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Oxytocin receptors influence the development and maintenance of social behavior in zebrafish (*Danio rerio*)

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Zebrafish are highly social teleost fish and an excellent model to study social behavior. The neuropeptide Oxytocin is associated different social behaviors as well as disorders resulting in social impairment like autism spectrum disorder. However, how Oxytocin receptor signaling affects the development and expression kinetics of social behavior is not known. In this study we investigated the role of the two oxytocin receptors, Oxtr and Oxtrl, in the development and maintenance of social preference and shoaling behavior in 2- to 8-week-old zebrafish. Using CRISPR/Cas9 mediated *oxtr* and *oxtrl* knock-out fish, we found that the development of social preference is accelerated if one of the Oxytocin receptors is knocked-out and that the knock-out fish reach significantly higher levels of social preference. Moreover, *oxtr*$^{-/-}$ fish showed impairments in the maintenance of social preference. Social isolation prior to testing led to impaired maintenance of social preference in both wild-type and *oxtr* and *oxtrl* knock-out fish. Knocking-out either of the Oxytocin receptors also led to increased group spacing and reduced polarization in a 20-fish shoal at 8 weeks post fertilization, but not at 4. These results show that the development and maintenance of social behavior is influenced by the Oxytocin receptors and that the effects are not just pro- or antisocial, but dependent on both the age and social context of the fish.

Many species, including humans, live in groups to enhance their fitness—their lifetime reproductive success. Living in a social context offers many benefits like improved predator and food detection[1], availability of mating partners, reduction of energy consumption[2] as well as the opportunity to learn vital behaviors from conspecifics[3]. In order to optimize cohabitation within a group, different forms of social behavior evolved.

The zebrafish (*Danio rerio*), a small teleost fish, is a powerful animal model used in biomedical research, including drug discovery[4], developmental biology[5,6] and neurobiology[7,8]. Furthermore, zebrafish exhibit a variety of behaviors including avoidance[9], foraging and hunting[10], responses to stress[11] and different forms of sociality[12–16]. Examples of sociality include mating behavior, aggressive behavior and other simpler behaviors that occur in groups. Zebrafish prefer to swim in cohesive shoals, a tendency that develops within the first weeks of age[17]. Swimming in close proximity to conspecifics, also called social preference, starts to develop as early as 1–2 weeks-post-fertilization (wpf)[18,19]. Although the development of shoaling behavior has been correlated with age-dependent changes in the dopaminergic and serotonergic system[20], the mechanisms underlying the development and maintenance of social preference and shoaling behavior are not well understood.

The nonapeptide Oxytocin is a highly conserved neuropeptide, present in humans and with only minor alterations in most other animals[21]. The zebrafish orthologue Isotocin (abbreviated Oxt) differs by only two amino acids from human Oxytocin[21]. In addition to its role in parturition[22] and lactation[23], Oxytocin has been described in the context of memory consolidation[24] and nocifensive behavior[25–28]. Intracerebral Oxytocin levels also influence anxiety in mice[29–31], rats[32,33] or humans[34,35]. Moreover, the association between Oxytocin and social behavior has been demonstrated in multiple studies[36–38] and the human Oxytocin receptor may influence social traits affected in neurodevelopmental disorders such as autism spectrum disorder[39,40].

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Social isolation for multiple weeks has an impact on different rodent behaviors including anxiety[41], aggression[42] and depressive-like behavior[43]. Gilles and Polston[44] reported that a two-week isolation of rats starting after weaning (postnatal day 21) led to increased pro- and antisocial behavior. They further described an inverse correlation between the number of oxytocinergic neurons in the anterior parvicellular division of the hypothalamic paraventricular nucleus (PVN) and the level of antisocial behavior[44]. After long-term social isolation, the application of Oxytocin was shown to reduce depression-like behavior of prairie voles[43] or aggressive behavior in male mice[42]. Furthermore, different studies using rats described the number of oxytocinergic neurons in PVN to be reduced[45] (postnatal day 38–48), increased[46] (postnatal day 74) or unchanged[44] (postnatal day 103–107) following social isolation. Isolation has also been reported to reduce the activity of Oxytocin receptors (OTR) in the nucleus accumbens of rats[46] and to affect the expression of OTR in specific brain regions to different degrees: a four-week long social isolation of prairie voles led to reduced hypothalamic expression of OTR[47], whereas six- and eight-week social isolation did not change the expression of OTR in murine hippocampus[48].

In mice, the Oxytocin receptor is expressed in different brain regions including the hippocampus, amygdala, suprachiasmatic nucleus and prelimbic cortex[49]. As teleost fish like zebrafish experienced a round of whole genome duplication approximately 320–350 million years ago[50], they possess two orthologous Oxytocin receptors, the Oxytocin receptor (Oxtr) and the Oxytocin receptor like (Oxtrl). Several studies[51–53] investigated how Oxytocin receptors influence social recognition or social preference in adult zebrafish by blocking both Oxtr and Oxtrl with antagonists[51,52] or knocking out the *oxtr*[53]. Often however, these studies came to contradictory conclusions, underscoring the complexity of Oxytocin's effects. The discrete impact of Oxtr or Oxtrl on the development and maintenance of social behavior in socially or isolation reared zebrafish remains to be elucidated.

Making use of CRISPR/Cas9 generated *oxtr* and *oxtrl* knock-out lines, the primary objective of this study was to investigate the role of the Oxytocin receptors in the development and maintenance of social behavior, specifically social preference and shoaling behavior in zebrafish (*Danio rerio*).

Results

To investigate the role of the Oxytocin receptors in development and maintenance of social behavior we compared the *oxtr*$^{-/-}$ and *oxtrl*$^{-/-}$ fish and their wild-type controls in two behaviors: social preference (assesses the fish's preference for a social versus a non-social area) and shoaling.

Social preference. To measure social preference, individual zebrafish larvae at different developmental stages were placed in a rectangular tank with one area where conspecifics could be seen through a transparent glass wall (Fig. 1a). Following a period of habituation, two conspecifics were added to the stimulus area of the tank and the behavior of the experimental fish was observed. We measured the time spent in proximity to conspecifics (social region-of-interest) as an indicator for social preference. In wild-type zebrafish, this preference developed incrementally over the first 4 wpf and persisted up to 8 wpf (Fig. 1b,c). The increase in social preference observed between 2 and 4 wpf was comparable to the results of previous studies[19,54], although not significant ($p = 8.03 \times 10^{-1}$). The *oxtr*$^{-/-}$ fish, by contrast, exhibited an early peak of maximal social behavior at 3 wpf. In contrast to the wild-type fish, this high sociability was not maintained but rather significantly ($p = 4.95 \times 10^{-2}$) decremented over 4–8 wpf (Fig. 1b,c). The development of social preference was also significantly ($p = 2.09 \times 10^{-4}$) altered in the *oxtrl*$^{-/-}$ fish, which also exhibited an early onset of social preference, reaching a peak precociously at 3 wpf (Fig. 1b,c). Furthermore, the maximal level of social preference exhibited by both Oxytocin receptor knock-out fish lines was substantially greater than the maximal level exhibited by the wild-type fish.

Isolation has been reported to alter social behavior in different species[55–58]. Therefore we next examined the effects of rearing in isolation on the development and expression of social preference in both wild-type and *oxtr*$^{-/-}$ and *oxtrl*$^{-/-}$ fish. We individually raised (in the absence of conspecifics) wild-type, *oxtr*$^{-/-}$ and *oxtrl*$^{-/-}$ fish from 2 days post-fertilization (dpf) to the day of experiment (Fig. 2a). In wild-type fish isolation rearing did not significantly ($p = 3.39 \times 10^{-1}$) affect the onset kinetics of social preference, but it dramatically diminished the maintenance of social preference typically observed at 8 wpf (Fig. 2b). Indeed, at 8 wpf, isolation-reared wild-type fish failed to exhibit any significant ($p = 3.26 \times 10^{-1}$) social preference. In contrast to socially reared wild-type fish, the increase in social preference from 2 to 4 wpf was significant after isolation rearing ($p = 1.22 \times 10^{-2}$). However, a direct comparison of same age socially and isolation-reared wild-type fish revealed no significantly different levels of social preference at 2 to 4 wpf. Isolation rearing had a similar effect on the *oxtr*$^{-/-}$ fish: isolation did not alter the onset kinetics of social preference but led to an accelerated decline in social preference measured at 8 wpf (Fig. 2b). Interestingly, the *oxtrl*$^{-/-}$ fish showed the same pattern, but the accelerated decline in social preference following isolation rearing was precociously evident at 4 wpf (Fig. 2b and c). In addition, RT-PCR of *oxt* expression in socially- and isolation-reared fish revealed that isolation led to significantly reduced *oxt* levels at 8 wpf, but not at 4 wpf (see Supplementary Fig. S1c). The expression of *oxtr* and *oxtrl*, however, was unaffected by isolation rearing (Supplementary Fig. S1d and S1e). These data show that the social preference develops following isolation rearing in wild-type, *oxtr*$^{-/-}$ and *oxtrl*$^{-/-}$ fish, but this social preference is not maintained. The isolation rearing induced decline in social preference is evident earlier in *oxtr*$^{-/-}$ and *oxtrl*$^{-/-}$ fish (Fig. 2c).

