The Drosophila DmGluRA is required for social interaction and memory

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Metabotropic glutamate receptors (mGluRs) have well-established roles in cognition and social behavior in mammals. Whether or not these roles have been conserved throughout evolution from invertebrate species is less clear. Mammals have eight mGluRs whereas Drosophila has a single DmGluRA, which has both Gi and Gq coupled signaling activity. We have utilized Drosophila to examine the role of DmGluRA in social behavior and various phases of memory. We have found that flies that are homozygous or heterozygous for loss of function mutations of DmGluRA have impaired social behavior in male Drosophila. Furthermore, flies that are heterozygous for loss of function mutations of DmGluRA have impaired learning during training, immediate-recall memory, short-term memory, and long-term memory as young adults. This work demonstrates a role for mGluR activity in both social behavior and memory in Drosophila.

Keywords: mGluR, memory, Drosophila, long-term memory, DmGluRA, learning

INTRODUCTION

The Metabotropic glutamate receptors (mGluRs) in mammals have been shown to be involved in memory formation, long-term depression (LTD), long-term potentiation in mammals and linked to autism spectrum disorders (ASDs) in humans (Sestan et al., 2003; Mukherjee and Manahan-Vaughan, 2012). In mammals there are eight mGluRs, which are divided into three groups. Canonically, previous studies have established that group I mGluRs in mammals activate the Gi pathway, while group II and group III mGluRs activate the Go/Gi signaling pathway (Niswender and Coon, 2010; Chaki et al., 2012; Mukherjee and Manahan-Vaughan, 2012). However, there is accumulating evidence that in mammals, due to promiscuity of coupling to Gi and Gq, group II mGluRs may activate the Gq signaling pathway and induce LTD in a manner dependent on phospholipase C (PLC) and inositol trisphosphate receptor (InsP3R) activity (Huang et al., 1997, 1999a,b; Otani et al., 1999, 2002), and group I mGluRs are capable of activating Gi (Kreibich et al., 2004). In Drosophila there is only one mGluR, DmGluRA, which is coupled to Gi and Gq signaling (McBride et al., 2007; Pan and Brodie, 2007; Pan et al., 2008; Carto and Brodie, 2009; Fossier and Brodie, 2012). Gi coupled signaling is able to engage and activate both the PI3K and ERK signaling cascades as well as increase glycogen synthase kinase-3 beta (GSK-3beta) activity and Gq mediated mGluR activation is also able to activate GSK-3beta activity (Fan et al., 2004; Huang et al., 2006; Beaulieu et al., 2009; Vaskaitis et al., 2010).
Drosophila DmGluRA expression has been demonstrated in the brain including expression in areas critically involved in short-term memory such as the antennal lobes (ALs) and mushroom bodies (MBs; McBride et al., 1999; Zars et al., 2000; Yu et al., 2004; Pesavento et al., 2008) and long-term memory in the MBs (McBride et al., 1999; Fauscal and Froot, 2001) where expression is particularly heavy (Ramakers et al., 2001; Pan and Broadsie, 2007; Pan et al., 2008). More recently a detailed analysis of DmGluRA protein expression in the central complex has been published, a region of the brain where the expression of several other metabotropic receptors implicated in mammalian learning was found (Kahsai et al., 2012). The MBs in the insect are thought to be analogous to the mammalian hippocampus as first postulated from structural similarity to the human hippocampus in 1850 by the French physiologist and anatomist Dujardin (Dujardin, 1850; Davis, 1993, 2011; Busto et al., 2010; Skoulakis and Grammenoudi, 2006). Additionally, DmGluRA protein has been demonstrated to play a role in signaling at the presynapse of the NMJ in Drosophila and therefore could be similarly affecting signaling at the presynapse in the brain (Pan and Broadsie, 2007; Pan et al., 2008; Banerjee et al., 2010).

