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Double and triple in situ hybridization for coexpression studies: combined fluorescent and chromogenic detection of neuropeptide Y (NPY) and serotonin receptor subtype mRNAs expressed at different abundance levels

Maria Bonn · Angelika Schmitt · Esther Asan

Abstract Multiple fluorescence in situ hybridization is the method of choice for studies aimed at determining simultaneous production of signal transduction molecules and neuromodulators in neurons. In our analyses of the monoamine receptor mRNA expression of peptidergic neurons in the rat telencephalon, double tyramide-signal-amplified fluorescence in situ hybridization delivered satisfactory results for coexpression analysis of neuropeptide Y (NPY) and serotonin receptor 2C (5-HT2C) mRNA, a receptor subtype expressed at high-to-moderate abundance in the regions analyzed. However, expression of 5-HT1A mRNA, which is expressed at comparatively low abundance in many telencephalic areas, could not be unequivocally identified in NPY mRNA-reactive neurons due to high background and poor signal-to-noise ratio in fluorescent receptor mRNA detections. Parallel chromogenic in situ hybridization provided clear labeling for 5-HT1A mRNA and additionally offered the possibility to monitor the chromogen deposition at regular time intervals to determine the optimal signal-to-noise ratio. We first developed a double labeling protocol combining fluorescence and chromogenic in situ hybridization and subsequently expanded this variation to combine double fluorescence and chromogenic in situ hybridization for triple labelings. With this method, we documented expression of 5-HT2C and/or 5-HT1A in subpopulations of telencephalic NPY-producing neurons. The method developed in the present study appears suitable for conventional light and fluorescence microscopy, combines advantages of fluorescence and chromogenic in situ hybridization protocols and thus provides a reliable non-radioactive alternative to previously published multiple labeling methods for coexpression analyses in which one mRNA species requires highly sensitive detection.

Keywords Triple in situ hybridization · Coexpression · NPY · 5-HT1A · 5-HT2C

Introduction

Functional properties of neurons in the context of specific neuronal circuitries are largely determined by their production of particular neuromediators on the one hand, and of specific receptors for neurotransmitters from afferent systems on the other hand. Within the entire brain there are numerous subsets of neurons showing differential production of neuropeptides, which have been implicated in various functions particularly in limbic telencephalic areas (Asan 1998; Muller et al. 2003; Karson et al. 2009; Criado et al. 2011). Morphological studies have indicated that monoaminergic afferents contact subsets of peptidergic neurons in limbic and other cortical and subcortical areas of the rat brain (Asan 1997, 1998; Eliava et al. 2003; Yilmazer-Hanke et al. 2004; Asan et al. 2005). Determination of monoamine receptor expression of target neurons is a necessary prerequisite for further studies assessing the functional impact of this innervation. Multiple immunolabeling or combined immunolabeling/in situ hybridization (ISH), which are methods of choice for these analyses since

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the results prove synthesis of the proteins to be detected, are often limited in their applicability due to the fact that specificity of many receptor antibodies is questionable (e.g., McDonald and Mascagni 2007), and that the sensitivity of the detection systems is sometimes not sufficient to detect low quantities of receptor proteins in neuronal membranes. Moreover, peptides and receptors are often localized to specific subcellular compartments (e.g., neuronal somata vs. processes), and thus an existing coexpression might not be identifiable using immunolabeling. ISH avoids these problems, since the mRNAs are concentrated in the cell body and specificity of cRNA probes can be comparatively easily checked. ISH using enzyme-linked detection systems and chromogenic substrates such as nitro blue tetrazolium (NBT) provides the highest sensitivity (Trinh le et al. 2007), and additionally allows monitoring of the chromogen deposition during the enzyme reaction to determine optimal signal-to-noise ratio. Therefore, chromogenic ISH (CISH) protocols can be reliably used for detection of rare mRNAs. However, multiple CISH with, for instance, alkaline-phosphatase (AP) and horseradish peroxidase (POD)-linked detection systems cannot usually be combined for coexpression studies since perikaryal colocalization of two or more colored chromogenic reaction products cannot be unequivocally recognized.

Thus, multiple ISH using conventional light and fluorescence microscopy has to date been carried out, for instance, using radioactively labeled probes combined with CISH, or multiple fluorescence in situ hybridization (FISH) (Barlier et al. 1999; Denkers et al. 2004; Son and Winzer-Serhan 2009). Although the sensitivity of radioactive ISH is high, the method has its limitations specifically concerning cellular resolution and signal-to-noise ratio. Conventional FISH protocols allow a sufficient detection only of mRNAs expressed at high abundance (Breininger and Baskin 2000). Recently, tyramide signal amplification (TSA™) methods have provided FISH protocols yielding satisfactory results for expression and coexpression studies of mRNAs with moderate abundance, for instance peptide coexpression in the central amygdaloid neurons (Reyes et al. 2008). A further increase in the detection sensitivity appears possible by applying TSA and additional amplification using an enzyme-labeled fluorescent substrate method (Breininger and Baskin 2000). In the course of studies aimed at unraveling interrelations between monoaminergic afferents and peptidergic neurons in limbic and sensory areas, we conducted a series of experiments to assess the serotonin receptor (5-HTR) mRNA expression of neuropeptide Y (NPY) mRNA-reactive neurons in the rat telencephalon. NPY has been implied in important CNS functions, ranging from the regulation of food intake and sleep to epilepsy and depression (Vezzani et al. 1999; Sperk et al. 2007; Dyzma et al. 2010; Morales-Medina

et al. 2010; Kubota et al. 2011). Functionally important interactions between the serotonergic system and telencephalic NPY neurons have been suggested, such as an increase in NPY mRNA production in the amygdala upon application of serotonin reuptake inhibitors, and appear to be important with respect to stress response or depression-like behavior (e.g., Luo et al. 2008; Christiansen et al. 2011). Application of double TSA-FISH provided satisfactory results for the detection of 5-HTR subtype mRNAs expressed at high-to-moderate levels in NPY mRNA-producing neurons. However, the method proved to be insufficiently sensitive to detect coexpression of rare 5-HTR subtype mRNAs. To solve this problem, the present study was designed to develop ISH protocols suited for specific and reproducible detection of mRNA species expressed at low and moderate abundance in peptidergic neurons of rat brain.

Materials and methods

Generation of cRNA probes

Primer sequences specific for rat NPY (Forward: 5′-CAAG CTCATTCCTCGCAGA-3′; Reverse: 5′-AACGACAACA AGGGAAATGG-3′; GenBank accession: NM_012614), rat serotonin receptor 1A (5-HT1A, GenBank accession: NM_012585.1; see Albert et al. 1990) and rat serotonin receptor 2C (5-HT2C, GenBank accession: NM_012765.3; see Julius et al. 1988) mRNA were chosen, cDNA fragments in a range of 400–910 bp of size were amplified and cloned in the dual promotor pCR®II (Invitrogen, Karlsruhe, Germany; for NPY fragment), the pGEM®-T Vector (Promega, Mannheim, Germany; for 5-HT1A fragment) or the BlueScript SK (+) phagemid (Agilent Technologies, Waldbronn, Germany; for 5-HT2C fragment). The identity of the respective cDNA inserts was verified by sequencing. For the generation of antisense and sense cRNA probes, plasmids were linearized for 2–3 h at 37°C in a water bath using the appropriate restriction endonucleases. Correct product size was documented by analysis of linearized plasmids on an agarose gel [0.8% agarose, 0.005% Gel Red™ (Biotrend, Cologne, Germany) in 1× TAE buffer consisting of 0.02 M Tris, 0.05 M EDTA, pH 8.3]. Linearized plasmids were purified with phenol/chloroform extraction (using TE-saturated phenol, Roth, Karlsruhe, Germany) followed by ethanol precipitation at −20°C over night by adding 0.1 Vol 3 M sodium acetate (pH 5.2) and 2.5 Vol ethanol. Plasmids were centrifuged at 4°C for 30 min, pellets were washed once with 80% ethanol, centrifuged at 4°C for 15 min and air-dried. From now, RNase-free environment was maintained up to post-hybridization steps and all solutions were made with 0.05%