An analysis of swim speed revealed significant differences between wildtype and mutant fish at 2 and 3 wpf (see Supplementary Figure S3a and S3b) these differences were not correlated with the alterations in social preference development described. For example, both 2- and 3-week old *oxtrl*$^{-/-}$ fish swam significantly faster than wildtype fish, but showed significantly enhanced (3 wpf) or decreased (2 wpf) social preference. Another example is the significantly reduced social preference of 4-week old isolation reared *oxtrl*$^{-/-}$ or *oxtr*$^{-/-}$ fish with an average swim speed comparable to wildtype zebrafish. Taken together, these data indicated that changes in swim speed induced by the loss of either Oxytocin receptor do not provide a straightforward explanation for the changes in social behavior observed.
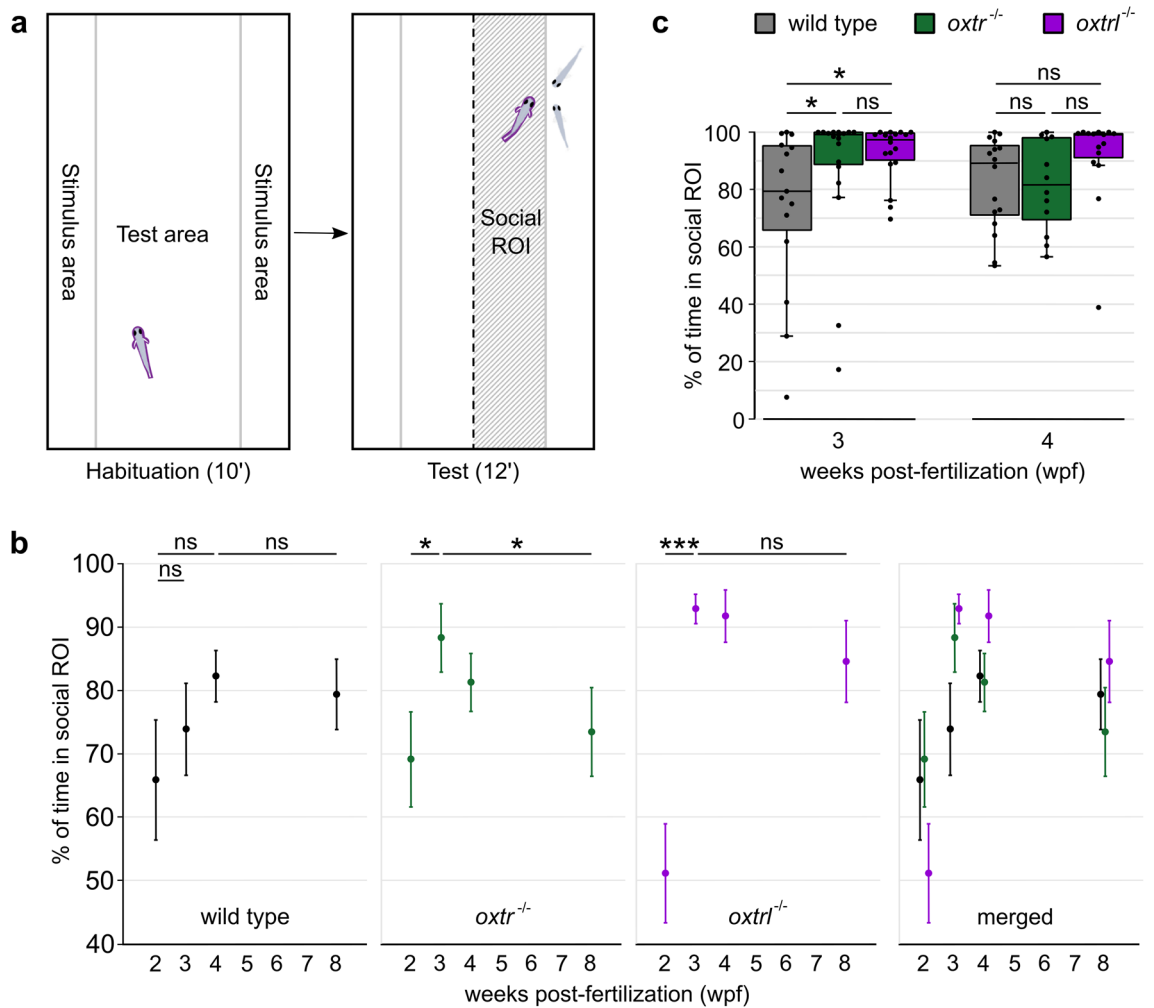
Figure 1. Oxytocin receptors affect the development, intensity and maintenance of social preference. (**a**) The behavioral chamber for social preference tests was composed of one test area and two stimulus areas, divided by transparent walls. After a habituation period, two stimulus fish were added to one stimulus area and the half next to the stimulus fish was defined as "social ROI" during analysis. (**b**) Social preference developed faster in both the *oxtr*$^{-/-}$ and *oxtrl*$^{-/-}$ fish and in *oxtr*$^{-/-}$ fish the maintenance of social preference was impaired. Values are reported as mean ± standard error of the mean (s.e.m.). Asterisks represent the statistical comparison of age in the different genotypes. n(social wildtype, 2 wpf) = 15, n(social wildtype, 3 wpf) = 15, n(social wildtype, 4 wpf) = 16, n(social wildtype, 8 wpf) = 13, n(social *oxtr*$^{-/-}$, 2 wpf) = 15, n(social *oxtr*$^{-/-}$, 3 wpf) = 18, n(social *oxtr*$^{-/-}$, 4 wpf) = 12, n(social *oxtr*$^{-/-}$, 8 wpf) = 16, n(social *oxtrl*$^{-/-}$, 2 wpf) = 18, n(social *oxtrl*$^{-/-}$, 3 wpf) = 18, n(social *oxtrl*$^{-/-}$, 4 wpf) = 15, n(social *oxtrl*$^{-/-}$, 8 wpf) = 11. Kruskal–Wallis-Test: p(social wildtype) = 9.31×10^{-1}, p(social *oxtr*$^{-/-}$) = 1.65×10^{-2}, p(social *oxtrl*$^{-/-}$) = 4.09×10^{-5}. Post-hoc Wilcoxon rank-sum test for socially reared *oxtr*$^{-/-}$: p(2wpf⟺3wpf) = 3.66×10^{-2}, p(2wpf⟺4wpf) = 6.11×10^{-1}, p(2wpf⟺8wpf) = 6.42×10^{-1}, p(3wpf⟺4wpf) = 7.40×10^{-2}, p(3wpf⟺8wpf) = 4.95×10^{-2}, p(4wpf⟺8wpf) = 6.42×10^{-1}. Post-hoc Wilcoxon rank-sum test for socially reared *oxtrl*$^{-/-}$: p(2wpf⟺3wpf) = 2.09×10^{-4}, p(2wpf⟺4wpf) = 3.54×10^{-4}, p(2wpf⟺8wpf) = 7.20×10^{-3}, p(3wpf⟺4wpf) = 9.86×10^{-1}, p(3wpf⟺8wpf) = 9.86×10^{-1}, p(4wpf⟺8wpf) = 9.86×10^{-1}. (**c**) At 3 and 4 wpf, the level of social preference was increased in the *oxtrl*$^{-/-}$ fish. Fish of the *oxtr*$^{-/-}$ line also showed enhanced social preference at 3 wpf, but did not maintain this high level at 4 wpf. Each dot represents one experimental fish. Asterisks represent the statistical comparison of genotype in two age groups. n(social wildtype, 3 wpf) = 15, n(social *oxtr*$^{-/-}$, 3 wpf) = 18, n(social *oxtrl*$^{-/-}$, 3 wpf) = 18, n(social wildtype, 4 wpf) = 16, n(social *oxtr*$^{-/-}$, 4 wpf) = 12, n(social *oxtrl*$^{-/-}$, 4 wpf) = 15. Kruskal–Wallis-Test: p(social 2 wpf) = 2.25×10^{-1}, p(social 3 wpf) = 2.35×10^{-2}, p(social 4 wpf) = 3.61×10^{-2}, p(social 8 wpf) = 2.87×10^{-1}. Post-hoc Wilcoxon rank-sum test for socially reared 3 wpf: p(wt⟺*oxtr*$^{-/-}$) = 2.93×10^{-2}, p(wt⟺*oxtrl*$^{-/-}$) = 2.93×10^{-2}, p(*oxtr*$^{-/-}$⟺*oxtrl*$^{-/-}$) = 7.00×10^{-1}. Post-hoc Wilcoxon rank-sum test for socially reared 4 wpf: p(wt⟺*oxtr*$^{-/-}$) = 9.26×10^{-1}, p(wt⟺*oxtrl*$^{-/-}$) = 5.34×10^{-2}, p(*oxtr*$^{-/-}$⟺*oxtrl*$^{-/-}$) = 5.34×10^{-2}. Number of replicates (n) and excluded n can be found in Supplementary Table S1, significance values in Supplementary Table S2. Significance is reported as *p < 0.05, **p < 0.01, ***p < 0.001.
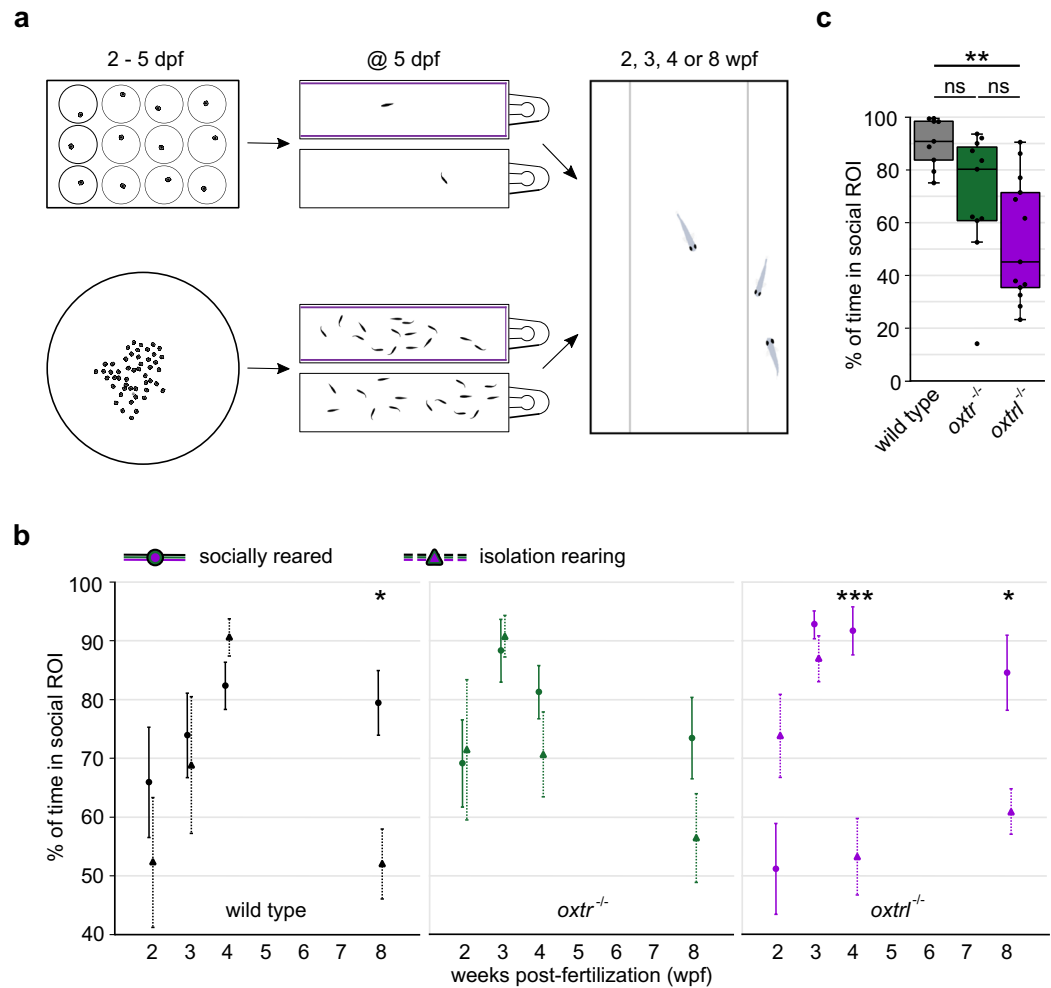
Figure 2. Isolation rearing impairs the maintenance, but not the development of social preference. (**a**) Rearing conditions: Experimental fish were raised in isolation (2–5 days post-fertilization (dpf) in 12-well plates, from 5 dpf until experiment in 1.1L tanks with visual barriers (here shown in purple) in every second tank) or with conspecifics (2–5 dpf in a dish with 145 mm diameter, from 5 dpf until experiment in 1.1L tanks with visual barriers in every second tank at densities of 10–15 fish/L). The social preference test was performed at 2, 3, 4 or 8 wpf. (**b**) The development of social preference was not influenced by isolation rearing. Similar to the phenotype observed for socially reared fish, knocking-out one of the Oxytocin receptors led to an accelerated development of social preference in isolated fish. Isolation rearing impaired the maintenance of social preference, but 4 wpf old $oxtr^{-/-}$ fish were less susceptible to isolation. Values are reported as mean ± standard error of the mean (s.e.m.). Asterisks represent the statistical comparison of rearing conditions (social vs. isolation) in different age groups of each genotype. Data for socially reared fish are also presented in Fig. 1b. n(isolated wildtype, 2 wpf) = 11, n(isolated wildtype, 3 wpf) = 11, n(isolated wildtype, 4 wpf) = 9, n(isolated wildtype, 8 wpf) = 16, n(isolated oxtr$^{-/-}$, 2 wpf) = 9, n(isolated oxtr$^{-/-}$, 3 wpf) = 18, n(isolated oxtr$^{-/-}$, 4 wpf) = 11, n(isolated oxtr$^{-/-}$, 8 wpf) = 10, n(isolated oxtrl$^{-/-}$, 2 wpf) = 13, n(isolated oxtrl$^{-/-}$, 3 wpf) = 12, n(isolated oxtrl$^{-/-}$, 4 wpf) = 13, n(isolated oxtrl$^{-/-}$, 8 wpf) = 17. Kruskal–Wallis-Test: p(wildtype) = 6.70×10^{-3}, p(oxtr$^{-/-}$) = 2.00×10^{-4}, p(oxtrl$^{-/-}$) = 7.79×10^{-9}, Post-hoc Wilcoxon rank-sum test for social vs. isolation reared wildtype: p(wildtype, 2wpf) = 3.39×10^{-1}, p(wildtype, 3wpf) = 9.59×10^{-1}, p(wildtype, 4wpf) = 3.39×10^{-1}, p(wildtype, 8wpf) = 1.64×10^{-2}, Post-hoc Wilcoxon rank-sum test for social vs. isolation reared $oxtr^{-/-}$: p(oxtr$^{-/-}$, 2wpf) = 4.09×10^{-1}, p(oxtr$^{-/-}$, 3wpf) = 4.09×10^{-1}, p(oxtr$^{-/-}$, 4wpf) = 4.09×10^{-1}, p(oxtr$^{-/-}$, 8wpf) = 2.76×10^{-1}, Post-hoc Wilcoxon rank-sum test for social vs. isolation reared $oxtrl^{-/-}$: p(oxtrl$^{-/-}$, 2wpf) = 9.09×10^{-2}, p(oxtrl$^{-/-}$, 3wpf) = 3.50×10^{-1}, p(oxtrl$^{-/-}$, 4wpf) = 3.58×10^{-4}, p(oxtrl$^{-/-}$, 8wpf) = 1.46×10^{-2}. (**c**) $oxtr^{-/-}$ and $oxtrl^{-/-}$ fish showed decreased social preference at 4 wpf after isolation rearing. Each dot represents one experimental fish. Asterisks represent the statistical comparison of genotype after isolation rearing at 4 wpf. n(isolated wildtype, 4 wpf) = 9, n(isolated oxtr$^{-/-}$, 4 wpf) = 11, n(isolated oxtrl$^{-/-}$, 4 wpf) = 13. Kruskal–Wallis-Test: p(isolated 2 wpf) = 1.85×10^{-1}, p(isolated 3 wpf) = 3.51×10^{-1}, p(isolated 4 wpf) = 1.80×10^{-3}, p(isolated 8 wpf) = 4.82×10^{-1}. Post-hoc Wilcoxon rank-sum test for isolation-reared 4 wpf: p(wt⇔oxtr$^{-/-}$) = 5.01×10^{-2}, p(wt⇔oxtrl$^{-/-}$) = 2.52×10^{-3}, p(oxtr$^{-/-}$⇔oxtrl$^{-/-}$) = 9.29×10^{-2}. Number of replicates (n) and excluded n can be found in Supplementary Table S1, significance values in Supplementary Table S2. Significance is reported as *p < 0.05, **p < 0.01, ***p < 0.001.

