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Chemoconnectomics: Mapping Chemical Transmission in *Drosophila*

Highlights

- The authors propose a new concept of the chemoconnectome (CCT) for chemical transmission
- Chemoconnectomics with genetic tools is a new approach for neural mapping
- CCT research in *Drosophila* will stimulate CCT studies in higher animals

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In Brief

Deng et al. propose the concept of the chemoconnectome (CCT) as the entirety of all neurotransmitters, neuromodulators, neuropeptides, and their receptors and the approach of chemoconnectomics to trace neural circuitry anatomically and functionally.

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Chemoconnectomics: Mapping Chemical Transmission in Drosophila

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SUMMARY

We define the chemoconnectome (CCT) as the entire set of neurotransmitters, neuromodulators, neuropeptides, and their receptors underlying chemotransmission in an animal. We have generated knockout lines of Drosophila CCT genes for functional investigations and knockin lines containing Gal4 and other tools for examining gene expression and manipulating neuronal activities, with a versatile platform allowing genetic intersections and logic gates. CCT reveals the coexistence of specific transmitters but mutual exclusion of the major inhibitory and excitatory transmitters in the same neurons. One neuropeptide and five receptors were detected in glia, with octopamine b2 receptor functioning in glia. A pilot screen implicated 41 genes in sleep regulation, with the dopamine receptor Dop2R functioning in neurons expressing the peptides Dilp2 and SIFa. Thus, CCT is a novel concept, chemoconnectomics a new approach, and CCT tool lines a powerful resource for systematic investigations of chemical-transmission-mediated neural signaling circuits underlying behavior and cognition.

INTRODUCTION

The connectome is the entire set of neural connections within the nervous system and its targets in an animal (Sporns et al., 2005; Bargmann and Marder, 2013). The connectome can be studied at multiple levels; electron microscopy (EM) can visualize the hard wiring of every neuron (Kasthuri et al., 2015; Morgan et al., 2016; White et al., 1986; Zheng et al., 2018), while magnetic resonance imaging (MRI) can visualize general patterns of connectivity (Glasser et al., 2016). Between them is the mesoconnectome, which can be studied by viral injections of markers or manipulators driven by cell-type-specific promoters (Beier et al., 2015; Lerner et al., 2016; Luo et al., 2018; Oh et al., 2014a; Watabe-Uchida et al., 2012). Each approach has advantages and disadvantages. EM and MRI lack molecular resolution and cannot manipulate molecular or cellular functions. While viral injections can overcome many of the above problems, it is unclear how many viral injections (at how many locations of the nervous system with how many promoters) are necessary. Thus, with the exception of the worm Caenorhabditis elegans, which contains only 302 neurons (White et al., 1986), the connectomes of larger nervous systems remain unclear.

Each neuron is endowed chemically with the presence of small-molecule transmitters, modulators, and peptides (Cooper et al., 2003). Here, we define the chemoconnectome (CCT) as all neurotransmitters, modulators, neuropeptides, and their receptors in an animal. Signaling between neurons and their target cells is mediated by chemical and electric transmission, with chemical transmission as the predominant mode (Loewi, 1921). Typically, a presynaptic neuron synthesizes and stores neurotransmitters in synaptic vesicles and, upon stimulation, releases transmitters and modulators, which diffuse across the synaptic cleft and act on receptors located on the cytoplasmic membrane of post-synaptic neurons or other target cells. Classical experiments have uncovered neurotransmitters, including acetylcholine (ACh) (Hunt and Taveau, 1906; Dale, 1914; Dale and Feldberg, 1934), noradrenaline (NA) (Oliver and Schäfer, 1895; Takamine, 1901, 1902; Elliot, 1904; Von Euler, 1946), histamine (Barger and Dale, 1910), 5-hydroxytryptophan (5-HT) (Erspamer and Vialli, 1937; Rapport et al., 1948), dopamine (DA) (Montagu, 1957; Carlsson et al., 1958), glutamate (Glu) (Curtis et al., 1959), and g-aminobutyric acid (GABA) (Roberts and Frankel, 1950; Udenfriend, 1950). Peptides have been discovered in the nervous system to serve as either transmitters or modulators (Von Euler and Gaddum, 1931; Hughes et al., 1975). Neurotransmitters and neuromodulators and neuropeptides act on receptors, of which some are ionotropic receptors and some are G-protein-coupled receptors (GPCRs). Abnormalities of neurotransmission have been implicated in multiple diseases, with 34% of US Food and Drug Administration (FDA)-approved drugs targeting GPCRs (Hauser et al., 2017). Thus, investigations of transmitters and receptors may provide insights in understanding human diseases and developing new drugs.