The role of DmGluRA in cognition has been previously demonstrated in studies of Drosophila models of Fragile X syndrome and Alzheimer’s disease. Fragile X is the leading inherited cause of intellectual disability and the leading known genetic cause of ASD. The FMR1 gene, the leading cause of Fragile X syndrome, has a role in long-term memory in the Drosophila model of Alzheimer’s disease that is based on mutations in the DmGluRA protein expression has been demonstrated in the brain including expression in areas critically involved in short-term memory such as the antennal lobes (ALs) and mushroom bodies (MBs; McBride et al., 1999; Zars et al., 2000; Yu et al., 2004; Pesavento et al., 2008) and long-term memory in the MBs (McBride et al., 1999; Fauscal and Froot, 2001) where expression is particularly heavy (Ramakers et al., 2001; Pan and Broadsie, 2007; Pan et al., 2008). More recently a detailed analysis of DmGluRA protein expression in the central complex has been published, a region of the brain where the expression of several other metabotropic receptors implicated in mammalian learning was found (Kahsai et al., 2012). The MBs in the insect are thought to be analogous to the mammalian hippocampus as first postulated from structural similarity to the human hippocampus in 1850 by the French physiologist and anatomist Dujardin (Dujardin, 1850; Davis, 1993, 2011; Busto et al., 2010; Skoulakis and Grammenoudi, 2006). Additionally, DmGluRA protein has been demonstrated to play a role in signaling at the presynapse of the NMJ in Drosophila and therefore could be similarly affecting signaling at the presynapse in the brain (Pan and Broadsie, 2007; Pan et al., 2008; Banerjee et al., 2010).

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short-term memory, but have age dependent impairments in LDT and short-term memory at 30 days of age (McBride et al., 2010). Pharmacologic treatment with mGluR antagonists starting before cognitive impairments begin prevents cognitive impairment. Furthermore, treatment with mGluR antagonists starting after the onset of cognitive impairments reverses cognitive impairments in this model, indicating mGluR involvement in modulating synaptic plasticity well into adulthood (McBride et al., 2010). This indicates that in the Alzheimer’s fly model, just as in the Fragile X fly model over active mGluR activity is contributing to memory impairment. More recently, under active mGluR activity has been implicated in phenotypes exhibited by tuberous sclerosis type 2 model mice (Auerbach et al., 2011). In spite of these findings, the involvement of DmGluRA in social interactions and memory in otherwise normal flies has remained unexplored in Drosophila. The purpose of this study was to examine the role of DmGluRA in social interactions and memory in Drosophila.

RESULTS

Social interaction can be examined in Drosophila in an ethologically relevant context by observing male courtship behavior directed toward female targets. Courting Drosophila males perform a characteristic sequence of behaviors: orienting toward and following the female, tapping her with his forelegs, vibrating one wing, lacking her genitalia, and attempting to copulate (Bastock, 1955, 1956; Sturtevant, 1915). The percentage of time that the male spends performing any of these behaviors toward a target female during a defined period of time is referred to as the courtship index (CI; Siegel and Hall, 1979).

We first examined the ability of young adult (6–10 days post-eclosion) homozygous null DmGluRA112 flies to perform naive courtship with virgin female targets as well as the DmGluRA2b, a precise excision control line. We found courtship behavior to be significantly impaired in the DmGluRA homozygous mutant flies, with the flies demonstrating almost no courtship activity (CIs of 3.2 ± 0.4), whereas the genetic background control flies demonstrated intact courtship behavior (CIs of 12.1 ± 0.8; Figure 1A). This demonstrated that the DmGluRA activity is required for social interaction since there was a significant impairment in naive courtship behavior compared to the control strain.