Shoaling. We also investigated another form of social behavior: shoaling. To measure shoaling we used a setup which allowed 20 zebrafish to move freely in a round tank (see Supplementary Fig. S2a) for 30 min. We tested socially reared wild-type, $oxtr^{-/-}$ and $oxtrl^{-/-}$ fish at ages of 4 and 8 wpf (younger fish could not be reliably tracked owing to their small size). To quantify shoaling, we analyzed the nearest-neighbor distance, inter-individual distance and farthest-neighbor distance of each fish as well as the cumulative shoal distance and the polarization parameter obtained from principal component analyses of individual videos (see "Materials and methods"). At 4 wpf, neither of the Oxytocin receptor knock-outs exhibited shoaling characteristics that were significantly different from their associated wild-type controls (p($oxtr^{+/+}$ vs. $oxtr^{-/-}$) = 4.74×10^{-1} (a), 4.09×10^{-1} (b), 4.74×10^{-1} (c) and 8.88×10^{-1} (d), p($oxtrl^{+/+}$ vs. $oxtrl^{-/-}$) = 3.17×10^{-1} (a), 2.59×10^{-1} (b), 1.29×10^{-1} (c) and 4.80×10^{-1} (d)) (Fig. 3a–d). At 8 wpf, however, both knock-out lines exhibited significant differences in their shoaling features, all consistent with the general phenotype of a less cohesive shoal (p($oxtr^{+/+}$ vs. $oxtr^{-/-}$) = 1.64×10^{-3} (a), 1.38×10^{-2} (b), 1.91×10^{-2} (c) and 4.95×10^{-2} (d), p($oxtrl^{+/+}$ vs. $oxtrl^{-/-}$) = 4.31×10^{-2} (a), 2.01×10^{-2} (b), 1.91×10^{-2} (c) and 4.95×10^{-2} (d)) (Fig. 3a–d). The cumulative shoal distance was not altered in both $oxtr^{-/-}$ and $oxtrl^{-/-}$ (see Supplementary Figure S2b). Moreover, knocking out one of the Oxytocin receptors did not lead to increased anxiety—represented by boldness (see Supplementary Figure S2c) and freezing (see Supplementary Figure S3c and S3d). Taken together, these two experiments revealed that both Oxytocin receptors play a crucial role in the development of social preference and shoal cohesion.

