

Development

# Development of GABAergic Inputs Is Not Altered in Early Maturation of Adult Born Dentate Granule Neurons in Fragile X Mice

Christine L. Remmers<sup>1</sup> and  Anis Contractor<sup>1,2</sup><https://doi.org/10.1523/ENEURO.0137-18.2018><sup>1</sup>Department of Physiology, Feinberg School of Medicine, Northwestern University, Chicago, IL 60611 and<sup>2</sup>Department of Neurobiology, Weinberg College of Arts and Sciences, Northwestern University, Evanston, IL 60208

## Abstract

Fragile X syndrome (FXS) is the most common form of inherited mental retardation and the most common known cause of autism. Loss of fragile X mental retardation protein (FMRP) in mice (*Fmr1* KO) leads to altered synaptic and circuit maturation in the hippocampus that is correlated with alterations in hippocampal-dependent behaviors. Previous studies have demonstrated that loss of FMRP increased the rate of proliferation of progenitor cells and altered their fate specification in adult *Fmr1* KO mice. While these studies clearly demonstrate a role for FMRP in adult neurogenesis in the hippocampus, it is not known whether the functional synaptic maturation and integration of adult-born dentate granule cells (abDGCs) into hippocampal circuits is affected in *Fmr1* KO mice. Here, we used retroviral labeling to birthdate abDGCs in *Fmr1* KO mice which allowed us to perform targeted patch clamp recording to measure the development of synaptic inputs to these neurons at precise time points after differentiation. The frequency and amplitude of spontaneous GABAergic events increased over the first three weeks after differentiation; however, this normal development of GABAergic synapses was not altered in *Fmr1* KO mice. Furthermore, the relatively depolarized GABA reversal potential ( $E_{\text{GABA}}$ ) in immature abDGCs was unaffected by loss of FMRP as was the development of dendritic arbor of the adult generated neurons. These studies systematically characterized the functional development of abDGCs during the first four weeks after differentiation and demonstrate that the maturation of GABAergic synaptic inputs to these neurons is not grossly affected by the loss of FMRP.

**Key words:** adult born neurons; fragile X; GABA; neurogenesis; synapse

## Significance Statement

Loss of fragile X mental retardation protein (FMRP) causes Fragile X syndrome (FXS), a devastating neurodevelopmental disorder that causes multiple alterations in the development of synapses and neurons. Previous studies have described a role for FMRP in neurogenesis and hippocampal-dependent conditioning tasks linked to neurogenesis. This study systematically assessed the functional development of GABAergic inputs to adult born dentate granule cells (abDGCs) during the first four weeks after differentiation of adult neural stem cells in *Fmr1* KO mice.

## Introduction

Fragile X syndrome (FXS) is the most common known genetic cause of autism and intellectual disability. FXS is caused by the expansion of the CGG repeat in the 5' UTR of the *Fmr1* gene, which leads to hypermethylation, tran-

scriptional silencing, and loss of expression of the protein product, fragile X mental retardation protein (FMRP; [Willemssen et al., 2011](#)). FMRP is an RNA-binding protein that regulates a large number of mRNAs, many of which encode synaptic proteins. Dysregulated expression of synaptic proteins is thought to perturb synapse maturation

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and plasticity; however, the specific mechanisms underlying synaptic and cognitive deficits in FXS remain unclear. There are a growing number of studies demonstrating that FMRP plays important roles in stem cells, including adult neural stem cells in the neurogenic niche in the subgranular zone (SGZ) of the hippocampus (Li and Zhao, 2014). The importance of adult-born dentate granule cells (abDGCs) to hippocampal function, memory processes, and potentially to several neuropsychiatric disorders (Gonçalves et al., 2016) has raised the possibility that alterations in neural stem cell proliferation and maturation and integration of abDGCs contribute to the pathology of FXS. Proliferation of adult neural progenitors is enhanced in mice lacking FMRP (*Fmr1* KO; Luo et al., 2010; but also see Eadie et al., 2009), and fate specification is altered with fewer neural progenitors differentiating into neurons (Luo et al., 2010). In addition, it has been reported that reduced survival of adult-born neurons leads to an overall reduction in the number of DGCs in *Fmr1* KO mice (Lazarov et al., 2012). Therefore, the evidence suggests that the number of abDGCs is reduced in *Fmr1* KO mice and that FMRP ablation specifically in adult neural stem cells results in cell autonomous effects on proliferation, fate specification, and hippocampal-dependent behaviors (Guo et al., 2011). Despite these known disruptions in neurogenesis in *Fmr1* KO mice, an outstanding question is whether FMRP loss also affects how abDGCs mature and integrate into the hippocampal network after differentiation.

In many neuronal types, FMRP and related proteins play a role in neuronal development and synaptic function. There are alterations in the development of synapses particularly during early developmental critical periods in the cortex in *Fmr1* KO mice (Nimchinsky et al., 2001; Cruz-Martín et al., 2010; Harlow et al., 2010). In several instances, these morphologic and functional changes normalize during development (Bureau et al., 2008); however, phenotypes associated with these circuits persist in adult *Fmr1* KO mice (Arnett et al., 2014; He et al., 2017). abDGCs also undergo critical periods of development during the first few weeks after differentiation when they are undergoing synapse formation and dendritic remodeling (Bergami et al., 2015) and have elevated plasticity (Ge et al., 2007b). However, it remains unknown whether

loss of FMRP results in disruptions in synapse formation on abDGCs during this early developmental postmitotic period when they are actively integrating into the hippocampal network.

