Modulation of Kv4.2/KChIP3 interaction by the ceroid lipofuscinosis neuronal 3 protein CLN3

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Edited by Mike Shipston

Voltage-gated potassium (Kv) channels of the Kv4 subfamily associate with Kv channel–interacting proteins (KChIPs), which leads to enhanced surface expression and shapes the inactivation gating of these channels. KChIP3 has been reported to also interact with the late endosomal/lysosomal membrane glycoprotein CLN3 (ceroid lipofuscinosis neuronal 3), which is modified because of gene mutation in juvenile neuronal ceroid lipofuscinosis (JNCL). The present study was undertaken to find out whether and how CLN3, by its interaction with KChIP3, may indirectly modulate Kv4.2 channel expression and function. To this end, we expressed KChIP3 and CLN3, either individually or simultaneously, together with Kv4.2 in HEK 293 cells. We performed co-immunoprecipitation experiments and found a lower amount of KChIP3 bound to Kv4.2 in the presence of CLN3. In whole-cell patch-clamp experiments, we examined the effects of CLN3 co-expression on the KChIP3-mediated modulation of Kv4.2 channels. Simultaneous co-expression of CLN3 and KChIP3 with Kv4.2 resulted in a suppression of the typical KChIP3-mediated modulation; i.e. we observed less increase in current density, less slowing of macroscopic current decay, less acceleration of recovery from inactivation, and a less positively shifted voltage dependence of steady-state inactivation. The suppression of the KChIP3-mediated modulation of Kv4.2 channels was weaker for the JNCL-related missense mutant CLN3R334C and for a JNCL-related C-terminal deletion mutant (CLN3ΔC). Our data support the notion that CLN3 is involved in Kv4.2/KChIP3 somatodendritic A-type channel formation, trafficking, and function, a feature that may be lost in JNCL.

Voltage-gated potassium (Kv) channels are critically involved in the control of neuronal excitability and action potential waveform (1). Members of the Kv4 subfamily, especially Kv4.2, carry a subthreshold-activating somatodendritic A-type current (I_{SA}) (2), which mediates synaptic filtering and controls the spread of dendritic excitation (3, 4). Notably, Kv4.2 channel-mediated I_{SA} is down-regulated in animal models of cortical malformations and epilepsy (5–11). Kv4 channels may form ternary complexes with auxiliary Kv channel–interacting proteins (KChIPs) (12) and dipeptidylaminopeptidase-related proteins (DPPs) (13). In heterologous expression systems, both auxiliary subunits cause an increase in Kv4 channel surface expression (12–16). Moreover, KChIPs and DPPs modulate Kv4 channel gating in a specific manner: KChIPs cause a slowing of macroscopic current decay and a positive shift in the voltage dependence of steady-state inactivation (12, 14, 15, 17), whereas DPPs cause an acceleration of macroscopic current decay and a negative shift of the voltage dependence of both activation and steady-state inactivation (13, 16, 18). Both auxiliary subunits cause an acceleration of recovery from inactivation (12–18). Among the four different known KChIP subtypes (19), KChIP3 seems to be special, because it is known to interact not only with Kv4 channels but also with DNA to act as a transcription repressor (KChIP3 is known to interact with DNA to aid with transcription repression). We also found that KChIP3-mediated modulation of Kv4.2 channels was weaker for the JNCL-related missense mutant CLN3R334C and for a JNCL-related C-terminal deletion mutant (CLN3ΔC). Our data support the notion that CLN3 is involved in Kv4.2/KChIP3 somatodendritic A-type channel formation, trafficking, and function, a feature that may be lost in JNCL.

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Results

**CLN3 impairs Kv4.2/KChIP3 complex formation**

It has been reported previously that CLN3 can bind to KChIP3 (23). Therefore, we asked whether co-expression of CLN3 may influence the binding of KChIP3 to the Kv4.2 channel protein. For this purpose, we expressed the epitope-tagged versions of Kv4.2, KChIP3, and CLN3, as well as untagged CLN3 in human embryonic kidney (HEK) 293 cells (see...
CLN3 effects on Kv4.2/KChIP3 channels

