1). To visualise this effect we constructed model time courses of fast and slow components of both currents (Fig. 3b). To analyse if TSPAN-13 may affect the Ca\textsuperscript{2+} dependent inactivation of the CaV2.2 channel we repeated these experiments using Ca\textsuperscript{2+} as a charge carrier (Fig. 3c and d). Ca\textsuperscript{2+} current through the CaV2.2 channel activated about twice as fast as the Ba\textsuperscript{2+} current (Fig. 3c versus 3a). Co-expression of TSPAN-13 enhanced the activation time constants at individual depolarisations (Fig. 3c), but this effect was not significant. Nevertheless, TSPAN-13 significantly decreased the steepness of the voltage dependence of Ca\textsuperscript{2+} current activation (Table S1 online). Inactivation of Ca\textsuperscript{2+} current through the CaV2.2 channel was not significantly altered by TSPAN-13 (Fig. 3d).

An accelerated voltage-dependent inactivation suggests that TSPAN-13 may affect the cumulative inactivation during action potential bursts. Indeed, the total inactivation at the end of a series of action-potential-like waveforms or at the end of a high-frequency series of short depolarising pulses was enhanced by TSPAN-13, nevertheless this effect was not significant (Fig. 3a, b, c online). Similarly, the facilitation of recovery from voltage-dependent inactivation by TSPAN-13 was not statistically significant (Fig. 3d and Table S2 online).

Further, we sought for effects of TSPAN-13 on gating currents, which reflect the activation process of the voltage sensor.

TSPAN-13 affects gating currents of CaV2.2 channels. We measured gating currents of CaV2.2 channels by depolarizing each cell to its reversal potential (Fig. 4a). The maximal charge \(Q_{\text{max}}\) evaluates as an area below the gating current trace and is indicative for the number of channels expressed in the cell membrane. This value was not affected by co-expression of TSPAN-13. In addition, the kinetics of the charge movement was not changed. The time constants of the decay of the gating current traces were \(0.54 \pm 0.02\) ms (\(n = 12\)) and \(0.58 \pm 0.02\) ms (\(n = 12\)) for control and TSPAN-13-transfected cells, respectively.

Next, we compared the relationship between the maximal conductance \(G_{\text{max}}\) and the maximal charge \(Q_{\text{max}}\) for each cell (Fig. 4b). This term reflects the efficiency of the coupling between the voltage sensor activation and the pore opening

We found that the \(G_{\text{max}}/Q_{\text{max}}\)
relationship for the recombinant CaV3.1 channel stably expressed in a HEK 293 cell line. Channel gating is in agreement with the observed shift of the voltage (unpaired two-tail t-test p < 0.01 nS/fC (n = 26); - cells transfected with an EGFP control plasmid (n = 15). (b) Averaged current-voltage (I-V) relationships constructed from peak current amplitudes measured as illustrated in panel A. ○ - cells transfected with an EGFP control plasmid (n = 26); ■ - cells transfected with a plasmid encoding TSPAN-13-EGFP (n = 22); ■ - cells co-transfected with a plasmid encoding TSPAN-13-EGFP (n = 19). (c) 1-V relationship for the recombinant CaV1.2 channel stably expressed in a HEK 293 cell. ○ - cells co-transfected with an EGFP control plasmid (n = 18); ■ - cells co-transfected with a plasmid encoding TSPAN-13-EGFP (n = 20). (e) 1-V relationship for the recombinant CaV2.2 channel stably expressed in a CHO cell line. ○ - cells co-transfected with an EGFP control plasmid (n = 16); ■ - cells co-transfected with a plasmid encoding TSPAN-13-EGFP (n = 15).

Point mutations within TSPAN-13 identify critical amino acid residues for channel modulation. The transmembrane segments S1 and S2 of tetraspanins contain highly conserved amino acid residues which have been shown to mediate tight interactions between both transmembrane domains12,13. The TSPAN-13 transmembrane segments S1 and S2 include several bulky amino acid residues which might be involved in these interactions. Thus, we mutated these candidate amino acids and analysed whether the Gmax/Qmax relationship is affected by expression of TSPAN-13 constructs containing single amino acid mutations in CaV2.2-expressing cells.