GABA synapses are the first inputs to form onto abDGCs and these synapses are initially excitatory due to a depolarized GABA reversal potential ( $E_{GABA}$ ) that results from a relatively high intracellular chloride concentration in young neurons (Overstreet Wadiche et al., 2005; Ge et al., 2006). These earliest GABA inputs are important for survival, dendritic development, and subsequent formation and unsilencing of glutamatergic synapses (Ge et al., 2006; Chancey et al., 2013; Song et al., 2013; Alvarez et al., 2016). The distinct characteristics of immature abDGCs endow these cells with properties that create unique roles for them in information processing (Ge et al., 2007a). Ablation of adult neurogenesis leads to impaired trace and contextual fear conditioning and pattern separation, indicating that newly generated neurons play a distinct role in learning that cannot be replicated by mature or developmentally born DGCs (Shors et al., 2001; Clelland et al., 2009; Nakashiba et al., 2012). Interestingly, these same behaviors that have been shown to require intact adult hippocampal neurogenesis are impaired in the *Fmr1* KO. *Fmr1* KO mice have deficits in trace fear conditioning (Zhao et al., 2005), and this phenotype can be recapitulated by specific deletion of *Fmr1* in adult neural stem cells (Guo et al., 2011). Therefore, while it is known that there are reductions in the number of abDGCs and correlated changes in behavior, it remains to be determined whether loss of FMRP results in changes in synaptic formation and maturation in abDGCs, which would alter their integration and activity in the hippocampal circuit.

Given the critical role of GABA signaling in early postmitotic maturation of abDGCs (Ge et al., 2006), and also the known alterations in GABA signaling during early postnatal development (He et al., 2014; Nomura et al., 2017) and in juvenile *Fmr1* KO mice (Paluszkiwicz et al., 2011; Martin et al., 2014; Zhang et al., 2017), we investigated whether the time course of development of inhibition onto abDGCs is altered in *Fmr1* KO mice.

## Materials and Methods

### Animals

All procedures related to the care and treatment of animals were performed in accordance with the Northwestern University Animal Care Committee's regulations. *Fmr1* KO mice (C57Bl/6) were maintained by breeding heterozygous females with WT or KO males. All experiments were performed blind to genotype in age-matched male littermates. Tail biopsies were used to perform *post hoc* genotyping of all mice used in the study.

### Retroviral birthdating

A replication incompetent retrovirus based on Moloney murine leukemia virus (MMLV) expressing RFP was prepared as described (Tashiro et al., 2006, 2015). Briefly, HEK-293 cells stably expressing GP2 were cotransfected with RFP and VSVG using Lipofectamine 2000. The media

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Correspondence should be addressed to Anis Contractor, Department of Physiology, Northwestern University Feinberg School of Medicine, 303 East Chicago Avenue, Chicago, IL 60611, E-mail: a-contractor@northwestern.edu.  
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were collected from transfected cells 3 and 6 d after transfection, filtered, and centrifuged at 25,000 rpm to precipitate the virus. Eight- to 10-week-old *Fmr1* WT or KO males were anesthetized using ketamine/xylazine and 1  $\mu$ l of virus was injected bilaterally into the SGZ of the dentate gyrus at a rate of  $\sim$ 0.3  $\mu$ l/min.

### Slice preparation and electrophysiology

We prepared 250- $\mu$ m coronal slices at 14, 21, and 28 ( $\pm$ 1) days postinjection (dpi). Slices were prepared using a Leica Vibratome in ice-cold high-sucrose artificial CSF (ACSF) containing the following: 85 mM NaCl, 2.5 mM KCl, 1.25 mM  $\text{NaH}_2\text{PO}_4$ , 25 mM  $\text{NaHCO}_3$ , 25 mM glucose, 75 mM sucrose, 0.5 mM  $\text{CaCl}_2$ , and 4 mM  $\text{MgCl}_2$ , equilibrated with 95%  $\text{O}_2$  and 5%  $\text{CO}_2$  and including 10  $\mu$ M DL-APV and 100  $\mu$ M kynurenatate. Slices were heated to 28°C in the same sucrose-ACSF, then the sucrose solution was gradually exchanged for recovery ACSF containing the following: 125 mM NaCl, 2.4 mM KCl, 1.2 mM  $\text{NaH}_2\text{PO}_4$ , 25 mM  $\text{NaHCO}_3$ , 25 mM glucose, 1 mM  $\text{CaCl}_2$ , 2 mM  $\text{MgCl}_2$ , 0.01 mM dL-APV, and 0.1 mM kynurenic acid.

After a 60-min recovery, individual slices were transferred to a recording chamber and continuously perfused with oxygenated ACSF containing 2 mM  $\text{CaCl}_2$  and 1 mM  $\text{MgCl}_2$  at an elevated temperature of 32°C. The dentate gyrus was visually identified and targeted recordings were made from RFP-expressing dentate granule cells. Recording electrodes were manufactured from borosilicate glass pipettes and had tip resistances of 4–6 M $\Omega$  when filled with internal recording solution. For whole-cell recordings, internal recording solution contained the following: 95 mM CsF, 25 mM CsCl, 10 mM Cs-HEPES, 10 mM Cs-EGTA, 2 mM NaCl, 2 mM Mg-ATP, 10 mM QX-314, 5 mM TEA-Cl, and 5 mM 4-AP, pH adjusted to 7.3 with CsOH. Data were collected and analyzed using pClamp 10 software (Molecular Devices). Neurons were voltage-clamped at  $-70$  mV to record spontaneous IPSCs (sIPSCs) and miniature IPSCs (mIPSCs), which were isolated by inclusion of D-APV (50  $\mu$ M), CNQX (10  $\mu$ M), and TTX (1  $\mu$ M) for mIPSCs. MiniAnalysis (Synaptosoft) was used to analyze sIPSCs and mIPSCs. For perforated patch recordings, the pipette solution contained the following: 150 mM KCl and 100 mM HEPES, pH adjusted to 7.2 with Tris-OH. The pipette tip was filled with gramicidin-free KCl solution and then backfilled with solution containing gramicidin (100  $\mu$ g/ml). GABAergic currents were evoked using a picospritzer to deliver a 50-ms puff of 10  $\mu$ M GABA in the presence of 50  $\mu$ M D-APV and 10  $\mu$ M CNQX. Responses were recorded at holding potentials between  $-100$  and 0 mV. The GABA reversal potential was calculated as the  $x$ -axis intercept of the best-fit line of the current–voltage plot.