Figure 1. CLN3 and Ca^{2+} can influence Kv4.2/KChIP3 binding. For co-IP experiments HEK 293 cells were transiently co-transfected with Kv4.2–GFP and KChIP3–Myc in the absence or presence of CLN3, GFP–CLN3, or HA–CLN3 (see "Experimental procedures"). A, co-expression of Kv4.2–GFP and KChIP3–Myc in the absence (lanes 2) or presence (lanes 4) of CLN3. GFP co-transfected with KChIP3–Myc (lane 1) and GFP–CLN3 co-transfected with KChIP3–Myc (lane 3) were used as negative and positive input controls, respectively. 10% of the input fractions (lanes 1–4) were separated by SDS-PAGE and analyzed by Myc immunoblotting (IB; see "Experimental procedures"). Note the prominent signal in the absence (lane 2) and the weaker signal in the presence of CLN3 (lane 4). Surprisingly, KChIP3–Myc could not be precipitated from extracts of cells co-expressing GFP–CLN3 and KChIP3–Myc in the absence of Kv4.2–GFP. B, HEK 293 cells were co-transfected with Kv4.2–GFP, KChIP3–Myc, and either empty pcDNA3.1 vector or HA–CLN3. The lysis, co-precipitation, and washing steps were performed in nominal Ca^{2+}-free buffers (−) or in the presence of different Ca^{2+} concentrations (15 nM and 50 μM; see "Experimental procedures"). Prior to co-IP analyses, aliquots of the input fractions were probed with antibodies against GFP and HA. Equal loading was confirmed by glyceraldehyde-3-phosphate dehydrogenase (GAPDH) immunoblotting. After immunoprecipitation of Kv4.2–GFP, bound KChIP3–Myc was detected by Myc immunoblotting. Co-IP of Kv4.2–GFP and KChIP3–Myc was tested in the absence (lanes 1–3) and presence of HA–CLN3 (lanes 4–6) and with different Ca^{2+} concentrations in the lysis and washing buffers. Lanes 1 and 4, nominal Ca^{2+}-free (−); lanes 2 and 5, 15 nM Ca^{2+}; lanes 3 and 6, 50 μM Ca^{2+}. Note the stronger signals in higher Ca^{2+} both in the absence and in the presence of HA–CLN3. The positions of molecular weight markers are indicated. The results shown in A and B were confirmed in three independent experiments.

CLN3 suppresses KChIP3-mediated modulation of Kv4.2 channels

Given the impairment of Kv4.2/KChIP3 binding by CLN3, we tested whether CLN3 may interfere with the typical KChIP3-mediated modulation of Kv4.2 channels. For this purpose we performed whole-cell patch clamp experiments on transfected HEK 293 cells (see "Experimental procedures"). Kv4.2-mediated currents were recorded on the first (d1) and on the second day (d2) after transfection (Fig. 2A). There was an increase in the current density mediated by homomeric Kv4.2 channels from 125 pA/pF on d1 (n = 7) to 216 pA/pF on d2 (n = 12), but co-expression of CLN3 had no effect on current density on either day (n = 9 and 12, respectively; Fig. 2B). KChIP3 co-expression, on the other hand, caused a strong increase in current density on both d1 and d2 (n = 15 and 9, respectively). If Kv4.2 was simultaneously co-expressed with KChIP3 and CLN3, the KChIP3-mediated increase in current density was strongly suppressed on d1 (n = 17) and virtually absent on d2 (n = 24; Fig. 2B; see also Table S1).

We asked whether the presence of CLN3 may also suppress the KChIP3-mediated modulation of the kinetics and voltage dependence of Kv4.2 channel inactivation and measured the “Experimental procedures”). Extracts from cells co-expressing Kv4.2–GFP and KChIP3–Myc in the absence or presence of GFP–CLN3 (or untagged CLN3) were prepared, and Kv4.2–GFP was precipitated using GFP–TRAP beads (Fig. 1A; see "Experimental procedures"). Analyses of bound proteins showed that Kv4.2–GFP, but not GFP alone, precipitated KChIP3–Myc (Fig. 1A, lanes 1 and 2), confirming the direct interaction between Kv4.2 and KChIP3. Unexpectedly, we obtained no co-immunoprecipitation (co-IP) signal for GFP–CLN3 + KChIP3–Myc (Fig. 1A, lane 3; see "Discussion"); however, in extracts from cells expressing Kv4.2–GFP + KChIP3–Myc in the presence of CLN3, the amount of precipitated KChIP3–Myc was strongly reduced compared with extracts from cells expressing Kv4.2–GFP + KChIP3–Myc in the absence of CLN3 (Fig. 1A, lanes 2 and 4; see also Fig. S2). These results, which were confirmed in three independent experiments, support the notion that the molecular interaction between Kv4.2 and KChIP3 is impaired in the presence of CLN3. Because the KChIP3/CLN3 interaction has been reported to be weaker in high Ca^{2+} (23), we performed co-IP experiments with either 0 nM (nominal Ca^{2+}-free), 15 nM or 50 μM Ca^{2+} in the lysis, co-IP, and wash buffer (Fig. 1B; see "Experimental procedures"). Co-IP analyses of extracts from cells expressing Kv4.2–GFP + KChIP3–Myc showed higher amounts of precipitated KChIP3–Myc with increasing Ca^{2+} concentrations, both in the absence (Fig. 1B, lanes 1–3) and in the presence of hemagglutinin (HA)–CLN3 (Fig. 1B, lanes 4–6). For all tested Ca^{2+} concentrations, the amount of precipitated KChIP3–Myc was lower in the presence of HA–CLN3 compared with extracts expressing Kv4.2–GFP + KChIP3–Myc in the absence of HA–CLN3 (Fig. 1B; see also Fig. S2). The results of three such experiments confirmed the impairment of Kv4.2/KChIP3 binding by CLN3, even in high Ca^{2+}. Notably, our data also suggest that Kv4.2/KChIP3 binding is per se Ca^{2+}-dependent.

