Double fluorescent knock-in mice to investigate endogenous mu-delta opioid heteromer subscellular distribution.

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ABSTRACT

The heteromerization of Mu (MOP) and delta (DOP) opioid receptors has been extensively studied in heterologous systems. These studies demonstrated significant functional interaction of MOP and DOP evidenced by new pharmacological properties and intracellular signalling in transfected cells co-expressing the receptors. Due to the lack of appropriate tools for receptor visualization, such as specific antibodies, the pharmacological and functional properties of MOP-DOP heteromers in cells naturally expressing these receptors remains poorly understood. To address endogenous MOP-DOP heteromer trafficking and signalling in vivo and in primary neuronal cultures, we generated a double knock-in mouse line expressing functional fluorescent versions of DOP and MOP receptors. This mouse model has successfully been used to map the neuroanatomic distribution of the receptors and to identify brain regions in which the MOP-DOP heteromers are expressed. Here, we describe a method to quantitatively and automatically analyze changes in the subcellular distribution of MOP-DOP heteromers in primary hippocampal culture from this mouse model. This approach provides a unique tool to address specificities of endogenous MOP-DOP heteromer trafficking.

INTRODUCTION

Opioid receptors belong to the subfamily of Class A G protein-coupled receptors (GPCRs). Four subtypes of opioid receptors mu (MOP), delta (DOP), kappa (KOP) and nociceptin (NOP) receptor respectively encoded by the *OPRM1*, *OPRD1*, *OPRK1* and *OPRL1* genes have been identified several decades ago (for review see [1-3]). These seven transmembrane domain receptors are functional in a monomeric form, but can also associate among themselves to generate a larger assembly or with different subtypes of opioids or non-opioid

receptors. In the latter case, the new entity is called heteromer and may exhibit specific functional properties.

In the case of the opioid system, MOP and DOP functional interactions are well documented Among others, they are essential for the development of opiate tolerance [4,5]. Numerous studies indicate that co-expression of the two receptors in heterologous systems promotes the formation of MOP-DOP heteromers, which affects binding and signaling properties [6,7]. However, in spite of a growing body of evidence in favor of the presence of MOP-DOP heteromers *in vivo*, the molecular mechanisms underlying functional interactions between these two receptors remain poorly characterized[8]. This is mainly due to the lack of appropriate tools, especially specific antibodies.

To deal with this issue and study MOP-DOP heteromers *in vivo*, we generated a double fluorescent knock-in mouse line co-expressing DOP and MOP receptors respectively fused to their C-terminus to the enhanced green fluorescent protein (DOP-eGFP) or mcherry (MOP-mcherry). The DOP-eGFP and MOP-mCherry functional fusions allow highly specific and simultaneous visualization of endogenously expressed receptors with subcellular resolution and proved to be unique tools for neuroanatomical studies [9]. Mapping of MOP and DOP receptors in the central and peripheral nervous systems indeed revealed MOP-DOP neuronal co-expression in discrete neuronal networks essential for survival such as the nociceptive pathway (see also mouse brain atlas at http://mordor.ics-mci.fr/). Specific targeting using the fluorescent tags also revealed MOP-DOP physical proximity in the hippocampus providing strong rationale for the existence of endogenous MOP-DOP heteromers [9]. In addition, the double fluorescent knock-in mice represent unique tools to explore the dynamics of this

complex under physiological or pathological conditions and to characterize the functional impact of MOP-DOP heteromers in the central and peripheral nervous system.

In this chapter, we describe optimized conditions for visualization of endogenous MOP-DOP heteromers in primary hippocampal neurons obtained from the double fluorescent knock-in mice. We also provide a protocol for automatic quantitative analysis of confocal images with an open source software to determine changes in receptor subcellular localization. This method allowed MOP-DOP heteromers monitoring and specific determination of their intracellular fate upon pharmacological activation.

MATERIAL AND METHODS

1. Animals

Double knock-in mice co-expressing fluorescent DOP and MOP receptors were obtained by crossing previously generated DOP-eGFP and MOP-mcherry knock-in mice. Briefly, DOP-eGFP knock-in mice expressing the delta opioid receptor fused to its C-terminus to the eGFP were generated by homologous recombination by inserting the eGFP cDNA into exon 3 of the delta opioid receptor gene, in frame and 5′ from the stop codon [10]. MOP-mcherry knock-in mice expressing the mu opioid receptor fused to its C-terminus to the red fluorescent protein mCherry were generated by homologous recombination following a procedure similar to the one used for DOR-eGFP knock-in mice [9]. The construct transfected into ES cells comprised a Gly-Ser-Ile-Ala-Thr linker followed by the cDNA sequence encoding the fluorescent protein (eGFP or mcherry). For subsequent clone selection, a resistance gene was included that corresponded to neomycin flanked by loxP sites for DOP-eGFP or to hygromycin flanked by FRT sites for MOP-mcherry (Figure 1). The resistance gene was removed by microinjection of a plasmid expressing the recombinase. Blastocysts