The low courtship activity of the DmGluRA mutants prevented us from examining memory in the homozygous mutant mGluR flies. Therefore to explore a possible role of DmGluRA in memory, we examined courtship and memory in flies heterozygous for this mutation. To do this we crossed both the DmGluRA null mutant (DmGluRA112) and precise excision control (DmGluRA2b) lines to Oregon R flies. We found that in the heterozygous state there was still an impairment in social interaction of the DmGluRA112 heterozygous flies compared to the DmGluRA2b controls, 65.2 ± 5.3 vs 85.3 ± 6.2, again indicating a role for DmGluRA function in social interactions in Drosophila (Figure 1B). It should be noted that courtship in the OreR background is significantly higher than in the original background, mainly due to visual acuity differences in detecting motion, since the original background is white eyed. The OreR/OreR controls, the precise excision heterozygotes (DmGluRA2b/OreR) controls and the mutant heterozygotes (DmGluRA112/OreR) all had similar...
eye color. Furthermore, both control genotypes displayed similar levels of naïve courtship activity. Although the heterozygous DmGluRA mutant flies displayed reduced naïve courtship activity, they still retained enough courtship activity to examine learning and various forms of memory using the conditioned courtship memory paradigm, an associative memory paradigm. In conditioned courtship, a male fly learns to modify his courtship behavior after experience with an unreceptive female (Siegel and Hall, 1979; Hall, 1994). Virgin females generally respond to a courting male by mating. However, recently mated females are unreceptive, display rejecting behaviors toward advances made by the male and have an overlapping but altered pheromonal profile that naïve males find less provocative than that of virgin female targets (Ejima et al., 2007). Normally, naïve male paired with a mated female target will initially court her, but his courtship activity soon decreases. This LDT is quantified, by comparing the CI during the first 10 min to the CI of the last 10 min period of a 1 h pairing with a previously mated female. In this paradigm wild-type flies typically show a ≥40% decrease in courtship activity (Joiner and Griffith, 1997; Kane et al., 1997). Heterozygous DmGluRA112 mutants display impaired LDT as young adults (Figure 2), similar to what has been previously observed in older DmGluRA112 mutant flies at 30 days of age (McBride et al., 2010). In contrast heterozygous DmGluRA112 controls and the OreR/OreR controls displayed intact LDT. This demonstrates a requirement for DmGluRA function in LDT.
FIGURE 2 | The learning-during-training phase of conditioned courtship is impaired in heterozygous DmGluRA112 mutant flies. Mean CIs (± SEM) are plotted; Ns are indicated above each bar for all groups. For levels of significance, *** p < 0.001. The initial and final courtship levels of control DmGluRA2b precise excision heterozygous flies (filled black bars), DmGluRA112 heterozygous mutant flies (open bars) and OreR background flies (striped bars) are compared. Control DmGluRA2b flies and control OreR background flies exhibited intact learning-during-training as demonstrated by a significant depression of courtship activity from the initial to the final interval of the training session, whereas heterozygous DmGluRA112 mutant flies did not demonstrate learning-during-training.

To assess immediate-recall memory, a male fly was placed in a training chamber with a previously mated female for 1 h, and subsequently paired with a virgin female within 2 min of completing training. A lower CI compared to naïve trained (untrained) flies is indicative of memory. Heterozygous DmGluRA112 mutants display impaired immediate-recall memory as young adults, as they are not able to suppress their courtship upon subsequent pairing with a virgin female target (Figure 3). In contrast heterozygous DmGluRA2b controls and the OreR/OreR controls displayed intact immediate-recall memory. This demonstrates a requirement for DmGluRA function in immediate-recall memory.

To assess short-term memory, a male fly was placed in a training chamber with a previously mated female for 1 h, and subsequently paired with a virgin female 60 min after completing training. A lower CI compared to naïve-trained flies is indicative of memory. Heterozygous DmGluRA112 mutants do not demonstrate a suppression of their courtship upon subsequent pairing with a virgin female target, therefore they do not demonstrate short-term memory (Figure 4). In contrast heterozygous DmGluRA2b controls and the OreR/OreR controls displayed a suppression of courtship after training and therefore demonstrated short-term memory. This demonstrates a requirement for DmGluRA function in short-term memory.

Finally we examined if DmGluRA function was required for long-term memory (McBride et al., 1999; Banerjee et al., 2010). To assess long-term memory, a male fly was placed in a training chamber containing food with a previously mated female for 7 h, and subsequently paired with a virgin female 4 days after completing training (McBride et al., 1999; Banerjee et al., 2010). Again, a lower CI compared to sham trained (naïve-trained) flies is indicative of memory. Heterozygous DmGluRA112 mutants do not demonstrate a suppression of their courtship upon subsequent pairing with a virgin female target, therefore they do not demonstrate long-term memory (Figure 5). In contrast heterozygous DmGluRA2b controls and the OreR/OreR controls displayed a suppression of courtship after training and therefore demonstrated long-term memory. This demonstrates a requirement for DmGluRA function in the formation of long-term memory.