### Two-photon laser scanning microscopy

Labeled abDGCs were patched in the whole cell configuration as described above with Alexa Fluor 488 dye (50  $\mu$ M) added to the internal solution. Dye was allowed to perfuse through the cell for  $\sim$ 20 min before image acquisition. Fluorescent images were acquired with picosecond pulsed excitation at 790 nm. Images of the soma and

dendrites were acquired with 0.19- $\mu\text{m}^2$  pixels with 10- $\mu$ s pixel dwell time with 1.0- $\mu$ m focal steps. Neuron Studio was used to create 3-D reconstructions of the dendrites and morphology analysis was performed in NeuronStudio (Wearne et al., 2005) and ImageJ.

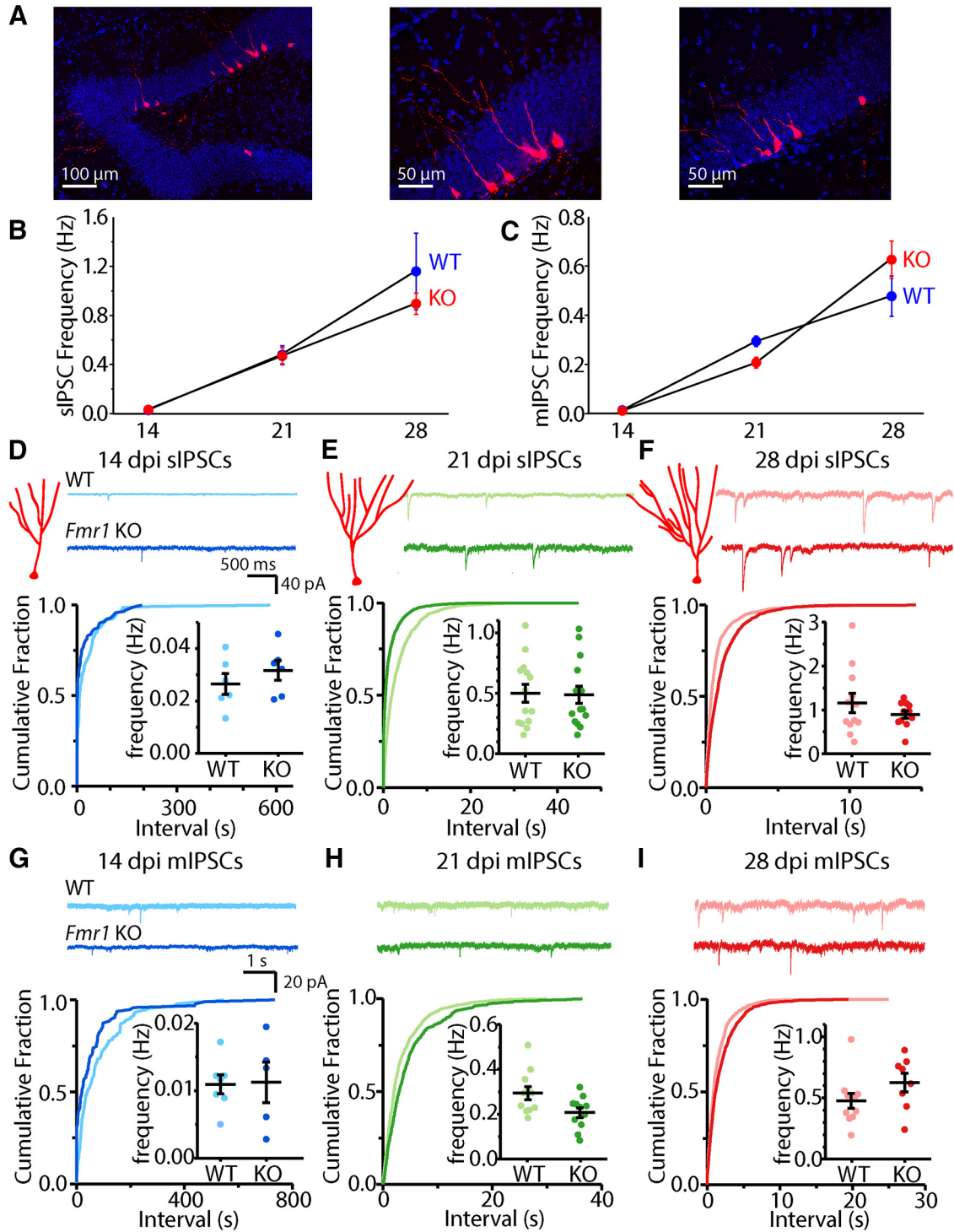
### Data analysis

Data analysis was performed using Microsoft Excel and OriginPro 2017 software. mIPSC and sIPSCs were analyzed using MiniAnalysis (Synaptosoft). Decay kinetics of mIPSC events was measured as the time to decay from 90% to 37% of the peak amplitude on the falling phase of the response. Comparisons were made with a Mann–Whitney  $U$  test, unless otherwise indicated. Differences were considered significant when  $p < 0.05$ . Data are shown as mean  $\pm$  SEM.