were implanted in pseudo gestant BalbC females. Chimeric mice were crossed with C57Bl6/J mice to obtain F1 heterozygous generation. Heterozygous animals were crossed to generate mice homozygous for *Oprd1*-eGFP or *Oprm1*-mcherry that are fertile and develop normally. Double knock-in animals were obtained by crossing the single knock-in mouse lines. The genetic background of all mice was C57/BL6/J: 129svPas (50:50 %).

Mice were housed in animal facility under controlled temperature (21 ± 2 °C) and humidity (45 ± 5 %) on a 12-h dark–light cycle with food and water ad libitum. All experiments were performed in accordance to the European legislation (directive 2010/63/EU acting on protection of laboratory animals) and the local ethical committee.

2. Primary hippocampal culture

2.1. Material and reagents:

- Double knock-in new born mice pups (P0-P3).
- 70% ethanol solution
- MilliQ water (autoclaved, or sterile filtered 0.22µm).
- Borate buffer *(see setup and procedures)*
- Poly-L-lysine hydrobromide (Sigma cat. No. P2623)
- 13 mm coverslips (Sigma, cat. no. P6407) coated with Poly-L-lysine *(see setup and procedure)*
- 24-well sterile culture plates (Falcon cat. no. 353047).
- Pasteur pipets (flamed at the extremity, cotton plugged and autoclaved).
- Hibernate minus phenol red (BrainBits SKU: HAPR)
- Papain (Worthington, cat. no. LS003126)

- Dulbecco's Modified Eagle's Medium (DMEM) with 4.5g glucose (GIBCO, cat. no. 71966-029)
- Neurobasal A (GIBCO, cat. no. A13710-01)
- Foetal Calf Serum FCS heat inactivated. **Caution:** *test several batches to determine the best one for your culture conditions.*
- Glutamax™ (GIBCO, cat. no. 35050061)
- L-glutamine (GIBCO, cat. no. 25030081)
- Penicillin-streptomycin (P/S) (cat. no. 15140122)
- DNAse (Sigma cat. no. DN25).
- B27 supplement (GIBCO, cat. no. 17504044)
- Trypan blue solution (Sigma, cat. no. T8154)
- Paraformaldehyde 32% solution diluted to 4% before use (*see setup and reagents)*.
- Phosphate buffer saline (PBS). (Sigma-Aldrich, cat. no. P5493)
- Cell strainer 70µm (Falcon, cat. no. 352350)

2.2. Setup and procedures

2.2.1. Borate buffer: dissolve boric acid 1,24g and Sodium tetraborate (borax) 1,9g in 400ml MilliQ H_2O. pH should be 8.4. Sterile filter (0.22µm) before use. **Caution:** *borax is a hazardous substance, manipulate cautiously and eliminate waste according to the safety rules fixed by your institution/government.*

2.2.2. Coverslips sterilisation and coating: put coverslips in a 100-mm petri dish, sterilise in 70% ethanol during 2 h under gentle agitation, let dry completely under laminar flow, and transfer to culture plates. Rinse once with MilliQ water. Coat coverslips with poly-L-lysine 25µg/ml final concentration in borate buffer; incubate at 37°C for 2h to overnight. Rinse 3 times with sterile water and pre-warm in DMEM medium. Coated coverslips may be

prepared several days before use, dried in the laminar flow hood and kept sealed at 4°C for up to 1 month.

2.2.3. Dissection medium: Prepare 5 mL of of ice cold Hibernate supplemented with 1X P/S and 0.5nM glutamax per animal and transfer 0.5 mL dissection medium in a 15-mL centrifugation tube per animal (2 hippocampi).

2.2.4. Enzyme solution: prior to dissection, prepare a fresh solution of Papain at 40U/mL concentration in Hibernate medium, incubate 5 minutes at 37°C in a water bath then keep on ice until use. Prepare 0.5 mL per animal (2 hippocampi).

2.2.5. Plating medium: Prepare 12 mL DMEM medium supplemented with 4.5 g/l glucose + 10% heat inactivated FCS + 2mM Glutamine + Pen/strep) per animal on the day of use.