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relevant parameters on d2. Kv4.2-mediated currents showed a multiphasic decay, which was best described by the sum of three exponential functions (Fig. 3, A and B). The mean time constants obtained for homomeric Kv4.2 channels were 13, 58, and 1480 ms with relative amplitudes of 74, 22, and 4%, respectively (n = 12). Surprisingly, CLN3 co-expression caused the Kv4.2-mediated currents to decay faster (n = 12; see “Discussion”), whereas KChIP3 co-expression caused the well-known slowing of initial current decay (n = 9) (12, 14, 17). If Kv4.2 was simultaneously co-expressed with both KChIP3 and CLN3, the KChIP3-mediated slowing of initial current decay was attenuated (n = 22; Fig. 3, A and B; see also Table S1). Next we studied the recovery from inactivation. The recovery kinetics were described by a single-exponential function with a mean time constant of 400 ms for homomeric Kv4.2 channels (n = 11). The recovery kinetics were not influenced by CLN3 co-expression (n = 8), but KChIP3 co-expression caused the well-known acceleration of recovery kinetics (n = 6; Fig. 3, C and D) (12, 14, 17). If Kv4.2 was simultaneously co-expressed with KChIP3 and CLN3, the recovery kinetics were in some cases biphasic, resulting in fast and a slow recovery components. This may be explained by different channel populations in the plasma membrane: one with fast kinetics (modified by KChIP3) and one with slow kinetics (not modified by KChIP3). A weighted recovery time constant, which was calculated for all Kv4.2 + KChIP3 + CLN3–expressing cells studied (n = 15), lay closer to the time constant of the slow component (Fig. 3D; see also Table S2). This indicates that CLN3 attenuated the KChIP3-mediated acceleration of recovery kinetics. Finally, we studied the voltage dependence of steady-state inactivation (Fig. 4). Inactivation was half-maximal at −64 mV with a slope factor of 7 mV for homomeric Kv4.2 channels (n = 9). The voltage dependence of steady-state inactivation was not influenced by CLN3 co-expression (n = 5) but strongly shifted in the positive direction and steepened by KChIP3 co-expression (n = 6). If Kv4.2 was simultaneously co-expressed with both KChIP3 and CLN3, the positive shift and the steepening of the voltage dependence were attenuated (n = 13; Fig. 4; see also Table S2).

Taken together, these data support the notion that CLN3 interferes with functional Kv4.2/KChIP3 interaction. As a consequence the pronounced KChIP3-mediated augmentation of Kv4.2 current density was completely abolished in the presence of CLN3 (Fig. 2B; d2). The typical KChIP3-mediated modulation of Kv4.2 gating parameters, on the other hand, was left intact to some degree in the presence of CLN3 (see Figs. 3 and 4 and “Discussion”). Therefore, we examined the dose dependence of the CLN3 effects in a stable Kv4.2-expressing cell line (62), which was transiently transfected with different amounts of KChIP3 and CLN3 cDNA (see “Experimental procedures” and Fig. S3). In a steady background of Kv4.2 expression (mean current density of 167 pA/pF, n = 6, when transfected only with empty pcDNA3.1 vector) either KChIP3 alone or KChIP3 together with CLN3 were expressed. The cDNA amounts for the transient transfection of the stable cell line were chosen to yield a KChIP3:CLN3 cDNA mass ratio of either 1:10 or 1:20 (see “Experimental procedures”). The results of these experiments (3 ≤ n ≤ 7) confirmed our findings obtained with transient transfection of Kv4.2 and indicated a dose dependence of the CLN3 effect on the functional Kv4.2/KChIP3 interaction. However, in the stable Kv4.2-expressing cell line, none of the CLN3 effects on the KChIP3-mediated channel modulation observed with a 1:10 ratio proved to be significant, and a 1:20 ratio significance was only reached for current density, for amp1 and amp2 of the macroscopic current decay kinetics, and for the voltage dependence of steady-state inactivation (Fig. S2 and Tables S1 and S2).

The CLN3 effect on functional Kv4.2/KChIP3 interaction is Ca2+-dependent

Based on our finding that the Kv4.2/KChIP3 complex is stabilized by Ca2+ both in the absence and in the presence of CLN3 (Fig. 1B), we conducted whole-cell patch-clamp experiments with different amounts of free Ca2+ (nominal Ca2+-free,
CLN3 effects on Kv4.2/KChIP3 channels

![Image of Figure 3](image-url)