## Results

### Development of spontaneous GABA currents in abDGCs in *Fmr1* KO mice

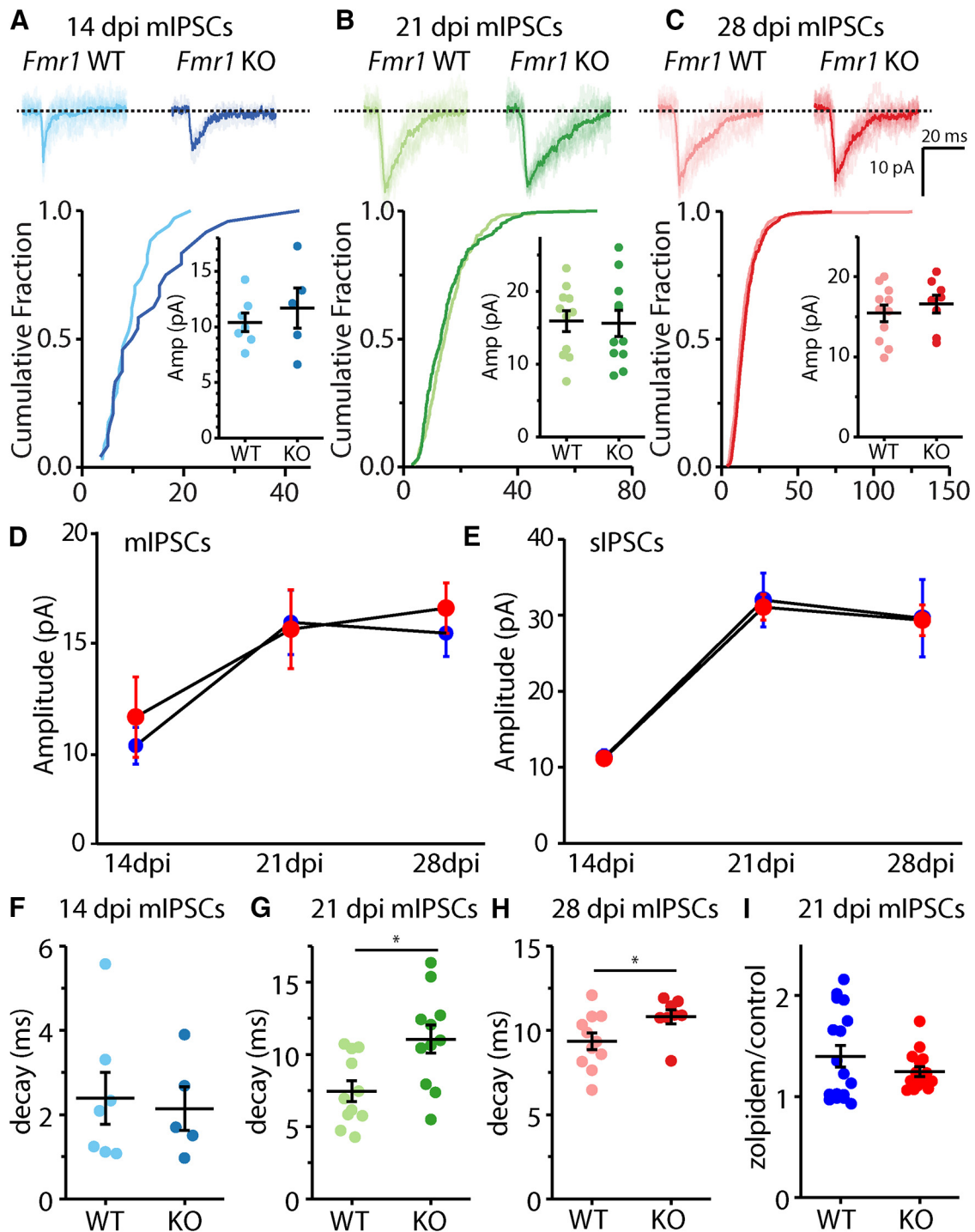
To identify and birthdate newborn DGCs, we injected a modified retrovirus expressing RFP into the SGZ of 8- to 10-week-old *Fmr1* KO mice and WT littermates (Tashiro et al., 2015). Retroviral injection clearly labeled neurons located in the SGZ of the dentate gyrus (Fig. 1A). Evoked IPSCs have been detected in abDGCs as early as 7 dpi (Ge et al., 2006) and in our recordings the earliest time point at which we consistently observed spontaneous inhibitory events was 14 dpi. Based on this we performed targeted patch-clamp recordings from RFP-expressing cells at 14, 21, and 28 dpi. We first measured the frequency of sIPSCs and mIPSCs at these time points. sIPSC frequency increased over time as abDGCs matured in both genotypes (Fig. 1B). Despite the known delays in maturation of properties of neurons in other cortical regions in *Fmr1* KO mice, we found that there was no difference in the sIPSC frequency at any postdifferentiation age of abDGC tested spanning this early period of development of these neurons (14 dpi WT:  $0.028 \pm 0.004$  Hz; 14 dpi KO:  $0.032 \pm 0.004$  Hz,  $n = 6/3$  (cells, animals respectively),  $p = 0.49$  Mann–Whitney  $U$  test; 21 dpi WT:  $0.480 \pm 0.074$  Hz,  $n = 14/7$ , 21 dpi KO:  $0.468 \pm 0.070$  Hz,  $n = 15/9$ ,  $p = 0.95$  Mann–Whitney  $U$  test; 28 dpi WT:  $1.16 \pm 0.031$  Hz,  $n = 12$ , 4 KO:  $0.90 \pm 0.086$  Hz,  $n = 11/6$ ,  $p = 0.70$  Mann–Whitney  $U$  test; Fig. 1B,D–F). In addition, we measured action potential independent spontaneous inhibitory events (mIPSCs) in abDGCs, which can be a good indicator of the number of inhibitory connections, or the release probability of those synapses. Again, we did not find a difference in the frequency of these events in comparisons between recordings in *Fmr1* WT and *Fmr1* KO mice at any of the ages tested (Fig. 1C,G–I). A comparison of the average mIPSC frequencies in each recording over time demonstrated an equivalent increase across this developmental period for abDGCs in both genotypes (Fig. 1C). Together, the lack of a difference in frequency of sIPSCs or mIPSCs during maturation of abDGCs indicates that there is no difference in the number of inhibitory synaptic connections in these neurons in the *Fmr1* KO mice during the first 4 weeks after differentiation.