Discussion

Many studies[59–61] have demonstrated a connection between Oxytocin and social behavior, but the exact role of the two Oxytocin receptors in development and maintenance of social behavior remained to be clarified. Our data indicate that preference for companions develops gradually in wild-type zebrafish in the first few weeks, reaching a maximum social preference at 4 wpf, which is then maintained at a high level until at least 8 wpf. If one of the Oxytocin receptors (Oxtr or Oxtrl) was deleted, the development of social preference was accelerated, reaching its maximal level precociously at 3 wpf. Although Autism spectrum disorder is caused by multiple factors like mutations in the Fragile × mental retardation 1 (*FMR1*) gene[62], intranasal application of Oxytocin can ameliorate some of the social impairments of autistic patients[40] and additionally, specific single-nucleotide polymorphisms (SNPs) of the human *OXTR*[39] gene are frequent across the ASD population and have been described as potential genetic risk factor for ASD[63]. We have shown here that the development of social preference is affected by Oxytocin receptors in a distantly-related vertebrate, the zebrafish. It is interesting to note, that a recent study reported that the development of social preference is also sped up in *fmr1*$^{-/-}$ zebrafish[64].

Isolation rearing of zebrafish did not affect the time course of the development of social preference, but significantly affected its maintenance. A recent study by Tunbak and colleagues[65] described decreased social preference in about 40% of fish after isolation rearing. They used wild-type fish with the genetic background AB and a behavioral chamber described previously[19], which, in contrast to the chamber used here, does not allow simultaneous visual access to both stimulus areas. Unfortunately, the age of fish was not specifically defined, but they classified the experimental fish as "juvenile", suggesting an age between 30 and 89 dpf[66]. In our experiments, isolation-reared wild-type fish exhibited social preference that was comparable to socially-reared fish at age 14 to 28 dpf, but they were less social after 8 weeks of isolation. These data suggest that reduced social preference is a response to isolation periods longer than 28 dpf but differences in experimental design (different isolation rearing environments, behavioral chambers, light conditions, number of stimulus fish) can influence the results of social preference tests.

The isolation-induced decline in social preference we observed for wild-type zebrafish in our study was accelerated in both of the Oxytocin receptor knock-outs. RT-PCR of *oxt* expression in socially- and isolation-reared fish revealed that isolation led to significantly reduced *oxt* levels at 8 wpf, but not at 4 wpf (see Supplementary Fig. S1c). Furthermore, and in contrast to socially-reared fish, isolation-reared fish did not show significantly higher *oxt* expression with increasing age. These data are consistent with the observed behavioral changes after isolation rearing. The expression of *oxtr* and *oxtrl*, however, was unaffected by isolation rearing (see Supplementary Figure S1d and S1e), which is contrary to a study reporting reduced expression of the Oxytocin receptor (OTR) in prairie voles following four weeks of social isolation[47]. In addition to the reduced *oxt* expression in isolated fish, another possible explanation for the dramatically reduced social preference level at 8 wpf might be the downregulation of *pth2*, as isolation leads to reduced expression of this neuropeptide[67] as well.

