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Figure 1

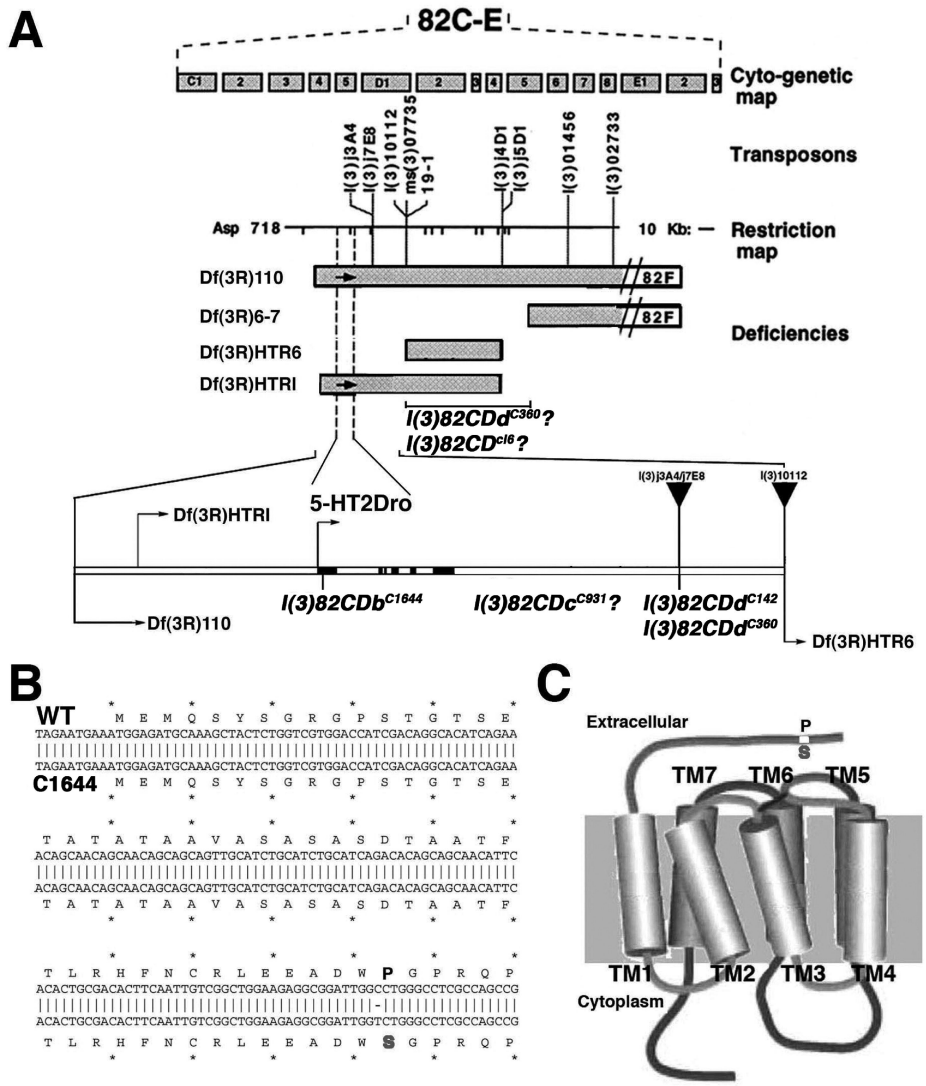

Figure1

Figure 2

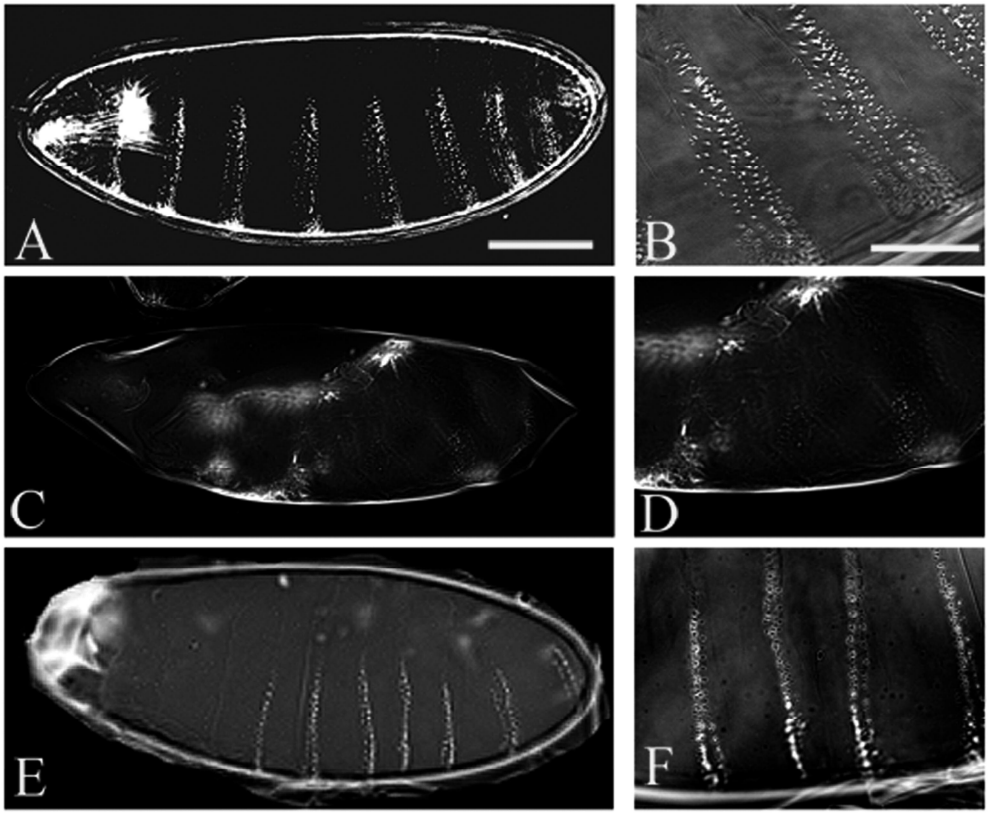

figure2

Figure 3

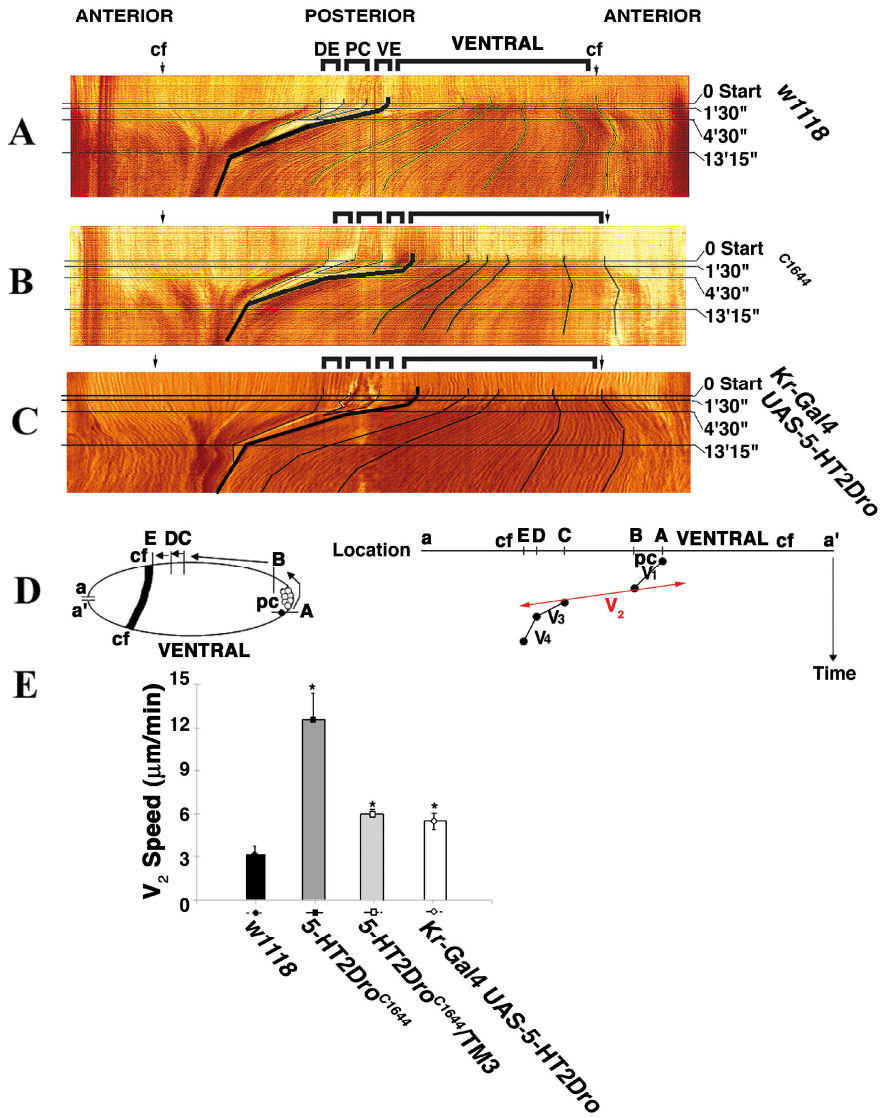

figure3

A gain of affinity point mutation in the serotonin receptor gene 5-HT$_{2Dro}$ accelerates germband extension movements during *Drosophila* gastrulation

Running title: Point mutation in 5-HT$_{2Dro}$ gene

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Abstract

Serotonin not only works as neurotransmitter in the nervous system, but also as a morphogenetic factor during early embryogenesis. In *Drosophila*, a previous report showed that embryos that lack the 5-HT_{2Dro} receptor locus, display abnormal gastrulation movements. In this work, we screened for point mutations in the 5-HT_{2Dro} receptor gene. We identified one point mutation that generates a gain of serotonin affinity for the receptor and affects germband extension, $5\text{-}HT_{2Dro}^{C1644}$. Embryos homozygous for this point mutation display a four-fold increase in the maximal speed of ectodermal cell movements during the rapid phase of germband extension. Homozygous $5\text{-}HT_{2Dro}^{C1644}$ embryos present a cuticular phenotype including a total lack of denticle belt. Identification of this gain of function mutation shows the participation of serotonin in the regulation of the cell speed movements during the germband extension and suggests a role of serotonin in the regulation of cuticular formation during early embryogenesis.

Keywords:

Serotonin; receptor; mutation; ectoderm; gastrulation; cuticle; affinity.

Introduction

In vertebrates, the biogenic amine serotonin (5-Hydroxytryptamine; 5-HT) affects a wide variety of central and peripheral functions involved in behavioral and physiological activities in addition to embryonic development. Numerous receptor subtypes mediate the many functions of 5-HT. At least 14 