In Drosophila, we and others have studied the roles of transmitters and receptors in a variety of behaviors (Zhou et al., 2008, 2012; Liu et al., 2011; Qian et al., 2017), usually one or a

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few neurotransmitters at a time. The CCT is a systematic approach that requires us to generate knockout (KO) and knockin (KI) lines targeting all known small-molecule transmitters, modulators, neuropeptides, and their receptors. 193 genes related to synthetases or transporters for transmitters, modulators, neuropeptides, their receptors, and non-olfactory orphan GPCRs were selected. KO lines for CCT enable studies of loss-of-function phenotypes of each transmitter or receptor. KI lines for CCT enable visualization of neurons expressing each transmitter or receptor. We have carried out proof-of-principle experiments to show that chemoconnectomics provides a powerful approach to study neural signaling. By highlighting the neurochemical features of transmitters, modulators, and neuropeptides, the CCT approach aims at, and its associated resources provide tools for, investigating functions of transmitters, modulators, neuropeptides, their receptors, and neurons expressing them. The KO lines allow examination of the functional involvement of each transmitter/modulator/neuropeptide and receptor in behaviors, while the KI lines allow examination of the expression patterns of transmitters, modulators, neuropeptides, and their receptors. Together, they facilitate the dissection of neurochemically deÞned circuitry. Chemoconnectomics complements, and has advantages over, macro-, meso-, and microconnectomics for studying neural circuitry and conventional mutagenesis for studying genes involved behavior. It has not escaped our notice that CCT should be generated in mammals to facilitate delineation of neural circuits underlying behaviors and cognition.

While neuropeptides can be targeted straightforwardly through their corresponding genes (Hewes and Taghert, 2001), small-molecule transmitters and modulators can be targeted indirectly by speciÞc enzymes or vesicular or cell surface transporters (Figure 1A). Receptors for all small-molecule transmitters and modulators and, for some neuropeptides, together with orphan GPCRs were targeted. In Drosophila, we have chosen 193 genes, including those encoding synthesizing enzymes for 10 of 11 small-molecule transmitters/modulators, those encoding 6 transporters, 40 neuropeptides, 32 ionotropic receptors, 59 metabotropic receptors, and 44 orphan GPCRs (Tables S1 and S2). Two of the small-molecule transmitters, Glu and glycine, were targeted not by synthesizing enzyme but by transporters (VGluT and GlyT).

We used the CRISPR-Cas9 system to generate KO lines (Ran et al., 2013) (Figure 1B) with an attP docking site introduced to generate an attP-KO line, which provides a versatile platform for further genetic modiÞcations, including KI and protein trapping by phiC31-mediated gene integration (Groth et al., 2004) (Figure 1C; Table S2).

We generated KI lines by fusing the Gal4 protein to the C terminus of a protein of interest with the stop codon for that protein removed and replaced with T2A. Gene expression could thus be visualized after crossing a Gal4 KI line to the UAS-mCD8::GFP reporter line (Figure 1D). Of the 128 gene KI Gal4 lines in which we have examined expression so far, 106 were detected in the brain. We generated KI lines for different isoforms if they exist for a single gene (Figure S1). As shown for the neuropeptide ion transport protein (ITP) and three GPCRs (Dh31-R, CCHa2-R, and DopR2), isoforms had distinct patterns.