The mutation of two phenylalanine residues located at positions 55 and 57 in segment S2 to alanine abolished the reduction of Gmax/Qmax, which was observed in the presence of TSPAN-13. In control cells, Gmax/Qmax was 0.18 ± 0.01 nS/IC while in the presence of TSPAN-13(F55A,F57A) Gmax/Qmax was 0.17 ± 0.01 nS/IC (no significant difference) (Fig. 4c). We further analysed whether a single mutation of F55A or F57A is sufficient to abolish the modulation of the Gmax/Qmax relationship. Indeed, the expression of TSPAN-13(F55A) or TSPAN-13(F57A) did not significantly affect Gmax/Qmax compared with controls (0.19 ± 0.01 nS/IC to 0.20 ± 0.01 nS/IC or 0.19 ± 0.01 nS/IC to 0.20 ± 0.01 nS/IC, respectively). We further estimated the protein expression level of several TSPAN-13 constructs to ensure that the missing modulatory effect of the TSPAN-13 point mutations is not simply caused by a reduced expression level. Four independent transfection experiments and Western blot analyses were performed using EGFP-tagged TSPAN-13 constructs (Fig. S4 online). Compared to TSPAN-13 the mutant constructs displayed expression levels that are not significantly different to each other. In fact we even observed a slightly higher expression of the TSPAN-13(F55A,F57A) mutant.

In summary, these data indicate that amino acid residues F55 and F57 located within S3 and S4 could be crucial for the functional modulation of CaV2.2 by TSPAN-13. However, since the deleted segments S3 and S4 include several bulky amino acid residues which might be involved in these interactions, we cannot rule out that the observed abrogation of CaV2.2 modulation (Fig. 5) is mediated by the C-terminal part of TSPAN-13. Further work needs to determine whether TSPAN-13 modulates CaV2.2 through a direct interaction between the two proteins or whether there is an indirect mechanism involving other molecules in the cell membrane.
Gmax/Qmax compared with controls (0.20 ± 0.01 nS/fC to 0.17 ± 0.01 nS/fC, (unpaired two-tail t-test p = 0.0020)). This reduction was similar to the effect of TSPAN-13 and makes an involvement of W33 in CaV2.2 modulation unlikely.

The N-terminus is important for membrane localization of TSPAN-13. Next, we analysed the importance of the N-terminus of TSPAN-13 for the modulation of CaV2.2, by constructing a deletion mutant of TSPAN-13 lacking the intracellular N-terminus. By co-transfection of TSPAN-13DNt, the TSPAN-13 mediated reduction of Gmax/Qmax in CaV2.2 expressing cells was abolished. While Gmax/Qmax was 0.20 ± 0.01 nS/fC in the presence of TSPAN-13DNt, corresponding controls showed similar values (0.21 ± 0.01 nS/fC; not significant). Importantly, the TSPAN-13DNt mutant did not co-localize with the membrane marker Akt-PH-mCherry (Fig. S). TSPAN-13DNt was rather found in intracellular compartments that potentially resemble the endoplasmic reticulum. We assume that the N-terminus of TSPAN-13 is required for its correct intracellular targeting and that modulation of CaV2.2 is abolished because of the absence of TSPAN-13DNt from the plasma membrane.

Tetraspanin CD81 does not modulate CaV2.2. In order to demonstrate that modulation of CaV2.2 is not a general property of all members of the tetraspanin family, we investigated the functional role of tetraspanin CD81. In another screening experiment using the split-ubiquitin system we searched for interaction partners of the L-type Ca2+1 channel CaV1.2 α1 subunit. Again, we used single domains as bait constructs and identified CD81 as a potential interaction partner of the CaV1.2 α1 subunit. A potential modulatory effect of CD81 on CaV2.2 was investigated by electrophysiological analysis. The corresponding Gmax/Qmax values were 0.22 ± 0.01 (n = 11) and 0.19 ± 0.01 (n = 9) for control cells and CD81-transfected cells, respectively. These values were not significantly different, indicating that modulation of CaV2.2 is specific for TSPAN-13.