diethyl pyrocarbonate (DEPC)-treated water. In vitro transcription was performed according to the manufacturer's instructions for 2–3 h at 37°C in a water bath using Biotin-, Fluorescein- or Digoxigenin (DIG)-RNA labeling mix (all Roche, Mannheim, Germany) and the appropriate RNA polymerases (Fermentas, Leon-Rot, Germany). For single labelings, cRNAs were labeled using Biotin-, Fluorescein- or DIG-RNA labeling mixes. For double labelings, both Fluorescein- and DIG-labeled cRNA probes were used for NPY and 5-HT2C mRNA detection as required, for 5-HT1A mRNA detection only DIG-labeled cRNA was used. For triple labelings, NPY cRNA was labeled using Biotin-RNA labeling mix, 5-HT2C cRNA with Fluorescein-RNA labeling mix and 5-HT1A cRNA with DIG-RNA labeling mix. After in vitro transcription, template DNA was removed by adding 20 U DNase I (Roche), incubation was carried out for 15 min at 37°C in a water bath and aliquots were run on an agarose gel (see above) to control success of in vitro transcription. Then, samples were purified with phenol/chloroform extraction (using water-saturated, stabilized phenol, AppliChem, Darmstadt, Germany) followed by precipitation as mentioned above. Air-dried pellets were dissolved in DEPC-treated water and concentration and riboprobe quality was assessed spectrophotometrically. Probes can be stored at −20°C for several months. To make sure that the different cRNA probes used in multiple ISH do not cross-react, we aligned the cRNA sequences of interest before conducting ISH experiments using "MultAlin", a program for multiple sequence alignment (Corpet 1988).

Animals and tissue preparation

For the subsequent experiments, brains of 3-month-old Wistar rats purchased from Charles River (Sulzfeld, Germany) were used. Animal experiments were carried out according to the German Law for the Protection of Animals, and were designed to minimize number and suffering of animals used. Anaesthetized animals were decapitated, the brains were dissected and cut into coronal slices of approximately 5-mm thickness. The slices were immediately placed in Tissue Freezing Medium® (Leica Microsystems, Wetzlar, Germany) on cork supports, snap frozen in liquid nitrogen-cooled 2-methylbutane and stored at −80°C until use. 10-μm-thick serial coronal sections containing hippocampus and amygdala (Bregma −2.12 to −3.3 mm; Paxinos and Watson 2007) were cut on a cryostat set at −25°C and thaw-mounted on Superfrost Plus® microscopic slides (Menzel, Braunschweig, Germany). Afterwards, sections were fixed for 5 min in freshly prepared 4% paraformaldehyde (PFA) in 0.01 M phosphate-buffered saline (PBS) at 4°C, transferred to chilled ethanol and stored at 4°C until usage.

In situ hybridization

If not mentioned otherwise, all working steps were performed at room temperature. Rehydration and washing were carried out by placing the microscopic slides in glass jars containing approximately 60 ml of the appropriate solutions. If heating of solutions was required, jars were placed in a water bath. For pre-hybridization, hybridization, antibody incubation and incubation during detection steps, the sections were covered with 200 μl of the appropriate solutions per slide and placed in a humid chamber.

Tissue was rehydrated in a graded series of ethanol (95, 80 and 70%, 2 min each), briefly rinsed in 2× sodium saline citrate buffer (SSC, Sigma-Aldrich, Munich, Germany), treated with 0.02 N HCl for 5 min, rinsed again in 2× SSC (2 × 5 min) and treated with freshly prepared acetylation solution containing 0.25% acetic anhydride and 0.2% HCl in 0.1 M triethanolamine buffer for 20 min, as previously described (Asan et al. 2005; Gutknecht et al. 2009). After rinsing in 2× SSC (2 × 5 min), sections were covered with pre-hybridization solution (50% deionized formamide, 4× SSC, 10% dextran sulfate, 1× Denhardt's solution, 250 μg/ml salmon sperm DNA), and a piece of parafilm was placed upon the section to evenly spread the solution. Incubation was carried out for 1 h at 57–58°C in a hybridization oven. Then, pre-hybridization solution was removed and replaced by one (for single FISH or CISH), two (for double FISH or FISH/CISH) or three cRNA probes of interest (for double FISH/CISH triple labeling) diluted in pre-hybridization solution at appropriate concentrations. Again, sections were covered with parafilm and hybridization was carried out for 16–20 h at 57–58°C followed by post-hybridization washes: (1) 3 × 5 min in 2× SSC, (2) 50% formamide in 2× SSC at 58°C for 30 min, (3) 2× SSC (3 × 5 min), (4) RNase buffer (50 mM NaCl, 10 mM Tris, 1 mM EDTA, 40 μg/ml RNase A) at 37°C for 30 min and (5) RNase buffer without RNase A at 58°C for 30 min as described by Gutknecht et al. (2009). Afterwards, tissue was rinsed in washing buffer (100 mM Tris, 150 mM NaCl, pH 7.5) for 5 min and then treated with 0.5% blocking reagent (Perkin Elmer) diluted in washing buffer for 30–120 min.

Detection protocols

For *single CISH*, sections were subsequently incubated in AP-conjugated anti-Fluorescein (1:600, Roche) or anti-DIG Fab fragments (1:600, Roche) diluted in antibody incubation buffer (0.25% blocking reagent, 0.15% Triton X-100, 100 mM Tris, 150 mM NaCl) for 2.5 h, rinsed in washing buffer (2 × 5 min) and AP buffer (100 mM Tris, 100 mM NaCl, 50 mM magnesium chloride, pH 9.5) for 5 min and subjected to chromogenic detection with NBT/5-bromo-4-chloro-3′-indolyphosphate (BCIP; Roche; stock

solution diluted 1:50 in AP buffer) for 16–20 h. Development of the enzyme reaction was carried out in the dark and chromogenic signal intensity was controlled by brief microscopic observation at regular intervals until the desired intensity of staining was achieved. Chromogenic reaction was terminated by washing in stop buffer (10 mM Tris, 0.1 mM EDTA, pH 7.5; 2 × 5 min).

For *single FISH*, three different detection protocols were applied: (1) blocked and hybridized sections were rinsed in washing buffer (3 × 5 min), incubated for 2.5 h in POD-conjugated anti-DIG (1:1,000, Roche) or POD-conjugated anti-Fluorescein Fab fragments (1:1,000, Roche) diluted in antibody incubation buffer, rinsed in washing buffer (3 × 5 min), reacted with TSA Biotin working solution (Perkin Elmer) for 30 min, rinsed in washing buffer (3 × 5 min) again and further incubated in the dark with AlexaFluor® 488-conjugated streptavidin (Invitrogen; 5 μg/ml washing buffer) for 30 min. (2) Sections incubated with POD-conjugated Fab fragments as described above were rinsed in washing buffer (3 × 5 min) and reacted with TSA Cy3 working solution (Perkin Elmer) for 7 min. (3) Sections reacted with a Biotin-labeled cRNA probe were incubated for 30 min with POD-conjugated streptavidin (Perkin Elmer, 1:500 in antibody incubation buffer) rinsed in washing buffer (3 × 5 min) and then reacted with TSA Cy3 working solution for 7 min. Since analyses described below showed that these three mRNA detection systems differed in sensitivity [(1) > (2) > (3)], we designated these techniques high sensitivity *(HS)-TSA-FISH*, moderate sensitivity *(MS)-TSA-FISH* and low sensitivity *(LS)-TSA-FISH*, respectively.