2.2.6. Growing medium: Prepare 12 mL Neurobasal medium supplemented with 2% B27, 2mM glutamax, 0.5mM glutamine and 1X P/S per animal on the day of use.

2.2.7. Phosphate-buffered saline (PBS): prepare 1L 1X PBS working solution from 10X stock solution by diluting with MilliQ water. Check the pH and adjust to 7.4 with 1M HCl or 1M NaOH solutions if needed. Sterile filter 0.22µm and keep at 4°C for up to 6 months.

2.2.8. Fixation solution: dilute paraformaldehyde (PFA) 32% solution to 4% final concentration in PBS 0.1M, adjust pH to 7.4 if needed. Prepare 500µl per well for use in 24-well plate. Keep up to 5 days at 4°C and up to 6 months at -20°C. ***Caution:*** *PFA a hazardous highly toxic substance, manipulate under flow hood and eliminate waste according to the safety rules fixed by your institution/government.*

2.3. Dissection and culture procedures

2.3.1. Dissection and cell dissociation

Decapitate pups. Transfer the head in a 33mm petri dish with 1.5 mL ice cold dissection medium and isolate the brain. Place the isolated brain in a new 33mm petri dish with 1.5mL

ice cold dissection medium. Remove the meninges, dissect to isolate the two hippocampi and keep them in 0.5mL dissection solution in a 15 mL-tube in ice. Add 0.5 mL of papain solution per tube to 0.5 mL dissection medium (1mL final / 2 hippocampi). Place tubes in a water bath at 37°C for 30 minutes with gentle shaking every 5-10 minutes. 5-10 minutes before digestion ends, add DNase at a final concentration of 1mg/mL.

Remove papain solution by decantation, add 1mL of Hibernate, and triturate with Pasteur pipet until the tissue is completely dissociated (about 15 to 20 times up-and-down are sufficient) ***(see note 1)***. Centrifuge at 1000g for 5 minutes at 22°C. Remove the supernatant, add 1mL plating medium to resuspend cells (3 to 5 gentle up-and-down with Pasteur pipet). Filter the cell suspension through a 70µm-cell strainer to remove any residual aggregates. Count cells by diluting 20µl of cell suspension in 80µl of 1:10 Trypan blue solution diluted in PBS. Place 20µl of this solution in a cell counting chamber and count cells excluding Trypan blue (viable cells) only.

2.3.2. Plating and feeding

Prewarm poly-L-Lysine coated plates in DMEM medium at 37°C. Remove DMEM and plate cells in 24-well plates at a density of 80 000 to 100 000 cells per well in a final volume of 500 µL.

Incubate in a humid incubator at 37°C and 5% CO2 and allow cells to adhere to the bottom of plates during one hour. Remove the plating medium by aspiration, rinse once with 500µl of prewarmed Neurobasal A medium, then add 500µl of growing medium and return back to the incubator. Let cells grow during at least twelve days with half of the medium replaced every four to five days.

3. Processing and pharmacological treatments

Pharmacological treatments should be realized between DIV 12 and DIV 15 after plating ***(see note 2)***. Ligands are added in a volume not exceeding 10% of the culture medium volume. Incubate cells during the appropriate time. At the end of the pharmacological treatment, remove the plate from the incubator and immediately place on ice, carefully aspirate the medium and wash twice with ice cold sterile filtered PBS. Remove PBS and add 500µL of PFA 4% in ice cold PBS and incubate during 20 minutes on ice. Remove PFA and rinse twice with ice cold PBS and proceed to immunostaining or keep sealed with Parafilm in 500µL PBS at 4°C up to 30 days ***(see note 3).***

4. Immunocytofluorescence (ICF):

4.1. Material and reagents

- Phosphate buffer Saline 0.1M, pH 7.4.
- Normal Goat Serum (NGS) (Sigma cat. no. S26)
- Tween20 (Euromedex cat. no. 2001-B)
- Primary and secondary antibodies (see table 1)
- ProLong™ Gold Antifade Mounting medium (Molecular Probes cat. no. P36935).
- DAPI (Sigma cat. no. D9542)
- Finepoint Forceps (Rubis Switzerland cat.no.1K920)
- Microscope glass slides.

4.2. Setup and reagents

4.2.1. **PBS Tween 20 solution (PBST)**: add 0.2% (V:V) of Tween 20 solution to 1X PBS solution (*see section 1.2.7*) , mix vigorously to complete dissolution and keep at 4°C. Bring at room temperature before use.

