Supplementary Figure 1. Spectroscopic characterization of CatCh. After light excitation, CatCh (black traces) enters a photocycle comparable to the WT (gray traces) in kinetics and the observable photointermediates. The figure depicts the spectral changes after 450 nm excitation with the characteristic wavelengths for the deprotonated Schiff-base, P390 (381 nm, top panel), for P520, dominant in the open state (541 nm, second
panel), and the ground state (440 nm, third panel). The first red-shifted intermediate, presumably P500, is not resolved and only detected as offset. The Schiff-base deprotonates in the microsecond time scale ($\tau=50 \mu$s), an event hardly observable due to the low amplitude at 381 nm, concomitant with a rise at 541 nm. The rise of the P520 intermediate occurs in the subsequent process ($t=1.5$ ms) before it decays ($t=9$ ms), thereby populating a second lasting species (P480). The ground state (D470) reverts in the following process ($t=10$ s). As for the opening and the closing kinetics in the current measurements, the L132C mutation causes no gross change in the functional states. The open state is determined mainly by the P520 intermediate, as can be assessed from the comparison with the currents after a 10 ns light flash [bottom panel, holding potential -60 mV (gray trace) and +60 mV (black trace)]. The main difference to the WT is the reduced P390 amplitude relative to the P520 amplitude. Thus, the L132C mutation does not affect the light reaction at the chromophore site. Note that the spectroscopic kinetics data of the photocycle was not altered in the presence of 50 mM Ca$^{++}$.

**Supplementary Figure 2.** Action spectrum of CatCh. Current amplitudes at different wavelengths ($\lambda$) were measured in by two-electrode-voltage-clamp in *Xenopus* oocytes in the absence of Ca$^{++}$ and normalized to the photon flux (n=6). Comparison of the ground state (–) and the action spectrum (■).
Supplementary Figure 3. Surface potential changes induced by Ca$^{++}$. It is known, that the voltage drop across the membrane depends on the applied potential difference ($\Psi'$) and is modified by the surface potential ($\Phi_0$). In general $\Phi_0$ depends on the negative surface charge density, which can be modified by screening with counterions. Therefore the activation of the voltage-gated sodium channels (and other voltage-sensitive channels) can be influenced by the change of the surface charge either on the external or internal side of the membrane. In our case, the Ca$^{++}$ conducted through CatCh neutralizes the negative surface charges on the inner membrane face of the neuron. By this a depolarizing effect on the membrane potential is induced, leading to the induction of action potentials at lower light intensities. A schematic drawing of this mechanism is depicted in a-c (after Hille, 2001). (a) In the dark, the CatCh channel is closed, the potential difference over the membrane, $E_M$ (applied external potential), is equivalent to the resting membrane potential (here set to $-60$ mV). For simplicity, $\Phi_0$ was set to $\Phi_0'$. (b) Upon light-activation of CatCh, the usual membrane depolarizing Na$^+$ influx occurs. However, the additional Ca$^{++}$ that enters the neuron increases the surface potential on the inner membrane face ($\Phi_0''$). The higher the Ca$^{++}$ influx, the more positive $\Phi_0''$ (indicated by double-headed arrow) and the smaller the voltage-drop across the membrane. This
facilitates activation of voltage-gated sodium channels. (c) By replacing extracellular Ca\(^{++}\) with Mg\(^{++}\), which does not permeate through CatCh and is already present to \(~4\) mM in the cytosol, only a minor depolarizing effect occurs. This is due to a weaker binding of Mg\(^{++}\) to the extracellular membrane side compared to Ca\(^{++}\), which slightly lowers the extracellular surface potential \(\Phi_0\). Note that the depolarizing effect of the surface potential increases with decreasing the slope of the voltage-drop across the membrane.

**Supplementary Note. Estimation of the Ca\(^{++}\)-influx into a neuron through CatCh during illumination.**

Estimations of the Ca\(^{++}\) current can be given based on either the Goldman-Hodgkin-Katz equation (A) or the ionic flux measurements shown in figure 2d of the main text (B).

(A)

\[
I(j) = \sum_j \left( \frac{z_j^2 \psi F^2}{RT} \right) \left( \frac{c_{j,i} - c_{j,a}}{1 - e^{-\frac{z_j F \psi}{RT}}} \right) - \frac{z_j F \psi}{RT}
\]

with \(\psi\) the applied potential and \(c_i\) or \(c_a\) the molar concentrations of the ion \(j\) with valence \(z_j\) in the intracellular (i) or extracellular (a) solution, respectively. Considering as intracellular and extracellular solutions the solutions used in the neuron patch-clamp experiments (intracellular: 129 mM potassium gluconate, 10 mM HEPES, 10 mM KCl, 4 mM MgATP and 0.3 mM Na\(_3\)GTP, titrated to pH 7.2; extracellular: 125 mM NaCl, 2 mM KCl, 2 mM CaCl\(_2\), 1 mM MgCl\(_2\), 30 mM glucose and 25 mM HEPES, titrated to pH 7.4), and the permeabilities of H\(^+\), K\(^+\) and Ca\(^{++}\) relative to sodium from the reversal potential shifts (see Fig. 2e main text; \(P_{H}= 4 \times 10^6\), \(P_{K}= 0.5\), \(P_{Ca}=0.24\)), the Ca\(^{++}\) current is given as \(0.7\%\) of the overall CatCh current.
The rate of $[\text{Ca}^{++}]_i$ change within a neuron, assuming the soma being a sphere with a diameter of 20 µm and the dendrites (dend) as cylinders of 1 µm diameter, is calculated by:

\[
I = -644 \text{pA} \quad \text{measured current}
\]
\[
C = 80 \text{pF} \quad \text{total capacitance}
\]
\[
C_m = 1 \cdot \mu\text{F} \cdot \text{cm}^{-2} \quad \text{specific capacitance}
\]
\[
i = \frac{I}{C} = -8.1 \text{pA} \cdot \text{pF}^{-1} \quad \text{total current density}
\]
\[
I_{Ca} = 0.007(-644 \text{pA}) = -4.5 \text{pA} \quad \text{calcium current from GHK-equation}
\]
\[
i_{Ca} = \frac{I_{Ca}}{C} = -0.056 \text{pA} \cdot \text{pF}^{-1} \quad \text{calcium current density}
\]

Volume (V) and surface (O) of neuron and the calcium flux into the compartments:

\[
V_{\text{soma}} = \frac{4}{3} \pi \cdot r^3 = \frac{4}{3} \pi \cdot 10^3 \mu\text{m}^3
\]
\[
O_{\text{soma}} = 4 \pi \cdot r^2 = 4 \pi \cdot 10^2 \mu\text{m}^2
\]
\[
V_{\text{dend}} = \pi \cdot h \cdot r^2 = \pi \cdot h \cdot 0.25 \mu\text{m}^2
\]
\[
O_{\text{dend}} = 2 \pi \cdot h \cdot r = 2 \pi \cdot h \cdot 0.5 \mu\text{m}
\]

Therefore, the rate of calcium concentration change can be formulated:

\[
\frac{\Delta c_{\text{Ca}}(\text{soma})}{\Delta t} = i_{\text{Ca}} C_m \frac{O_{\text{soma}}}{V_{\text{soma}}} \cdot F \cdot z = 0.056 \frac{\text{pA}}{\text{pF}} \cdot \frac{1}{\mu\text{F}} \cdot \frac{3}{10 \mu\text{m} \cdot 96500 \text{C} \cdot \text{mol}^{-1} \cdot 2} = 0.9 \frac{\mu\text{mol}}{\text{L} \cdot \text{s}}
\]
\[
\frac{\Delta c_{\text{Ca}}(\text{dend})}{\Delta t} = i_{\text{Ca}} C_m \frac{O_{\text{dend}}}{V_{\text{dend}}} \cdot F \cdot z = 0.056 \frac{\text{pA}}{\text{pF}} \cdot \frac{1}{\mu\text{F}} \cdot \frac{2}{0.5 \mu\text{m} \cdot 96500 \text{C} \cdot \text{mol}^{-1} \cdot 2} = 12 \frac{\mu\text{mol}}{\text{L} \cdot \text{s}}
\]

Note that the GHK-equation gives only a rough estimation for the fractional Ca$^{++}$ current at the resting potential of a neuron as it does not reflect binding or saturation.
(B) Therefore, we may compare the estimation with the experiments from the flux measurements in HEK293 cells (main text Fig. 2d). Going from 140 mM sodium to 90 mM calcium \((c_{\text{ca}}(\text{rev}))\) in the extracellular solution, we observed a decrease to approximately one third of the sodium driven current (37 %). To get an upper estimate of the Ca\(^{++}\) current in the neuron experiments \((c_{\text{ca}}(\text{meas})=2\text{ mM})\), we make additional simplifications and considerations: First, we neglect other ionic currents, especially protons, that contribute to the measured current of \(-644\text{ pA} \) and relate the fractional Ca\(^{++}\) current only to sodium. Likewise, we do not consider Ca\(^{++}\) buffering or clearance within the neuron. Second, we take Ca\(^{++}\) saturation into account as it has been measured for wild-type ChR2. Here, a \(K_m\)-value of 18 mM was determined\(^3\), while the sodium current does not saturate in the used concentration range.