For *double FISH*, first HS-TSA-FISH was applied to detect one of the cRNA probes. Reacted sections were then subjected to a POD block in 0.02 N HCl for 15 min and rinsed in washing buffer (3 × 5 min) followed by MS-TSA-FISH to detect the second cRNA probe. For *FISH/CISH*, the appropriate combinations of POD- and AP-conjugated Fab-fragments were applied simultaneously. Fluorescent labeling of cRNA was carried out using HS-TSA-FISH, followed by chromogenic detection with NBT/BCIP, which was carried out as described for single CISH. For *triple labelings (double FISH/CISH)*, sections were first subjected to LS-TSA-FISH. After POD block, double FISH/CISH was performed as described above. For all variations, except for single CISH, staining of nuclei was carried out for 5 min using 300 nM DAPI (Roche) diluted in washing buffer.

Protocol optimization experiments

For each cRNA probe optimal hybridization temperature was determined in single CISH labelings. With NPY and 5-HT2C probe, identical results were obtained between 57 and 60°C, whereas 5-HT1A probe delivered optimal and identical results only between 57 and 58°C. Furthermore,

different cRNA probe concentrations between 2 and 20 ng/μl were tested at different hybridization temperatures and best results were achieved using 10 ng/μl for rat NPY and rat 5-HT2C and 15 ng/μl for rat 5-HT1A. Also antibody concentrations, specificity, and incubation times for all probes were optimized in single CISH single labelings. To compare the influence of the hapten used for probe labeling and its detection method on ISH sensitivity, CISH and HS-TSA-FISH were carried out for each cRNA probe using both Fluorescein- and DIG-labeled cRNA probes. Additionally, HS-TSA-FISH results were compared with MS- and LS-TSA-FISH results if necessary.

During the test experiments for multiple labelings, signal intensities for CISH and HS-TSA-FISH were compared to those observed in single labelings to check whether chromogen development and signal interfered with FISH signal detectability and vice versa. For these experiments, sections were covered with coverslips in buffer after the different FISH protocols, and the fluorescence signal intensities and distribution were briefly studied. Subsequently, the coverslips were removed by dipping the slides in washing buffer, and the reaction sequence was continued. During the final step of CISH detection, the sections were incubated in NBT/BCIP solution and regularly observed using brief exposition with low illumination until the signal was comparable to signals detected in parallel single CISH labeling. After chromogen deposition was terminated, the fluorescent signal in the HS-TSA-FISH/CISH double-labeled sections was studied again and compared to the observations before chromogenic reaction and to parallel single HS-TSA-FISH reactions. Experiments indicated that simultaneous hybridization of different cRNA probes at 57–58°C and application of probe concentrations in the range determined for single labelings yielded high sensitivity and signal-to-noise ratio for combined detections of all mRNA species.

Controls

Probe specificity was checked by substitution of the respective antisense cRNA probes with an equivalent amount of labeled sense cRNA probe and by omission of cRNA probes. In multiple ISH reactions, cross controls (mix of antisense and sense cRNA probes followed by detection using the double/triple detection protocols) were carried out for all probe combinations and detection protocols and the results were compared with single ISH for the antisense cRNA probes using the appropriate detection protocol for these probes.

Mounting and microscopic analysis of reactions

After detection procedure and rinsing in washing buffer (2 × 5 min), sections were mounted using Aquatex® (for

single CISH; Merck, Darmstadt, Germany) or Fluoro-Gel (for single and double FISH; Science Services, Munich, Germany) and observed with a Zeiss axioscope (Zeiss, Oberkochen, Germany) equipped with bright field illumination and appropriate fluorescence filter systems for AlexaFluor® 488, Cy3 and DAPI fluorescence detection. To determine which mounting medium was best suited for FISH/CISH-reacted sections, the stability of the chromogenic and fluorescence signals under bright field and epifluorescence illumination over time was compared after mounting FISH/CISH-reacted sections in Aquatex® (Merck, Darmstadt, Germany), Fluoro-Gel (Science Services, Munich, Germany) and, additionally, using a 60% glycerol/PBS mixture containing 1.5% *n*-propyl gallate (NPG) as an anti-fading additive (Valnes and Brandtzaeg 1985). Slides were stored until analysis in light-tight boxes at 4°C. Microphotographs were made using a digital Spot camera (Visitron Systems, Puchheim, Germany) and the Spot Advanced software (Diagnostic Instruments, Inc., Sterling Heights, MI, USA). Identified neurons were only evaluated as reactive for the different mRNA species if the signals were associated with a morphologically recognizable (CISH-reactions) and/or DAPI-labeled nucleus (FISH and FISH/CISH-reactions).

Results

Comparative single labelings

Optimal cRNA probe concentration, hybridization temperature and conditions for all cRNA probes were determined in CISH single labelings. After appropriate adaptation of these conditions, CISH for all studied mRNA species showed high signal intensity, high signal-to-noise ratio and lack of labeling in sense controls (Fig. 1a, b, e, f, i, j, m, n). The signal distribution patterns in HS-TSA-FISH and corresponding CISH reactions were similar (Fig. 1a, c, e, g, i, k, m, o); however, the signal-to-noise ratio was generally lower due to background labeling (small fluorescent spots) of different intensity, as shown in FISH sense controls (Fig. 1, forth column). While for FISH experiments, the choice of hapten in cRNA labeling (Fluorescein or DIG) had no impact on signal distribution and intensity, highest signal-to-noise ratio was achieved using DIG-labeled probes and AP-conjugated anti-DIG Fab fragments in CISH (not shown). Therefore, only this detection system was applied for CISH in multiple labelings in order to ensure optimal detection sensitivity.

CISH detection of NPY mRNA yielded clear signals in all telencephalic regions studied, with a distribution consistent with the pattern described in the literature (e.g., Quidt de and Emson 1986). Signal intensity was similar in CISH and HS-TSA-FISH preparations, for instance in numerous neurons of the hilus of the dentate gyrus (DG; Fig. 1a, c). In isocortical areas and cortex-like amygdaloid nuclei such as the lateral nucleus (La), numerous neurons displaying high, moderate and low NPY-mRNA content were detectable (Fig. 2a). Comparison of HS-, MS- and LS-TSA-FISH for NPY mRNA showed that MS-TSA-FISH was only slightly less sensitive than HS-TSA-FISH, detecting numerous NPY mRNA-positive neurons displaying high-to-low mRNA content (Fig. 2b). Using LS-TSA-FISH, comparatively fewer NPY-positive cells were observed, indicating that with this method neurons with comparatively low content of NPY mRNA were below detection limits (Fig. 2c).

CISH for 5-HT2C mRNA showed numerous reactive neurons displaying moderate-to-high levels of mRNA for instance in the DG granular cells, in cortical areas and in the amygdala (e.g., La; Fig. 1e), and similar results were observed using HS-TSA-FISH (Figs. 1g, 2d). Again, the results were consistent with the pattern described in the literature for expression of this receptor subtype (e.g., Mengod et al. 1990). Background labeling, particularly using HS-TSA-FISH, appeared somewhat higher than in ISH for NPY mRNA (cf. Fig. 2a, d). This was even more apparent when 5-HT2C mRNA was detected using MS-TSA-FISH, where the signal-to-noise ratio was too low to allow unequivocal identification of labeled neurons (Fig. 2e). LS-TSA-FISH was not tested for this cRNA probe.