different receptors have been molecularly characterized in mammals. All receptors except 5-HT_3 belong to the G protein coupled receptor family and activate different signaling pathways. In insects, biogenic amines are similarly involved as neuromodulators and neurotransmitters, in addition to their role in cross-linking proteins and chitin during the sclerotization of the cuticle (Wright, 1987). In *Drosophila*, four 5-HT receptors have been described (Witz et al., 1990; Saudou et al., 1992; Colas et al., 1995) in addition to the transporter (Park et al., 2006).

We previously reported that the G-protein coupled receptor 5-HT_{2Dro} is an ortholog of the mammalian 5-$HT_{2A, B, C}$ receptor subfamily. 5-HT_{2Dro} receptor mRNA and protein expression starts after cellularization at the blastoderm stage, when major gastrulation movements begin. Further experiments demonstrated that the 5-HT_{2Dro} receptor is functional during early developmental stages. Concomitant with 5-HT_{2Dro} receptor expression, there is also a detectable transient peak of 5-HT synthesis (Colas et al., 1995). Moreover, the 5-HT_{2Dro} receptor mRNA is expressed in a pattern similar to the pair-rule gene Fushi tarazu (Pankratz and Jäckle, 1993). In contrast to other pair-rule genes whose expression surrounds the embryo entirely, the seven stripes of 5-HT_{2Dro} mRNA are restricted to the presumptive ectoderm.

We previously demonstrated that embryos homozygous for the deficiency *Df(3R)HTRI*, that removes the 5-HT_{2Dro} receptor locus, display abnormal morphogenetic movements during the germband extension (GBE) and die at the end of embryogenesis or early first instar larval stage (Colas et al., 1999b). However, a contribution of other genes present in the 55 kb of DNA deleted in *Df(3R)HTRI* could not be completely ruled out. In this work, we looked for a

point mutant in the 5-HT_{2Dro} receptor gene in order to validate the functions of 5-HT in GBE process.

