Fig. 7 Characterizations and validations of GCaMP-X_N targeting nuclear Ca^{2+}. **a** Design of GCaMP-X_N. Based on the design principle of GCaMP-X, similar to GCaMP-X_C, CBM was fused into N-terminus of GCaMP; and a nuclear localization signal (NLS) was tagged onto C-terminus of GCaMP. **b**, **c** Basic validations of GCaMP3-X_N with pCREB signals and neurite outgrowth. Representative images of pCREB immunostaining (**b**, left), statistical summary of pCREB intensities (**b**, right), tracing of neurite morphology (**c**, upper), Sholl analysis and statistical summary of neurite length (**c**, lower) were compared among neurons transfected with YFP, NLS-GCaMP3-NLS or GCaMP3-X_N. **d** Different Ca^{2+} dynamics resulted from neurons expressing NLS-GCaMP3-NLS or GCaMP3-X_N. Confocal images representing Ca^{2+} fluorescence at three phases: before, during and at the end of extracellular stimuli of 40 mM $[K^+]_o$ (upper). Ca^{2+} response ($\Delta F/F_0$) (lower left) and its rising speed ($\Delta F/F_0 \cdot s^{-1}$, normalized change of fluorescence per second, lower right) were averaged from multiple neurons (number indicated within parentheses), to compare NLS-GCaMP3-NLS with GCaMP3-X_N. **e**, **f** Simultaneous monitoring of cytosolic and nuclear Ca^{2+} dynamics. Representative confocal images indicative of Ca^{2+} fluorescence (upper in green) and time-dependent responses ($\Delta F/F_0$, lower) are to compare Ca^{2+} dynamics in the cytosol vs. the nucleus of the same neuron upon 40 mM $[K^+]_o$ stimuli. Cytosolic Ca^{2+} was monitored by GCaMP3-X_C for both cases, whereas nuclear Ca^{2+} was either by GCaMP3-X_N (**e**) or by NLS-GCaMP3-NLS (**f**). Neurons were loaded with Hoechst 33342 to label the nuclei of neurons (upper, blue). Standard error of the mean (S.E.M.) and Student's t-test (two-tailed unpaired with criteria of significance: $*p < 0.05$; $**p < 0.01$; and $***p < 0.001$) were calculated when applicable, and n.s. denotes "not significant"

design assure the exclusion of the above possible perturbations? According to this study, apoCaM interference is eliminated by CBM central to GCaMP-X design, indicative of an effective approach to avoid unwanted binding with apoCaM targets as well as their subsequent Ca^{2+}/CaM binding/signaling. Other apoCaM-associated signaling events, e.g., of enzymes and cytoskeletal proteins, should also be effectively protected by our strategy[9]. Native protein targets of Ca^{2+}/CaM binding should not be affected by either GCaMP or GCaMP-X, considering that the Ca^{2+}/CaM motif preferentially binds onto the M13 motif within the same sensor because of their intramolecular closeness[11] and high affinity ($K_d \approx 4$ nM for Ca^{2+}/CaM)[37]. Therefore, in combination of CBM and M13, spatial restrictions are applied onto the CaM motif under all Ca^{2+} conditions, thus effectively reducing its

overall mobility and concentration. Both apo and Ca^{2+} forms of CaM or GCaMP-X are protected, greatly enhancing its resistance against any potential intermolecular binding with endogenous signaling proteins.

Due to compartmentalized nature of cellular calcium, a variety of organelle-targeted GECIs are developed, e.g., to address the questions specifically for nuclear calcium[34]. Nuclear presence of GCaMP has been considered as a sign of cell damages[5,7], also confirmed in this study by nucleus-accumulated GCaMP or by NLS-GCaMP-NLS. However, the reasons for such damages remain unclear. Instead of any direct interference with Ca^{2+}/CaM-binding proteins, it is more likely that GCaMP struck in the nucleus impairs certain critical apoCaM-related nuclear signaling thus eventually causing neural damages. Meanwhile, the