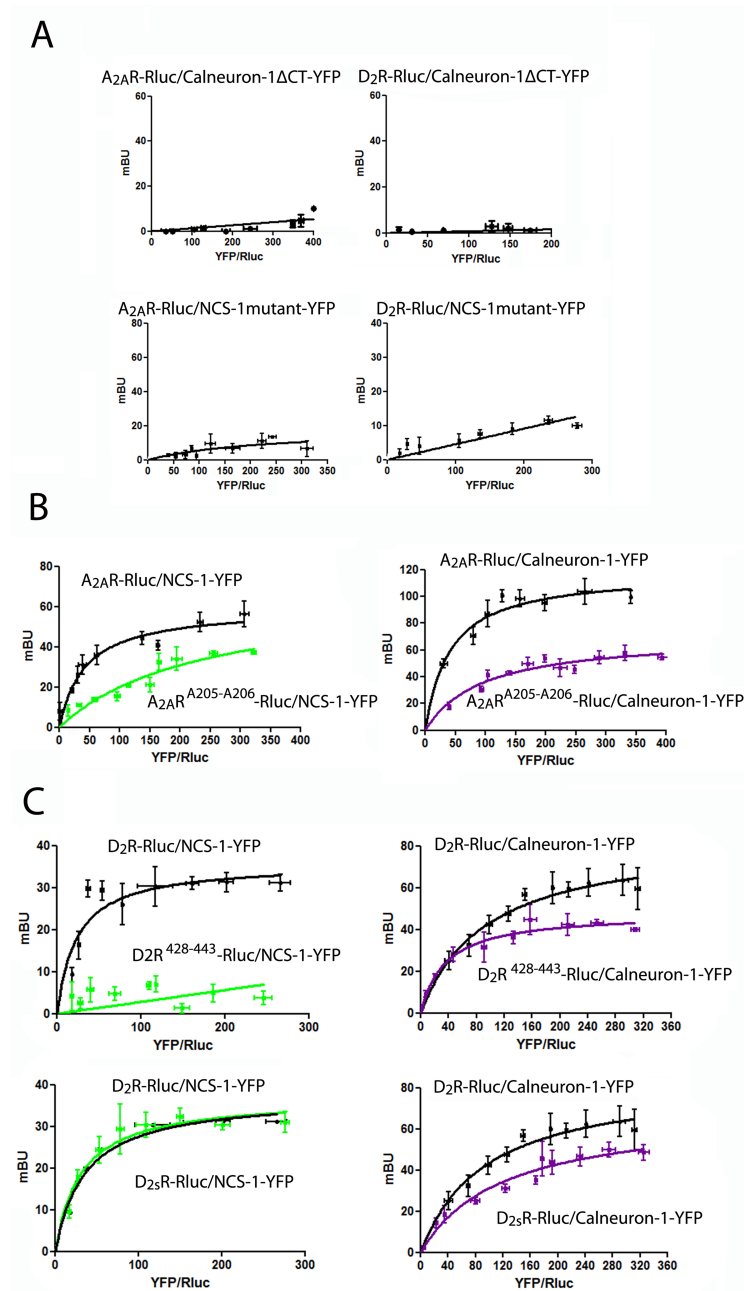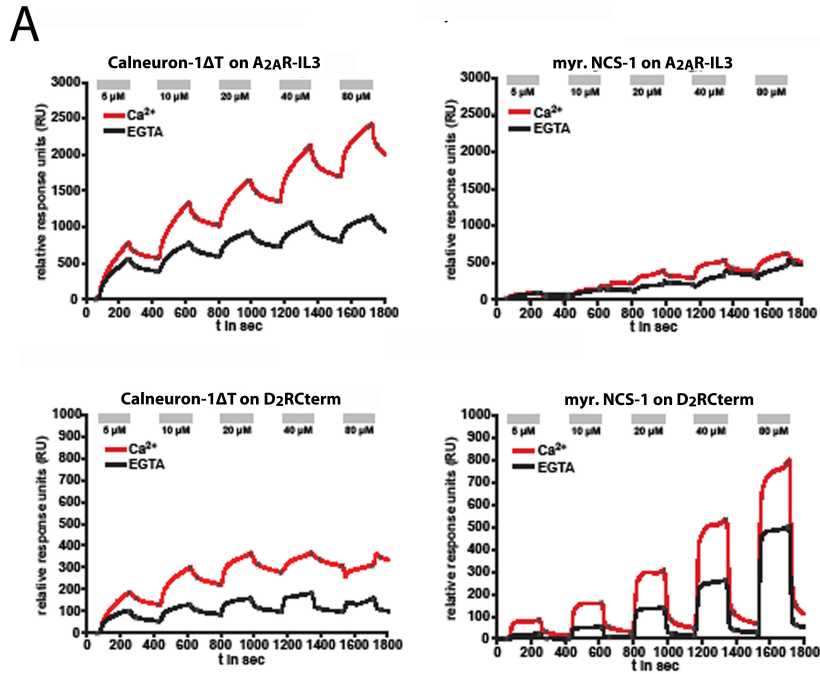**Figure S1, related to Figures 2 and 3. Molecular determinants involved in NCS-1 or calneuron-1 interaction with $A_{2A}R$ and D_2R**