4.2.2. **Blocking solution:** add 5% of Normal Goat Serum (NGS) to the PBST solution. Prepare the day of use.

4.3. Method

Incubate fixed cells in 250 µL of blocking solution for one hour under gentle agitation at room temperature (20-22°C). Then remove the blocking solution by aspiration and incubate 2h at RT or overnight at 4°C with primary antibodies in blocking solution (250µL/well) under gentle agitation. Wash three times in PBST and incubate for two hours protected from light with specific secondary antibodies diluted in blocking solution (Table 1) (250µL/well). Wash three times in PBST, incubate 5 minutes in DAPI solution (1µg/mL in PBS) for nuclei staining and then wash three times in PBS followed by 1 wash in MilliQ H_2O. Remove coverslips from wells with finepoint Forceps, let coverslips dry completely at room temperature protected from light and mount on glass slides with ProLong™ Gold Antifade mounting medium. Keep for up to one year at -20° protected from light.

5. Confocal microscope

Images were acquired with a laser-scanning confocal microscope Leica SP5 using 63X /NA 1.4 oil immersion lens and X5 numerical zoom. The Pinhole was adjusted to 1 airy Unit and the gain was adjusted without offset for each filter on a specific scanning plan allowing specific acquisition without saturation. Image acquisition was performed according to Nyquist parameters in XY with an average frame of 3 in a sequential scan mode to avoid cross talk between different wavelengths. Z-stacks were obtained by scanning the whole neuron thickness with step of 1 µm in z.

6. Image analysis with ICY open source software

231 Confocal images were analyzed with ICY software (http://icy.bioimageanalysis.org/).
232 Quantification was performed on a single plane extracted from a z-stack. The analysis
233 combined two sequential steps. The first one consists in isolating each neuron to define
234 regions of interest (ROI). The second one involves the detection of the spots in each channel
235 and the determination of the amount of co-localisation in each ROI.

236 **6.1. ROI definition**

237 Each neuron was carefully delineated using the "free-hand area" tool. This initial ROI is
238 filled with the "fill holes in ROI" plugin to define the total cell area (ROI $_{total}$). ROIs were then
239 processed to generate two ROIs corresponding to the cell periphery and the cytoplasm
240 (detailed protocol available online *http://icy.bioimageanalysis.org/*). Based on staining in basal
241 conditions, we estimated that most of the plasma membrane staining was found over an 8
242 pixels thickness. Therefore, we automatically eroded with the "Erode ROI" plugin the ROI
243 $_{total}$ by 8 pixels and subtracted this new ROI (ROI $_{cyto}$) to ROI $_{total}$ to obtain a ROI
244 corresponding to the cell periphery (ROI $_{peri}$).

245 **6.2. Spots detection and co-localisation**

246 To detect specific signal in each ROI, we used the "spot detector" plugin which rely on the
247 wavelet transform algorithm [11]. By carefully setting the sensitivity threshold and the scale
248 of objects to detect, it allows detection of spots even in images with low signal to noise ratio.
249 In our conditions, the sensitivity threshold was fixed between 50 and 60 % and the scale of
250 objects set at 2 (pixel size 3) for mu and delta receptors. Once parameters were defined,
251 images were images were processed with the tool "protocol" in Icy which is a graphical
252 interface for automated image processing. Data including the number of spots detected in
253 each channel and ROIs, the number of co-localized objects and the ROI area were

automatically collected in excel files. Objects were considered co-localized if the distance of their centroid was equal to or less than 3 pixels. The protocol is available online (*NewColocalizer with binary and excel output v1_batch.xml*). To obtain histograms we calculated object densities for each receptor reported to the surface of each ROI. Membrane to cytoplasm density ratios were calculated to illustrate the subcellular distribution of each receptor. The extend of co-localisation was calculated according to the following formula for each ROI [$\% \, colocalisation = 100X \, (\frac{colocalised \, MOP \, and \, DOP \, objects}{\sum(\, detected \, MOP \, and \, DOP \, objects)})$].

7. Statistical analysis

Statistical analyses were performed with Graphpad Prism V7 software (GraphPad, San Diego, CA). Normality of the distributions and homogeneity of the variances were checked before statistical comparison to determine appropriate statistical analysis. In our case, data were not normally distributed and the non-parametric Mann Witney test was used to compare receptor densities in the plasma membrane and cytoplasm in basal conditions or after agonist treatment. The extend of receptor co-localisation was compared using two-way ANOVA with repeated measures followed by post-hoc Sidak's test for multiple comparisons. Basal group was compared to agonist treated group (first factor) within cytoplasm and plasma membrane localisation (second factor).