Our results differ from the work of Landin and co-workers, who tested the social preference of adult and 3-week-old zebrafish after application of the Oxytocin receptor antagonist L-368,899, a specific inhibitor of both Oxtr and Oxtrl[52]. At both developmental stages they detected a decreased social preference. Unlike the Landin et al. study, a previous study by Zimmermann and colleagues did not find a significant decrease in social preference after intraperitoneal injection of L-368,899. The conflicting results in the two studies were attributed to the use of different concentrations of the antagonist, 0.01 ng/g[51] and 100 μg/g[52]. In contrast to these two studies, we used CRISPR/Cas9 generated genetic mutants lacking only one of the two Oxytocin receptors allowing us to dissect their distinct individual roles throughout development. Both receptors influenced the development of social preference, but in contrast to $oxtrl^{-/-}$, $oxtr^{-/-}$ fish did not maintain a high level of social preference as they became older. Wircer and colleagues[68] described a subpopulation of oxytocinergic neurons, which are located in the posterior tuberculum and express *oxtr*. Ablation of these neurons led to reduced social preference in adult zebrafish[68]. Interestingly, a study by Ribeiro and colleagues[53] showed impaired social recognition but unchanged social preference in a single Oxytocin receptor ($oxtr^{wz16/wz16}$) knock-out zebrafish line. Difference in the results obtained between Ribeiro et al. and our study may be due to the difference in experimental condition or age as they used a different behavioral chamber for the social preference test and three- to six-month old fish. *oxtrl* knock-out fish were not tested in their study. In another study, Ribeiro and co-workers described a non-significant tendency
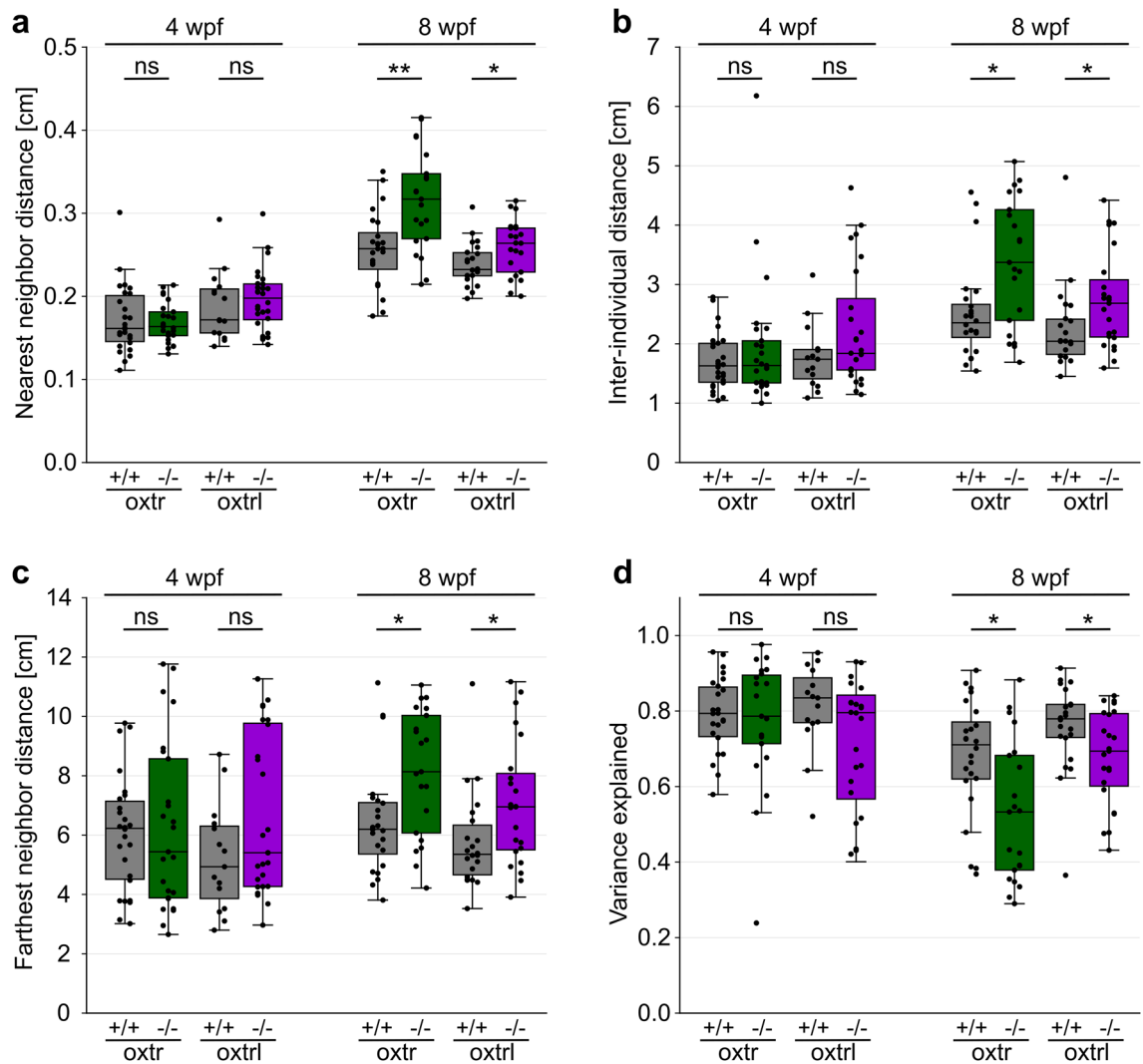
Figure 3. Shoal cohesion and polarization is positively influenced by the Oxytocin receptors at 8 wpf. (**a**) The absence of either Oxytocin receptor led to increased nearest neighbor distance at 8 wpf, but not at 4 wpf. n for shoals: see below. Kruskal–Wallis-Test for nearest-neighbor distance: $p(4wpf) = 1.69 \times 10^{-2}$, $p(8wpf) = 4.72 \times 10^{-5}$, Wilcoxon rank-sum test for the nearest-neighbor distance of wild-type vs. mutant genotype: $p(oxtr^{+/+} \Leftrightarrow oxtr^{-/-}, 4wpf) = 4.74 \times 10^{-1}$, $p(oxtrl^{+/+} \Leftrightarrow oxtrl^{-/-}, 4wpf) = 3.17 \times 10^{-1}$, $p(oxtr^{+/+} \Leftrightarrow oxtr^{-/-}, 8wpf) = 1.64 \times 10^{-3}$, $p(oxtrl^{+/+} \Leftrightarrow oxtrl^{-/-}, 8wpf) = 4.13 \times 10^{-2}$. (**b**) The inter-individual distance was enlarged in both Oxytocin receptor KO lines at 8 wpf, but not at 4 wpf. n for shoals: see below. Kruskal–Wallis-Test for inter-individual distance: $p(4wpf) = 2.07 \times 10^{-1}$, $p(8wpf) = 2.40 \times 10^{-3}$, Wilcoxon rank-sum test for the inter-individual distance of wild-type vs. mutant genotype: $p(oxtr^{+/+} \Leftrightarrow oxtr^{-/-}, 4wpf) = 4.09 \times 10^{-1}$, $p(oxtrl^{+/+} \Leftrightarrow oxtrl^{-/-}, 4wpf) = 2.59 \times 10^{-1}$, $p(oxtr^{+/+} \Leftrightarrow oxtr^{-/-}, 8wpf) = 1.38 \times 10^{-2}$, $p(oxtrl^{+/+} \Leftrightarrow oxtrl^{-/-}, 8wpf) = 2.01 \times 10^{-2}$. (**c**) *oxtr*$^{-/-}$ and *oxtrl*$^{-/-}$ showed increased farthest neighbor distance at 8 wpf, but not at 4 wpf. n for shoals: see below. Kruskal–Wallis-Test for farthest-neighbor distance: $p(4wpf) = 4.79 \times 10^{-1}$, $p(8wpf) = 2.65 \times 10^{-3}$, Wilcoxon rank-sum test for the farthest-neighbor distance of wild-type vs. mutant genotype: $p(oxtr^{+/+} \Leftrightarrow oxtr^{-/-}, 4wpf) = 4.74 \times 10^{-1}$, $p(oxtrl^{+/+} \Leftrightarrow oxtrl^{-/-}, 4wpf) = 1.29 \times 10^{-1}$, $p(oxtr^{+/+} \Leftrightarrow oxtr^{-/-}, 8wpf) = 1.91 \times 10^{-2}$, $p(oxtrl^{+/+} \Leftrightarrow oxtrl^{-/-}, 8wpf) = 1.91 \times 10^{-2}$. (**d**) Coordinated swimming, represented by the variance explained, was reduced in both knockout groups at 8 wpf but remained unaffected at 4 wpf. n for shoals: see below. Kruskal–Wallis-Test for variance explained: $p(4wpf) = 5.64 \times 10^{-1}$, $p(8wpf) = 7.00 \times 10^{-4}$, Wilcoxon rank-sum test for the variance explained of wild-type vs. mutant genotype: $p(oxtr^{+/+} \Leftrightarrow oxtr^{-/-}, 4wpf) = 8.88 \times 10^{-1}$, $p(oxtrl^{+/+} \Leftrightarrow oxtrl^{-/-}, 4wpf) = 4.80 \times 10^{-1}$, $p(oxtr^{+/+} \Leftrightarrow oxtr^{-/-}, 8wpf) = 4.95 \times 10^{-2}$, $p(oxtrl^{+/+} \Leftrightarrow oxtrl^{-/-}, 8wpf) = 4.95 \times 10^{-2}$. Number of shoals (containing 20 fish each) per group: $n(oxtr^{+/+}, 4wpf) = 26$, $n(oxtr^{-/-}, 4wpf) = 25$, $n(oxtrl^{+/+}, 4wpf) = 15$, $n(oxtrl^{-/-}, 4wpf) = 28$, $n(oxtr^{+/+}, 8wpf) = 24$, $n(oxtr^{-/-}, 8wpf) = 21$, $n(oxtrl^{+/+}, 8wpf) = 22$, $n(oxtrl^{-/-}, 8wpf) = 23$. Each dot represents one shoal. Asterisks represent the statistical comparison between mutant and wild-type genotype at different ages. Number of replicates (n) and excluded n can be found in Supplementary Table S1, significance values in Supplementary Table S2. Significance is reported as *$p < 0.05$, **$p < 0.01$, ***$p < 0.001$.