Our basic constructs allow ßexibility and versatile applications, facilitating the generation of Flp, Lex A, and p65AD (Figure 4A) lines, as well as superfold GFP (sfGFP) lines for protein localization (Pédelacq et al., 2006). With sfGFP fused to Dop1R1, a DA receptor (DR) in Dop1R1::sfGFP, we detected its expression in the mushroom bodies (MBs) (Figures 1F and 1G). Similarly, we detected MB expression of a 5-HT receptor (in 5HT1A::sfGFP) (Figures 1H and 1I). We compared Gal4 and LexA KI lines for 10 genes; Gal4 and LexA KIs for the same gene usually had very similar patterns (those shown in Figures 1D and 1E are for CCHa2), although the patterns observed for Gal4 and LexA were quite different for CCAPR (Figure S2).

Neuronal Distribution of CCT Genes

Our KI lines make it possible to examine the distribution of neurons expressing neuropeptides as well as those for synthetases or transporters of small-molecule transmitters and receptors. 11 small molecules (ACh, GABA, Glu, Gly, DA, 5-HT, octopamine (OA), histamine (HA), tyramine (TA), adenosine (Ado), and D-serine) have been reported in Drosophila (Dolezelova et al., 2007; Kong et al., 2010; Yamazaki et al., 2014).

We have generated the following KI Gal4 lines to cover small-molecule transmitters: ACh by genetic tagging of choline acetyltransferase (ChAT) (Greenspan, 1980; Itoh et al., 1986; Kitamoto et al., 1992), GABA by tagging glutamic acid decarboxylase (GAD1) (Jackson et al., 1990) and the vesicular GABA transporter (VGAT) (Fei et al., 2010), glycine by tagging glycine transporter (GlyT) (Frenkel et al., 2017), Glu by tagging the vesicular Glu transporter (VGluT) (Daniels et al., 2004), DA by tagging tyrosine hydroxylase (TH) (Budnik and White, 1988; Neckameyer and Quinn, 1989; Mao and Davis, 2009) and the DA transporter (DAT) (Porzgen et al., 2001), 5-HT by tagging tryptophan hydroxylase (TrH) (Neckameyer and White, 1992) and the cytoplasmic 5-HT transporter (SerT) (Corey et al., 1994; Demchyshyn et al., 1994), OA by tagging the TA b hydroxylase (TbH) (Monastirioti et al., 1996), and HA by tagging the histidine decarboxylase (HDC) (Burg et al., 1993). TA cannot be speciÞcally tagged, but TDC2-Gal4 covers the combined pattern of TA and OA (Cole et al., 2005). We have also generated LexA KI lines for all of these genes and Flp KI lines for ChAT, VGluT, GAD1, TrH, and TH.

Neurons expressing these genes were visualized after the Gal4 KI lines were crossed to the UAS-mCD8::GFP reporter line (Figure 2). Distinct patterns have been observed for ChAT (Figure 2A), VGluT (Figure 2B), VGAT (Figure 2C), HDC (Figure 2D), DAT (Figure 2E), TH (Figure 2F), SerT (Figure 2G), and TbH (Figure 2H). The pattern of SerT is similar to that of TrH reported by us recently (Qian et al., 2017).

We generated KO and KI lines targeting the neuropeptide genes known from previous reports and predicted from bioinformatics (Broeck, 2001; Hewes and Taghert, 2001; Nässel and Winther, 2010). Expression patterns of neuropeptides are