RESULTS AND DISCUSSION

In this chapter, we have presented an optimized method for monitoring the subcellular distribution of endogenous MOP and DOP receptors. To this aim, we combined the use of a genetically modified mouse line co-expressing functional fluorescently tagged receptors, optimized primary neuronal culture protocol and automatic quantitative analysis of confocal

images with an open source software. Importantly, the image analysis procedure can be easily implemented in any laboratory since data processing does not require extensive mathematical developments or program writing with specialized software.

Individual distribution of MOP and DOP receptors

High magnification confocal images analyzed as single focal plan revealed discontinuous and punctate distributions for DOP-eGFP and MOP-mCherry that were predominantly located at the cell surface in basal conditions (Figure 2-A). Images also revealed a perinuclear cytoplasmic localization of both receptors that likely correspond to receptor stock in the endoplasmic reticulum. These observations were consistent with data from the literature describing a predominant and membrane localization of DOP [10,12] and MOP [13] but also substantial localization in perikarya [10].

Quantification using the ICY software indicated a higher density of fluorescent objects at the cell surface for both MOP and DOP receptors (Figure 2-B) that was three times higher compared to the cytoplasm (Fig. 2-C). Activation by the MOP-DOP agonist CYM51010 let to the appearance of high intensity punctate structures in the cytoplasmic and a dramatic decrease in the plasma membrane labelling of both receptors (figure 2-A). Accordingly, the ratio corresponding to the density of fluorescent objects density at the plasma membrane compared to the cytoplasm dropped dramatically from 3 to 1 for both MOP and DOP receptors which supports internalization of the two receptors in vesicle-like structures.

These results are in agreement with previous reports using the density of fluorescence to estimate changes in DOPeGFP subcellular distribution *in vivo*. In these studies, the ratio of fluorescence density between the plasma membrane and cytoplasm was about 1.5 in basal

conditions and significantly decreased following agonist stimulation [10,14]. We therefore tested our quantification method using images of DOR-eGFP neurons in the hippocampus acquired with similar parameters in confocal microscopy. Using the protocol described above, we found that the density in DOP-eGFP objects under basal conditions was around 3 similar to our results in primary neuronal cultures. We also established that this value corresponded to a ratio in fluorescence density between the plasma membrane and the cytoplasm of about 1.7, similar to previously reported ratios [10]. Moreover, we quantified the ratio of fluorescence density between the plasma membrane and the cytoplasm using the set of images used in Erbs et al 2016, and calculated a similar increase of about 10% in DOP-eGFP expression at the plasma membrane after chronic morphine treatment in the neurons of the hippocampus [15]. Altogether, these results fully validate the quantification method developed using ICY software.

Co-localization of MOP and DOP receptors and detection of MOP-DOP heteromers

Our analysis revealed substantial colocalization of MOP and DOP associated signals under basal conditions. In fact, more than 22% of the MOP and DOP objects were co-localized within the plasma membrane whereas cytoplasmic colocalization was fairly low (around 10%) (Figure 2-D). After selective MOP-DOP activation with the agonist CYM51010, the cytoplasmic colocalization was increased by 87% to reach 18.7%. A two-fold decrease in the percentage of MOP-DOP colocalization within the plasma membrane was also observed after CYM51010 treatment (Figure 2-D). These observations support a view in which MOP and DOP receptors remain associated upon specific activation of the heteromers and undergo common intracellular fate.

It is however important to note, that due to the resolution limit of confocal microscopy, MOP-DOP physical interaction has to be assessed by other experimental approaches such as co-localization in electron microscopy or disruption of the physical contact by a transmembrane peptide. The latter has been successfully performed for MOP-DOP receptors *in vivo*. Indeed, a peptide corresponding to the MOP TM 1 or to the DOP second intracellular loop in fusion with the cell transduction domain of the human immunodeficiency virus (HIV) TAT protein interfered with MOP-DOP co-immunoprecipitation [16-18]. The recently described proximity ligation assay [19] provides high spatial resolution and represents another attractive option to address physical proximity.

In addition, the resolution of the images did not enable to distinguish the pool of receptors associated to the plasma membrane from receptors located in the sub-membrane compartment which represents another limitation of our analysis. Total internal reflection fluorescence microscopy (TIRFM) would be required to differentiate the two compartments and precisely monitor the first steps in receptor internalization.

MOPmcherry-DOPeGFP co-localisation studies could also be combined with identification of the intracellular compartments using specific antibodies. This would enable for fine mapping of the receptors in the vesicular structures and cellular compartments and would provide detailed information on the intracellular fate of MOP-DOP heteromers.