**Figure 3. Effect of KChIP3 and CLN3 on Kv4.2 inactivation kinetics.** A, currents mediated by Kv4.2 and Kv4.2/KChIP3 in the absence (black traces) or presence of CLN3 (gray traces). Current traces were leak-subtracted and normalized to peak. Insets, initial current decay kinetics shown on an expanded time scale. Note that CLN3 accelerated the decay kinetics of Kv4.2-mediated currents in the absence of KChIP3 (upper traces). The cross-over of normalized current traces, typically caused by KChIP co-expression, is still seen in the presence of CLN3, but the KChIP3-mediated slowing of the initial current decay is strongly attenuated by CLN3 (lower traces; the Kv4.2 trace in the absence of KChIP3 and CLN3 is shown as a reference). B, time constants of current decay ($\tau_1$, $\tau_2$, and $\tau_3$) and their relative amplitudes (amp1, amp2, and amp3, in %) obtained by triple-exponential fitting of the macroscopic current decays. C, the kinetics of recovery from inactivation were obtained by plotting relative current amplitudes ($I_{hod}/I_{norm}$, no leak subtraction) against the interpulse duration and fitting the data with a single-exponential or, if necessary, with a double-exponential function. D, recovery time constants obtained from single-exponential fitting (circles) and weighted time constant based on double-exponential fitting (diamond). The data in B and D are presented as means ± S.D., and the number of observations ($n$) is indicated for each group; the data in C are presented as means ± S.E. Statistical analyses were done with one-way ANOVA and Dunnett’s post hoc testing. Asterisks indicate values significantly different in the presence of CLN3 compared with Kv4.2 alone or Kv4.2 + KChIP3. *p < 0.05; **p < 0.0001 (see also Tables S1 and S2).

15 nM Ca$^{2+}$, and 50 μM Ca$^{2+}$) in the pipette solution to manipulate in a controlled manner the cytoplasmic Ca$^{2+}$ concentration (see “Experimental procedures”). The relevant electrophysiological parameters were measured on d2 for Kv4.2/KChIP3 channel complexes in the absence and in the presence of CLN3, and the data obtained with 15 nM Ca$^{2+}$ ($n$ ≤ 9 and $n$ ≤ 24, respectively) in the patch-pipette served as control (Fig. 5). We first tested the Ca$^{2+}$ dependence of Kv4.2/KChIP3 channels in the absence of CLN3 and found no significant differences for the well-known KChIP3-mediated modulation if Ca$^{2+}$ was increased to 50 μM ($n$ ≤ 14). However, the KChIP3-mediated modulation in the absence of CLN3 was less pronounced in recordings with a nominal Ca$^{2+}$-free solution in the patch-pipette ($n$ ≤ 20), especially for the recovery kinetics and the voltage dependence of steady-state inactivation (Fig. 5, D and E). Intriguingly, for Kv4.2 + KChIP3 + CLN3, we observed a weaker CLN3 effect on the KChIP3-mediated channel modulation in 50 μM Ca$^{2+}$ ($n$ ≤ 14; i.e. larger current density, slower current decay, faster recovery kinetics, and more positive voltage dependence of steady-state inactivation as compared with 15 nM Ca$^{2+}$; Fig. 5, B–E). For the inactivation kinetics (i.e. macroscopic current decay and recovery from inactivation; Fig. 5, C and D), but not for other parameters, the CLN3 effect on the KChIP3-mediated modulation was also weaker under nominal Ca$^{2+}$-free conditions (n ≤ 9) as compared with 15 nM Ca$^{2+}$. The results of these experiments are summarized in Tables S3a and S3b. Taken together, the data suggest a Ca$^{2+}$ dependence for both the KChIP3-mediated channel modulation per se and the CLN3-mediated attenuation of KChIP3-mediated channel modulation but apparently within different concentration ranges (see “Discussion”).

JNCL-related CLN3 mutants exert weaker effects on functional Kv4.2/KChIP3 interaction

We tested two JNCL-related CLN3 mutants (the missense mutant CLN3R334C and the C-terminal deletion mutant CLN3ΔC; see Fig. S1 and “Experimental procedures”) with our standard pipette solution (15 nM Ca$^{2+}$; Fig. 6). The relevant electrophysiological parameters were measured on d2 for Kv4.2 + KChIP3 + CLN3 (WT, $n$ ≤ 24), Kv4.2 + KChIP3 + CLN3R334C ($n$ ≤ 16), and Kv4.2 + KChIP3 + CLN3ΔC ($n$ ≤ 17). We asked, first, whether the electrophysiological parameters obtained with the CLN3 mutants differ from the ones obtained with WT CLN3, and second, whether the functional Kv4.2/KChIP3 interaction is still significantly affected by the CLN3 mutants (i.e. whether the electrophysiological parameters significantly differ from the ones obtained with maximal KChIP3-mediated modulation in the absence of CLN3). There was a trend toward weaker effects on Kv4.2/KChIP3 current densities, which proved to be significant for CLN3ΔC; however, similar to WT CLN3, both mutants still significantly affected the KChIP3-mediated modulation of current densities (Fig. 6B). The effects on Kv4.2/KChIP3 inactivation kinetics (macroscopic onset and recovery) were significantly weaker than the CLN3 WT effects for both CLN3R334C and CLN3ΔC, and the data showed no significant differences from the ones obtained with maximal KChIP3-mediated modulation (Fig. 6B). The effects on Kv4.2/KChIP3 functional interaction were less pronounced for the tested CLN3 mutants than for WT CLN3. For the current density data, a trend is seen suggesting that functional surface expression of Kv4.2/KChIP3 channels is still strongly...

suppressed by CLN3<sub>B334C</sub> similar to WT CLN3, whereas the suppression is moderate, albeit still significant, for CLN3ΔC.