For 5-HT1A mRNA, ISH signals in the CA pyramidal and the granule cells of the DG, which have been shown to express high levels of this receptor mRNA (Miquel et al. 1991), were comparatively strong using CISH (Fig. 1i), and were also recognizable using HS-TSA-FISH (Fig. 1k). However, unspecific background labeling was significantly higher in HS-TSA-FISH compared to CISH. Thus, while CISH showed very little labeling for instance in the hilus of the DG, numerous small fluorescent dots were observed in this region in HS-TSA-FISH preparations (Fig. 1i, k). In other regions such as the amygdala, where 5-HT1A mRNA expression is reported to be comparatively low (Miquel et al. 1991), detection of 5-HT1A antisense probes with CISH was precise and specific since the signals, although very discrete, were clearly detectable and could be assigned to specific cells (Fig. 1m). HS-TSA-FISH detection merely showed an increased density of fluorescent dots in the vicinity of DAPI-stained nuclei compared to the sense controls (Fig. 1o, p). Since these fluorescent dots were frequently quite dispersed, HS-TSA-FISH signals in regions with closely spaced neurons and comparatively low expression levels of 5-HT1A could often not be assigned to defined cell bodies, preventing unequivocal identification of labeling in specific cells. Thus, satisfactory
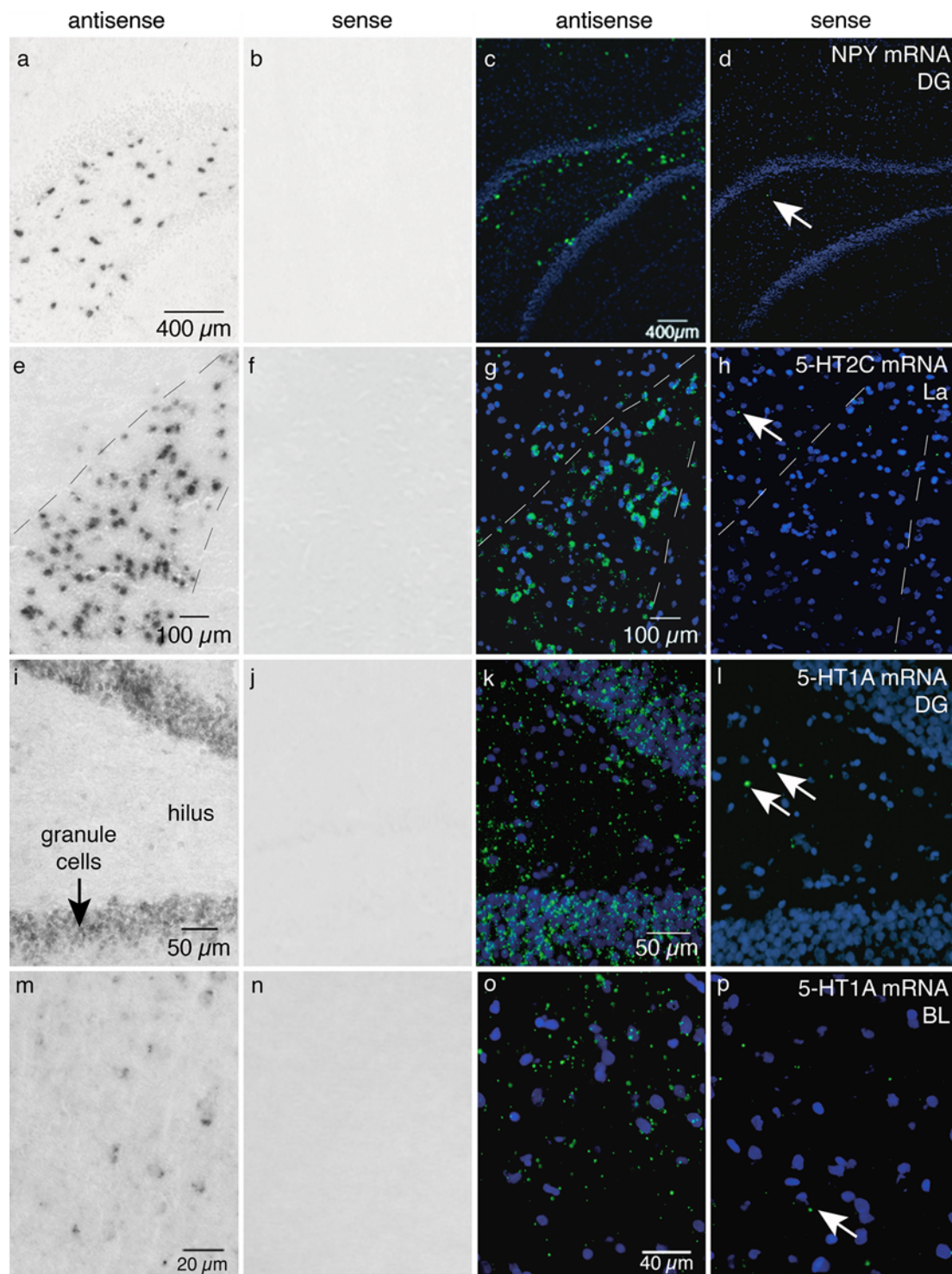
Fig. 1 Comparative single ISH for NPY and serotonin receptors in different brain areas. Both CISH (**a**) and FISH (**c**) signals are strong and clear for NPY mRNA in the DG. **e**, **g** Signal intensities of 5-HT2C mRNA-reactive neurons in the La range from high-to-moderate in CISH (**e**), equivalent signals are found in HS-TSA-FISH (**g**). **i**, **m** CISH signals for 5-HT1A mRNA are of moderate and low intensity but clearly identifiable with high signal-to-noise ratio in granule cells of the DG (**i**) and in neurons of the BL (**m**), respectively. **k**, **o** Corresponding HS-TSA-FISH signals are weak in the DG granule cells with high background of multiple fluorescent dots in the hilus (**k**), and dispersed between nuclei in the BL (**o**), preventing unequivocal identification of labeled cells in the latter. Sense controls in CISH are virtually devoid of labeling (**b**, **f**, **j**, **n**), but contain occasional small fluorescent dots in HS-TSA-FISH (arrows in **d**, **h**, **I**, **p**). In all FISH images (third and fourth column) nuclei were stained using DAPI. *Scale bars* in (**a**, **c**, **e**, **g**, **i**, **k**, **m**, **o**) are also valid for (**b**, **d**, **f**, **h**, **j**, **l**, **n**, **p**), respectively
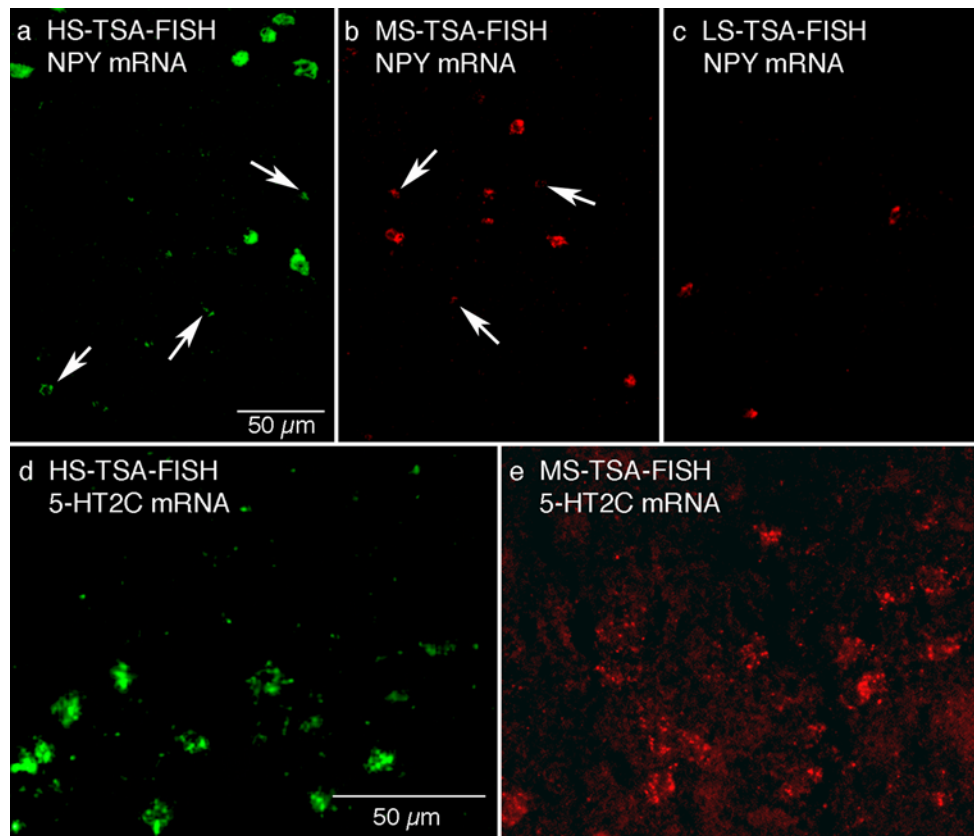
Fig. 2 Comparison of different FISH protocols for NPY and 5-HT2C mRNA in La. For NPY mRNA, best results were achieved applying high sensitivity (HS)- and moderate sensitivity (MS)-TSA-FISH (**a**, **b**). *Arrows* indicate low-expressing NPY mRNA-positive cells. With low sensitivity (LS)-TSA-FISH, only high-expressing NPY mRNA-positive neurons were detectable (**c**). For 5-HT2C mRNA, only HS-TSA-FISH was sufficiently sensitive (**d**); using MS-TSA-FISH, signal-to-noise ratio was low (**e**). *Scale bar* in **a** is also valid for **b** and **c**, *scale bar* in **d** also for (**e**)