CONCLUSION

Double knock-in mice represent unique tools to investigate endogenous MOP-DOP neuroanatomical distribution but also to explore functional dynamics in physio-pathological conditions. As described here, the use of double fluorescent knock-in mice combined with

confocal imaging and ICY software analyses enables easy quantification of receptor sub-cellular distribution and co-localisation, hence specific MOP-DOP trafficking. Importantly, the field of application of the method described here is not restricted to the study of MOP-DOP heteromers but can be applied to address the heteromerization of any other pair of GPCRs. Moreover, the analysis with the ICY software is not restricted to the study of receptors but is widely applicable to monitor the co-localisation of any two objects detected independently and can be applied to any type of high-resolution images.

NOTES

1- Cell dissociation is a critical step; if the trituration is too gentle, the tissue will not dissociate, if too vigorous cells will break.

2- Because re-expression of DOP-eGFP and MOP-mCherry is only detectable from day in vitro (DIV) 10 in primary neurons, pharmacological treatments should be performed between DIV12 and DIV15 to ensure full expression of the receptors.

3- Paraformaldehyde fixation decreases the fluorescence intensity of eGFP and mCherry and amplification by immunostaining is recommended. The DOP-eGFP construct can also be used for *ex vivo* real-time imaging of receptor internalization by confocal microscopy [10]. However, due to the low expression level of endogenous MOP receptors and their weak expression at the plasma membrane in basal conditions, real-time monitoring of MOP-mcherry remains presently below the detection limit.

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CONFLICT OF INTEREST

The authors declare no conflict of interest

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TABLE 1: Primary and secondary antibodies

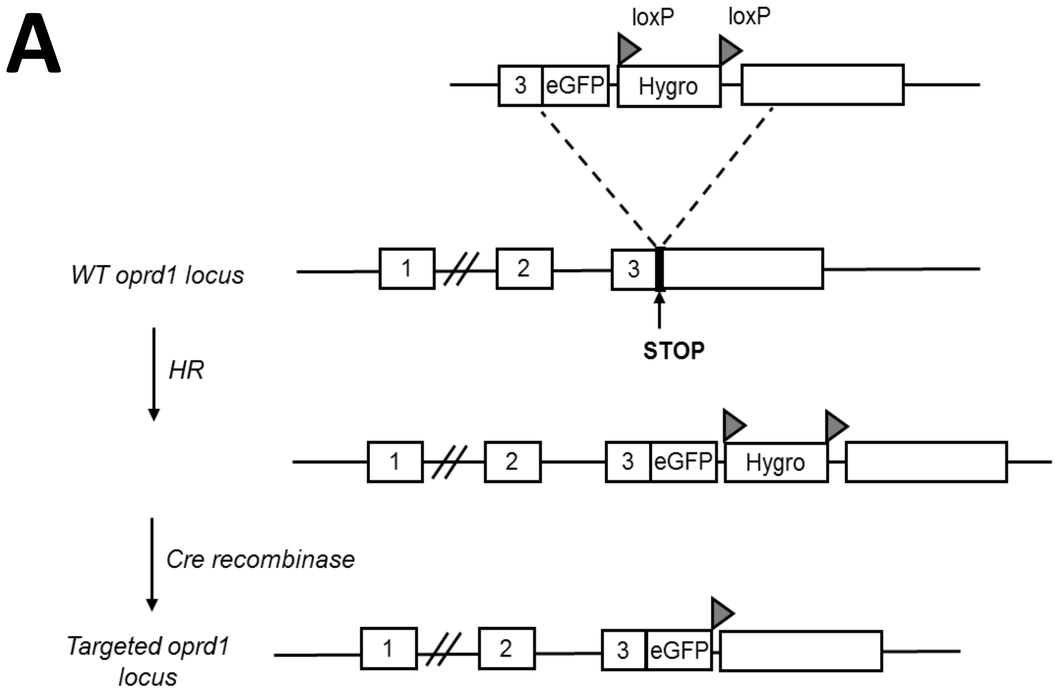
Antigen	Antibody	Supplier reference	Dilution
eGFP	Chicken IgY	AVES-GFP1020	1/1000
mCherry	Rabbit IgG	Clontech-632496	1/1000
Chicken IgY	Goat anti chicken Alexa Fluor 488	Molecular Probes-A11039	1/2000
Rabbit IgG	Goat anti rabbit Alexa Fluor 594	Molecular Probes-A11012	1/2000