**Figure 1.** Frequency of sIPSCs and mIPSCs is not altered in abDGCs in *Fmr1* KO. **A**, Representative images of 21 dpi abDGCs virally labeled with RFP. Average sIPSC (**B**) and mIPSC (**C**) frequencies across all time points measured in *Fmr1* WT (blue) and *Fmr1* KO mice (red). **D**, Schematic of dendritic morphology of abDGCs and representative traces (top panel), cumulative distribution of interevent-interval and average frequency of each recorded neuron (inset) of sIPSCs (bottom panel) at 14 dpi, 21 dpi (**E**) and 28 dpi (**F**). **G**, **H**, **I**, Representative traces, cumulative distribution of interevent-intervals and average frequency of mIPSCs in each recorded abDGC (inset) at 14, 21, and 28 dpi, respectively.

The amplitude of spontaneous events, particularly mIPSCs, is an indicator of the strength of individual synapses and is expected to increase as abDGCs undergo maturation

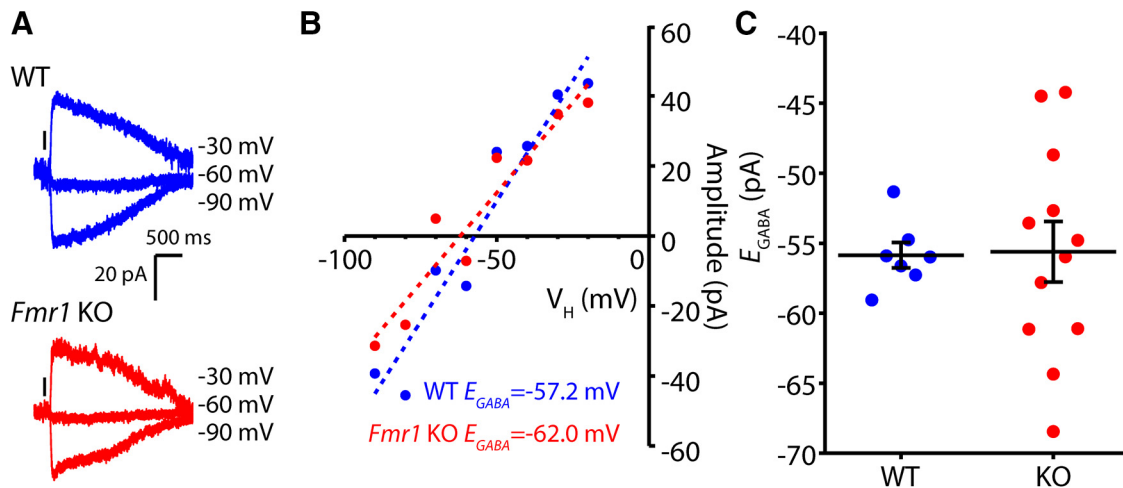
(Ge et al., 2006). Comparison of the mIPSC amplitudes at each of the days postdifferentiation spanning this period again did not reveal any difference between re-



**Figure 2.** mIPSC amplitude is unaffected by loss of FMRP, but mIPSC decay is slower in abDGCs in *Fmr1* KO. **A**, Representative traces of individual and averaged mIPSC events (top) and cumulative distribution and average amplitudes mIPSCs in each recording measured at 14 dpi, 21 dpi (**B**) and 28 dpi (**C**) abDGCs. Average mIPSC amplitude (**D**) and sIPSC amplitude (**E**) across all time points measured in *Fmr1* WT (blue) and KO (red). Average decay of mIPSCs in 14 dpi (**F**), 21 dpi (**G**), and 28 dpi (**H**) abDGCs. **I**, Average mIPSC decay with zolpidem normalized to decay pre-zolpidem (\* $p < 0.05$ , Mann-Whitney *U* test).

cordings in each of the genotypes (WT 14 dpi:  $10.4 \pm 0.827$  pA,  $n = 7/3$ ; KO 14 dpi:  $11.7 \pm 1.81$  pA,  $n = 5/5$ ,  $p = 0.64$ , Mann-Whitney *U* test; WT 21 dpi:  $15.9 \pm 1.45$  pA,  $n = 12/5$ , KO 21 dpi:  $15.6 \pm 1.78$  pA,  $n = 12/5$ ,  $p = 1.00$ , Mann-Whitney *U* test; WT 28 dpi:  $15.4 \pm 1.04$  pA,  $n = 11/4$ , KO 28 dpi:  $16.6 \pm 1.13$  pA,  $n = 8/3$ ,  $p = 0.49$ ,

Mann-Whitney *U* test; Fig. 2A–C). In addition, and consistent with no genotype-mediated changes in synaptic strength during the development of abDGCs in *Fmr1* KO mice, there were no differences in the amplitude of sIPSCs (Fig. 2E). In recordings of both mIPSCs and sIPSCs there was an equivalent increase of the average amplitude



**Figure 3.**  $E_{GABA}$  in 21 dpi abDGCs in *Fmr1* KO and WT. **A**, Representative traces of GABA responses from perforated-patch recordings in 21 dpi abDGCs in *Fmr1* WT (top, blue) and *Fmr1* KO (bottom, red) measured at three holding potentials ( $-30$ ,  $-60$ ,  $-90$  mV). The response to a 50-ms puff of  $10 \mu\text{M}$  GABA was measured at several holding potentials in the presence of  $50 \mu\text{M}$  D-APV and  $10 \mu\text{M}$  CNQX. **B**, Representative current–voltage curves constructed from responses in two cells (blue WT, red KO).  $E_{GABA}$  was calculated as the x-axis intercept of the best-fit line of the current–voltage plot. **C**, Grouped  $E_{GABA}$  data from all recordings.

of events in older neurons in both genotypes (Fig. 2D,E). Interestingly, at 14 dpi, the average amplitude for both mIPSCs and sIPSCs was similar, suggesting that at this time point presynaptic inhibitory neurons make a single synaptic connection per axon. However, the amplitudes diverged at 21 dpi, with sIPSC amplitudes larger than those of mIPSCs, indicating that single presynaptic axons make multiple contacts onto abDGCs in these older neurons (Fig. 2D,E).