A. BRET experiments in HEK-293T cells in cells transfected with $A_{2A}R$-Rluc cDNA (0.2 µg; left panels) or D_2R-Rluc cDNA (0.3 µg; right panels) and calneuron-1ΔCT-YFP cDNA (0.4 µg to 1.2 µg; bottom panels) or myristoylation-deficient YFP-NCS-1 cDNA (0.2 µg to 0.8 µg; bottom panels). **B.** BRET saturation experiments in HEK-293T cells transfected with mutant $A_{2A}R^{A205-A206}$-Rluc cDNA (0.2 µg) and increasing amounts of NCS-1-YFP cDNA (0.2 µg to 0.8 µg; green curve) or calneuron-1-YFP cDNA (0.4 µg to 1.2 µg; purple curve) and compared

with control cells transfected with $A_{2A}R$ cDNA (0.2 μg; black curves). **C.** BRET saturation experiments in HEK-293T cells transfected with mutant $D_2R^{428-443}$-Rluc cDNA (0.25 μg; top graphs) or $D_{2S}R$-Rluc cDNA (0.25 μg; bottom graphs) and increasing amounts of NCS-1-YFP cDNA (0.2 μg to 0.8 μg; green curves) or calneuron-1-YFP cDNA (0.4 μg to 1.2 μg; purple curves) and compared with control cells transfected with D_2R cDNA (0.3 μg; black curves). BRET, expressed as miliBRET units (mBU), is given as a function of 1000 x the ratio between the fluorescence of the acceptor (YFP) and the luciferase activity of the donor (Rluc). Values are means ± S.E.M. of 5 to 7 different experiments.

Figure S2, related to Figures 2 and 3. The NCS-1 and calneuron-1 binding to $A_{2A}R$ third intracellular loop and to D_2R C-terminal domain determined by surface plasmon resonance

A. Net sensograms corresponding to the interaction of His_6SUMO-calneuron-1ΔCT and myristoylated NCS-1 on immobilized GST-$A_{2A}R$-IL3 and D_2R-Cterm using surface plasmon resonance (SPR). Surface plasmon resonance was performed in single cycle mode using increasing amounts of injected His_6SUMO-calneuron-1ΔCT (calneuron-1ΔCT) or myristoylated NCS-1 (myr. NCS-1), shown in the grey boxes on top of each sensogram. Sensograms were recorded in single cycle mode at a flow rate of 10 μl/min and with 180 s analyte injections at concentrations of 5 μM, 10 μM, 20 μM, 40 μM and 80 μM. All runs were

performed in HBS-P buffer supplemented with 500 μM Ca^{2+}/1 mM Mg^{2+} (red traces) or 2 mM EGTA/1 mM Mg^{2+} (black traces) Net sensograms were calculated by subtracting response units obtained after analyte injections on control surfaces (GST for GST-A_{2A}R-IL3 and blank CM5 surface for D_2R-Cterm) and in case of His_6SUMO-calneuron-1ΔCT also of response units obtained from injections of the His_6SUMO-tag only. The molar binding activity (MBA) was calculated from the stability point of last injection considering the molecular mass of the analyte as well as the amount of ligand immobilized on the surface of the sensor chip surface as described by Catimel at al. (1997). Stability points used to calculate the molar binding activity (MBA) are shown in grey crosses on the individual traces. In all cases higher response units (RUs) were obtained in the presence of Ca^{2+}. **B.** Molar binding activities.
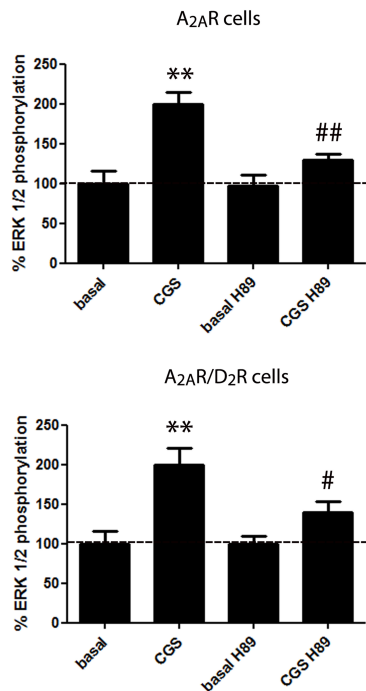