for less social preference in adult *oxtr* wild-type and knock-out fish after rearing in presence of *oxtr* knock-out conspecifics[69]. In our experiments, we reared wild-type fish with wild-type and knock-out fish with knock-out conspecifics. Different studies[70–73] with *Oxtr* knock-out mice have shown decreased social behavior and impaired social recognition with enhanced aggression. Remarkably, the deficits in social behavior were also present in heterozygous *Oxtr*^{+/−} mice[73]. In these studies, adult mice were tested. In a very recent study[74], Nunes, Gliksberg and colleagues observed impaired social affiliation in adult zebrafish after chemical ablation of oxytocinergic neurons in the dorsorostral part of the neurosecretory preoptic area at early developmental stages (4–6 dpf and 12–14 dpf), but later ablation (20–22 dpf and 90 dpf) did not affect social preference of adult fish. As the ablated oxytocinergic neurons fully recovered within 42 days[74], the described impaired social preference is likely due to the requirement of these oxytocinergic neurons at early developmental stages. The receptor knock-out fish we used in this study lacked Oxtr and Oxtrl, respectively, during all developmental stages. For oxtr^{−/−}, we observed a significant reduction in social preference between the peak of maximal social preference at 3 wpf and 8 wpf. It is possible that this significant decrease persists until adulthood, resulting in impaired social preference of adult *oxtr*^{−/−}, but this remains to be tested with knock-out fish. In comparison to oxtrl^{−/−} fish, the maintenance of social preference was impaired at higher degrees in oxtr^{−/−} fish, suggesting that this receptor might be more important for the Oxytocin influenced development of adult affiliative behavior in larval stages.

Our data suggest no alteration in anxiety or aggression levels in *oxtr*^{−/−} and *oxtrl*^{−/−} fish at the ages tested, as two indicators of anxiety—boldness (see Supplementary Figure S2c) and freezing (see Supplementary Figure S3c and S3d)—were not different between wildtype and mutant fish. In addition, the frequency of aggressive behavior, like biting, was not changed. In line with the Ribeiro et al. study[53], a study with Oxytocin receptor knock-out prairie voles[75] showed impaired social recognition in *Oxtr*^{−/−}, but did not find significant changes in social behavior. Our results further show that Oxtr and Oxtrl do not have same functions since *oxtr*^{−/−} and *oxtrl*^{−/−} knockouts exhibit different degrees of social preference throughout development. Importantly, when considering the role of oxytocin receptors, one may need to evaluate the potential role of Vasotocin, the zebrafish orthologue to mammalian Vasopressin, which can also bind to Oxtr and Oxtrl, though with a much lower affinity than Oxt[52].

As *oxtrl*^{−/−} fish show enhanced social preference level at 3 and 4 wpf, we expected them to shoal more tightly, with decreased group spacing compared to wild-type. The increase in social preference was not, however, accompanied by changes in the shoaling parameters of nearest-neighbor, inter-individual, farthest-neighbor distances and the polarization parameter "variance explained" in either the *oxtr*^{−/−} and *oxtrl*^{−/−} at 4 wpf. And yet, at 8 wpf the nearest-neighbor, inter-individual, and farthest-neighbor distances were significantly increased while the variance explained significantly decreased in both *oxtr*^{−/−} and *oxtrl*^{−/−}. These data indicate less polarized shoaling behavior with increased group spacing in *oxtr*^{−/−} and *oxtrl*^{−/−}. In line with our results, intraperitoneal injection of L-368,899 also led to enhanced nearest-neighbor, inter-individual, and farthest-neighbor distances in shoals of four adult fish[52], supporting a pro-cohesive role of the two Oxytocin receptors in the organization of shoals with fish older than 8 wpf. The major differences between the shoaling experiments of Landin and colleagues and ours are the age (adult vs. 8 or 4 wpf), shoal size (4 fish vs. 20 fish per shoal) and the behavioral chamber (trapezoid, 1.8 L vs. round, 2.4 or 3.0 L). Furthermore, in Landin et al., both Oxytocin receptors were blocked by antagonist injection 1 h prior to experiment whereas in our experiments only one of the two Oxytocin receptors was knocked-out since the time of fertilization. Although not significant, the first signs of reduced *oxtrl*^{−/−} shoaling capability were visible as early as 4-weeks of age. As shoaling behavior develops continuously until adulthood[17], the most parsimonious explanation for both sets of data is that Oxytocin receptors are likely to be important for shoal organization at later stages of development, whereas other signaling pathways may be able to compensate at 4 wpf. Similar to the social preference, both Oxytocin receptors modulate shoaling behavior, but to different degrees. Since shoaling parameters are also influenced by predator avoidance[76,77] and experience[78], shoaling should not be considered as an exclusive expression of social behavior. Moreover, shoaling and schooling are regulated by both visual and mechanosensory[79] (lateral line) inputs.

In a study by Tang and colleagues[80] the shoaling behavior of 90 CRISPR/Cas9 generated knock-out lines (for different genes) was compared (6 adult fish per shoal). This study revealed the influence of multiple genes on collective behavior of zebrafish—in particular on swim speed, group spacing and polarization. Tang et al. further showed that a single gene does not necessarily affect swimming speed, group spacing and polarization, but can influence only one or two of these shoaling parameters. Our data are consistent with this finding as we did not detect significant changes in the cumulative shoal distance at 8 wpf (see Supplementary Fig. S2b), whereas the group spacing and polarization parameters were significantly altered at this age. At first glance, the observations that the Oxytocin receptor mutant fish develop a preference for social companions precociously, yet exhibit poor coordination and shoaling behavior at 8 wpf may seem inconsistent. However, this may be explained by the observations of a decline in preference for social companions over ontogeny. *oxtr*^{−/−} mutants, for example, show a larger effect in diminished shoaling parameters at 8 wpf compared to *oxtrl*^{−/−} which agrees with the larger reduction in the preference for social companions at this age. The modest or no change in shoaling parameters at 4 wpf could reflect either a technical limitation of this assay at an early age, or, that there may be a "ceiling effect" to detect improved shoaling characteristics.

Taken together, our results show that the Oxytocin receptors play an important role in the development and maintenance of zebrafish social behavior and the impact of Oxytocin signaling depends on the age and environment of the fish.

Materials and methods

The materials and methods section follows the recommendations in the ARRIVE2.0 guidelines[81].

Study design, sample size and exclusion criteria.   The number of biological replicates for each experiment can be found in Supplementary Table S1.

For the investigation of social preference, we planned to test 18 fish per group. The sample size was determined prior to experiments using the E-equation method[82]. Unfortunately, some experimental fish died during rearing and therefore, 15 to 18 fish were tested per group. Data was excluded from further analysis if the test fish's average swim speed was below a threshold (73/411). This threshold was calculated for each age group by determining the average swimming speed of each genotype group (wild type, $oxtr^{-/-}$ or $oxtrl^{-/-}$) and multiplying the smallest with 0.6. The fish of three genotype groups ($oxtr^{+/+};oxtrl^{+/+}$ = "wild type", $oxtr^{-/-};oxtrl^{+/+}$ = "$oxtr^{-/-}$" and $oxtr^{+/+};oxtrl^{-/-}$ = "$oxtrl^{-/-}$") were reared either in isolation or with conspecifics at 10–15 fish/L density and were tested for their social preference at age of 2, 3, 4 or 8 wpf. To keep the testing conditions as similar as possible, the different genotypes were tested without unnecessary delay (performing all experiments of one age group within one month excect for 8 wpf (groups were tested within four months) and the isolation and socially reared fish of one genotype were tested on the same day, whenever possible.