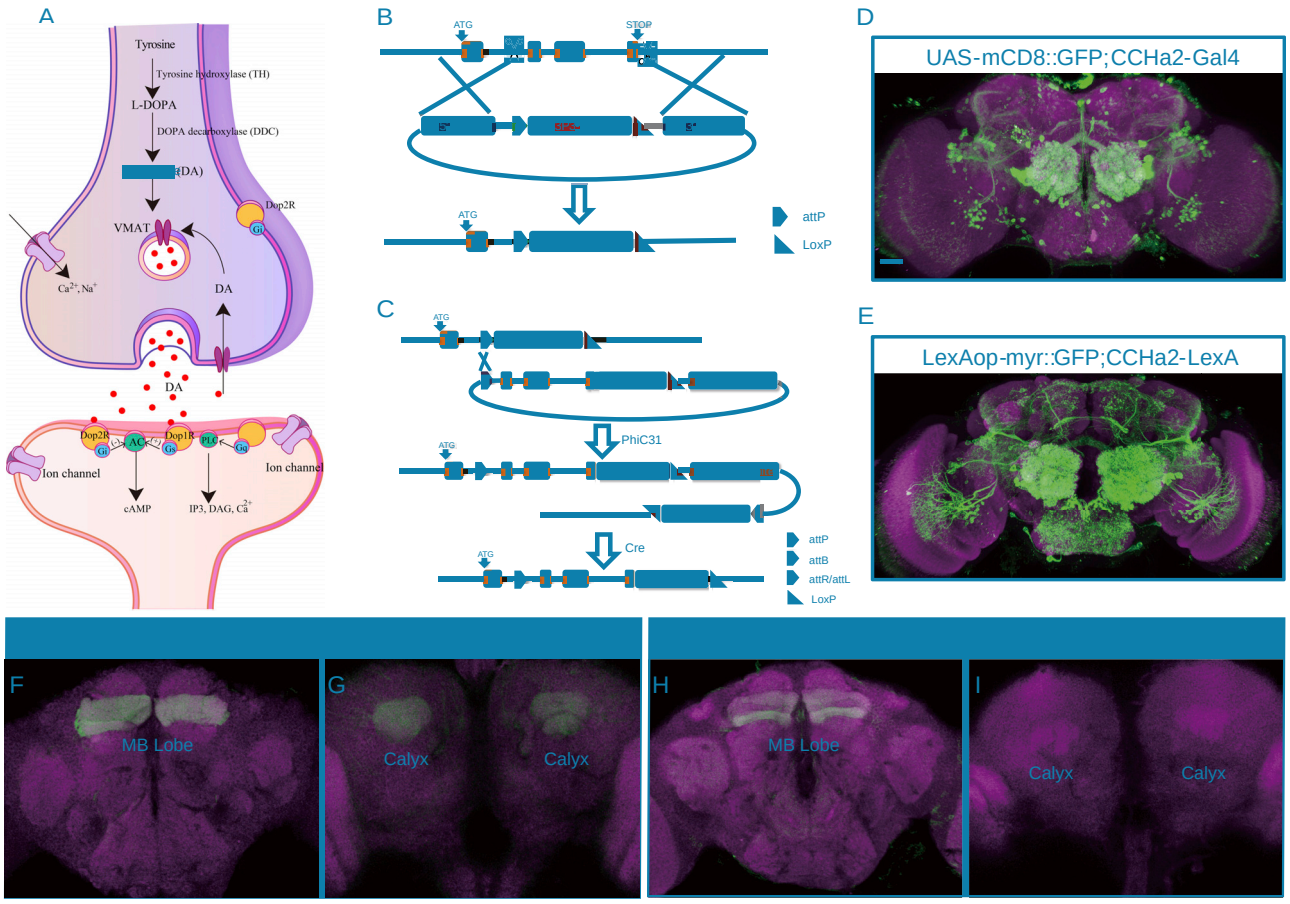

Figure 1. KO and KI Lines in Drosophila CCT

(A) A dopaminergic synapse: DA synthesis and transport and DRs.

(B) Homologous-recombination-mediated gene targeting. To generate a KO, most of the exons were replaced by the 3P3-RFP cassette through homologous recombination, and CRISPR-Cas9 was used to increase the targeting efÞciency. In addition, an attP site before the 3P3-RFP cassette was inserted into the non-transcriptional region to allow for further modiÞcations.

(C) Site-speciÞc integration system mediated KI. The KI cassette including the genomic region deleted in the KO lines and KI elements such as the Gal4 or LexA coding sequence were introduced through PhiC31-mediated attP/attB recombination. loxP sites were also introduced.