In wildtype gastrulae, peaks of both 5-HT_{2Dro} receptor expression and 5-HT synthesis coincide precisely with the onset of GBE. We previously reported that the peak of 5-HT synthesis is dependent on the maternal deposition of biopterins, cofactors of tryptophan hydroxylase (the limiting enzyme in 5-HT synthesis) and on the zygotic expression of both tryptophan hydroxylase and DOPA decarboxylase (Colas et al., 1999a). Mutant embryos with an impaired peak of 5-HT synthesis display a particular cuticular organization called "double line" (Colas et al., 1999b), which was similarly observed in *Df(3R)HTRI* homozygous embryos and in *shotgun* embryos mutated in the DE-cadherin locus (Colas et al., 1999b). Whether the cuticular phenotype is a direct consequence of a gastrulation defect remained to be determined.

GBE results in an approximate doubling in length of the antero-posterior axis of the *Drosophila* embryo (Zallen and Wieschaus, 2004). As in other species, this process of convergent extension relies mostly on cell intercalation (Schoenwolf and Alvarez, 1989). Irvine and Wieschaus proposed that ectodermal cell intercalation, the main process in GBE, could be driven by adhesion strength, which would be different in adjacent segments (Irvine and Wieschaus, 1994). Differences in adhesion strength between cells of alternate segments would control cell intercalation speed. In fact, cells do not delaminate or migrate across the tissue but, instead, rearrange specific contacts after an ordered spatial–temporal pattern of junction remodeling. It has been proposed that axis elongation in Drosophila occurs through stereotyped cell-shape changes across a uniform field (Bertet et al., 2004). However, this model stipulates a single round of intercalation, whereas multiple rounds are required for full elongation. Recently, the asymmetric distribution of cytoskeletal and junctional proteins has been proposed to contribute directly to polarized cell behavior during tissue elongation (Bertet et al., 2004; Zallen and Wieschaus, 2004; Blankenship et al., 2006).

DE-cadherins localize to the apical part of the lateral membranes of ectodermal cells and are involved in cell adhesion (Tepass et al., 1996; Oda et al., 1998; Greaves et al., 1999). DE-cadherin regulation seems to be important for the GBE process. Zallen and Wieschaus have demonstrated that striped expression of the pair-rule genes are both necessary and sufficient to orient planar polarity (Zallen and Wieschaus, 2004). The F-actin asymmetry was reported as the primary planar polarity in the GBE, followed by the segregation of Myosin II and Bazooka into complementary surface domains. DE-cadherin and Armadillo/beta-catenin would then be recruited to sites of new cell contacts prior to Bazooka association (Blankenship et al., 2006). The local destabilization of DE-cadherin–based adhesion indeed may cause the local destabilization of polarized cell behavior during tissue elongation. Regulators of these phenomenon remain to be identified.

In this work, we screened for mutations in the 5-HT_{2Dro} receptor gene and identified one point mutant that generates an increase in 5-HT affinity for the receptor. Homozygous embryos for this mutation present a defect in cell speed movements during GBE accompanied by cuticular defects.

Results

***l(3)82CDb^{C1644}* encodes a mutant 5-HT$_{2Dro}$ receptor with increased affinity for 5-HT**

To understand the embryonic functions of 5-HT, we became interested in mapping mutants in the 5-HT$_{2Dro}$ receptor. The gene encoding this receptor is localized on the right arm of the third chromosome at 82C5 (Colas et al., 1995). Using EMS, Meagraw and Kaufman (FlyBase) screened several homozygous lethal mutants in the 82C4-D5 region. Five mutants, *l(3)82CDb^{C1644}, l(3)82CDc^{C931}, l(3)82CDd^{C142}, l(3)82CDd^{C360}* and *l(3)82CDc^{l6}*, were mapped in this interval, since they all complement with *Df(3R)6-7* (82D5-82F3-6) but not with *Df(3R)110* (82C4-82F3). To check whether some of these point mutants affect the 5-HT$_{2Dro}$ receptor gene, we refined further the mapping using *Df(3R)HTRI* (82C4-82D5) that deletes *5-HT$_{2Dro}$* and the *Df(3R)HTR6* (82D2-82D5) that leaves the gene intact (figure 1A) (Colas et al., 1999b). *L(3)82CDb^{C1644}, l(3)82CDc^{C931}, l(3)82CDd^{C142}* and *l(3)82CDd^{C360}* were lethal with *Df(3R)HTRI* but viable with *Df(3R)HTR6* indicating that they are in the region of interest. However, *l(3)82CDc^{l6}* that is viable with *Df(3R)HTRI,* does not map at the 5-HT$_{2Dro}$ locus and were not further analyzed. We found that *l(3)82CDd^{C142}* and *l(3)82CDd^{C360}* define a distinct complementation group in 82C5, since they fail to complement the lethal insertion *j3A4* localized in the vicinity of *5-HT$_{2Dro}$* (Colas et al., 1999b).

We then focused on the two remaining mutants *l(3)82CDb^{C1644}* and *l(3)82CDc^{C931}*. To assess whether they correspond to mutant alleles of the *5-HT$_{2Dro}$* gene, we extracted genomic DNA from single embryo to check by PCR the presence of the balancer chromosome. We took advantage of a reporter gene (twi-LacZ) of the balancer chromosome to distinguish the homozygous mutant embryos from the others. We sequenced all the coding exons of the 5-HT$_{2Dro}$ receptor gene of homozygous *l(3)82CDb^{C1644}* and *l(3)82CDc^{C931}* embryos. The sequence revealed that the *l(3)82CDb^{C1644}* chromosome had a mutation in the first coding exon of *5-HT$_{2Dro}$* gene (Exon2), while the *l(3)82CDc^{C931}* issued from the same genetic screen had a

wildtype sequence (fig.1B). The point mutation in *l(3)82CDb*^{C1644} changes the proline 52 into a serine in the N-terminus of the receptor (fig.1B-C).

Up to now, the role of the N-terminus part of the GPCR that recognize biogenic amine is not established. In mammals, the N-terminus domain of the 5-HT receptors is not directly involved in ligand binding. So it was intriguing that a mutation in this domain leads homozygous lethal mutation. We investigated how this point mutation influenced the receptor function. We performed transient expression of the mutated 5-HT_{2Dro} receptor in COS-1 cells and binding experiments on membrane preparations. This experiment showed that several of the tested ligands, including 5-HT, the endogenous physiological ligand, have an increased affinity for the mutant receptor compared with wildtype receptor. The Ki of 5-HT was 200 nM for the wildtype 5-HT_{2Dro} receptor and only 11 nM for the mutant receptor, while expression was similar (table 1). These data indicate that the mutation in the N-terminus domain of the 5-HT_{2Dro} receptor confers to the receptor a gain of 5-HT affinity.