Figure 3 | Activation and inactivation kinetics of the current through CaV2.2 channels stably expressed in CHO cells. (a) Examples of current traces recorded by 10 ms long depolarisations from a holding potential of −80 mV to potentials increasing from −10 mV up to +50 mV with a step of 10 mV. Ba2+ was used as charge carrier. Cells were either transfected with an EGFP control plasmid (n = 18) or with a plasmid encoding TSPAN-13-EGFP (n = 19). Current traces activated by depolarisations to +10 mV and higher were fitted by a single exponential and corresponding time constants were averaged and plotted in the graph. The significance of the difference between time constants determined for individual depolarisations was tested by the unpaired two-tail t-test (+10 mV, p = 0.018; +20 mV, p = 0.0017; +30 mV, p = 0.0016; +40 mV, p = 0.0027; +50 mV, p = 0.0100). (b) Averaged current traces recorded by 2 s long depolarisations from a holding potential of −80 mV to +20 mV (peak of an IV). Ba2+ was used as charge carrier. Cells were either transfected with an EGFP control plasmid (solid line; n = 22) or with a plasmid encoding TSPAN-13-EGFP (dashed line; n = 20). Each trace was fitted by a sum of two exponentials. The right part of the panel shows both, the fast and slow components which were simulated using the averaged time constants and relative amplitudes listed in table 1. (c) Examples of current traces recorded using the same protocol as in (a). Ca2+ was used as charge carrier. Cells were either transfected with an EGFP control plasmid (n = 11) or with a plasmid encoding TSPAN-13-EGFP (n = 14). Current traces activated by depolarisations to +10 mV and higher were fitted by a single exponential and corresponding time constants were averaged and plotted in the graph. (d) Averaged current traces recorded by 2 s long depolarisations from a holding potential of −80 mV to +20 mV (peak of an IV). Ca2+ was used as charge carrier. Cells were either transfected with an EGFP control plasmid (solid line; n = 8) or with a plasmid encoding TSPAN-13-EGFP (dashed line; n = 12).
**Table 1** | Effect of TSPAN-13 on the inactivation kinetics of 

| Channel Type | $\alpha_1$ (%) | $\tau_{\text{fast}}$ (ms) | $\tau_{\text{slow}}$ (ms) | n |
|--------------|----------------|--------------------------|--------------------------|---|
| $\alpha_1$-control-EGFP | 60 ± 3* | 122 ± 6 | 483 ± 22 | 22 |
| $\alpha_1$-TSPAN-13-EGFP | 69 ± 2* | 126 ± 2 | 494 ± 29 | 20 |
| $\alpha_2$-control-EGFP | 54 ± 4 | 93 ± 18 | 358 ± 37 | 8 |
| $\alpha_2$-TSPAN-13-EGFP | 55 ± 5 | 106 ± 17 | 367 ± 46 | 12 |

*Significantly different from control, p < 0.0255

**Discussion**

In this study, we took advantage of the specific qualities of the yeast split-ubiquitin system to further define the interactome of voltage-gated $\alpha_2$-Ca$^{2+}$ channels and have characterized TSPAN-13 as an interaction partner of the $\alpha_2$-Ca$^{2+}$ channel $\alpha_1$ subunit. The tetraspanin family of membrane proteins is defined by its consensus structure comprising four transmembrane segments, two characteristic extracellular loops and a conserved CCG motif. The small and large extracellular loops are located between transmembrane segments S1&2 and S3&4, respectively. The large extracellular loop has been described to be essential for the function of different tetraspanins by facilitation of protein-protein interactions. A multitude of interactions between ubiquitously expressed tetraspanins themselves and other membrane proteins typically leads to the formation of the so-called tetraspanin web. Tetraspanin-enriched microdomains are involved in a variety of cellular processes as diverse as cell migration, intracellular trafficking, cell fusion and signalling. However, an interaction of tetraspanins with voltage-gated Ca$^{2+}$ channels has not been described so far.

In order to demonstrate a broad expression of TSPAN-13 in the brain, we performed an RT-PCR analysis. TSPAN-13 encoding mRNA was found in the hippocampus, neocortex and cerebellum. Currently an investigation of TSPAN-13 expression at the protein level was not accomplishable since tetraspanins are extremely homologous to each other, thereby limiting the availability of specific antibodies. Furthermore, many tetraspanins are not sufficiently immunogenic to yield reliable antibodies. Hence, a detailed characterization of tetraspanin-associated proteins and functions is difficult to accomplish and studies are currently restricted to a few tetraspanin members.