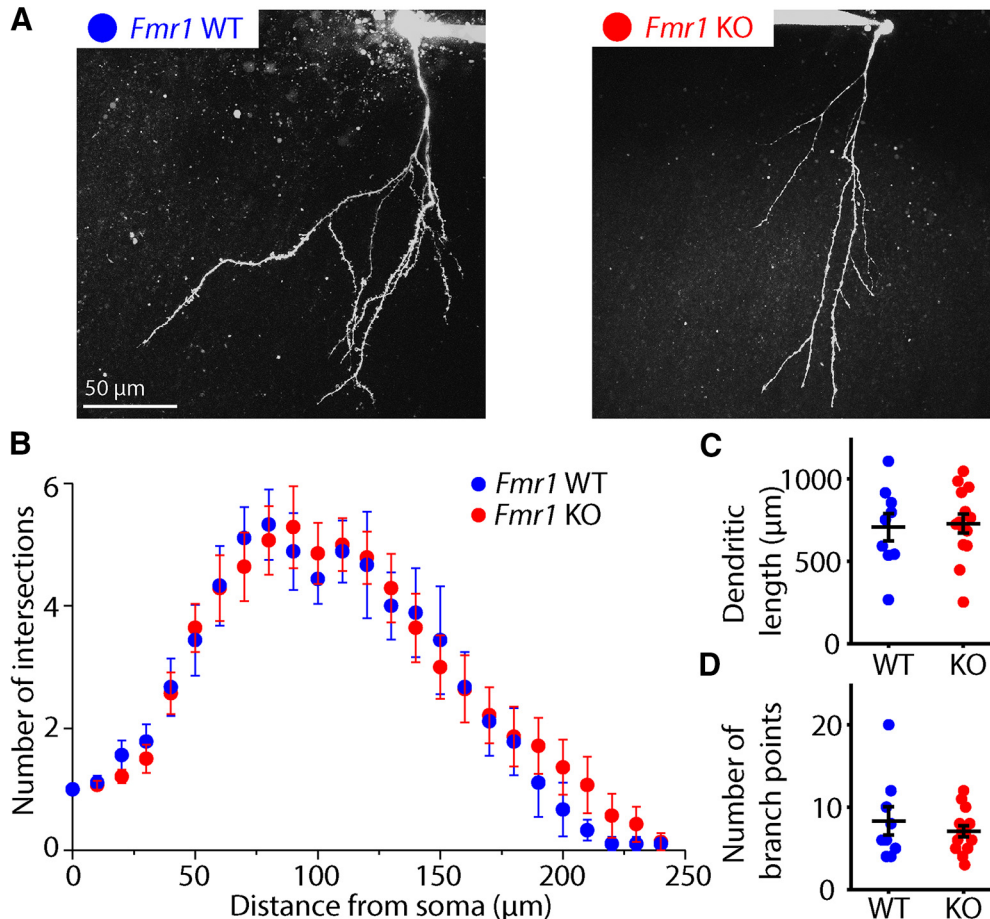
Measuring the decay of mIPSCs, we found that there was a significant increase in the decay time course as abDGCs matured in both genotypes that could be indicative of a change in the subunit composition of GABA<sub>A</sub>Rs (Overstreet Wadiche et al., 2005). At the youngest time measured mIPSC decay was not significantly different in *Fmr1* KO (WT 14 dpi:  $2.39 \pm 0.612$  ms,  $n = 7/3$ ; KO 14 dpi:  $2.15 \pm 0.517$  ms,  $n = 5/4$ ,  $p = 1.00$ , Mann–Whitney  $U$  test; Fig. 2F). However, at older ages of abDGCs, the decay of mIPSCs in *Fmr1* KO neurons was slower (WT 21 dpi:  $7.45 \pm 0.722$  ms,  $n = 12/5$ ; KO:  $11.1 \pm 0.987$  ms,  $n = 12/5$ ,  $p = 0.008$ , Mann–Whitney  $U$  test; WT 28 dpi:  $9.36 \pm 0.492$  ms,  $n = 11/4$ ; KO 28 dpi:  $10.8 \pm 0.410$  ms,  $n = 8/3$  cells,  $p = 0.041$ , Mann–Whitney  $U$  test; Fig. 2G,H). In addition, sIPSC decay was not significantly different in *Fmr1* KO (WT 14 dpi:  $1.66 \pm 0.275$  ms,  $n = 6/3$ ; KO 14 dpi:  $1.57 \pm 0.127$  ms,  $n = 6/3$ ;  $p = 0.7$ , Mann–Whitney  $U$  test; WT 21 dpi:  $12.6 \pm 1.19$  ms,  $n = 14/8$ ; KO 21 dpi:  $16.0 \pm 1.39$  ms,  $n = 15/9$ ,  $p = 0.08$ , Mann–Whitney  $U$  test; WT 28 dpi:  $14.2 \pm 1.32$  ms,  $n = 12/4$ ; KO 28 dpi:  $13.3 \pm 0.80$  ms,  $n = 11/6$  cells,  $p = 0.651$ , Mann–Whitney  $U$  test).

Previous studies have found that the decay kinetics of sIPSCs are slower in abDGCs than in mature granule cells because of the incorporation of  $\alpha 1$  subunit into postsynaptic GABA<sub>A</sub> receptors as neurons mature (Overstreet Wadiche et al., 2005). We tested whether the increased decay of the mIPSCs in abDGCs in *Fmr1* KO mice might reflect a lower  $\alpha 1$  subunit incorporation by measuring the effect of zolpidem, an  $\alpha 1$ -specific

positive allosteric modulator, on the decay kinetics of mIPSCs. mIPSCs were recorded at 21 dpi and the decay measured before and after the addition of  $0.5 \mu\text{M}$  zolpidem (Fig. 2I). We found that zolpidem lengthened mIPSC decay in 21 dpi abDGCs in both *Fmr1* KO and WT to the same degree suggesting that  $\alpha 1$  subunit content does not underlie the differences in decay observed in the *Fmr1* KO mice (decay zolpidem/decay control WT:  $1.40 \pm 0.11$ ,  $n = 17/7$ ; KO:  $1.25 \pm 0.05$ ,  $n = 15/7$ ,  $p = 0.87$ , Mann–Whitney  $U$  test).