(D and E) Expression of UAS-mCD8-GFP driven by CCHa2-Gal4 (D), and of LexAop-myr::GFP driven by CCHa2-LexA (E). Green, GFP; magenta, nc82. Scale bar, 30 mm.

(F and G) Protein localization revealed by sfGFP fused in-frame to Dop1R1 at MB lobe region (F) and calyx region (G).

(H and I) Protein localization revealed by sfGFP fused in-frame to 5-HT1A receptor at MB lobe region (H) and calyx region (I).

Green, sfGFP; magenta; nc82. Scale bar, 30 mm.

shown in Figures 2 and S1ÐS4 Neuropeptides were usually detected in limited regions (Figures 2IÐ2T), whereas their receptors were detected in broader patterns (Figures 2YÐ2Bb and S3). Neurons expressing SIFaR, CNMaR, or NPFR (Figures 2Y, 2Z, 2Aa, and 2Bb) were more in number and innervated more broadly than neurons expressing SIFa, CNMa, or NPF (Figures 2J, 2M, and 2N).

Two Glu receptors (GluRIA and GluRIB) were detected in the MBs and antennal lobe (AL), and GluRIB was also detected in the supraesophogeal ganglion (SOG) and the visual system (Figures 2U and 2V). There are Þve genes encoding GABA receptors, two for GABA-A (Ffrench-Constant et al., 1991; Henderson et al., 1993) and three for GABA-B (Mezler et al., 2001). GABA-B-R1 was found in the AL, visual system, MBs and ellipsoid body (EB);GABA-B-R3 was found in the EB, but not in the MB (Figures 2W and 2X).

Glial Expression of CCT Genes

Genes for small-molecule transmitters, modulators, and neuropeptides were detected in neurons (Figures 1DÐ1H and 2). Are any of these genes expressed in glial cells?

So far, we have not detected the expression of small-molecule transmitters or modulators in glia. However, we have detected the expression of one neuropeptide and Þve receptors in glial cells. The expression of the RD isoform of the ITP neuropeptide gene was found in glia and neurons (Figure 3A). The RC isoform of the receptor for Dh31 neuropeptide was also detected in glia and neurons (Figure 3B). An isoform of the