We found that the modulatory effects of TSPAN-13 are specific for N-type channels and do not occur with L- and T-type $\alpha_2$-Ca$^{2+}$ channels. This interaction was not identified by a recently published quantitative proteomic approach of $\alpha_2$-Ca$^{2+}$ channels. We assume that different methodical approaches favour the identification of certain protein-protein interactions based on their biochemical character.

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**Figure 4** | Effect of TSPAN-13 on gating currents of CaV2.2 channels expressed in CHO cells and determination of the $G_{\text{max}}/Q_{\text{max}}$ ratio.

(a) Examples of current traces recorded by depolarization from a holding potential of −80 mV to the potential corresponding to the reversal potential of the investigated cell. Data represent the average of five runs. Cells were either transfected with an EGFP control plasmid or with a plasmid encoding TSPAN-13-EGFP. (b) $G_{\text{max}}$ versus $Q_{\text{max}}$ for control cells (○) and cells transfected with TSPAN-13 (■). Straight lines represent linear fits of experimental data. (c) $G_{\text{max}}/Q_{\text{max}}$ ratio for TSPAN-13 and various TSPAN-13 constructs. Number of cells tested is indicated at each column. ** - significantly different from control, p < 0.01. Abbreviations: FL-TSPAN-13, full-length-TSPAN-13; δ, regions that are deleted in TSPAN-13 constructs.

**Figure 5** | Intracellular localisation of full-length TSPAN-13-EGFP and mutant forms of TSPAN-13-EGFP expressed in HEK293 cells using pAkt-PH-mCherry as membrane marker. In contrast to full-length TSPAN-13, the Δ(S3-C-term) mutant and TSPAN-13(F55A,F57A) mutant, the N-terminal TSPAN-13 deletion mutant does not localise to the plasma membrane. First column: TSPAN-13-EGFP constructs used in this study; second column: EGFP fluorescence of TSPAN-13 constructs; third column: red fluorescence of pAkt-PH-mCherry membrane marker; forth column: merged images. Confocal images were recorded 48 h after transfection.
hydrophobic interaction between transmembrane segments may be lost due to stringent solubilisation and/or co-immunoprecipitation conditions. In fact, it is the strength of our study, to identify interaction partners which could not be detected by other methods. For sure each method has its advantages and disadvantages and probably only their combination gives a more complete map of the CaV2.2 interactome.

The interaction between TSPAN-13 and CaV2.2 could be located to the membrane-spanning domain IV of the CaV2.2 a1s1 subunit. The regions of TSPAN-13 required for interaction and modulation of CaV2.2 could be narrowed down to a truncated version of TSPAN-13 encompassing the N-terminus and transmembrane segments S1 and S2. Sequence analysis of TSPAN-13 segments S1 and S2 indicates the presence of highly conserved amino acids which have been shown to mediate tight interactions between both transmembrane domains12,13. Mutations of these key residues perturbed intramolecular tetraspanin interactions causing protein destabilization12.

Importantly, we identified single hydrophobic amino acids located within transmembrane segment S2 to be crucial for the interaction between TSPAN-13 and CaV2.2. Because of the hydrophobic nature of domain IV of CaV2.2 and S2 of TSPAN-13, we assume that the interaction between both proteins is hydrophobic and takes place inside the membrane environment.

While membrane localization of full-length TSPAN-13 was in agreement with previous studies, where a TSPAN-13-EGFP fusion protein also localized to the plasma membrane in breast cancer cells23, TSPAN-13Nt was rather found in intracellular compartments, presumably the endoplasmic reticulum. Therefore, we assume that the N-terminus of TSPAN-13 is required for its correct intracellular targeting and that the lack of CaV2.2 modulation is caused by the absence of TSPAN-13Nt from the plasma membrane.

The specific interaction between TSPAN-13 and CaV2.2, which we observed in the yeast two hybrid system and by co-immunoprecipitation has also specific functional consequences. Our electrophysiological analysis revealed that TSPAN-13 suppressed the amplitude of current through CaV2.2 channels in NG108-15 cells. It modulated the voltage-dependent activation and inactivation kinetics of CaV2.2 expressed in the CHO cells in a complex way. Reduction of the Gmax/Qmax ratio by an unaltered Qmax suggests a lowered opening probability which is consistent with the observed decrease in the current amplitude. An altered voltage-dependence of τact(V) is consistent with modulation of channel gating24,25.