and unequivocal 5-HT1A mRNA detection was only possible using CISH. MS- and LS-TSA-FISH were not tested for 5-HT1A mRNA since based on the findings for NPY mRNA detection using these different methods (see above), sufficient sensitivity was not expected.

Multiple ISH

Mounting media and microscopic analysis

Mounting media might exert differential effects on diffusion or stability of the chromogen and on fluorescence detectability and fading properties of the fluorescent dye. For CISH and FISH preparations, different mounting media are recommended, such as Aquatex® and Fluoro-Gel, respectively. Another common mounting medium for fluorescent preparations is a 60% glycerol/PBS mixture containing 1.5% NPG as an anti-fading additive (Valnes and Brandtzaeg 1985). Since in pilot experiments deterioration of signal quality and increased deposition of unspecific signals had been observed in FISH/CISH preparations mounted with different media, we conducted a

systematic study to determine the most suitable mounting medium and identified possible pitfalls in the analysis procedures by testing the influence of these media and of different illumination conditions (bright field, fluorescence excitation using filter systems for DAPI, AlexaFluor® 488 and Cy3-detection) on signal detectability and unspecific staining over time. Results for bright field and DAPI illumination are shown in Fig. 3. In sections mounted with Aquatex®, which provided optimal stability for CISH preparations under all illumination conditions and over time (Fig. 3a–b′), fluorescence of AlexaFluor® 488 and DAPI was rapidly bleached under all illumination conditions in FISH preparations (Fig. 3c–d′). Fluorescence fading of FISH signals in FISH/CISH preparations was reduced after mounting with Fluoro-Gel (not shown) and there was no increased unspecific staining after bright field illumination (Fig. 3e, e′) or after illumination using filter systems for AlexaFluor® 488 and Cy3-detection (not shown). However, Fluoro-Gel led to increases in unspecific, especially nuclear staining, particularly in DAPI-stained FISH/CISH preparations after 20 min of UV illumination (DAPI filter; Fig. 3f, f′). Five weeks after
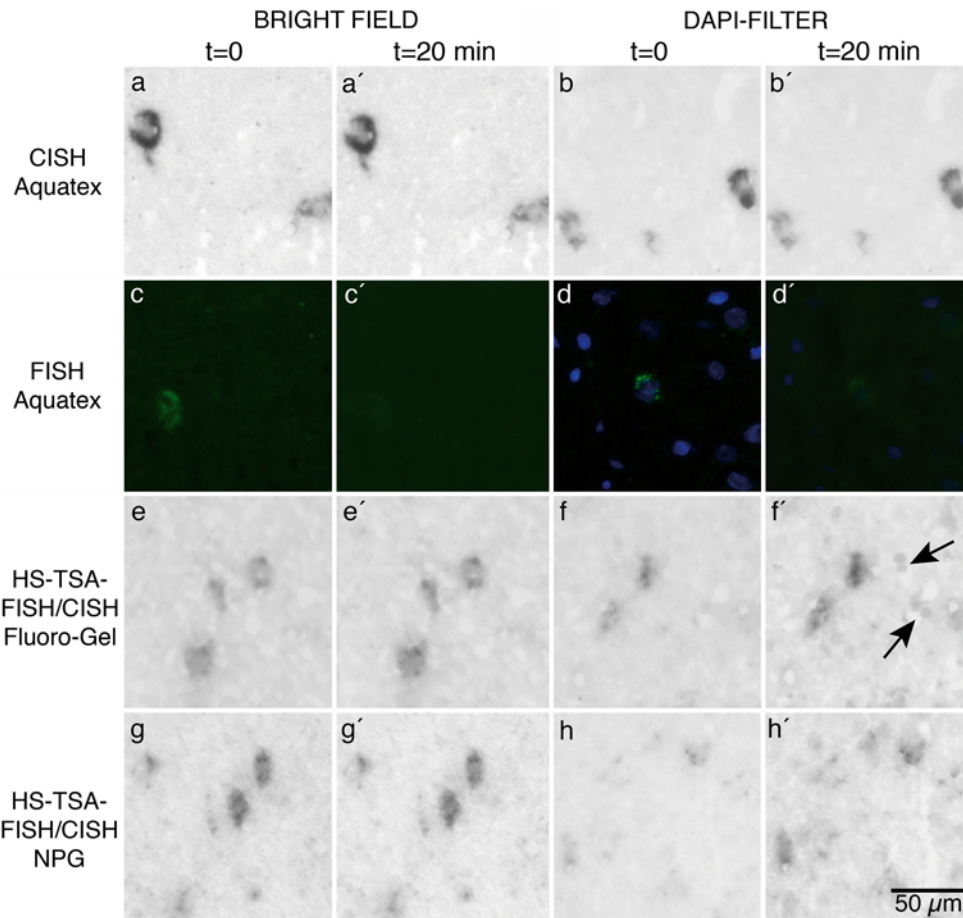
Fig. 3 Selected images documenting the impact of different mounting media and illumination conditions on signal detectability and unspecific staining over time in different cortical areas (for further details see text). (**a**, **a′**, **b**, **b′**) Mounting of CISH preparations with Aquatex®, as recommended, yielded clear signals, illumination was without effect. (**c**, **c′**, **d**, **d′**) FISH preparations mounted with Aquatex® showed rapid fluorescence fading. (**e**, **e′**, **f**, **f′**, **g**, **g′**, **h**, **h′**) HS-TSA-FISH/CISH sections mounted with Fluoro-Gel (**e**, **f**) and especially NPG (**g**, **h**) showed increased unspecific staining of the CISH reaction product after prolonged illumination particularly using UV-excitation (DAPI-Filter). *Scale bar* in (**h′**) is also valid for (**a–h**)

mounting with Fluoro-Gel the NBT/BCIP signal got weaker and started to become blurry. In FISH/CISH preparations mounted with NPG-containing medium, there was no increased unspecific staining after bright field illumination (Fig. 3g, g′) but these preparations displayed very high unspecific background staining after 20 min illumination for fluorescence excitation at all wavelengths, for instance after UV illumination (Fig. 3h, h′).