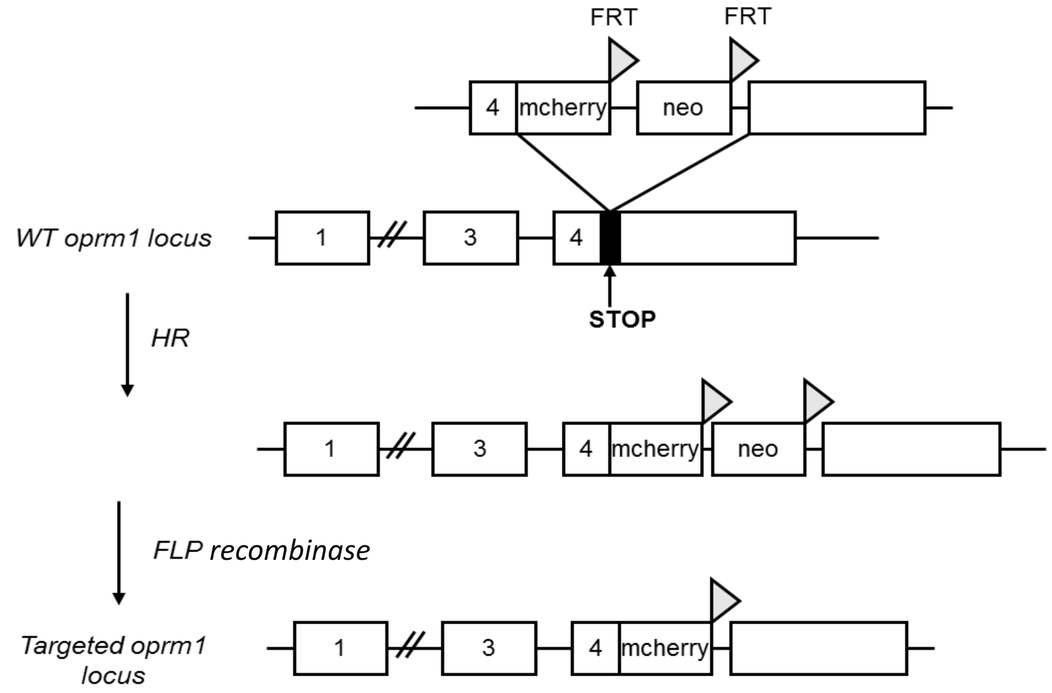
FIGURE LEGENDS

Figure 1: Double Knock-in DOP-eGFP/MOP-mCherry mouse engineering. **A)** Construction of the DOP-eGFP mouse. A cDNA sequence corresponding to the eGFP cDNA, and the FRT flanked neomycine (neo) cassette was inserted by homologous recombination (HR) into the *Oprd1* locus. HR was followed Cre recombinase treatment in ES cells. **B)** Construction of the MOP-mcherry mouse. A cDNA sequence corresponding mCherry cDNA, and the loxP flanked hygromycine (hygro) cassette were inserted by HR to the *Oprm1* locus. HR was followed by FRT recombinase treatment in ES cells. **(C)** Double knock-in mice were obtained by crossing homozygote DOP-eGFP and MOP-mCherry mice.

Figure 2: MOP-DOP heteromer visualisation and quantification. **A)** Representative confocal images illustrating MOP-mcherry and DOP-eGFP co-localisation in basal conditions