### Maturation of the GABA reversal potential ( $E_{GABA}$ ) in abDGCs

As abDGCs mature the chloride reversal potential becomes progressively hyperpolarized in a similar manner to what occurs in other developing neurons (Ge et al., 2006; Chancey et al., 2013). The chloride equilibrium potential in large part determines the reversal potential for GABA<sub>A</sub> receptors ( $E_{GABA}$ ) and therefore affects the strength of inhibitory transmission.  $E_{GABA}$  reaches its mature value 4 weeks after differentiation in abDGCs (Ge et al., 2006). Prior analysis of *Fmr1* KO mice has demonstrated that  $E_{GABA}$  maturation is delayed in the developing cortex and hippocampus of *Fmr1* KO mice (He et al., 2014; Tyzio et al., 2014). Therefore, we measured  $E_{GABA}$  using perforated patch recording from abDGCs 21 d after differentiation. In WT abDGCs the reversal potential was still depolarized from the mature value at this age (WT  $E_{GABA}$ :  $-55.9 \pm 0.91$  mV,  $n = 7/3$ ). However, this value did not differ from that recorded in abDGCs in *Fmr1* KO animals (KO  $E_{GABA}$ :  $-55.6 \pm 2.17$  mV,  $n = 12/6$ ,  $p = 0.967$ , Mann–Whitney  $U$  test; Fig. 3A–C). Therefore, while the reversal potential for GABA is still relatively immature and depolarized in 21 dpi abDGCs, there is no effect of the loss of FMRP on  $E_{GABA}$ , as has been reported in other developing neurons.



**Figure 4.** Morphology of 21 dpi abDGCs in *Fmr1* KO and WT. **A**, Representative two-photon images of 21 dpi abDGCs filled with Alexa Fluor 488 in slices from *Fmr1* WT (right) and *Fmr1* KO (left) mice. **B**, Sholl analysis of dendritic complexity of 21 dpi abDGCs in *Fmr1* WT (blue) and *Fmr1* KO mice (red). Average total dendritic length (**C**) and total number of branch points (**D**) of 21 dpi abDGCs in *Fmr1* WT (blue) and *Fmr1* KO (red) mice.

#### Development of dendrites of abDGCs in *Fmr1* KO mice

Dendritic morphology of abDGCs resembles that of mature DGCs as early as 21 dpi, when distal dendrites reach the outer molecular layer and significant arborization is observed (Zhao et al., 2006). Dendritic spines begin to form around 16 dpi, consistent with the fact that glutamatergic signaling is rarely observed before 14 dpi (Ge et al., 2006; Zhao et al., 2006). We thus sought to determine if loss of FMRP would lead to alterations in dendritic morphology in 21 dpi abDGCs during this critical period of their development. abDGCs were filled with a morphologic dye and imaged using a two-photon microscope (Fig. 4A). Quantification of their dendritic complexity at 21 dpi, as assessed by Sholl analysis did not uncover any significant difference between the genotypes (two-way ANOVA for unbalanced design,  $p = 0.999$  for genotype  $\times$  radius interaction; Fig. 4B). In addition, measurement of total dendritic length did not reveal any difference in 21 dpi abDGCs in *Fmr1* KO (WT:  $701 \pm 94.2 \mu\text{m}$ ,  $n = 8/5$ ; KO:  $729 \pm 57.5 \mu\text{m}$ ,  $n = 15/8$ ,  $p = 0.781$ , Mann–Whitney  $U$  test), and there was no difference in the number of dendritic branch points in *Fmr1* KO at 21 dpi (WT:  $8.63 \pm$

$1.92$ ,  $n = 9/5$ , KO:  $7.20 \pm 0.66$ ,  $n = 14/8$ ,  $p = 0.917$ , Mann–Whitney  $U$  test; Fig. 4C,D).

#### Discussion

In this study, we set out to systematically describe the development of abDGCs in the Fragile X mouse model, focusing on the formation of GABAergic inputs to these neurons during the first few weeks after differentiation. There are multiple studies that have found that loss of FMRP can delay neuronal development in the Fragile X brain, therefore, a similar delay in development of abDGCs could have an impact on how these neurons become connected to hippocampal circuits, and how they contribute to circuit function. Studying GABA synapse development is particularly relevant as these are the first synapses to form onto abDGCs and GABA also produces a trophic effect on abDGCs (Ge et al., 2006; Song et al., 2013; Alvarez et al., 2016). Surprisingly, our data indicate that the development of inhibitory signaling onto abDGCs during the first four weeks is mostly unaffected by the loss of FMRP in the *Fmr1* KO mice. Given the range of impairments in GABA signaling that have been described in the hippocampus of *Fmr1* KO mice including altered expression of GABA receptor subunits and GAD 65/67, and the

alterations in  $E_{\text{GABA}}$  in *Fmr1* KO mice (Braat and Kooy, 2015a), it is surprising that GABA signaling appears to be unaltered in developing abDGCs during the critical period of their maturation.

### Critical period development and chloride homeostasis in Fragile X

At the cellular level Fragile X is a complex disorder because loss of FMRP leads to the dysregulation of expression of many neuronal proteins (Tang et al., 2015). The mouse model has been particularly useful in describing these complex alterations in neuronal development (Contractor et al., 2015). An important aspect that has emerged from some of these studies is the alterations in synaptic development of neurons during early critical periods in the cortex (Cruz-Martín et al., 2010; Harlow et al., 2010; Nomura et al., 2017). While these numerous delays in both excitatory and inhibitory neuron maturation have been well documented, up until now, it has not been established whether the development of abDGCs shows similar alteration in maturation to the developmentally generated neurons.