In the 30-min long shoaling experiment, shoaling parameters were analyzed in a shoal of 20 fish at age of 4 or 8 wpf in three different genotypes ($oxtr^{+/+};oxtrl^{+/+}$ = "$oxtr^{+/+}$" or "$oxtrl^{+/+}$", $oxtr^{-/-};oxtrl^{+/+}$ = "$oxtr^{-/-}$"and $oxtr^{+/+};oxtrl^{-/-}$ = "$oxtrl^{-/-}$"). In contrast to social preference, we detected a significant difference in some shoaling parameters between the homozygous wild-type cousins of $oxtr^{-/-}$ and the homozygous wild-type cousins of $oxtrl^{-/-}$. Therefore, we analyzed the wild-type zebrafish as two genotype groups, "$oxtr^{+/+}$" and "$oxtrl^{+/+}$", respectively. We tested 23–28 socially reared (10–15 fish/L) shoals per group.

Test fish were used only once, sacrificed and genotyped after the experiment. If the fish/shoal was identified as heterozygous (22/611) it was excluded from further analysis.

Experimental animals and generation of knock-out.   The $oxtr$ (ZDB-GENE-110805-2/NCBI mRNA Reference Sequence: NM_001199370.1) and $oxtrl$ (ZDB-GENE-110805-1/NCBI mRNA Reference Sequence: NM_001199369.1) genes were mutated by Ajay Mathuru and Caroline Kibat using the sgRNA:Cas9 system described in [83]. Two CRIPSR targets (GGAAGTTACCGTGTTGGCCT and GGCTGATAAGCTTTAAAATA for $oxtr$; GTGCGTCCTTGTGGCCATCC and GGGGGGATTTTGTTCAGCCC for $oxtrl$) for each gene were identified using ZiFit (http://zifit.partners.org/zifit/). Customized sgRNAs with 20 nucleotide sequence complementary to a target site were synthesized by first cloning the target sequences into the expression construct pDR274 (Addgene #42,250) and then in vitro transcribed using T7 promoter according to the manufacturers protocol (Thermo Fisher # AMB13345).The Cas9 mRNA was transcribed from linearized plasmid MLM3616 (Addgene #42,251) following the manufacturers protocol (Thermo Fisher # AMB1344). The sgRNA:Cas9 RNAs cocktail (containing 12.5 ng/µL sgRNA and 300 ng/µL Cas9) were injected into single-cell embryos of AB wild-type background. The efficiency of the CRISPR targets and quality of the Cas9 endonuclease were determine 24 h post injection of sgRNA:Cas9 by PCR on 10% of the injected embryos. The remaining embryos were raised to adulthood and genotyped at three months post injection. A total of 64 individuals were genotyped. The sequence of the genotyping primer set is in detailed in the genotyping methods section. The PCR products were cloned into pGEMT (Promega #1360) and sequenced to verify the mutations. The F0 mutants were then then outcrossed to Danio Reds (https://doi.org/10.1016/S0006-291X(03)01282-8). F1 fish were in-crossed to establish germline transmitting homozygous mutant lines.

The lines are named $oxtr^{ync02}$ and $oxtrl^{ync03}$, in this paper abbreviated as $oxtr^{-/-}$ and $oxtrl^{-/-}$, respectively. The homozygous wild-type cousins of these fish were used as wild-type control. Moreover, these wild-type cousins were used for regular out-crossing of the homozygous knockouts (KO) to keep the genetic background of the experimental fish as similar as possible. We could not test $oxtr^{-/-}$ $oxtrl^{-/-}$ double KO fish as their generation by breeding failed, possibly because the presence of at least one oxytocin receptor is vital at specific developmental stages. When evaluating the expression of the knocked-out receptor using specific primers by real time PCR, amplification signals were detected in the wild-type but not in the mutant fish, indicating the successful knock-out. Furthermore, a compensation of the knocked-out receptor by its orthologue Oxytocin receptor was not detectable (see Supplementary Fig. S1b).

Experimental fish were bred by incrossing homozygous mutant or wild-type fish in a 1:1 sex ratio per batch. Each group consisted experimental fish of multiple clutches (up to 17) and the clutch size was 300–400 eggs (no difference between wild-type and mutant fish). On the day of experiment the test fish were 2, 3, 4 or 8 wpf old, depending on the age group. We used experimental fish with a total body length comparable to the total body lengths described in the Zebrafish Book[66]: 6 mm (2 wpf), 8 mm (3 wpf), 10 mm (4 wpf) and 14 mm (8 wpf). No differences in development or growth were detected comparing mutant and wild-type fish. In the social preference test the stimulus fish (two per test fish) had the same age and similar size than the test fish. As it is difficult (juveniles) to impossible (larvae) to distinguish males and females at the developmental stages used in this study, fish became experimental fish independent of their sex.

Zebrafish housing and husbandry.   Up to 5 dpf, the larvae were kept in a 28.5 °C incubator with a 14/10 light–dark cycle. At 2 dpf, eggs were either individually isolated in a 12-well plate (3 mL E3 medium [5 mM NaCl, 17 mM KCl, 0.33 mM CaCl$_2$, 0.33 mM MgSO$_4$] per 22 mm diameter well) or kept in pertri dishes (145 mm diameter, filled with 150 mL E3 medium) in groups of 50.

From 5 dpf on, they were raised isolated (including visual barriers placed in every second tank, see Fig. 2a, center) in 1.1L ZebTEC tanks or in groups of mixed sexes at densities of 10–15 fish/L in 1.1L ZebTEC tanks (social preference test) or 3.5L ZebTEC tanks (shoaling test).

From 5 dpf on, zebrafish were housed in a ZebTEC Active Blue Stand Alone System, with the water temperature of 28.5 °C ± 1 °C, the pH = 7.4 ± 0.3 and the conductance = 650 µS ± 100. The light–dark cycle in the

fish facility is 14 h-light/10 h-dark with a 30 min twilight phase. Fish were fed three times per day: Up to 10 dpf with vinegar eelworms (*Turbatrix aceti,* bred in house), CAVIAR 50–100 (SAFE) and crushed Gemma Micro 75 (Skretting), from 10 to 20 dpf with vinegar eelworms, brine shrimp (*Artemia salina, Ocean nutrition V154019*) and CAVIAR 50–100, from 21 to 56 dpf with brine shrimp and CAVIAR 100–200 (SAFE).

Experimental procedures.   *Social preference test.*   Behavioral chamber (height 10 mm) for testing social preference consisted of the test area (25×75 mm (for 2 to 4 wpf) and 50×75 mm (for 8 wpf)) and the stimulus areas (8×75 mm (for 2 to 4 wpf) and 16×75 mm (for 8 wpf)). The behavioral chamber was placed on a screen, providing white backlight illumination and a camera positioned above recorded the fish's movements with 20 (3–8 wpf) or 30 (2 wpf) frames per second using the program "Pylon recorder". To reduce visual and acoustic disturbances, the setup was surrounded by a black, sound-absorbing box (LBH: 450×670×850 mm). Prior to the experiment the test and stimulus fish were moved from their home tank to a 1L breeding tank (TECNI-PLAST, Part Number: ZB10BTE) with a nursery insert (TECNIPLAST, Part Number: ZB300BTI) and kept in a 28.5 °C incubator (test and stimulus fish in different tanks). After each test, the behavioral chamber was cleansed with hot water (~60 °C) and refilled with fresh ZebTEC Stand Alone system water (28.5 °C). The fish were moved using a disposable 3 ml pipette (the tip was cut off to generate a sufficient big diameter) by elevating the nursery insert and then transferred to the center of test area (test fish) or edge of stimulus area (stimulus fish) of the behavioral chamber. At 8 wpf the chamber was covered with the lid of a 145 mm dish to prevent fish from jumping out. The test fish habituated to the chamber without any other fish for ten minutes followed by a 12 min test phase in which two stimulus fish of same age and size were placed to one of the stimulus areas. A transparent wall between test and stimulus area allowed visual access to the stimulus fish whereas a white opaque wall determined the rear side of the stimulus area.