Figure S3, related to Figure 7. G-protein-cAMP-PKA-dependent A_{2A}R-mediated MAPK activation
ERK1/2 phosphorylation in HEK-293T cells transfected with A_{2A}R-Rluc cDNA (0.3 μg) alone (top graph) or A_{2A}R-Rluc cDNA (0.3 μg) and D_2R-YFP cDNA (0.5 μg) (bottom graph). Cells were pre-treated or not with the PKA inhibitor H98 (10 μM for 30 min) and were stimulated with the A_{2A}R agonist CGS 21680 (CGS; 100 nM). ERK1/2 phosphorylation levels are expressed as percentage over basal, represented by a dotted line. Values are given as means ± S.E.M. of 6 different experiments. One way ANOVA followed by a Bonferroni multiple comparison *post hoc* test: **p < 0.01, compared to basal; #p < 0.05 and ##p < 0.01, compared to CGS alone.
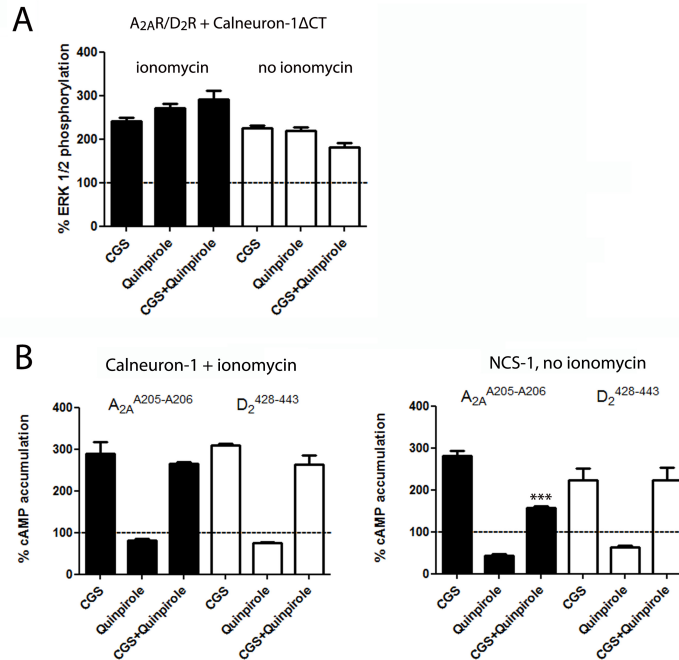
Figure S4, related to Figure 4. Specificity of NCS-1- and calneuron-1-mediated modulation of the signaling of A$_{2A}$R-D$_2$R heteromer in transfected cells
A. ERK1/2 phosphorylation in HEK-293T cells transfected with A$_{2A}$R-Rluc cDNA (0.3 µg), D$_2$R-YFP cDNA (0.4 µg) and calneuron-1ΔCT cDNA (0.6 µg). Cells were not stimulated (basal, dotted line) or stimulated with the A$_{2A}$R agonist CGS 21680 (CGS; 100 nM), the D$_2$R agonist quinpirole (1 µM) in the absence (black bars) or the presence (white bars) of ionomycin (1 µM). **B.** In the left graph, cAMP accumulation in HEK-293T cells transfected with A$_{2A}$R$^{A205-A206}$-Rluc mutant cDNA (0.3 µg), D$_2$R-YFP cDNA (0.4 µg) and calneuron-1 cDNA (0.6 µg) (black bars) or A$_{2A}$R-Rluc cDNA (0.3 µg), D$_2$R$^{428-443}$-YFP mutant cDNA (0.4 µg) and calneuron-1 cDNA (0.6 µg) (white bars) in the presence of ionomycin (1 µM). In the right graph, HEK-293T cells transfected with A$_{2A}$R$^{A205-A206}$-Rluc mutant cDNA (0.3 µg), D$_2$R-YFP cDNA (0.4 µg) and NCS-1 cDNA (0.5 µg) (black bars) or A$_{2A}$R-Rluc cDNA (0.3 µg), D$_2$R$^{428-443}$-YFP mutant cDNA (0.4 µg) and NCS-1 cDNA (0.5 µg of cDNA transfected) (white bars) in the absence of ionomycin. Cells were stimulated with the A$_{2A}$R agonist CGS 21680 (CGS; 100 nM) or the D$_2$R agonist quinpirole (1 µM) or both. In **A**, values are expressed as the percentage of basal (100%, dotted line) and are means ± S.E.M. of 4 to 6 different experiments. In **B**, cAMP accumulation is expressed as the percentage of basal or forskolin values (dotted line) and are means ± S.E.M. of 4 to 6 different experiments. One way ANOVA followed by Dunnett's multiple comparison *post hoc* test: ***$p < 0.001$ compared to CGS alone.
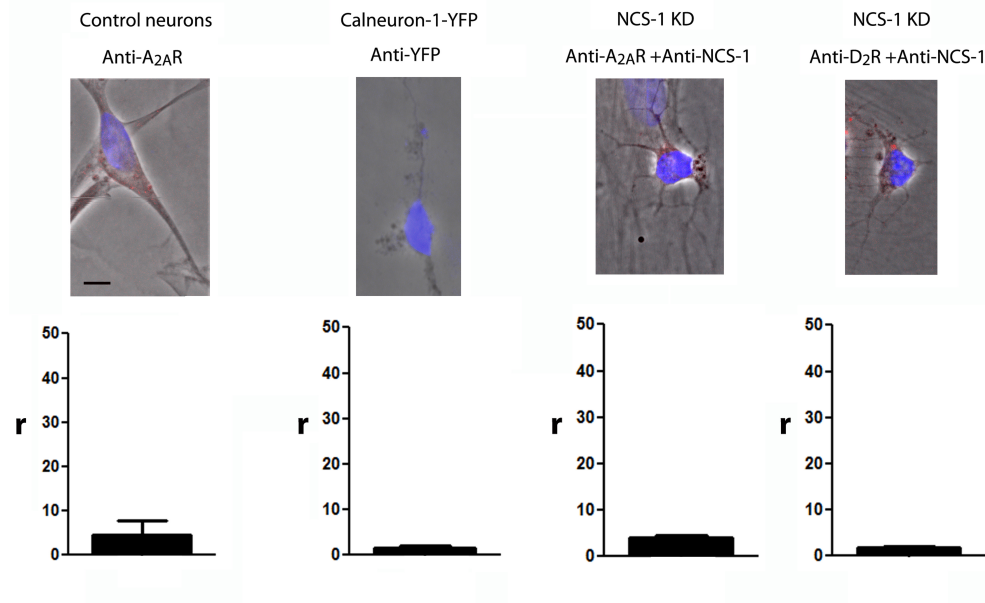
Control neurons	Calneuron-1-YFP	NCS-1 KD	NCS-1 KD
Anti-A$_{2A}$R	Anti-YFP	Anti-A$_{2A}$R +Anti-NCS-1	Anti-D$_2$R +Anti-NCS-1