Discussion

We tested whether CLN3, via its putative interaction with KChIP3, may influence Kv4.2/KChIP3 interaction and, thus, the expression level and inactivation gating of A-type potassium channels. The combined results of our co-IP and whole-cell patch-clamp experiments support this hypothesis and suggest that JNCL-related mutant CLN3 proteins have less influence on Kv4.2/KChIP3 interaction.

KChIP3 is a multifunctional neuronal calcium sensor involved in apoptosis

KChIPs belong to the neuronal calcium sensor (NCS) superfamily of Ca<sup>2+</sup>-binding EF-hand proteins, and it was postulated that these auxiliary Kv4 channel β-subunits may regulate A-type currents and, thus, neuronal excitability in response to changes in cytoplasmic Ca<sup>2+</sup> (12, 22). The data directly supporting this idea exist for cerebellar neurons, in which Kv4.2/KChIP3 complexes make a major contribution to I<sub>SA</sub> (27). Ca<sup>2+</sup> entering these cells through low voltage-activated Ca<sup>2+</sup> channels is thought to bind to KChIP3, thereby promoting its modulatory functions including a positive shift in the voltage dependence of Kv4.2 channel steady-state inactivation. This guarantees a high I<sub>SA</sub> availability at physiological membrane potentials (27). Our patch-clamp data obtained with Kv4.2/KChIP3 in the absence of CLN3 with a nominal Ca<sup>2+</sup>-free and a 15 mM Ca<sup>2+</sup> pipette solution, respectively, reflect this form of Ca<sup>2+</sup>-dependent modulation of A-type potassium channels (Fig. 5, D and E). The observed Ca<sup>2+</sup> effects on the KChIP3-mediated modulation of Kv4.2 channel gating may involve a stabilization of the Kv4.2/KChIP3 complex, as suggested by our co-IP data obtained in different Ca<sup>2+</sup> concentrations (Figs. 2B and 6).

Before the identification of KChIP3 as Kv4 channel β-subunits, proteins identical to KChIP3 had been identified in two different contexts as “DREAM” (a Ca<sup>2+</sup>-dependent transcription repressor) (20) and “calsenilin” (a Ca<sup>2+</sup>- and presenilin-binding protein) (21). KChIP3 (DREAM) binds to the downstream regulatory element of the prodynorphin gene (20), which relates KChIP3 to pain sensing. The other previously identified function relates KChIP3 (calsenilin) to intracellular Ca<sup>2+</sup> homeostasis and brain pathophysiology, because mutations in the KChIP3-binding partner presenilin are associated with familial Alzheimer’s disease (28). It is thought that, via its interaction with presenilin, KChIP3 controls other presenilin-binding partners (22). Because presenilin is tightly associated with endoplasmic reticulum (ER) Ca<sup>2+</sup> release channels, KChIP3 is involved in the control of intracellular Ca<sup>2+</sup> release (reviewed in Ref. 22). In fact, overexpression of KChIP3 modifies ER Ca<sup>2+</sup> release and may lead to apoptosis (29, 30). Presenilin is also part of the γ-secretase enzyme complex, which mediates the γ-cleavage of amyloid precursor protein to produce Aβ peptides (reviewed in Ref. 22). Because of its interaction with presenilin, KChIP3 functions as a Ca<sup>2+</sup> sensor for the γ-secretase, which mediates enhanced enzyme activity at elevated Ca<sup>2+</sup> levels (31). The presenilin/γ-secretase enzyme complex is integral to the proapoptotic activity of KChIP3 (32).