5-*HT*$_{2Dro}$^{C1644} homozygous embryos display specific cuticular alterations.

L(3)82CDb^{C1644} was EMS-induced on a Rucuca chromosome that presents several non-lethal mutations: *ru h*^{1} *th st cu sr e*^{s} *ca*. In order to analyze the phenotype induced by the mutation in the 5-HT_{2Dro} receptor, we first generated a recombinant chromosome that was only mutated in the 5-HT_{2Dro} gene. After recombination of both arms of the third chromosomes, we verified that the recombinant *5-HT*$_{2Dro}$^{C1644} chromosome was still lethal against the original *l(3)82CDb*^{C1644} strain and *Df(3R)HTRI.* This recombined *5-HT*$_{2Dro}$^{C1644} chromosome was then used for all subsequent experiments. We analyzed the timing of the lethality in *5-HT*$_{2Dro}$^{C1644} mutant embryos.

In order to distinguish the phenotypes, we used a GFP-labeled balancer chromosome to follow the fate of each embryo. With this technique, we observed that about 20% of homozygous *5-HT*$_{2Dro}$^{C1644} embryos hatched and died shortly during the first instar larvae

(table 2). The majority of the homozygous *5-HT$_{2Dro}^{C1644}$* embryos died at the embryonic stages. We analyzed the cuticular phenotype of the embryos that never hatched in the *5-HT$_{2Dro}^{C1644}$* strain (figure 2, table 2). We verified that the homozygous balancer embryos displayed mainly wildtype cuticles (table 2). The *5-HT$_{2Dro}^{C1644}$* homozygous embryos presented either "double line" or "ghost" phenotypes (figure 2C-D, 2E-F, table 2). Among the unhatched homozygous *5-HT$_{2Dro}^{C1644}$* embryos 43% present wild-type cuticle, 30% double-line and 22% ghost cuticles. The "double line" phenotype is characterized by the presence of two rows of denticles in each segment (figure 2E-F) and has been previously observed in homozygous *Df(3R)HTRI* embryos that lack the 5-HT$_{2Dro}$ receptor (Colas et al., 1999b). Moreover, we observed that the "ghost" phenotype with no denticle at the surface of the embryo was mainly represented in *5-HT$_{2Dro}^{C1644}$* embryos (figure 2C-D, table 2). This phenotype could be distinguished from the non-fertilized embryos as the cuticle had been secreted. Interestingly, the transheterozygous *5-HT$_{2Dro}^{C1644}$/Df(3R)HTRI* embryos hatched but lead to small larvae that all died quickly with no obvious cuticular defects, showing a partial cuticular rescue. These data revealed that the *5-HT$_{2Dro}^{C1644}$* mutation affects significantly the cuticular development.

5-HT$_{2Dro}^{C1644}$ embryos display altered cell speed movements during GBE.

A previous study showed that *Df(3R)HTRI* homozygous embryos presented defects in GBE (Colas et al., 1999b). We thus investigated the GBE in *5-HT$_{2Dro}^{C1644}$* homozygous and heterozygous mutant embryos. Following the previously described method (Colas et al., 1999b), we video-recorded the rapid phase of the GBE and followed the movements of ectodermal cells around the midline of single embryos. After recording, each embryo was PCR-genotyped. The absence of the balancer chromosome marker was indicative of homozygous mutant chromosome. Using image analysis of the digital video recording, we assessed the speed of cell movements, near the ventral and dorsal midline of the embryo. This was made by using a developed software that allowed the "peeling off" of a single superficial

layer of pixels from each recorded video image, then laying them flat after opening from the most rostral point. Accumulating these layers over time allowed the visualization and quantification of the speed and extent of individual cell movement at the surface of the embryo and the evaluation of the synchronization of these movements. Taking the initiation of extension movements as a reference time point, the speed and synchronization of the extension movements can be directly evaluated. The figure 3 displays representative results of these investigations on individual genotyped embryo. As the X-axis represents the position of the cell along the embryo and the Y-axis the time scale, the slope of the curves represents their speed. We followed the migration of ectodermal cells, ventral to the pole cells because they were easy to distinguish in each embryos (fig. 3D). Interestingly, we observed an initial contraction of the ventral ectoderm reflecting the mesoderm invagination in wildtype embryos before ectodermal cells start moving (fig. 3A) that could not be detected in $5\text{-}HT_{2Dro}^{C1644}$ homozygous embryos (fig. 3B). For cells close to the pole cells, the highest cell speed (V_2) was recorded in $5\text{-}HT_{2Dro}^{C1644}$ homozygous embryos and was four times higher than in the wildtype embryos (V_2= 12.5±1.8, n=6 vs. 3.3±0.5 microm/min, n=6) (fig. 3E). Calculating the speed of similar cells in $5\text{-}HT_{2Dro}^{C1644}/TM3$ heterozygous embryos (V_2= 5.9±0.3 microm/min, n=5) showed that it was only two times faster (fig. 3E). The data shows that 5-HT and 5-HT_{2Dro} receptors are involved in cell speed regulation during the GBE. Moreover, the intermediary phenotype observed for the heterozygous $5\text{-}HT_{2Dro}^{C1644}/TM3$ embryos supports the notion that the mutation leads to a gain of function for the 5-HT_{2Dro} receptor.

Locally over-expressing 5-HT_{2Dro} receptor embryos display altered cell speed movements during GBE.

A transgenic strain that overexpresses a wildtype 5-HT_{2Dro} receptor under the control of the Krüppel promoter, has been previously produced (*Kr-Gal4,UAS-5-HT_{2Dro}*) (Colas et al., 1999b). This transgenic strain displays an overexpression of the wildtype 5-HT_{2Dro} receptor in

the central region of the embryos, where the endogenous receptor expression is low (Colas et al., 1999b). Cell speed calculation in *Kr-Gal4,UAS-5-HT*$_{2Dro}$ embryos during GBE showed that the maximal speed was similar to heterozygous *5-HT*$_{2Dro}^{C1644}$*/TM3* embryos (V_2= 5.7±0.7, n=3 vs. 5.9±0.3 microm/min, n=5) (figure 3C-E). These data reveals that defects of GBE in heterozygous *5-HT*$_{2Dro}^{C1644}$ mutant embryos can be phenocopied by *5-HT*$_{2Dro}$ local overexpression.