Therefore, Fluoro-Gel was used for mounting of all sections subjected to FISH, and, sections were analyzed within a few days after preparation. During analysis, single or double fluorescence-labeled neurons were localized at low magnification first and, if applicable, colocalization of fluorescence was briefly evaluated. Then, the CISH signal was recorded in the same area at higher magnification. Finally, photographs were taken using bright field illumination and fluorescence excitation. To avoid increase of unspecific nuclear staining, the DAPI signal was watched as briefly as possible, and photographs of the DAPI reaction were taken at the end of analysis.

Detection specificity and sensitivity for the different mRNA species

Test experiments comparing detectability of the reaction products for the different mRNA species in multiple and in parallel single labelings showed that, under optimal conditions, detection specificity and labeling efficiency in double labelings were similar to those achieved in parallel single labelings using the same detection protocols for the individual mRNAs (cf. Figs. 1, 2 and 4, 5). Since with LS-TSA-FISH comparatively fewer NPY-positive cells were observed than using MS-, HS-TSA-FISH or CISH (see above), triple labelings were only qualitatively evaluated.
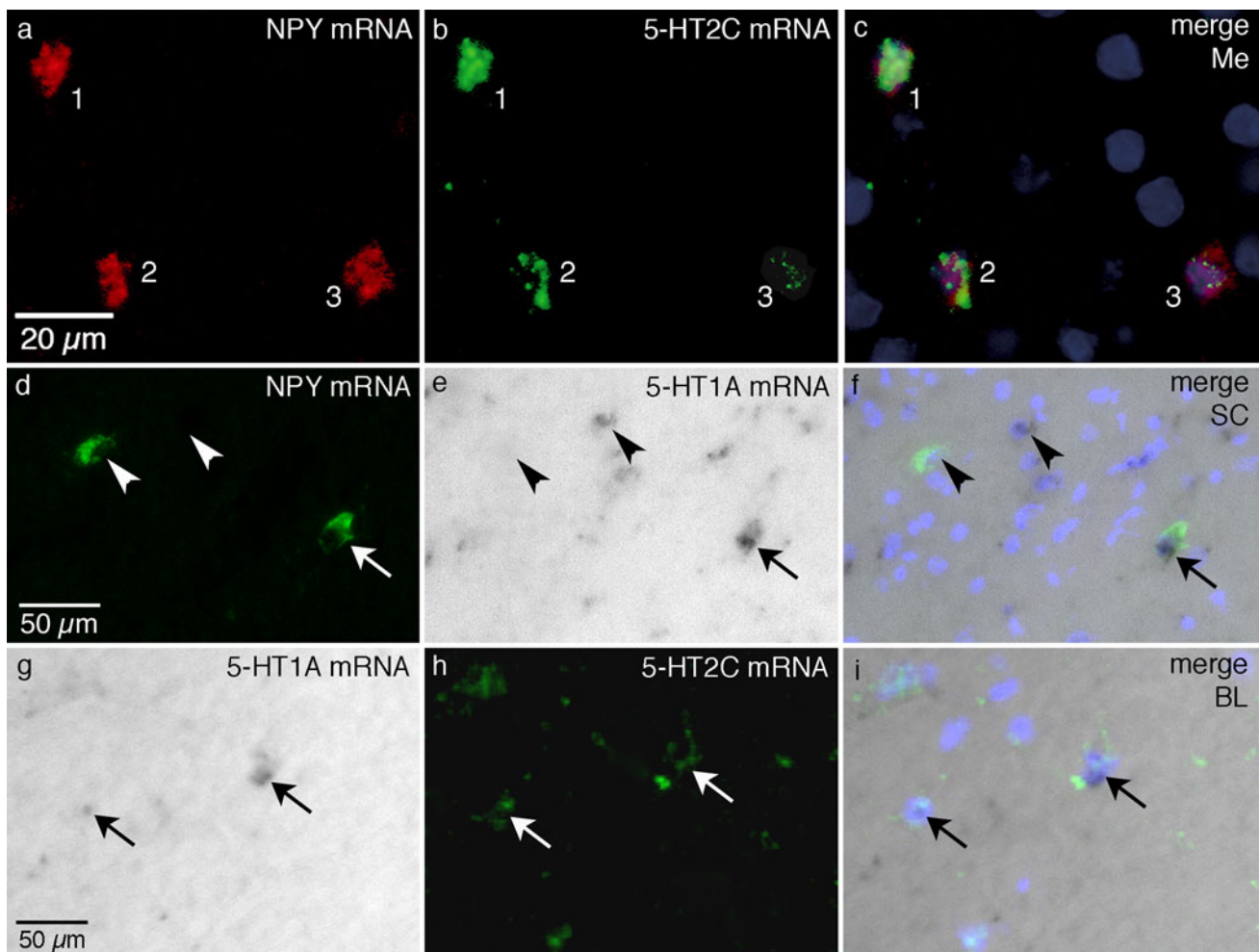
Fig. 4 Coexpression of NPY, 5-HT2C and 5-HT1A mRNA in different brain areas. **a** MS-TSA-FISH for NPY mRNA and **b** HS-TSA-FISH for 5-HT2C mRNA in the Me at high (*1*), moderate (*2*) and low (*3*) expression level. In (**c**) both images are merged with DAPI staining and coexpression of both mRNAs is visible. **d** HS-TSA-FISH for NPY mRNA and **e** CISH for 5-HT1A mRNA in the SC. In (**f**) both images are merged with DAPI staining and coexpression of both mRNAs is visible (*arrow*). *Arrowheads* in (**d–f**) point to single-labeled NPY and 5-HT1A mRNA-positive cells. **g** CISH for 5-HT1A mRNA and **h** HS-TSA-FISH for 5-HT2C mRNA in the BL. In (**i**) both images are merged with DAPI staining and coexpression of both mRNAs is visible (*arrows*). *Scale bars* in (**a**, **d**, **g**) are also valid for (**b**, **c**), (**e**, **f**) and (**h**, **i**), respectively

Results of multiple labelings

Double TSA-FISH delivered satisfactorily sensitive labeling for coexpression studies of NPY and 5-HT2C mRNA in different brain regions applying MS- and HS-TSA-FISH, respectively. Coexpression of 5-HT2C mRNA was found in subpopulations of NPY mRNA-producing neurons of different areas. The relative frequency of 5-HT2C mRNA expressing among the entire population of NPY-producing neurons differed in a region-specific way. Thus, although systematic quantitative studies were not carried out, it appeared that while most NPY mRNA-reactive neurons of the medial amygdaloid nucleus (Me) showed receptor mRNA-reactivity (Fig. 4a–c), the subpopulation of 5-HT2C coexpressing NPY mRNA-reactive neurons in cortical areas, for instance the somatosensory cortex (SC), was lower (Fig. 5a–c). Additionally, the method enabled identification of different expression levels of the receptor in NPY neurons (Fig. 4a–c). In all regions analyzed, there were additional 5-HT2C mRNA-reactive neurons devoid of NPY mRNA (not shown).