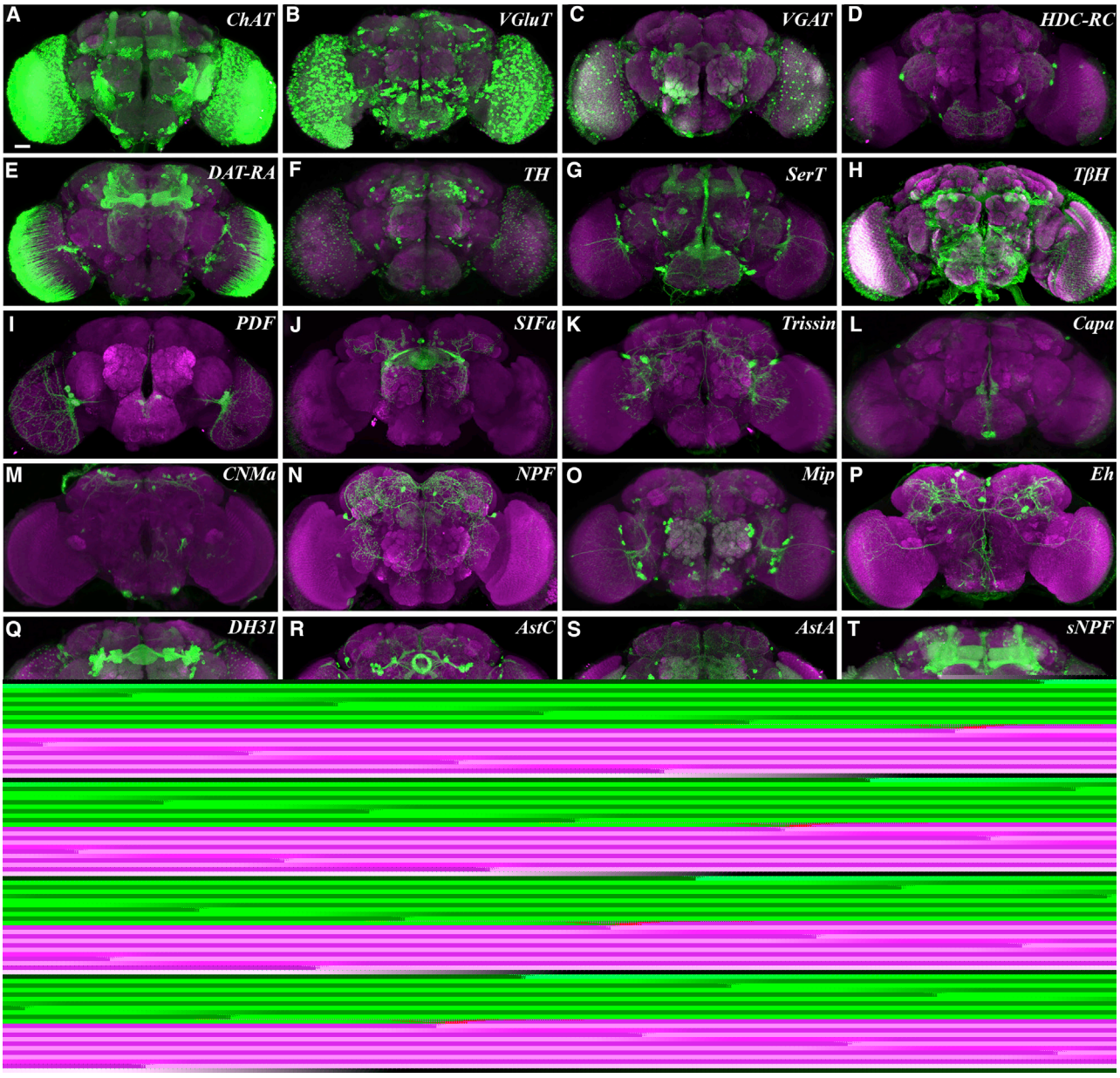

Figure 2. Distribution of Neurons Expressing CCT Genes Revealed by KI Lines

(AÐH) Expression patterns of genes encoding ChAT, a marker for Ach (A), VGluT for Glu (B), VGAT for GABA (C), HDC-RC for HA (D), DAT-RA for DA (E), TH for DA (F), SerT for 5-HT (G), and TβH for OA (H). The HDC-RA/B isoform was not detected in the brain.

(IÐT) Expression patterns of neuropeptides in the brain for PDF (I), SIFa (J), Trissin (K), Capa (L), CNMa (M), NPF (N), Mip (O), Eh (P), DH31 (Q), AstC (R), AstA (S), and sNPF (T).

(UÐX) Expression patterns of gene encoding GluR1A (U) and GluR1B (V), GABA-B-R1 (W), and GABA-B-R3 (X).

(YÐBb) Expression patterns of neuropeptide receptors neurons in the brain: SIFaR (Y), CNMaR (Z), NPFR-RB/D (Aa) isoform, and NPFR-RA/C isoform (Bb)

Dop1R1 receptor was found in glia and neurons (Figure 3C). With the help of multiple glial Gal4 lines, we detected ITP-RD in perineurial, cortical astrocyte-like, and ensheathing glia, Dh31R-RC in subperineurial and cortical glia, and Dop1R1-RB/C in cortical and ensheathing glia (Figure S4). The Ado receptor (AdoR) (Figures S3D and S5) and Lk receptor (LkR) (unpublished data) were also detected in glia. AdoR inhibits male-male aggression (Figure S5), though we have not determined whether it functions in neurons or glia.

The octopamine b2 receptor (Octb2R) was detected in both neurons and glia (Figures 3EÐ3J). Nuclear GFP expression driven by Octb2R-Gal4 overlapped with immunostaining by the anti-Repo antibody, a pan-glial marker (Figures 3EÐ3G). Overlap was also detected between Octb2R and immunostaining of the