Discussion

The mutation in the N-terminal domain of 5-HT$_{2Dro}$ receptor is a gain of 5-HT affinity.

We identified a lethal mutation in a *Drosophila* strain characterized by a point mutation in the 5-HT$_{2Dro}$ receptor gene that changes proline 52 to serine in the N-terminal domain of the receptor, increasing 20 fold its affinity for 5-HT. The complete deletion of the N-terminal domain of the 5-HT$_{2Dro}$ receptor also led to a gain of 5-HT affinity (Colas et al., 1997). These data indicate that the N-terminal domain of the 5-HT$_{2Dro}$ receptor is involved in binding of 5-HT. Classically, biogenic amines including 5-HT bind to amino-acid side chains within the transmembrane domains of their receptors (Manivet et al., 2002). However, in *Drosophila*, most biogenic amine receptors have a long N-terminal domain (Onai et al., 1989; Saudou et al., 1990; Witz et al., 1990; Colas et al., 1995; Gotzes and Baumann, 1996). Few studies have been performed to understand the role of these long domains. Our study demonstrates for the first time a role for the long N-terminal domain of the serotonergic receptor 5-HT$_{2Dro}$. Since this mutant receptor is more sensitive for its endogenous ligand 5-HT, 20 fold less 5-HT could be sufficient to reach signaling activity in mutant receptor comparable to that of fully stimulated wildtype receptor.

The mutant 5-HT$_{2Dro}$ receptor GBE phenotype can be phenocopied by wildtype 5-HT$_{2Dro}$ receptor ectopic expression.

5-HT$_{2Dro}^{C1644}$ homozygous mutant embryos display an increase in GBE movements compared with wildtype. As previously shown, 5-HT is transiently synthesized in *Drosophila* embryos, with a peak of synthesis at 3h15 after fertilization (Colas et al., 1999a). Since the mutated 5-HT$_{2Dro}$ receptor found in *5-HT$_{2Dro}^{C1644}$* strain could be activated by 20 fold less 5-HT, it could thus reach a threshold of activity earlier than wildtype receptor, stronger signaling and/or its activity may last longer. *5-HT$_{2Dro}^{C1644}$* homozygous embryos seem to respond to an early signal inducing cell movements since GBE starts before detectable mesodermal invagination.

However, the precise measure of the time elapsed between fertilization and the onset of GBE is hardly accessible, thus limiting the possibility to document this putative time shift.

A transgenic fly strain with a restricted ectopic expression of 5-HT_{2Dro} cDNA under the Krüppel gene promoter (*Kr-Gal4, UAS-5-HT_{2Dro}*) also generates gastrulation defects, supporting a role of 5-HT signaling in GBE (Colas et al., 1999b). This transgenic strain displays (i) an overexpression of the 5-HT_{2Dro} receptor in the central region of the embryos, where the endogenous receptor expression is low with a local disruption of the receptor segmental expression and (ii) a change in the timing of the 5-HT_{2Dro} receptor expression as Krüppel is expressed before 5-HT_{2Dro}. In *Kr-Gal4, UAS-5-HT_{2Dro}* embryos, the first defects appear as abnormal anterior initiation of the extension movements before the posterior initiation of movements (Colas et al., 1999b). Cell movements studies during GBE show that cell speed defects of heterozygous *5-HT_{2Dro}^{C1644}*/TM3, and *Kr-Gal4, UAS-5-HT_{2Dro}* embryos are similar. The cuticular phenotype of *Kr-Gal4, UAS-5-HT_{2Dro}* embryos is weaker than *5-HT_{2Dro}^{C1644}*. Indeed, they display either wildtype or mild denticles alterations, like heterozygous *5-HT_{2Dro}^{C1644}/TM3* embryos. Moreover, the *Kr-Gal4, UAS-5-HT_{2Dro}* strain is viable, indicating that the overexpression of the receptor mimics the phenotype of *5-HT_{2Dro}^{C1644}/TM3* embryos. The phenotype of homozygous point mutant is thus stronger than the overexpression of the wildtype *5-HT_{2Dro}^{C1644}*. The difference between homozygous and heterozygous *5-HT_{2Dro}^{C1644}* embryos and the similar phenotype of heterozygous *5-HT_{2Dro}^{C1644}* and *Kr-Gal4, UAS-5-HT_{2Dro}* strongly support the notion that the mutation in 5-HT_{2Dro} confers a gain of function to the receptor in respect to GBE movements. In mutants depleted of 5-HT (synthesis mutant) (Colas et al., 1999a) or of 5-HT_{2Dro} receptor {*Df(3R)HTRI*} (Colas et al., 1999b), although altered, GBE movements still occurs. 5-HT and the 5-HT_{2Dro} receptor seem thus to be involved in regulating the speed of the cell movements during the GBE process but apparently not the GBE or mesodermal invagination movements themselves.

GBE defects in 5-HT_{2Dro}^{C1644} could be due to cell adhesion defects.

The change in cell speed in the 5-HT_{2Dro} receptor mutant most probably reflects alterations in ectodermal cell intercalation that was initially proposed to depend upon the difference of adhesion strength between neighboring cells (Irvine and Wieschaus, 1994). Cell intercalation of epithelial cells would be driven by simple remodeling of contacts in an ordered directional pattern allowing progressively exchange places with neighboring cells (Bertet et al., 2004; Zallen and Wieschaus, 2004). In this model, axis elongation in Drosophila occurs through stereotyped cell-shape changes across a uniform field (Bertet et al., 2004). However, multiple rounds are required for full elongation and intercalating cells locally organize to generate multicellular structures (rosette) that form and resolve in a directional fashion. Directionality is disrupted in mutants that lack antero-posterior patterning and fail to elongate. Multicellular structures, and not individual cells or cell interfaces, represent thus the functional units of cell behavior during tissue elongation. The asymmetric distribution of cytoskeletal and junctional proteins has been proposed to contribute directly to polarized cell behavior during tissue elongation (Blankenship et al., 2006). F-actin enrichment at antero-posterior interfaces is the earliest evidence of planar polarity. Myosin II subsequently accumulates at these interfaces and may coordinate the contraction of linked edges to drive multicellular (rosette) formation. These transient structures resolve in a directional fashion to create contact between cells that were previously separated along the dorso-ventral axis. DE-cadherin association is an early step in new contact formation and coincides with formation of a transient F-actin structure. Bazooka may be recruited later to new interfaces during their eventual stabilization (Blankenship et al., 2006).