TSPAN-13 facilitated the voltage-dependent channel gating when Ba2+ was used as a charge carrier. Ca2+-dependent inactivation of the CaV2.2 channel remains controversial. While it is generally accepted that calmodulin interacts with CaV2.2 channels26,27, its effects on current kinetics were reported by some authors28,29, but questioned by others30. In our settings Ba2+ and Ca2+ currents inactivated with similar kinetics. TSPAN-13 did not alter the inactivation kinetics of the Ca2+ current suggesting that it probably does not interfere with the IQ motif.

The Ca2+ current activated faster than the Ba2+ current and was modulated differently by TSPAN-13. The lowered slope factor of the voltage-dependence of τact(V) is consistent with a lowered sensitivity of the channels voltage sensor24,25.

Activation kinetics of CaV2.2 channels is modulated by G-proteins which constitutively inactivate the channel and this inactivation is relieved by a strong depolarisation called prepulse facilitation30,31. However, prepulse facilitation was not altered by TSPAN-13 (Fig. S5 online), therefore we can exclude an interaction between TSPAN-13 and G-proteins.

We assume that the physiologic effect of TSPAN-13 on CaV2.2 channels may be much more pronounced as indicated by our characterization based on a recombinant system. In fact in NG108-15 cells we observe a significant suppression of the current through CaV2.2 channels, this suppression is not significant in CHO cells expressing CaV2.2 and TSPAN-13. In our view these results indicate that the pure recombinant system lacks further important interaction partners which are present in NG108-15 cells. Therefore our recombinant electrophysiological experiments may only partially describe the actual effect of TSPAN-13 on CaV2.2 channels.

Another member of the tetraspanins, CD81 was not able to modulate CaV2.2 channels. We assume that the modulation by TSPAN-13 represents a novel mechanism for regulation of CaV2.2 Ca2+ channel activity, thereby affecting presynaptic transmitter release. The presence of TSPAN-13 in defined synaptic membrane compartments could exclusively influence CaV2.2 mediated transmitter release.

Methods
Split-ubiquitin yeast two-hybrid screening. The Split-ubiquitin system (DUALmembrane kit 3) together with a mouse brain cDNA library (pNubGx) were purchased from Dualsystems Biotech (Zurich, Switzerland). Construction of bait vectors and screenings were performed according to the manufacturer’s instructions. In particular, domains IV and the C-termini of CaV2.2 and CaV2.1 were cloned into the pBT3-N vector. Since it turned out that CaV2.1 Ca2+ channel constructs did not express in yeast, we used yeast codon-optimized cDNA-fragments of CaV2.1. Each screening experiment was performed with about ~1·5 × 107 transformants. Library plasmids were isolated from HIS3- and ADE2-positive yeast cells and identified by nucleotide sequencing. To confirm protein-protein-interaction we performed additional assays using PCR-generated full length cDNAs (pPRSN-vector) of the corresponding proteins. To analyse this interaction of the individual TSPAN-13 constructs in more detail, we performed a β-galactosidase filter-lift assay according to the manufacturer’s instructions.

Pellet-β-galactosidase assay. The pellet-β-galactosidase assay allows for a quantitative estimation of the interaction strength between two proteins expressed in the same two hybrid system. This assay was developed by Mo¨ckli and Auerbach and is described in . Briefly, yeast colonies were grown to an OD600 of 0.8 to 1.0 and then harvested by centrifugation. Yeast cells were broken by repeated freeze-thaw cycles and resuspended in water. The pellet-β-galactosidase assay was performed in 96-well plates by an overlay of the cells with X-Gal-staining solution. Development of blue color was observed using a flat-bed scanner and quantified by the program Multi Gauge V3.0 (Fujifilm, Düsseldorf, Germany).

RT-PCR analysis. Whole brain and hippocampus, neocortex and cerebellum were prepared from cercal dissociated adult male mice. Total RNA was isolated using the RNeasy Midi and Mini kit (Qiagen, Hilden, Germany) according to the manufacturer’s protocol. cDNA synthesis was performed by the Transcriptor High Fidelity cDNA synthesis kit (Roche Diagnostics, Mannheim, Germany). Oligonucleotide primers for PCR of TSPAN-13 cDNA were selected from different exons to exclude amplification of genomic DNA. The following primers were used: 5’-CCACCGCCACGATGGTGTGCG-3’ and 5’-CAAGCCGATCCACCTCGTGC-3’. PCR products were verified by sequence analysis.