\[
\begin{align*}
I &= -644 \cdot \text{pA} & \text{measured current} \\
C &= 80 \cdot \text{pF} & \text{total capacitance} \\
C_m &= 1 \cdot \mu\text{F} \cdot \text{cm}^{-2} & \text{specific capacitance} \\
i &= \frac{1}{C} = -8.1 \cdot \text{pA} \cdot \text{pF}^{-1} & \text{total current density}
\end{align*}
\]

\[
\begin{align*}
I_{\text{ca}} &= \frac{I_{\text{ca}(\text{max})}}{I_{\text{ca}(\text{max})}} \cdot \frac{c_{\text{ca}}(\text{meas})}{c_{\text{ca}}(\text{meas}) + K_m} = 0.37 \cdot (-644 \cdot \text{pA}) \cdot \frac{2\text{mM}}{(2\text{mM} + 18\text{mM})} = -29 \cdot \text{pA} \\
i_{\text{ca}} &= \frac{I_{\text{ca}}}{C} = -0.363 \cdot \text{pA} \cdot \text{pF}^{-1}
\end{align*}
\]
Therefore, the rate of calcium concentration change can be formulated:

$$\frac{\Delta c_{\text{Ca}(\text{soma})}}{\Delta t} = i_{\text{Ca}} \cdot C_m \cdot \frac{O_{\text{soma}}}{V_{\text{soma}} \cdot F \cdot z} = 0.363 \frac{\text{pA}}{\text{pF}} \cdot 1 \frac{\mu F}{\text{cm}^2} \cdot \frac{3}{10 \times 96500 \text{C} \cdot \text{mol}^{-1} \cdot 1.2} = 5.6 \frac{\mu \text{mol}}{\text{L} \cdot \text{s}}$$

$$\frac{\Delta c_{\text{Ca}(\text{dend})}}{\Delta t} = i_{\text{Ca}} \cdot C_m \cdot \frac{O_{\text{dend}}}{V_{\text{dend}} \cdot F \cdot z} = 0.363 \frac{\text{pA}}{\text{pF}} \cdot 1 \frac{\mu F}{\text{cm}^2} \cdot \frac{2}{0.5 \times 96500 \text{C} \cdot \text{mol}^{-1} \cdot 1.2} = 75 \frac{\mu \text{mol}}{\text{L} \cdot \text{s}}$$

As demonstrated, the Ca++ current density in neurons during CatCh activity lies between –0.056 to –0.363 pA pF⁻¹. We therefore argue that the Ca++-influx through CatCh lies well within the physiological range. We would further like to emphasize that the Ca++-influx through CatCh is still small compared to sodium and potassium and is only 6-fold increased compared to WT ChR2, which is also Ca++ permeable. For WT ChR2, long-term studies have been performed by numerous research groups in multiple neuronal systems and no long-term Ca++-effects were observed, neither on cell health nor on synaptic plasticity. CatCh might even be less damaging than WT ChR2 as it does not require borderline phototoxic light intensities for neuronal activation.

**Supplementary Discussion. Possible effects of CatCh activity on synaptic plasticity.**

Comparison of the Ca++ current density during CatCh activity (see above) with that during NMDA receptor activity, which results in LTP and can cause excitotoxicity, results in an ~1000-fold increased Ca++ current density at the synapse during NMDA receptor activity compared to that during CatCh activity. This is because the Ca++ current density during NMDA receptor activity is *per se* by a factor 10 increased (3.3 pA pF⁻¹)⁴ compared to CatCh. Additionally, the NMDA receptor density at the synapse is ~10⁴ NMDA receptors/µm² ⁵ and is thus about 100-fold higher than the CatCh expression density in our experiments of ~10² CatCh molecules/µm² (as determined from a single channel conductance of 63 fS and an open probability of 0.4). We therefore argue that any effects of the Ca++-permeability of CatCh 1. on synaptic plasticity 2. or possible excitotoxicity would require an at least ~1000-fold increased expression of CatCh.
More likely effects of CatCh on synaptic plasticity result from an elevated probability of “coincidence detection” (if a neuron is already depolarized at the same time as it receives a synaptic input, NMDA will be activated as it has no magnesium block, resulting in LTP), than as a result of its own Ca\(^{++}\) conductance. However, such effects would be inherent to all light-activated cation channels, including WT ChR2, and is not restricted to CatCh. As WT ChR2 was proven to allow accurate neuronal control over prolonged periods by numerous research groups in multiple neuronal systems, effects on LTP is not considered to be a problem.

The consideration of a localized current source also applies to the pre-synapse, which contains high Ca\(^{++}\) channel densities. If CatCh slightly contributes to a rise in presynaptic Ca\(^{++}\), this would only further enhance its efficiency in inducing plasticity, as light stimuli would in this case slightly enhance the level of Ca\(^{++}\) induced transmitter release.

In a study using WT ChR2 to optically induce synaptic plasticity\(^6\) it was concluded that the additional Ca\(^{++}\) influx seen during light-induced action potentials was mainly due to activation of voltage-gated Ca\(^{++}\) channels during the WT ChR2 inherent after-depolarization following an action potential, not to Ca\(^{++}\) influx through the pore of WT ChR2 itself. CatCh does not have an extended after-depolarization (see Fig. 4i main text), thus there is probably less activation of voltage-gated Ca\(^{++}\) channels upon light stimulation. However, if the 6-fold increase in Ca\(^{++}\) conductance would suffice to increase the release probability of CatCh-positive axons, CatCh would be a welcomed tool that would finally allow controlled optical manipulation of pre-synaptic events.

SUPPLEMENTARY REFERENCES