We previously reported that the 5-HT_{2Dro} receptor is expressed with a pair rule-like pattern in ectodermal cells and that the apico-basal localization of Armadillo (β-catenin ortholog), in the ectodermal cells, that links cadherins with the cytoskeleton is altered in homozygous *Df(3R)HTRI* embryos (Colas et al., 1999b). Moreover, GBE defects are detected

both in the *5-HT$_{2Dro}^{C1644}$* gain of function strain and in *Df(3R)HTRI* deficient strain. 5-HT via the 5-HT$_{2Dro}$ receptor appears thus to be able to influence the polarized adhesion signals controlling the GBE process. DE-cadherin and Armadillo/beta-catenin being recruited to sites of new cell contacts prior to Bazooka association (Blankenship et al., 2006), the 5-HT-dependent DE-cadherin–based adhesion may thus regulate the stabilization/destabilization of polarized cell behavior during tissue elongation.

Putative link between GBE defects and 5-HT$_{2Dro}$ receptor

In embryos injected with Y-27632, a pharmacological inhibitor of Rho-kinase and specific regulator of Myosin-II, intercalation fails to occur (Bertet et al., 2004). Interestingly, the 5-HT$_{2Dro}$ receptor is orthologous to mammalian 5-HT$_{2A, B, C}$ receptors, which are involved in smooth muscle cell contraction via a Rho-dependent regulation of myosin light chain kinase (Nishikawa et al., 2003). The 5-HT$_{2Dro}$ receptor may thus transduce extracellular signal (5-HT) to the cytoskeleton and regulate either the timing or intensity of cell intercalation, Rho/Rho kinase being a likely effector of the 5-HT$_{2Dro}$ receptor although the precise transduction of this pathway remains to be determined in *Drosophila*.

Many *5-HT$_{2Dro}^{C1644}$* homozygous embryos that did not hatch display a “ghost” phenotype that is nearly absent in other studied strains. The previously described “ghost” and “double line” cuticular phenotypes in *Df(3R)HTRI* (Colas et al., 1999b) have been proposed to result from variations in intensity of GBE defects, the “ghost” phenotype reflecting earlier and/or stronger defects. In both strains, alterations of differential adhesion between cell expressing or not the 5-HT$_{2Dro}$ receptor may explain the cuticular phenotypes. Interestingly, by *in situ* hybridization, 5-HT$_{2Dro}$ probe showed no 5-HT$_{2Dro}$ mRNA misexpression in *5-HT$_{2Dro}^{C1644}$* mutant embryos and by immunohistochemistry, we observed no defects in Fushi tarazu or Engrailed, expression in *5-HT$_{2Dro}^{C1644}$* embryos at different developmental stages (data not shown). These observations support an indirect role of 5-HT$_{2Dro}$ in cuticular

formation. It has to be noticed that cell speed appears more variable in *5-HT*$_{2Dro}^{C1644}$ homozygous embryos (higher SEM, figure 3D), suggesting higher variability in the control of GBE. Together, these observations support the notion that this gain of function mutation in the 5-HT$_{2Dro}$ receptor increases variability in the embryonic development and that 5-HT is likely involved in setting the time and/or the speed of GBE process by polarizing adhesion signals via the 5-HT$_{2Dro}$ receptor transduction.

Experimental procedures

Fly stocks

The following strains have been used in this study. Point mutant lines: *l(3)82CDb*^{C1644} Stocks 0004830, *l(3)82CDc*^{C931} Stocks 0004831, *l(3)82CDd*^{C142} Stocks 0004832, *l(3)82CDd*^{C360} Stocks 0004833, *l(3)82CDc*^{16} Stocks 0004839. Deficiency lines: *Df(3R)HTRI, Df(3R)HTR6, Df(3R)110, Df(3R)6-7* (Colas et al., 1999b). Blue balancers: *TM3 Sb Ser P[hb-lacZ] and TM3 Ser P[twi-lacZ];* Green balancers: *TM3 Sb Ser P[hb-GFP]*.

To obtain *5-HT*$_{2Dro}$^{C1644} strain, we used *kar*^{2}*ry*^{506}*, ru,h*^{1}*,th,st,cu,sr,e*^{s}*,Pr,ca (Ruprica), Ki,kar*^{2}*ry*^{506}*, D*^{1}*,kar*^{2} *ry*^{506} and *w1118* background chromosomes.

We proceeded the crossings as follows to obtain the *5-HT*$_{2Dro}$^{C1644} strain:

1) Recombination of the left arm of the third chromosome :

l(3)82CDb^{C1644}/TM3 Ser and kar²ry506/kar²ry506 generated l(3)82CDb^{C1644}/kar²ry506

l(3)82CDb^{C1644}/kar²ry506 and Ruprica/TM6 Bri,Tb generated recombined l(3)82CDb^{C1644}*/Ruprica

recombined l(3)82CDb^{C1644}*/Ruprica and Df(3R)HTRI/TM3 Ser, Sb generated l(3)82CDb^{C1644}*/TM3 Ser, Sb

2) Preliminary crossing to prepare the second recombination :

pr, pwn FLP38/CyO ; Ki, kar²ry506 and kar²ry506/kar²ry506 generated +/CyO ; Ki, kar²ry506/kar²ry506

3) Recombination of the right arm of the third chromosome :

l(3)82CDb^{C1644}*/TM3 Ser, Sb X and +/CyO ; Ki, kar²ry506/kar²ry506 generated l(3)82CDb^{C1644}*/Ki, kar²ry506

l(3)82CDb^{C1644}*/Ki, kar²ry506 and D, kar²ry506/TM3 Ser, Sb generated recombined l(3)82CDb^{C1644}**/D, kar²ry506

l(3)82CDb^{C1644}**/D, kar²ry506 and Df(3R)HTRI/TM3 Ser generated l(3)82CDb^{C1644}**kar²ry506/TM3 Ser

This stock was used in this study as 5-HT_{2Dro}^{C1644}.