The intriguing finding that KChIP3 can also interact with CLN3 (23) may add another aspect to the involvement of KChIP3 in brain pathophysiology, because mutated CLN3 is found in JNCL (24). On the other hand, it may shed more light on the hitherto not well-defined function of CLN3 (25). KChIP3 may represent a Ca<sup>2+</sup> sensor for CLN3, similar to what is postulated for the other known KChIP3-binding partners. Chang et al. (23) found that the KChIP3/CLN3 interaction is disturbed in the presence of high Ca<sup>2+</sup> (50 μM), possibly reflecting a regulatory mechanism similar to the Ca<sup>2+</sup>-dependent dissociation of KChIP3 (DREAM) from DNA (20). Notably, KChIP3 and CLN3 seem to be opponents in the control of cell survival, because in contrast to KChIP3, CLN3 is anti-apoptotic. Overexpression of CLN3 suppresses Ca<sup>2+</sup>-induced neuronal cell death, whereas down-regulation of CLN3 has the opposite effect, and additional down-regulation of KChIP3 in the same cells again prevents cell death (23). It is possible that the proapoptotic activity of KChIP3 can be neutralized by an interaction with CLN3. Moreover, KChIP3 has been reported to be up-regulated in CLN3 knockdown cells and in the brains of CLN3 knockout mice but down-regulated by CLN3 overexpression (23). From these data it has been concluded that CLN3 can negatively regulate cellular levels of KChIP3 expression.
KChIP3-mediated modulation of Kv4.2 channel inactivation gating, or both. Surprisingly, we found that CLN3 significantly accelerated the decay kinetics of Kv4.2-mediated currents even if KChIP3 was not co-expressed. Thus, CLN3 may either be able to directly interact with Kv4.2 channels, or it may activate other KChIP3-unrelated regulatory mechanisms, which influence Kv4.2 channel macroscopic inactivation. Although numerous CLN3 interaction partners have been identified (25), it is not known whether other KChIP or Kv4 isoforms interact with CLN3. This was not studied further because neither any of the other gating parameters examined nor current densities were influenced by CLN3 in the absence of exogenous KChIP3. On the other hand, we were able to show that CLN3 co-expression suppressed all aspects of the KChIP3-mediated Kv4.2 channel modulation. Thus, our experimental results fully confirm the formulated working hypothesis; however, they provide no direct information on the mode of CLN3 action. There are different not mutually exclusive possibilities: similar to the findings of Chang et al. (23), CLN3 may negatively regulate KChIP3 expression levels in HEK 293 cells. Although not systematically investigated, our Western blot data do not support this notion but rather suggest higher KChIP3–Myc levels in the presence of GFP-tagged and untagged CLN3 as compared with the KChIP3–Myc + GFP control (Fig. 1A). Higher KChIP3–Myc levels were also seen with Kv4.2–GFP co-expression. We think that both CLN3 (or GFP–CLN3) and Kv4.2–GFP co-expression exerted a stabilizing effect on KChIP3–Myc in our experiments. Such a stabilizing effect was indirectly shown by the finding that KChIP expression levels, especially KChIP3, are actually down-regulated in Kv4.2 and Kv4.3 knockout mice (33, 34).

Our combined results support the notion that CLN3, instead of decreasing exogenous KChIP3 expression levels, directly influences Kv4.2/KChIP3 complexes or their formation. The absence of a co-IP signal for KChIP3–Myc + GFP–CLN3 was unexpected and apparently contradicts the findings of Chang et al. (23). However, these authors used a glutathione S-transferase–KChIP3 fusion protein and untagged CLN3 instead of KChIP3–Myc and GFP–CLN3, respectively, as in our study. Because our data demonstrated undisturbed complex formation between KChIP3–Myc and Kv4.2–GFP (Fig. 1A), we suspect that the GFP tagging of CLN3 interfered with KChIP3–Myc association. On the other hand, our co-IP data clearly show that both untagged and HA-tagged CLN3 interfere with Kv4.2–GFP/KChIP3–Myc complex formation (Fig. 1A and Fig. S2).

CLN3 may compete with Kv4.2 for KChIP3 binding already during co-expression and co-trafficking (35) and withdraw KChIP3 from complex formation with Kv4.2. Alternatively, CLN3 may interact with mature Kv4.2/KChIP3 channel complexes in the plasma membrane to exert its effects. CLN3 is mainly located in the late endosomal/lysosomal compartment but has also been reported to reside, among others, in the ER, trans-Golgi network and plasma membrane (reviewed in Refs. 25 and 35). Given the numerous subcellular locations reported for CLN3 (35, 36), effects on immature Kv4.2/KChIP3 channels caused by co-expression and co-trafficking and effects on

**Mode of CLN3 action on A-type potassium channels**

The KChIP3/CLN3 interaction put forward by Chang et al. (23) represented the starting point for the present study. We hypothesized that, similar to the suppression of the proapoptotic activity of KChIP3, CLN3 may also suppress the KChIP3-mediated modulation of Kv4.2 channel surface expression, the
mature Kv4.2/KChIP3 channels in the plasma membrane are equally possible.

Our data suggest that CLN3 may disturb Kv4.2/KChIP3 complex formation, thereby negatively influencing the trafficking of Kv4.2 channels to the cell surface. It should be noted in this context that current density, which is a good correlate of channel surface expression, showed the highest CLN3 sensitiv-