Coexpression of 5-HT1A mRNA could not be identified in NPY mRNA-reactive neurons unequivocally using double TSA-FISH due to the high background labeling in 5-HT1A FISH (see above). Therefore, HS-TSA-FISH/CISH was carried out for this combination of cRNA probes and yielded clear colocalization results, e.g., in the SC (Figs. 4d–f, 5). Again, 5-HT1A mRNA-reactive neurons represented a subpopulation of NPY mRNA-producing neurons and the number of 5-HT1A mRNA-positive cells
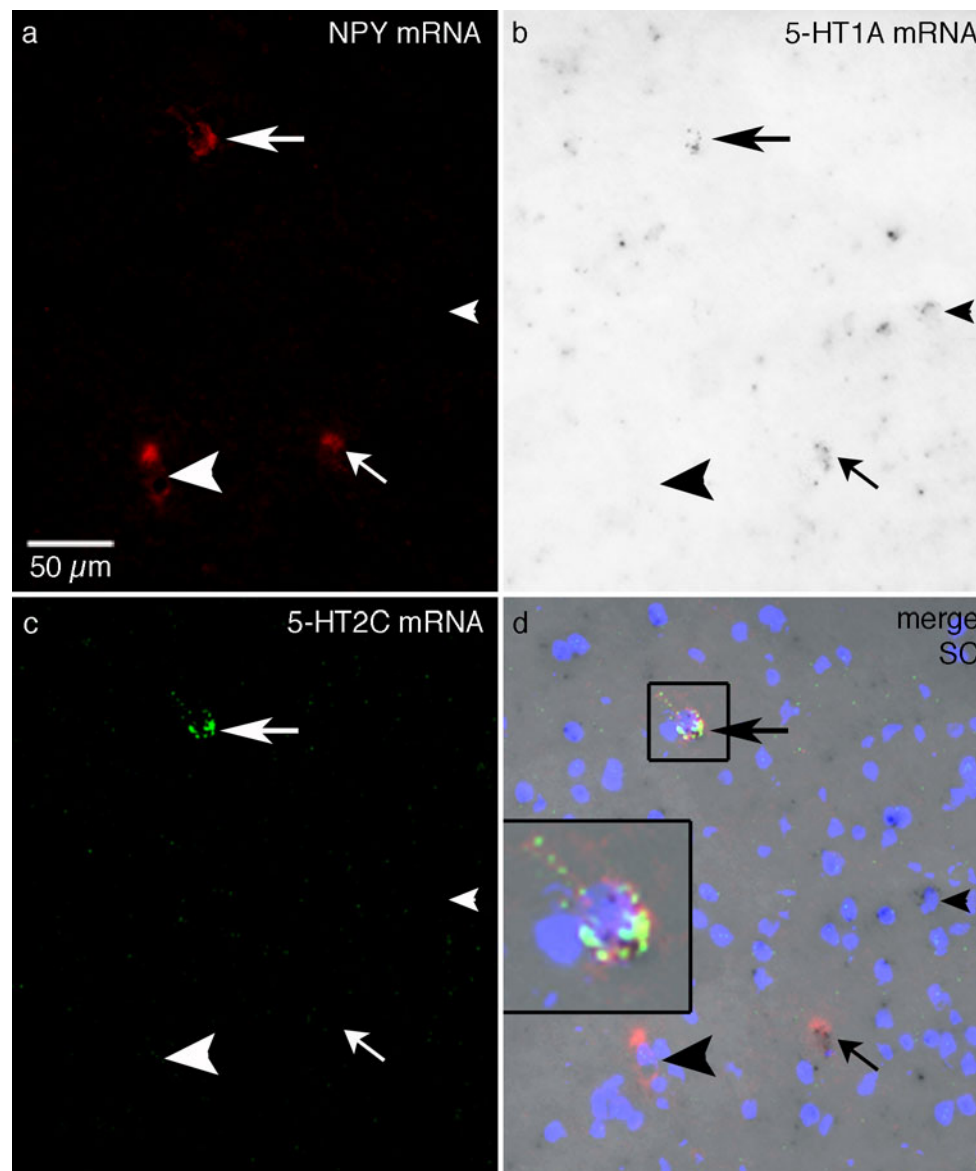
Fig. 5 Triple ISH for **a** NPY, **b** 5-HT1A and **c** 5-HT2C mRNA in the SC. **d** Shows a merging of the three images with DAPI staining. *Large arrows* point to a triple-labeled cell, shown at higher magnification in the inset in (**d**), *smaller arrows* to a cell double-labeled for NPY and 5-HT1A mRNA. *Large* and *small arrowheads* indicate cells single-labeled for NPY and 5-HT1A mRNA, respectively. *Scale bar* in (**a**) is also valid for (**b–d**)

was much higher than that of double-labeled neurons (Figs. 4d–f, 5a, b, d). HS-TSA-FISH/CISH was also successfully applied to detect coexpression of 5-HT1A and 5-HT2C mRNA, for instance in the basolateral nucleus of the amygdala (BL; Fig. 4g–i).

From these well-established double labelings, we developed a novel variation of triple ISH applying a combination of double TSA-FISH and CISH. Although it is likely that not all NPY-producing neurons were detected in these preparations since the LS-TSA-FISH method was used for NPY mRNA detection, we were able to provide unequivocal proof of coexpression of both 5-HTR mRNAs in NPY mRNA-reactive neurons, e.g., in the SC (Fig. 5). Specificity

of the mRNA detection in the triple labelings was supported by the fact that in the same sections neurons coexpressing only NPY and 5-HT1A or only NPY and 5-HT2C mRNA (cf. Fig. 4a–c) in addition to single-labeled NPY and 5-HT1A mRNA-reactive neurons were observed (Fig. 5).

Discussion

In the present study, we developed a protocol combining TSA-FISH with highly sensitive CISH, and succeeded in a comparatively simple, specific and reproducible detection of coexpression of low- and moderately abundant 5-HTR

subtype mRNAs in NPY mRNA-producing neurons. This protocol complements conventional double TSA-FISH protocols suited to study coexpression of high to moderately expressed receptor mRNA species. Additionally, a combination of double FISH and CISH provided a novel variation of triple ISH to detect coexpression of multiple mRNAs in individual neurochemically identified neural cell populations. Using these techniques, we succeeded in documenting expression of 5-HT1A and/or 5-HT2C receptors in subpopulations of NPY mRNA-producing neurons of cortical and subcortical rat telencephalic areas. The application of combined FISH/CISH presents a practicable option for coexpression studies of mRNAs expressed at different abundance levels, has a broad applicability and can be analyzed by conventional light and fluorescence microscopy.

Methodical considerations

To date, most double ISH methods, which have been applied for coexpression studies in the nervous system, have been limited in their applicability for different reasons.

Combinations with radioactive ISH suffer from handling problems and low cellular resolution (Hara et al. 1998), non-amplified FISH methods often possess comparatively low sensitivity (Raap et al. 1995), and sometimes even TSA-FISH is not sufficiently sensitive as described in the present study. More recent attempts to extend the sensitivity of FISH for detection of rare mRNA species by applying further amplification steps of TSA-FISH, for instance using an additional enzyme-labeled fluorescent method, suffer from rather high unspecific staining due to the frequent formation of unspecific fluorescent crystals and require continuous adaptation and control of reaction times and conditions (Breininger and Baskin 2000). The difficult handling might be a reason why these amplification methods have not become widely used. A significant advantage of the present protocol for combined TSA-FISH and CISH for simultaneous detection of multiple mRNA species is the fact that the individual ISH techniques combined are well established, and that CISH detection provides particularly high sensitivity. An additional advantage is the manageability of the chromogenic reaction since the rather slow development of the signal in the NBT/BCIP reaction can be