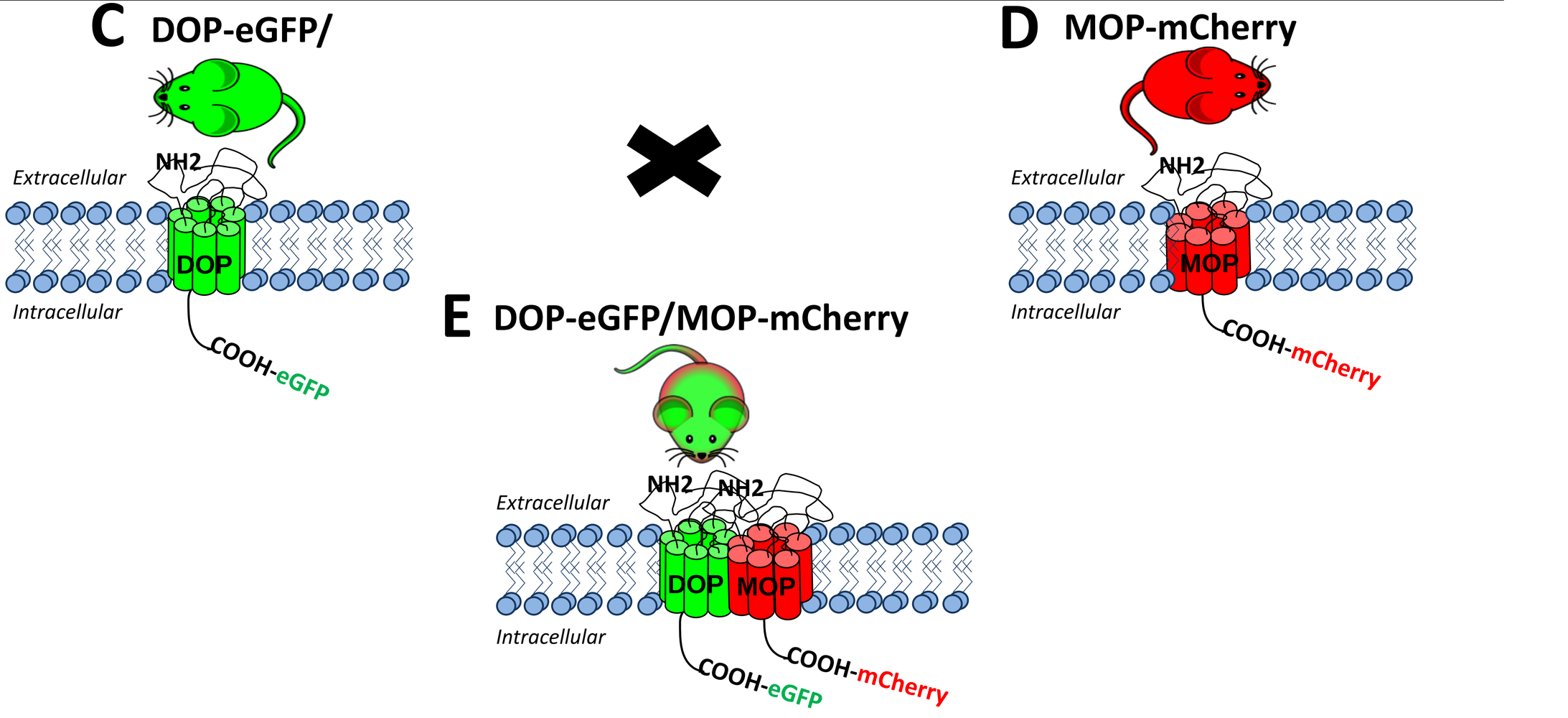
or after treatment during 60 minutes with the MOP-DOP agonist CYM51010 400nM. Scale bar: 5µm. **B)** Image quantification with ICY software illustrating each receptor distribution. In basal conditions, MOP and DOP densities are significantly higher in the plasma membrane compared to cytoplasm. Mann Witney test, *p-value <0.05. CYM51010 treatment for 60 minutes led to changes in receptor subcellular distribution with no more statistical difference in receptor densities between the plasma membrane and cytoplasm (Mann Witney test, p-value > 0.05). **C)** Changes in receptors distribution. Plasma membrane to cytoplasm ratio of MOP or DOP spots densities in basal conditions or after with 400nM CYM51010 for 60 minutes reflects receptor redistribution. Mann Witney test, ****p-value <0.001; **** p-value <0.0001. **D)** Quantification of MOP-DOP co-localisation upon agonist activation with 400nM CYM51010 for 60 minutes reveals MOP-DOP co-internalisation. Two-way ANOVA with repeated measures, post-hoc Sidak's test. *p-value <0.05 for basal cytoplasm *vs* CYM51010 cytoplasm; **p-value <0.01 for basal membrane *vs* CYM51010 membrane. ### p-value <0.001 for basal membrane *vs* basal cytoplasm; NS: p-value >0.05 for CYM51010 membrane vs CYM51010 cytoplasm.





Adapted from Scherrer et al 2006 PNAS

Adapted from Erbs et al 2015 Brain Struc funct.



A

Basal

CYM51010 60 minutes

MOP-mcherry

DOP-eGFP

DAPI-MERGE

5µm

B

MOP

DOP

objects/µm²

1.0
0.8
0.6
0.4
0.2
0.0

*
NS
*
NS

Membrane
Cytoplasm

Basal
CYM51010
Basal
CYM51010

C

Objects density
(membrane/cytoplasm)

4
3
2
1
0

****
**

Basal
CYM51010

MOP
DOP

D

MOP-DOP colocalisation (%)

30
20
10
0

###
NS
*
**

Membrane
Cytoplasm

Basal
CYM51010