### Figure 6. Effects of WT and mutant CLN3 on Kv4.2/KChIP3 functional interaction.

Kv4.2/KChIP3 channels were co-expressed with WT and mutant CLN3 and functionally characterized. The missense mutant CLN3R334C and the C-terminal deletion mutant CLN3ΔC were tested. A, currents recorded for Kv4.2 + KChIP3 + CLN3 (WT, trace 1), Kv4.2 + KChIP3 + CLN3ΔC (trace 2), and Kv4.2 + KChIP3 + CLN3Δ3ΔC (trace 3). Inset on the right, initial decay kinetics are shown on an expanded time scale (currents normalized to peak and superimposed). The functional parameters obtained for the three groups are shown. B, current densities. C, time constants of macroscopic current decay and their relative amplitudes. D, weighted time constants of recovery from inactivation. E, voltages of half-maximal inactivation (V_{1/2 inact}) and corresponding slope factors (k). All data are presented as means ± S.D., the number of observations (n) is indicated for each group, and individual data points are shown in B (gray circles). Horizontal and vertical broken lines, mean values for Kv4.2 expressed alone (no KChIP3-mediated modulation); horizontal and vertical solid lines, mean values for Kv4.2/KChIP3 in the absence of CLN3 (maximal KChIP3-mediated modulation). Statistical analyses were done with one-way ANOVA and Dunnett’s post hoc testing. Asterisks indicate values significantly different from Kv4.2/KChIP3 co-expressed with WT CLN3. *p < 0.05; § symbols indicate a significant difference from the maximal KChIP3-mediated modulation, §, p < 0.05; §§, p < 0.0001 (see also Tables S1 and S2).
**CLN3 effects on Kv4.2/KChIP3 channels**

**A role for KChIP3 and somatodendritic A-type potassium channels in JNCL?**

Knockout mice with targeted disruption of the Cln3 gene (Cln3<sup>−/−</sup>) and knockin mice, which harbor the most common alteration of the human CLN3 gene (Cln3<sup>Δex7/8</sup>), have been created to be used as preclinical disease models for JNCL (39, 40). Both models exhibit the hallmarks of JNCL, including intracellular accumulation of autofluorescent storage material, astrocytosis, microglial activation, neuronal loss, and neurological deficits (39, 40). Given the typical neurological phenotype of JNCL, the previously shown interaction between the CLN3 protein and the Kv4 channel β-subunit KChIP3 (23) combined with the results of the present study leads to the question of whether KChIP3 and somatodendritic A-type potassium channels may play a role in JNCL.

A cellular hallmark of the neuronal ceroid lipofuscinoses (NCL) including JNCL is the accumulation of autofluorescent ceroid lipopigments with subunit c of mitochondrial ATP synthase or sphingolipid activator proteins A and D as major protein components (41). Autophagy, the process in which such intracellular macromolecules are normally digested during organelle turnover, requires the fusion of autophagic vacuoles with late endosomes and lysosomes. This process is disrupted in CLN3 deficiency leading to the accumulation of autophagic vacuoles accompanied by disturbed Ca<sup>2+</sup> homeostasis (42, 43). The latter may represent an important mechanistic link between CLN3 and KChIP3 in JNCL, because the reported vulnerability of CLN3-deficient cells to Ca<sup>2+</sup>-induced cytotoxicity following treatment with thapsigargin or with the Ca<sup>2+</sup>-ionophore A23187 is thought to be mediated by KChIP3 (23). CLN3 deficiency may lead to increased availability of KChIP3, which by its known interaction with presenilin may in an unphysiological manner influence ER Ca<sup>2+</sup> channels (22, 30). Apparently, despite its important functions as a neuronal calcium sensor, too-high levels of free KChIP3 may be detrimental to cell function. In accordance with this notion, the down-regulation of KChIP3 expression levels observed in Kv4.2 knockout mice has been previously interpreted as a feedback mechanism to ensure that free KChIPs do not accumulate (33). Thus, a so-far-unconsidered role of CLN3, which is reduced or lost in JNCL, may be to keep cellular levels of free KChIP3 below a critical proapoptotic level at physiological and moderately increased cytoplasmic Ca<sup>2+</sup> concentrations. A strong increase in cytoplasmic Ca<sup>2+</sup> may augment the proapoptotic activity of KChIP3, a mechanism expected to be exaggerated by CLN3<sup>−/−</sup> mice. Because JNCL patients exhibit deficits in motor coordination and cerebellar atrophy (50–52), cerebellar neurons, especially cerebellar granule cells, have moved into the focus of preclinical JNCL research. In both Cln3<sup>−/−</sup> and Cln3<sup>Δex7/8</sup> mice, cerebellar granule cells show a higher vulnerability to α-amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid (AMPA) receptor-mediated excitotoxicity (53, 54). Altered AMPA receptor trafficking and enhanced AMPA receptor function have been initially suggested to underlie the increased AMPA receptor-mediated excitotoxicity in Cln3<sup>−/−</sup> mice (54). However, a more recent detailed examination of the physiology of the mossy fiber–granule cell synapse performed by Studniarczyk et al. (55) found no differences in postsynaptic AMPA receptor expression or function but rather presynaptic alterations in Cln3<sup>−/−</sup> mice. In particular, the authors reported altered short-term plasticity under conditions of reduced extracellular Ca<sup>2+</sup>, which may be associated with disturbed Ca<sup>2+</sup> handling and sensing (55). Moreover, a reduced density of synaptic vesicles and decreased numbers of membrane adjacent synaptic vesicles in Cln3<sup>−/−</sup> mice were found in that study (55). In accordance with this, Grünewald et al. (56) found severely affected excitatory and inhibitory synaptic transmission, including the loss of GABAergic interneurons in the amygdala, hippocampus, and cerebellum of Cln3<sup>−/−</sup> mice. Cerebellar network activity depends critically on Kv4 channels and their fine-
CLN3 effects on Kv4.2/KChIP3 channels