Figure S5, related to Figures 5 and 6. Negative controls for PLA experiments in neuronal primary cultures

In situ proximity ligation assays *(*PLA) in primary cultures of rat striatal neurons with calneuron-1-YFP cDNA (1 µg) or NCS-1 shRNA cDNA (1.5 µg). PLA was performed with primary antibodies against A$_{2A}$R, GFP, A$_{2A}$R and NCS-1 or D$_2$R and NCS-1. Confocal microscopy images are shown (superimposed sections) in which very few red clusters appear in neurons detected by phase contrast. Scale bars = 20 µm. Cell nuclei were stained with Hoesch (blue). Bars represent the quantification of ratio *r* values (number of red spots/cell containing spots) corresponding to the different conditions. Data are means ± SEM of counts in 20-30 different neurons of three independent preparations.
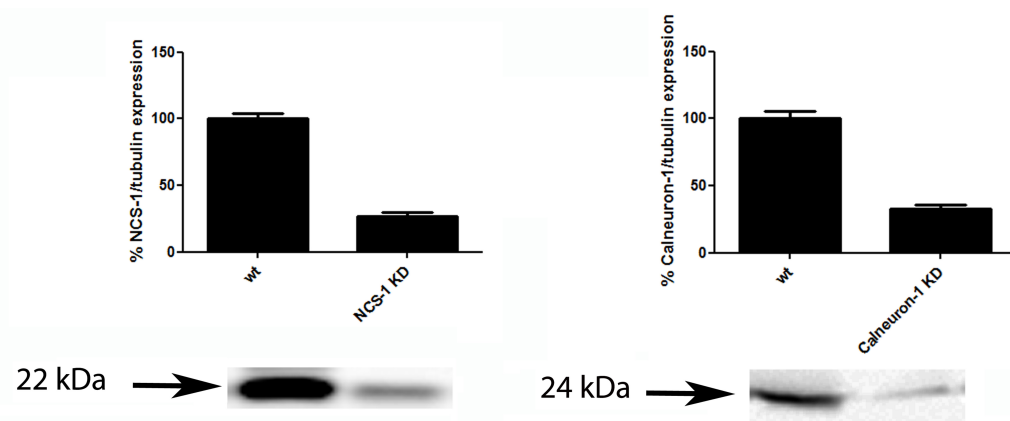