To ensure that the decreased courtship activity of the homozygous and heterozygous DmGluRA112 mutants was not the result of specific impairment in not being able to complete the various phases of courtship, we measured the percentage of flies
FIGURE 4 | The short-term memory of conditioned courtship is impaired in heterozygous DmGluRA112 mutant flies. Short-term memory was measured by placing a trained male in a holding chamber for 60 min, then subsequently placing him in a testing chamber with a virgin female target for a 10 min courtship interval. The resulting CI is compared to the CI obtained for naïve courtship. Mean CIs (±SEM) are plotted; Ns are indicated above each bar for all groups. For levels of significance, ***p < 0.001. Control DmGluRA2b flies (filled black bars) and control OreR background flies (striped bars) exhibited intact short-term memory as demonstrated by a significant depression of courtship activity in the trained versus the naïve groups. The DmGluRA112 mutant flies (open bars) did not demonstrate a suppression of courtship activity after training and therefore had impaired short-term memory.

that progressed through the stages of courtship. The homozygous DmGluRA112 mutants demonstrated the ability to progress through all of the stages of courtship in a 10 min testing period. Both the homozygous DmGluRA112 mutants and the homozygous control precise excision DmGluRA2b had a significantly lower percentage of flies progressing to the licking/attempted copulation stage compared to the flies that were crossed to the OreR background (p < 0.05 by chi square). However, the DmGluRA112 mutants and the homozygous control precise excision DmGluRA2b did not differ from each other in the percentage of flies that reached this final step (Figure 6A). Both of the heterozygous lines as well as the OreR background control reached similar percentages of achieving all stages of courtship (Figure 6A). Since both the homozygous and heterozygous DmGluRA112 mutant flies were observed to be capable of performing all of the steps of courtship, the lack of courtship activity does not appear to be secondary to some type of impairment that is rendering them incapable of completing all of the steps of courtship behavior. To ensure that the decreased courtship activity of the homozygous and heterozygous DmGluRA112 mutants was not the result of locomotor activity impairments, we examined locomotor function in the dishes utilized for the conditioned courtship testing (McBride et al., 2005, 2010). We did not find differences in spontaneous line crossing between homozygous or heterozygous DmGluRA112 mutant flies vs homozygous or heterozygous DmGluRA2b control flies or the OreR/OreR control flies (Figure 6B). Additionally, neither the homozygous nor heterozygous flies in any of the genotypes displayed gross impairments in olfaction or vision (Figures 6C,D).

DISCUSSION

Although a role for mGluRs signaling is well established in memory processes in mammals, the role of the only Drosophila mGluR, DmGluRA, has remained relatively unstudied with regard to social interaction or memory (Serajee et al., 2003; Mukherjee and Manahan-Vaughan, 2012). The expression of DmGluRA in the Drosophila brain is in areas critically involved in social behavior and memory including the ALs and the MBs, thus may contribute...
FIGURE 6 | Analysis of courtship quality, locomotor activity, olfaction, and visual acuity in DmGluRA mutants. (A–D) The Ns for all genotypes in all panels is > 19. Filled black bars indicate control homozygous males (DmGluRA\textsuperscript{2b} precise excision, 2b/2b); Open bars indicate DmGluRA\textsuperscript{112} homozygous mutant males (112/112); Striped bars indicate control heterozygous males (DmGluRA\textsuperscript{2b} precise excision, 2b/OreR); Stippled bars indicate DmGluRA\textsuperscript{112} heterozygous mutant males (112/OreR); Gray bars indicate OreR background males. (A) The quality of courtship that was performed by naïve males was further analyzed by binning the number of males that advanced to particular phases of courtship for each genotype that was shown in Figure 1. All of the genotypes demonstrated that they could perform each phase of courtship for each genotype that was shown in Figure 1. (B) Locomotor activity was measured by a line crossing assay. (C) To measure olfactory capabilities we used the olfactory trap assay (Orgad et al., 2000; McBride et al., 2005). No differences were found between any of the genotypes tested with this assay at the 36 or 60 h time points. (D) To examine the visual capabilities of the genotypes and various treatment groups, we used the Y maze test (Orgad et al., 2000; McBride et al., 2005). No significant differences were detected between any of the treatments.
to these behavioral and cognitive processes (Ramaekers et al., 2001; Pan and Brodier, 2007; Pan et al., 2008).