Fig. 6 Method guide for planning multiple ISH combining FISH and CISH
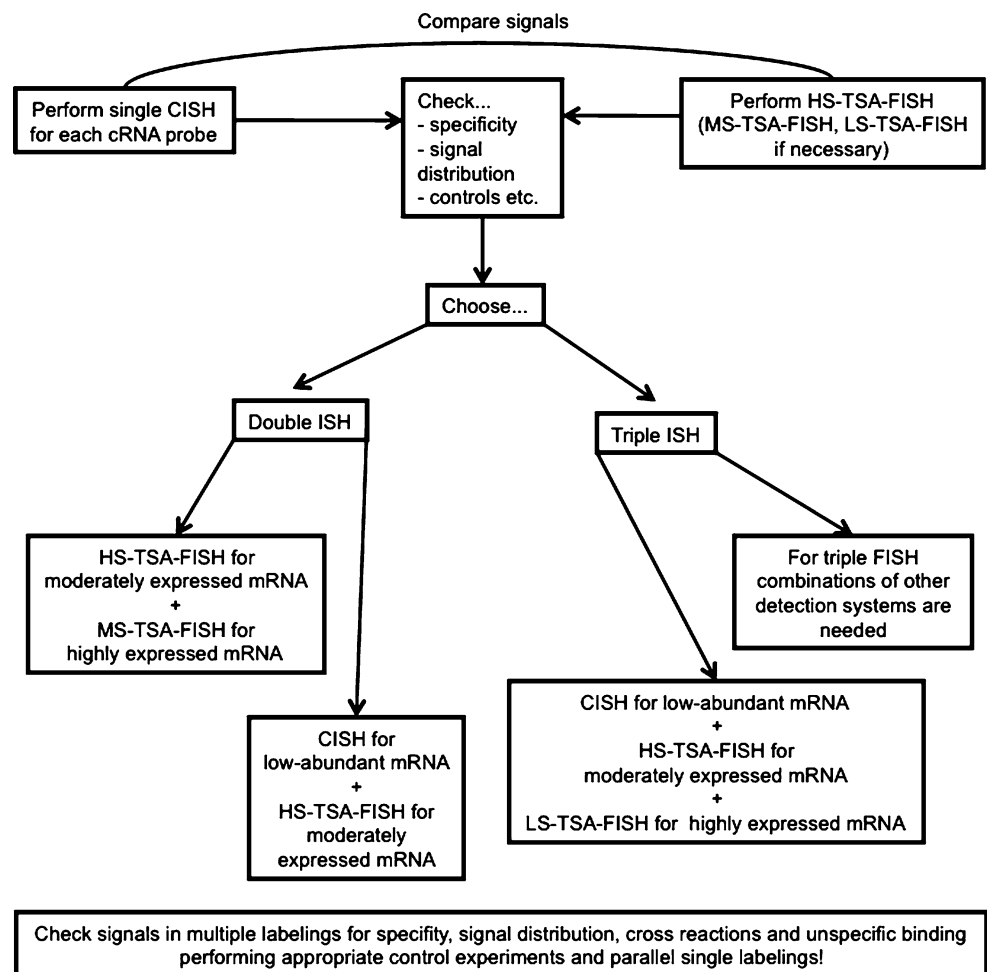
controlled without any risk of development of unspecific reaction products, and the development can be terminated as soon as the optimal signal-to-noise ratio is achieved. Therefore, the working protocol is straightforward, comparatively simple and reproducible, and can be easily adapted to simultaneous detection of other mRNAs of interest. Additionally, the high and reproducible detection sensitivity in double labelings enables semi-quantitative evaluation of coexpression incidence. Thus, in our experiments, semi-quantitative evaluation of the relative number of NPY neurons coexpressing 5-HT1A or 5-HT2C mRNA among all NPY neurons in the different brain areas appears possible. For triple labelings, the protocol takes only about 1 h longer than HS-TSA-FISH/CISH double labelings and no additional IHC procedure or radioactive isotopes are required. Another positive aspect of the protocol is the fact that tissue can be inspected by conventional light and fluorescence microscopy, and thus by eye. Besides allowing for intermittent monitoring of the procedure after all steps, visual examination ensures brief UV and visible light exposition times, thus reducing bleaching and unspecific chromogen deposition. Moreover, problems of unmixing of autofluorescence and of different fluorescence excitation spectra occurring when multiple fluorophores are applied and analyzed using confocal laser systems (e.g., quantum dots and organic fluorophores; Chan et al. 2005), are avoided. However, before double and triple ISH experiments are performed, some important caveats have to be observed to provide optimal results using this method. Figure 6 shows a concept for planning optimal experimental design. Thus, coexpression studies should be preceded and accompanied by comparative single HS-TSA-FISH and CISH. These experiments serve to establish receptor mRNA expression levels and detectability using different probes and detection protocols as described for the probes used in the present experiments and are a necessary prerequisite for choosing appropriate ISH protocols for multiple labeling. If applicable, double FISH is preferable to FISH/CISH combinations since it presents even less pitfalls. For instance, limited stability of the NBT/BCIP under UV light in combination with a fluorescence mounting medium can complicate analysis and documentation. Additionally, semi-quantitative analyses of multiple ISH results can only be carried out if the detection sensitivity of mRNA species to be analyzed is comparable in single and multiple labelings. Thus, since in our triple labelings NPY mRNA detection obviously did not detect all NPY-producing neurons, only qualitative analyses should be carried out on these preparations. Also, since each cRNA probe requires a different range of hybridization temperature, multiple labelings yielding identical sensitivity for each probe compared to corresponding single labelings might sometimes not be achievable. Then, only qualitative evaluation should be done.

However, if these caveats are considered, the described method provides a significant technical improvement for investigations not only into the receptor complement of identified neuronal subtypes, but also into coexpression of proteins expressed at different abundance levels in other tissues.

Functional implications

Sensory and limbic cortical and subcortical regions of the rat brain are innervated by serotonergic afferents arising from the dorsal and median raphe nuclei, which influence projection cells and interneurons via synaptic contacts and in a paracrine mode (e.g., Beaudet and Descarries 1976; Papadopoulos et al. 1987; Jacobs and Azmitia 1992; Vertes et al. 1999; Michelsen et al. 2007; Muller et al. 2007). Serotonergic signal transduction, particularly via the 5-HT1A and 5-HT2C receptor subtypes, has been shown to be relevant for processing of sensory and emotionally relevant stimuli, and imbalances in these systems are thought to be involved in the etiopathology of affective disorders such as depression and anxiety (Lowry et al. 2005; Ogren et al. 2008; Iwamoto et al. 2009). Via activation of the two receptor subtypes, serotonin exerts differential influence on the target neuron. 5-HT1A activation has been reported to produce cell hyperpolarization due to the opening of G protein-gated inwardly rectifying potassium channels (Luscher and Slesinger 2010). In contrast, 5-HT2C activation facilitates cell depolarization by inhibition of specific potassium channels promoting potassium outflow (Weber et al. 2008). Previous studies have documented expression of 5-HT1A and 5-HT2C in pyramidal cells and GABAergic interneurons in limbic and other cortical areas of the rat brain (Aznar et al. 2003; Amargós-Bosch et al. 2004; Puig et al. 2010). Coexpression of both receptor subtypes has to date only been indicated for cortical pyramidal cells (Yuen et al. 2008). The present findings documenting the expression of 5-HT1A and 5-HT2C mRNA in subpopulations of cortical and subcortical NPY-producing neurons provide the first morphological evidence for a functional link between serotonergic and NPY systems. Additionally, our first triple ISH experiments supply proof that some NPY mRNA-producing cortical interneurons coexpress both 5-HTR. Since NPY neurons in cortical areas represent a small subpopulation of GABAergic interneurons, the results complement previous studies and show that, like pyramidal cells, interneurons may express multiple 5-HTR subtypes. The effects of serotonin on NPY neurons producing one or both 5-HTR may depend on extracellular serotonin concentration and sensitization/desensitization of these receptors. It also appears possible that NPY neurons bearing different 5-HTR might be innervated by different subsets of serotonergic afferents

(see Lowry et al. 2005). The methods documented in the present study enable further detailed analyses of the entire 5-HTR mRNA expression of NPY-producing neurons particularly in limbic brain areas. These data will provide the basis for electrophysiological and connectivity studies of serotonin-NPY interactions, and may thus further our understanding of the role of these important neuromediator systems in CNS functions.

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