Plasmids and co-immunoprecipitation (coIP). For cell-imaging and patch-clamp experiments full-length mouse TSPAN-13 and mutants were PCR-generated and cloned into pEGFP-N1 (Clontech, Saint-Germain-en-Laye, France). CoIPs were performed using N-terminal Myc-tagged-TSPAN-13 pcDNA3-vectors (Invitrogen Darmstadt, Germany). CHO cells and CHO cells stably expressing CaV2.2 were co-transfected with vectors encoding Myc-TSPAN-13. For each coIP-experiment five 10 cm dishes were rinsed with ice-cold PBS pH 7.4 (containing proteinase-inhibitor complete 1: 50 Roche (Roche, Mannheim, Germany)), washed and homogenized. After centrifugation (5 min at 800 × g at 4°C) supernatants were separated and membrane-enriched protein fractions were isolated by an additional centrifugation-step (16000 × g for 2 h at 4°C). Pelleted membranes were resuspended in 1×422-buffer (Logoharp, Freiburg, Germany) and final protein concentration was adjusted to 1.25 µg/µl. Solubilisation was carried out at 4°C for 16 h under continuous movement, afterwards insoluble material was removed by centrifugation (110 min at 16000 × g at 4°C). Aliquots of solubilized protein (supernatants) and pellets were resolved in SDS-loading buffer and checked for solubilisation efficiency.

Equal amounts of solubilized proteins (~400–600 µl, c = 1.25 µg/µl) were added to 50 µl DynaBeads® Protein G (Invitrogen, Darmstadt, Germany) and 10 µg anti-Myc antibody (Cell-signalling, Frankfurt, Germany) or 10 µg mouse-IgG control (Merck, Darmstadt, Germany). After immunoprecipitation (2.5 h at 4°C), beads were washed and proteins were eluted with SDS-buffer (10 min at 45°C) following manufacturer’s instructions.

Western blot analysis. Solubilized proteins and eluates obtained from coIP were separated on 12% SDS-polyacrylamide gels, resolved for 1% and 2% (w/v) agarose gels with 10x Tris-buffered-gel running buffer. After electrophoresis, proteins were transferred to nitrocellulose membranes (Carl-Roth, Karlsruhe, Germany) and blotted with the primary Ab (anti-Myc, Cell-signalling, Frankfurt, Germany) or anti-GFP antibody (Abcam Cambridge, United Kingdom). After washing, the secondary antibodies were applied and proteins were detected using an imaging system (LAS-3000 mini, Fujifilm-Europe, www.nature.com/scientificreports
maximal conductance (except for measurement of T-type calcium currents where it was reversal potential estimated individually for each cell. At such conditions gating

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fetal calf serum (FCS), 100 U/ml penicillin–streptomycin and 1 mM sodium

Cell lines and cultivation

Streptavidin-HRP (Cell Signaling, Frankfurt, Germany) was used to detect

antibodies were obtained from mouse IgG-POX from Biomol (Hamburg, Germany).

(TEA-Cl), 125; and pH 7.4 (TEA-OH). For measurement of Cav2.2 currents in CHO

CaCl2, 10; HEPES, 10; glucose, 10; TEA-Cl, 130; MgCl2, 1; CsCl, 5 and pH 7.0 (TEA-

EGTA, 10; MgCl2,3 ; Na2–ATP, 3; GTP-Tris, 0.6; HEPES, 10; and pH 7.2 (CsOH).

Patch pipettes were made from borosilicate glass (Sutter Instrument, Novato,

and seeded onto 13 mm round glass coverslips (VWR International, Darmstadt,

differentiation. Biochem. Biophys. Res. Commun. 423, 55–59 (2012).

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R.M. and T.W. acquired, analysed and interpreted data; wrote the paper. L.Li and A.B. acquired, analysed and interpreted data. B.L. provided critical experimental expertise and technology. J.C. provided critical reagent and helped acquire data. L.La provided critical experimental expertise and technology; designed electrophysiological experiments and interpreted data; wrote the paper. N.K. conceived and designed experiments and interpreted data; wrote the paper.

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