Herein, we demonstrate that the DmGluR function is required for social interaction. We found that in the homozygous and heterozygous DmGluRA112 mutants there is impairment in social behavior. This data fits well with the previous finding that a mutation in mGluRs is linked to autism in humans (Serajee et al., 2003). Heterozygous DmGluRA112 mutants display impaired learning during training as young adults, thereby demonstrating a requirement for DmGluRA function in LTD, which may be analogous to working memory in mammals. Also heterozygous DmGluRA112 mutants display impairments in immediate-recall memory, short-term memory, and long-term memory as young adults. These findings fit well with the known role of mGluRs in short-term and long-term memory formation in mammals (Mukherjee and Manahan-Vaughan, 2012). In our study the deficits in social behavior and memory did not appear to be caused by impairments in vision, olfaction, locomotion, or the capability to perform complicated coordinated motor tasks such as copulation or flight, thus DmGluRA function appears to be specific for social and cognitive tasks.

This study illustrates an evolutionarily conserved role of the mGluRs in synaptic plasticity and memory formation which is an important finding in the context of using lower organisms to model cognitive diseases such as Fragile X or Alzheimer’s disease. Indeed, these are two disease models where antagonizing the Drosophila DmGluR has been demonstrated to rescue social and/or memory impairments (McBride et al., 2005, 2010, Choi et al., 2010). It was in the Drosophila model of Fragile X that pharmacological blockage of DmGluRA protein function was first demonstrated to rescue social interaction, immediate-recall memory, and short-term memory representing the first time pharmacologic treatment rescued social impairments in an animal model of autism or memory impairments in an animal model of intellectual disability (Rubin, 1999b; McBride et al., 2005, 2012). Additionally, it was demonstrated that treatments initiated in development as well as in adulthood demonstrated efficacy in rescuing social interactions and memory. The finding that adulthood treatments could ameliorate phenotypes associated with developmental disorders was paradigm shifting (Rubin, 1999a; State, 2010) and has now been demonstrated in other models of developmental disorders (Rubin, 1999c; Li et al., 2008; Guy et al., 2007). This strategy of decreasing mGluR activity to rescue cognition in the Fragile X model was later confirmed in mouse model of Fragile X by genetic and pharmacologic manipulation (Yoo et al., 2005; Dolen et al., 2007; Choi et al., 2011), and has met with some early success in trials with Fragile X patients (Jacquemont et al., 2011). This demonstrates the important role of DmGluRA function not just in development, but also in adulthood, warranting additional studies.

In Drosophila or cell culture models of Alzheimer’s disease antagonizing mGluRs has been efficacious in rescuing phenotypes associated with the models including cognitive impairment and Abeta secretion (Kim et al., 2010; McBride et al., 2009). Furthermore, both agonist and antagonists of mGluRs are under development for the treatment of depression in humans (Chaki et al., 2012; Mukherjee and Manahan-Vaughan, 2012). The current work along with the extensive mammalian literature on the involvement of mGluRs in memory illustrate that caution should be observed when looking at the mGluRs as receptors to modulate for the rescue of disease specific symptoms, because they may have unwanted effects on other aspects of cognition.