Shoaling experiment.   The shoaling setup (see Supplementary Fig. S2a) consisted of a white round behavioral chamber with a diameter of 70 cm which was surrounded by 29.5 °C water (heated by a pump) to prevent cooling of the water inside the behavioral chamber during the 30 min of experiment. A high-resolution camera (Basler acA4112-30um), positioned 73 cm over the chamber, recorded the fish with 30 frames per second using the program "Pylon recorder" and 1200 white LEDs in the ceiling provided the necessary illumination (approximately 650 Lux). A white box surrounding the setup reduced visual and acoustic disturbances of the fish. For each replicate the chamber was completely emptied, dried off and refilled with 2.4 L (4 wpf)/ 3.0 L (8 wpf) fresh 28.5 °C warm Stand Alone system water. In the morning of an experimental day, the home tanks were moved from the ZebTEC stand alone to a 28.5 °C incubator. Approximately 5 min before the experiment started, 20 fish of same age and similar size were transferred from their home tank and to a 1L breeding cage (TECNIPLAST) with a nursery insert (TECNIPLAST, Part Number: ZB300BTI). With the nursery insert all 20 fish of one shoal were moved simultaneously to the center of the behavioral chamber. The recording of shoaling behavior started immediately and continued for 30 min.

Genotyping.   In order to identify homozygous knockouts (KO) and homozygous wild types (wt) for generation of experimental fish, fin clip PCR was performed according to the ZIRC genotyping protocols (https://zebrafish.org/wiki/protocols/genotyping). Additionally, each experimental fish was genotyped after the experiment. The following primer sequences were used.

	Forward	Reverse	Band size
oxtr	TGGAGGACATCTTCAAGGA	CTTCCCTCGGTGCTTCAGGA	916 bp (wt), 413 bp (KO)
oxtrl	TGATCCTCTGGCCCATTAAC	TGGTCCATAAAAGCGAAAGG	314 bp (wt), 304 bp (KO)

The PCR products were loaded on a 1% (*oxtr*) or 2% (*oxtrl*) agarose gel and run at 100 V for 30 min (*oxtr*) or > 2 h (*oxtrl*). But as the difference between *oxtrl*$^{+/+}$ and *oxtrl*$^{-/-}$ is only 10 base pairs, PCR products were also sequenced using the forward primer.

Real-time PCR.   RNA was extracted from isolated brains as described before[67] and using the QuantiTect Reverse Transcription Kit (QIAGEN Cat. No. 205311) 200 ng of RNA was transcribed into cDNA. 5μL of 1:10 diluted cDNA template were mixed with 1.3μL primers (10 μM) and 6.25 μL SYBR Green PCR master mix (Applied Biosystems/Thermo Fisher Art. No. 4309155). The cycling parameters were 10 min at 95 °C, followed by 40 cycles of denaturation (15 s at 95 °C) and amplification (60 s at 60 °C). A Real Time PCR System (Applied Biosystems/Thermo Fisher Art. No. 4376600) was used for qRT-PCR. The fluorescence threshold was set to 0.9 for all experiments and genes and we used TATA box binding protein (*tbp*) as reference gene. We used the ΔC_T method and the following primer sequences.

	Forward	Reverse
oxt	AAGCTCTCGGTGTCAGCCTT	GGCATACACTGTCGAATGGG
oxtr	CCAACCTCTTCGTTCTTTACAC	ACAAAATCTCCCCAGCAATC
oxtrl	AAACCTGTGCGTCCTTGTG	ATAAAAGCGAAAGGTGATGTCC
tbp	GTACTCACAGGTGCCAAGGT	GATTGCGTAGGTCACCCCAG

Outcome measures.   In the social preference test, the location of the test fish was measured in each frame of habituation and during the last 10 min of test, as the stimulus fish needed up to 2 min after being transferred to the stimulus area to exhibit normal social behavior. During analysis the test area was divided into two regions of interest (ROI). The "social ROI" was an area next to the stimulus fish whereas the "antisocial ROI" was on the opposite site of the test area. The percentage of total time was determined, in which the fish was located in the "social ROI". The average swimming speed in absence (habituation) or presence (test) of stimulus fish was calculated and used as exclusion criteria for fish with reduced motion (e.g. because of freezing).

In the shoaling experiments all twenty fish of each shoal were tracked using TRex[84] (tracking threshold 15 (4 wpf) and 50 (8 wpf)) and the nearest-neighbor distance, the inter-individual distance and the farthest-neighbor distance of each fish were calculated and cumulative shoal distance and variance explained as parameter for coordinated swimming were determined. For determination of the variance explained, x- and y-components of all individual trajectories (20 per shoal) were first assembled in a $40 \times 54{,}000$ (30 min with 30 frames per second) matrix, whose covariance matrix was then eigen decomposed to obtain principal components (PCs). We found the first two PCs corresponded to the x- and y-component of the shoal centroid trajectory over time. Principal component analysis (PCA) is a dimensionality reduction approach that can be used to quantify the degree of linear correlation in a dataset. If individual features are highly correlated, they can be compressed in one PC while virtually no data are lost. This can be measured with the variance of the original dataset that is explained by the derived PCs. In our case, the higher the variance explained by the first two PCs, the higher the correlation between the individual fish's movement and the movement of the shoal overall (see Supplementary Fig. S2d). Additionally, we analyzed the variance explained from 20 fish of randomly chosen movies, expecting no coordinated swimming. In line with this expectation the variance explained was very low and increased continuously with increasing number of fish from the same movie (see Supplementary Fig. S2e). Thus, the variance explained can be used to quantify coordinated swimming of the fish.

Statistical methods.   Significance is reported as follows: *$p < 0.05$, **$p < 0.01$, ***$p < 0.001$.

All significance values can be found in Supplementary Table S2.

Normal distribution of data was checked using the Kolmogorov-Smirnoff-Test which revealed a non-normal distribution. Unpaired data was tested with a Kruskal–Wallis Test followed by a post-hoc Mann–Whitney-U Test (= Wilcoxon rank-sum test). The statistical analyses were carried out using python (from scipy.stats: kstest, kruskal and mannwhitneyu) or MATLAB (kstest, signrank, kruskalwallis and ranksum). In the social preference test n = 332 fish were included in the analysis (79 excluded), in the shoaling experiment n = 184 shoals were included in the analysis (18 excluded).

Blinding and randomization.   The investigators were not blinded to the genotype of the experimental fish, but both experiments were recorded with an overhead camera and analyzed using a tracking software (custom written tracking and analysis script for social preference and TRex[84] plus a custom written python script for shoaling) to provide an unbiased data analysis. The test fish were chosen randomly from their home tank group and in the social preference test the location of stimulus fish was changed between replicates in a random order. The experiments were performed from 8:00 AM to 5:00 PM and the order of genotype groups per day was changed between experimental days to minimize a potential circadian confounder. Furthermore, the position of tank during rearing was chosen in a way that all replicates of one experimental group were reared in all possible heights/light conditions.

Ethical statement.   All procedures were conducted in accordance with the institutional guidelines of the Max Planck Society and were approved by the Regierungspräsidium Darmstadt, Germany (governmental ID: V 54-19 c20/15-F126/1013 and V54-19 c20/15-F126/1016).

Data availability
The datasets generated and analyzed during this study and the custom written scripts are available under the public repositories GitHub (https://github.com/GemmerA/OxytocinReceptorSocial) and Edmond (https://edmond.mpdl.mpg.de/imeji/collection/_pyBtaEsY6X9liD).

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Author contributions

A.G. and E.M.S. conceived the project. A.G., S.R. and E.M.S. designed the experiments, C.K. and A.S.M. generated the *oxtr* and *oxtrl* knock-out fish. A.G. and K.M. conducted the experiments, bred and genotyped the experimental fish, L.A. and T.E. wrote the python script for analysis of the shoaling experiments, A.G., L.A. and T.E. analyzed the data, A.G. and E.M.S. wrote the manuscript and prepared the figures. All authors reviewed the manuscript.

Competing interests

The authors declare no competing interests.

Additional information

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