DNA extraction, PCR, sequencing

Unique embryos have been genotyped by PCR. After collection of the embryos (from 3 to 5 hours), DNA was extracted as described (Colas et al., 1999b) and 1/10 of the DNA from a single embryo was amplified using regular PCR amplification conditions for 38 cycles. In order to identify homozygous mutants, embryos were genotyped by PCR using oligonucleotides for Atonal (5'CGTTGGATCCAGCAACATAACACCACCATA3' and 5'ATCGGGATCCGGGAATTCAGCGCAGCAATC3') or (Twist-beta-galactosidase) (5'GCGTCAGTTGCGTTCCGTAAGTGC3' and 5'CAGTTTGAGGGGACGACGACAGTA3') genes.

In order to identify point mutations in homozygous embryos, each exon of the 5-HT_{2Dro} receptor had been amplified by PCR using following amplimers:

exon 1: 5'GGAATTCCAAAACAAGTATTGTTGATGCTG3' and 5'GGGGTACCTTATCTACTATATTTTACTTGC3';

exon 2, 3 and 4: 5'GGGGTACCAATTTTTTTGAGATTCACATATA3' and 5'GGGGTACCGCAGTAAGTCACCACCACGTAATT3';

exon 5: 5'GGTGGAAAATTATTCGAAAGT3' and 5'GCGATTCACCACCAAAATAATTTA3';

exon 6: 5'GGGGTACCCTTAACTTTTTGGTACTACTAATA3' and 5'GGGGTACCTGGCACTGGATCCGCGTCTGGGCT3'.

Embryos, whose DNA was not amplified by twist beta-galactosidase amplimers but by atonal (a marker of the third chromosome) primers, were considered as homozygous for a mutation on the third chromosome. Indeed, twist beta-Galactosidase is present on the balancer chromosome. This technique allowed the unambiguous genotyping of all individual embryos.

PCR products of the 5-HT_{2Dro} receptor were purified with Geneclean kit and directly sequenced.

Binding experiment

[^{125}I] DOI was used as a radioligand to investigate the pharmacological properties of membrane fractions from 5-HT_{2Dro}R transiently transfected COS-1 cells (Colas et al., 1995). Briefly, cell membranes were prepared by four cycles of homogenization (Brinkman P10 disrupter) and centrifugation (48,000 x g for 15 min). The assay was established to achieve steady state conditions and to optimize specific binding (Kellermann et al., 1996). 50 µg of membrane proteins were incubated with 5 nM [^{125}I]-DOI at 4°C for 60 min. The incubation medium (200 microl) contained 50 microl of radioligand, 50 microl of buffer or of competing drug, and 100 microl of membrane suspension (Protein concentration, 50 microg/ml). The mixture was incubated at 30°C for 30 min. Assays were terminated by vacuum filtration through glass fiber filters (GF/B), which had been pretreated with 0.1% polyethyleneimine, followed by 4 washes of 5 ml of ice- cold buffer. Filters were dried rapidly and their radioactivity determined by liquid scintillation counting. Non specific binding, determined in the presence of 10 microM unlabeled DOI, represented about 30% of total binding. Competition studies for [^{125}I] DOI binding were performed by adding increasing concentrations of test drug to the reaction. Data were analyzed using The GrapPad software. Pharmacological profiles of transiently expressed receptors were determined over three independent experiment performed in triplicate.

Cuticle preparation

Embryos were laid on an agar plate, observed at 25°C, 60% humidity. The percent of hatching larvae was evaluated after 24, 48 h and 72 h (25°C) after egg laying and expressed as the percentage of non-hatched embryos in relation to the total number embryos and larvae. After

72 h, the number of non-hatched embryos has been counted. About 25 % of *l(3)82CDc*^{C931} embryos (larval lethal on the same chromosome), 25% of *5-HT*$_{2Dro}$^{C1644}*/Df(3R)HTRI,* and 40 % of *5-HT*$_{2Dro}$^{C1644} embryos died before hatching. Cuticles from non-hatched embryos were prepared as described in (Colas et al., 1999b). Percentages in table 2 correspond to the proportion of a phenotypic class in relation to the total number of non-hatched embryos.

Time lapse video experiment

Flies laid on apple juice agar for 1 hour, aged for 2 hours, then overlaid with halocarbon 3S oil (Prolabo Voltalef) were staged according to (Campos-Ortega and Hartenstein, 1985). The development of randomly selected single embryo was video-recorded for 1 hour (one picture every 15 seconds, i.e. 241 pictures/hour) using a Leica MZ12 binocular and digital Sony camera under transmitted light. A surrounding superficial pixel layer of each picture of the video recorded embryos was selected and, after opening at the most rostral point and flattening, accumulated over time. (see supplementary data for detailed procedure). This allows a direct visualization of the movements of individual cells initially located around the midline, the quantification of their speed during gastrulation movements and their relative synchronization (speed = slope of the curve). We followed a set of ventral ectodermal cells close to the invaginating endoderm since they can be easily distinguish on each figures. We illustrated the different speed of individual cells (V_1 to V_4) as changes in the slope of the curve. The main inflexion point of the curve, the most reliably identifiable point, corresponds to V_2. Each embryo was individually PCR-genotyped after video recording using balancer chromosome as markers (Colas et al., 1999b). False colors correspond to different grey levels of the recorded images. Statistical significance was assessed by ANOVA and NeumanKeul posthoc test.

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Figure Legends

Figure 1: *l(3)82CDb^{C1644}* is an allele of *5-HT$_{2Dro}$* that encodes a P52S mutant receptor. (A) The *l(3)82CDb^{C1644}*, *l(3)82CDc^{C931}*, *l(3)82CDd^{C142}*, *l(3)82CDd^{C360}* and *l(3)82CDc^{l6}* complementation groups are represented. Their location were ordered and refined by deletion mapping with *Df(3R)HTRI* and *Df(3R)HTR6*. Only *l(3)82CDb^{C1644}* and *l(3)82CDc^{C931}* mapped in the vicinity of *5-HT$_{2Dro}$*. (B) The sequence of the second exon of the *5-HT$_{2Dro}$* gene (first coding exon) is presented for wildtype (WT) and *5-HT$_{2Dro}$^{C1644}* chromosomes. A C to T transition in *l(3)82CDb^{C1644}* changes the proline 52 into a serine within the long N-terminus domain of the receptor. (C) Schematic drawing indicating the location of the point mutation in the receptor molecule of the *l(3)82CDb^{C1644}* strain. TM: transmembrane domain.