At first pass our results demonstrating that reduction of DmGluRA activity negatively impacts social behavior and cognition may seem counterintuitive, because antagonism of this receptors signaling can enhance memory in specific disease models. First, we have previously found that treatment with mGluR antagonists does not enhance memory in wild-type flies, indeed they impair memory and social activity (McBride et al., 2005; Choi et al., 2010). Also, toward this point it is important to keep in mind the way the molecular signaling occurs during memory formation. DmGluRA is predominantly coupled to G_{i}, thereby suppressing cAMP signaling. There is well documented literature in the fly field that indicates that cognition is impaired if cAMP levels are either too high, or too low. The dun mutant has severe memory impairments and results from too much cAMP. The dun mutation would be analogous to the DmGluRA mutants, with too much cAMP. In contrast, the rat mutation leads to too little cAMP and also results in memory impairment (Skoulakis and Grammenoudi, 2006). This would be analogous to the fly models of Alzheimer’s disease and fragile X syndrome, where the problem is too little cAMP and it is corrected by treatment with mGluR antagonists which should correct the cAMP to a level where normal memory can occur (McBride et al., 2005, 2010; Choi et al., 2010, 2011).

In conclusion, this work demonstrates that in Drosophila, just as in mammals, proper DmGluRA function is required for social behavior and various aspects of cognition including LTD, immediate-recall memory, short-term memory, and long-term memory.

MATERIALS AND METHODS

BEHAVIORAL TRAINING AND TESTING

Virgin male flies were collected under ether anesthesia within 4 h of eclosion. Virgin XX, y; f (attached X) females were collected on the day of eclosion and kept in food vials in groups of 10–15. Flies were aged in a 12 h light/dark cycle before behavioral training and testing. All testing was performed during the relative light phase. Mated females were 5 days old and observed to mate with a male the night before training. The virgin females that were used as targets were 4 days old (Joiner and Griffiths, 1997; McBride et al., 1999, 2005).

For courtship behavior testing, males of the appropriate genotype were collected within 4 h of eclosion and kept in isolation before testing. All flies were kept in 12 h light/dark cycles at 25°C and 50–75% relative humidity and were aged 6–10 days post-eclosion before training. All male subjects were transferred to fresh control food the day before testing (McBride et al., 1999, 2005, 2010). Male flies were assigned to random groups for behavior training and testing, which was performed blind (Siegel and Hall, 1979; Kane et al., 1997; McBride et al., 1999). The total amount of time a male was engaged in courtship activity while paired with an unanesthetized target female during a test period of 10 min or until successful copulation occurred was scored. A CI was calculated as the percentage of total observation time spent courting (Siegel and Hall, 1979). Testing of naive courtship, LTD, immediate-recall...
and short-term memory were performed as previously described (McBride et al., 1999, 2005). For the naive courtship analysis, the male was sham trained for 1 h in the training chamber without the addition of the female. The male was then transferred to the mating chamber containing a virgin female. Males were monitored for courtship activity that included orienting, following of the female, wing extension and vibration, tapping of female with his foreleg, genital licking and attempted copulation for a period of 10 min, or until copulation occurred.

Measurement of immediate-recall was made by pairing a naive male with a non-receptive female for a single 1 h training session and then placing him in a second chamber with a receptive female within 2 min of completing training. Short-term memory was assessed by taking a male that had been trained with a non-receptive female for 1 h and placing him in isolation for 1 h before pairing with a virgin, receptive, female. At least 16 animals were tested for each genotype during analyses of naive courtship, learning during training, immediate recall, short-term memory, and long-term memory.

The training paradigm for assessment of long-term memory is derived from McBride et al. (McBride et al., 1999; Banerjee et al., 2010). Males were paired with a non-receptive female for seven continuous hours and then kept in isolation for 4 days before testing. Sham-trained males were treated identically, except for the exposure to the training female. The observers were blind to the genotypes of the animals for all courtship studies (Banerjee et al., 2010; Szydlowska et al., 2010). Locomotor, olfaction, and visual acuity testing was done as in the study by McBride et al. (Griffith et al., 1993; McBride et al., 2005; Orgad et al., 2000).

Drosophila STRAINS

The Drosophila strains were cultured as in the study by McBride et al. (2005). The DmGluRA mutant and control lines used during this study were obtained from the Bloomington Drosophila Stock Center. Males were generated from virgins and long-term memory.

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