22 kDa ➡

24 kDa ➡

Figure E6, related to Figures 5 and 6. Silencing NCS-1 or calneuron-1 on rat striatal neurons

Rat striatal primary cultures not transfected (wt) or transfected with NCS-1 shRNA cDNA (NCS-1 KD; 1.5 µg) or calneuron-1 shRNA cDNA (calneuron-1 KD, 1.5 µg). Membranes from primary cultures were analyzed by SDS-PAGE and immunoblotted with anti-NCS-1 or anti-calneuron-1 antibodies. Representative Western blots are shown.

SUPPLEMENTAL EXPERIMENTAL PROCEDURES

Resonance Energy Transfer experiments

For Bioluminescence Resonance Energy Transfer (BRET) experiments, HEK-293T cells transiently co-transfected with a constant amount of cDNA encoding for the protein fused to RLuc and with increasingly amounts of cDNA corresponding to the protein fused to YFP (see figure legends) were used after 48 h transfection. To quantify protein-YFP expression, fluorescence of cells (20 μg protein) was read in a Fluoro Star Optima Fluorimeter (BMG Labtechnologies, Offenburg, Germany) equipped with a high-energy xenon flash lamp, using a 10 nm bandwidth excitation filter at 400 nm reading. For BRET measurements, 5 μM of coelenterazine H (Molecular Probes, Eugene, OR) was added to the equivalent of 20 μg of cell suspension. After 1 minute, the readings were collected using a Mithras LB 940 that allows the integration of the signals detected in the short-wavelength filter at 485 nm and the long-wavelength filter at 530 nm. To quantify protein-RLuc expression, luminescence readings were also performed after 10 minutes of adding 5 μM coelenterazine H. The net BRET is defined as [(long-wavelength emission)/(short-wavelength emission)]-Cf where Cf corresponds to [(longwavelength emission)/(short-wavelength emission)] for the donor construct expressed alone in the same experiment. Data were fitted to a non-linear regression equation, assuming a single-phase saturation curve with GraphPad Prism software (San Diego, CA, USA). BRET is expressed as miliBRET units, mBU (net BRET x 1000).

For Sequential Resonance Energy Transfer (SRET) assays, HEK-293T cells were transiently co-transfected with constant amounts of cDNAs encoding for both proteins fused to RLuc and GFP[2] and with increasingly amounts of cDNA corresponding to the protein fused to YFP. In SRET, the oxidation of the Rluc substrate DeepBlueC by protein-Rluc triggers protein-GFP[2] excitation (BRET), which triggers a subsequent excitation of protein-YFP (FRET). Emission of YFP after addition of DeepBlueC is only possible if the three fusion proteins are in close proximity (<10 nm), allowing sequential bioluminescent and fluorescent resonance energy transfer to occur. Cells were used 48 h post-transfection. Using aliquots of transfected cells (20 μg of protein), different determinations were performed in parallel: (i) Quantification of protein-YFP expression was performed as indicated in BRET experiments. (ii) For quantification of protein-Rluc expression cells were distributed in 96-well microplates (Corning 3600, white plates with White bottom), and luminescence was determined 10 min after addition of 5 μM coelenterazine H in a Mithras LB 940 multimode reader. (iii) For SRET, cells were distributed in 96-well microplates (Corning 3600, white plates with white bottom), and 5 μM DeepBlueC (Molecular Probes, Eugene, OR) was added. The SRET signal was collected using a Mithras LB 940 reader with detection filters for short wavelength (410 nm) and long wavelength (530 nm). By analogy with BRET, net SRET is defined as ((long wavelength emission)/(short wavelength emission)) - Cf, where Cf corresponds to long wavelength emission/short wavelength emission for cells expressing protein-Rluc and protein-GFP[2]. Linear unmixing was done for SRET quantification, taking into account the spectral signature to separate the two fluorescence emission spectra. SRET is expressed as miliSRET units, mSU (net SRET x 1000). Data were fitted as in BRET experiments.