Figure 2: Cuticular phenotype of *5-HT$_{2Dro}$^{C1644}* mutant embryos. (A, B) Cuticular phenotype of wildtype embryos. (C, D) "Ghost" cuticular phenotype of *5-HT$_{2Dro}$^{C1644}* embryos. "Ghost" embryos present no denticle but clear differentiated cuticle. (E, F) "Double line" phenotype of *5-HT$_{2Dro}$^{C1644}* embryos. Note that in each segment, there are only 2 rows of denticles. The scale bar in A, C, E corresponds to 80 microm while it corresponds to 20 microm in B, D, F.

Figure 3: Quantification of cell movements during GBE in wildtype, *5-HT$_{2Dro}$^{C1644}* and *Kr-Gal4, UAS-5-HT$_{2Dro}$* embryos. The gastrulation of embryos has been video recorded and image analysis allowed the direct visualization and quantification of ectodermal cell movements along their relative synchronization The X-axis is the flat projection of a pixel layer at the periphery of the embryos for each video frame, i.e. at a determinate time. This allows to visualize the location of ectodermal cells present around the midline of the embryo before cell movements start (1 pixel layer correspond to 1 picture). As we recorded one picture

every 15 seconds, we accumulated all flat projections along th Y-axis (thus being the timescale). As a consequence, each line on the figures corresponds to the trajectory of one cell along the periphery of the embryos. We analyzed 3-5 ectodermal cells directly close to the pole cells (bold lines) to calculate the cell speed movements from the beginning of the GBE. 1 pixel corresponds to 0.2 microm on the X-axis and to 15 seconds on the Y-axis (see supplementary data). (A) On a representative wildtype (w^{1118}) embryo, mesoderm invagination is first visualized by slight ventral ectoderm contraction before the ectodermal extension starts (time t=0 min). Then, ventral ectodermal cells begin to move from the anterior to the posterior position. The cephalic furrow (cf) can easily be observed. Cells surrounding the pole cells (PC) correspond to the ventral (VE) and dorsal (DE) endoderm that will form the posterior midgut after invagination. (B) In the video recording of a representative $5\text{-}HT_{2Dro}^{C1644}$ homozygous mutant embryo, ectodermal cells start their extension before contractions triggered by mesodermal invagination could be observed. (C) A representative *Kr-Gal4,UAS-5-*HT_{2Dro} embryo displays milder defects than homozygous $5\text{-}HT_{2Dro}^{C1644}$ embryos. The bold line starting initially close to the ventral endoderm (VE) corresponds to the cell tracing used to calculate speed presented in E. (D) In an embryo that undergoes GBE, A, B, C, D and E correspond to the position of the cell at the time of a change in its speed (Vi) during elongation. These points are noted on the X-axis of the right graph. (E) In wildtype (w^{1118}) embryos, at the beginning of GBE, cells move slowly and accelerate until pole cells invaginate and then cell speed decrease until posterior cells reach the dorsal cephalic furrow, V_2 corresponds to the maximal speed of the cell. (*) corresponds to significantly different speeds from wildtype ($p<0.05$). All embryos have been individually PCR genotyped after video recording using balancer chromosome markers. False colors correspond to different grey levels of the recorded images. (See supplementary data for the corresponding video recordings).

Table 1: 5-HT$_{2Dro}$ pharmacological properties.

Transfection	*5-HT$_{2Dro}$* cDNA	*5-HT$_{2Dro}^{C1644}$* cDNA
Agonists		
Dexfenfluramine	17±1	5.0±0.3$^{\#}$
5-HT	200±16	11±1$^{\#}$
α-methyl 5-HT	420±23	370±18
5-CT	1700±72	28 000±900*
Tryptamin	2040±83	1950±62
Antagonists		
Ritanserin	8.1±1.1	7.0±0.8
Ketanserin	40±5	8.1±0.7$^{\#}$
Mesulergine	78±6	71±5
Bufotenine	200±12	1350±45*
Yohimbine	310±18	340±16

Affinity determined as competition of ^{125}I-DOI is expressed as Ki±SEM in nM for each compound for wildtype *5-HT$_{2Dro}$* or mutant *5-HT$_{2Dro}^{C1644}$* receptor cDNA expressed transiently in COS-1 cells. # significant increase compared to wildtype; * significant decrease compared to wildtype; $P<0.05$, n=9. Following transfection of identical amount of plasmid encoding cDNAs, the expression levels of wildtype and mutant receptors were not significantly different (similar Bmax). 5-HT: 5-Hydroxytryptamine; 5-CT: Carboxytryptamine.

Table 2: Cuticular phenotypic classes of non-hatched embryos in percent.

	5-HT$_{2Dro}^{C1644}$	*5-HT$_{2Dro}^{C1644}$/Df(3R)HTRI*	*l(3)82CDc^{C931}*
n	**204**	**208**	**487**
Wildtype	79	96	95
Ghost	8	4	1
Double line	11	0	2
Others	2	0	2

Cuticles were prepared as described in Material and Method and observed with bright field microscope. The fly strains were maintained with the same TM3 balancer chromosome. The *l(3)82CDc^{C931}* embryos were used as control of the homozygous balancer phenotype since homozygous *l(3)82CDc^{C931}* are late larval lethal. Transheterozygous *5-HT$_{2Dro}^{C1644}$/Df(3R)HTRI* and homozygous *l(3)82CDc^{C931}*embryos are hatching as weak first instar larvae that are dying with apparent wildtype cuticle. Part (62.5% for *5-HT$_{2Dro}^{C1644}$*) and the near totality (*5-HT$_{2Dro}^{C1644}$/Df(3R)HTRI* and *l(3)82CDc^{C931}*) of the non-hatched embryos correspond to homozygous TM3 balancer chromosome on the same genetic background. The percent of homozygous *5-HT$_{2Dro}^{C1644}$* embryos that hatch and die as first-instar larvae is thus about 20%, and the percent of unhatched homozygous *5-HT$_{2Dro}^{C1644}$* embryos presenting wild-type, double-line or ghost cuticular phenotypes is about 43, 30, 22% respectively. Using a GFP -labeled balancer chromosomes, we confirmed that the “ghost” phenotype represented about 1/4 of all *5-HT$_{2Dro}^{C1644}$* homozygous embryos. Wildtype, “ghost”, and “double line” phenotypes are described in figure 2. Values are presented as percent of embryos with a cuticular phenotype in relation to those that hatch 72 hours after egg laying and (n) corresponds to the total counted embryos.