24 h after the start of transfection, the growth medium was aspirated, and the cells were washed with ice-cold PBS, scraped in 1.5 ml of ice-cold PBS, and centrifuged for 5 min at 1,000 x g at 4 °C. The cell pellets were lysed in 100 μl of ice-cold lysis buffer (10 mM Tris-Cl, pH 7.5, 0.5% Nonidet P-40, 150 mM NaCl, 0.5 mM EDTA, protease inhibitors) and placed on ice for 30 min. Where indicated, lysis, dilution and wash buffers lacked EDTA were supplemented with Ca2+ at a final concentration of 15 nM and 50 μM, respectively. The cell lysates were centrifuged at 20,000 x g for 15 min at 4 °C, and the supernatants transferred to a new tube. Then 150 μl of ice-cold dilution buffer (10 mM Tris-Cl, pH 7.5, 150 mM NaCl, 0.5 mM EDTA, protease inhibitors) was added to the lysate, and aliquots (input) were removed for Western blotting analysis. Based on calculations with the program WEBMAXC extended (RRID: SCR_018807), the free Ca2+ concentration in the dilution...
buffer was adjusted to 0 nm (nominal Ca\(^{2+}\)-free), 15 nm, or 50 µM. The lysates were mixed with 25 µl of GFP–TRAP bead slurry (Chromotek) and incubated for 2 h at 4 °C on a rotating wheel. After centrifugation at 2,500 \( \times \) g, the supernatants were removed, and the beads were washed three times with 500 µl of ice-cold dilution buffer. GFP–TRAP beads were resuspended in 75 µl of 2× SDS sample buffer and boiled for 10 min at 95 °C. Aliquots of the input and the eluates were separated by SDS-PAGE and analyzed by Myc immunoblotting. Immunoreactive bands were visualized by enhanced chemiluminescence detection using a molecular imager (model ChemiDoc XRS system, Bio-Rad).

**Data analysis**

Quantification of immunoreactive band intensities was performed using the software QuantityOne 4.5.0 (Bio-Rad). The current traces were analyzed with PulseFit (Heka Electronics), and the obtained data were further processed with KaleidaGraph (Synergy Software). Macroscopic current decay kinetics were described by the sum of three exponential functions (64), the kinetics of recovery from inactivation by a single-exponential function or by a double-exponential function with a weighted time constant. The voltage dependence of steady-state inactivation was described by a Boltzmann function of the form \( I/I_{\text{max}} = 1/(1 + \exp (-V - V_{1/2 \text{ inac}})/\kappa) \), where \( V \) is the prepulse voltage, \( V_{1/2 \text{ inac}} \) is the prepulse voltage that causes half-maximal inactivation, and \( \kappa \) is the slope factor of the voltage dependence. Statistical analyses were done with Kaleidagraph and Prism (GraphPad Software). Comparison of band intensities for two groups and current densities for one group on d1 and d2 was done using unpaired Student’s \( t \) test. Comparison of electrophysiological parameters for more than two groups on d2 was done using one-way analysis of variance (ANOVA) with Dunnett’s post hoc testing.

**Data availability**

All data and statistical analyses are summarized in the supporting tables.

**Acknowledgments**—We thank Annett Hasse and Margrit Hölzel for cell line maintenance and Frank Stehr (NCL-Stiftung, Hamburg, Germany) and Thomas Braulke for discussion.

**Author contributions**—C. S. and S. S. data curation; C. S. and S. S. formal analysis; C. S. and S. S. investigation; C. S. and S. S. methodology; C. S., S. S., and R. B. writing-review and editing; R. B. conceptualization; R. B. funding acquisition; R. B. writing-original draft; R. B. project administration.

**Funding and additional information**—This work was supported by Grants BA 2055/4 and BA 2055/6 from the Deutsche Forschungsgemeinschaft (to R. B.).

**Conflict of interest**—The authors declare that they have no conflicts of interest with the contents of this article.

**Abbreviations**—The abbreviations used are: Kv channel, voltage-gated potassium channel; KChIP, Kv channel-interacting protein; DPP, dipeptidyl-aminoopeptidase-related protein; DREAM, downstream regulatory element antagonist modulator; (J)NCL, (juvenile) neuronal ceroid lipofuscinosis; HEK, human embryonic kidney; IP, immunoprecipitation; IB, immunoblotting; d1 and d2, first and second day after transfection; NCS, neuronal calcium sensor; AMPA, \( \alpha \)-amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid; GST, glutathione S-transferase; ER, endoplasmic reticulum; HA, hemagglutinin; ANOVA, analysis of variance; CLN3, ceroid lipofuscinosis neuronal 3.

**References**