Among the known effects in developing neurons is the delay in the maturation of the GABA reversal potential in both the somatosensory cortex (He et al., 2014) and the hippocampus (Tyzio et al., 2014). In abDGCs,  $E_{\text{GABA}}$  has been demonstrated to be depolarized during early maturation in the weeks following differentiation (Tozuka et al., 2005; Ge et al., 2006) in a manner similar to developing neurons in other regions of the brain in perinatal mice. This occurs because of the relatively elevated intracellular chloride concentrations in abDGCs established by the juvenile chloride transporter NKCC1 (Ge et al., 2006). Disrupting chloride homeostasis in abDGCs leads to a profound alteration in the formation of synapses and in the maturation of the dendritic arbor. As there is growing evidence of altered  $E_{\text{GABA}}$  in developing neurons, we also measured  $E_{\text{GABA}}$  in abDGCs 21 d after differentiation when this measure is still maturing and not at its adult value (Ge et al., 2006). We confirmed that in 21 dpi abDGCs in both WT and *Fmr1* KO mice, the measured  $E_{\text{GABA}}$  is still relatively depolarized, but the values of  $E_{\text{GABA}}$  were not significantly different between the genotypes. Therefore, this crucial measure that regulates the strength of GABA signaling, and has a major impact on neuronal development, is not affected in abDGCs in *Fmr1* KO mice.

### GABA and Fragile X

The sequence of the formation of inputs and neurotransmitter signaling onto abDGCs broadly reflects that of developing neurons in other brain regions, including the early establishment of tonic and phasic GABAergic signaling followed by the establishment of excitatory connections (Ge et al., 2007a). GABA has an established role in brain development affecting proliferation (LoTurco et al., 1995; Haydar et al., 2000), migration, and maturation of progenitors and neurons (Represa and Ben-Ari, 2005; Wang and Kriegstein, 2009). After postnatal development, there are multiple known defects in GABA signaling associated with Fragile X, including age- and region-specific changes in GABA<sub>A</sub> receptor subunit ex-

pression (Braat and Kooy, 2015b), changes in tonic and phasic GABA currents onto excitatory neurons (Centonze et al., 2008; Curia et al., 2009; Olmos-Serrano et al., 2010; Zhang et al., 2017) as well as defects in interneurons themselves (Gibson et al., 2008; Nomura et al., 2017). Expression of several GABA<sub>A</sub> receptor subunits is reduced in the cortex, hippocampus, or forebrain of *Fmr1* KO mice (El Idrissi et al., 2005; D'Hulst et al., 2006; Gantois et al., 2006; Adusei et al., 2010). Expression of the glutamate decarboxylase enzyme (GAD) responsible for converting glutamate to GABA is increased in the hippocampus, but decreased in the amygdala of *Fmr1* KO mice (El Idrissi et al., 2005; Olmos-Serrano et al., 2010). Functionally, both the frequency and amplitude of mIPSCs and sIPSCs is reduced in the amygdala of juvenile *Fmr1* KO mice (Olmos-Serrano et al., 2010); however, a similar alteration is not observed in the subiculum of older mice (Curia et al., 2009). In some cases, these alterations in GABAergic signaling occur only early in postnatal development (Adusei et al., 2010; Nomura et al., 2017). Thus, loss of FMRP clearly affects GABA signaling in the postnatal mouse brain, but whether this is also the case for abDGCs was not known. By mapping spontaneous events by recording both sIPSCs and mIPSCs at three time points after differentiation of abDGCs, we were able to establish how inhibitory synapses form onto these neurons. We found that in both genotypes the frequency of IPSCs increases over time as would be expected if new synapses were being formed over the post differentiation period we analyzed. Interestingly, the amplitude of both the sIPSC and mIPSC events increased between 14 and 21 dpi. The increase in the mean quantal size at this developmental time point suggests that individual synapses become stronger. At the earliest time points, the amplitude of both mIPSCs and sIPSCs is equivalent suggesting that presynaptic axons of the inhibitory cells make a single contact whereas by 21 dpi the mean amplitude of sIPSCs is double the mean amplitude of quantal events, suggesting that the sIPSCs represent the release at multiple synapses. While again we did not observe any genotype differences in these parameters, we did observe consistent lengthening of the decay kinetics of mIPSCs in *Fmr1* KO mice which were significant in recordings from both 21 dpi and 28 dpi neurons. Prior work that compared the decay kinetics of inhibitory events in abDGCs and mature granule neurons found that the sIPSCs in abDGCs are slower because of the subunit composition of postsynaptic GABA<sub>A</sub> receptors (Overstreet Wadiche et al., 2005). This study found that the zolpidem sensitivity of inhibitory events was increased in mature neurons suggesting that the  $\alpha 1$  subunit is increasingly incorporated into neurons as they mature (Overstreet Wadiche et al., 2005). Given this, we considered the possibility that a reduction in the incorporation of the  $\alpha 1$  subunit in abDGCs in *Fmr1* KO mice could underlie the prolonged decay of mIPSCs in *Fmr1* KO. However, we did not observe a significant difference in the effect of zolpidem on mIPSC decay in 21 dpi abDGCs between *Fmr1* KO and WT, indicating that a reduction in  $\alpha 1$  expression is unlikely to underlie the changes in mIPSC decay. It is possible that



alterations in expression of other GABA receptor subunits or even differences in the location of GABAergic synapses on developing neurons may underlie the observed change in mIPSC decays recorded by a somatic electrode. Interestingly, while prior work has reported that abDGCs lack expression of the GABA<sub>A</sub>  $\alpha$ 1 subunit (Overstreet Wadiche et al., 2005), we found zolpidem had a significant effect on most mIPSCs in the 21 dpi birth-dated neurons in both genotypes. This may reflect differences in the populations of neurons that were analyzed in the previous study which recorded from POMC-GFP labeled neurons which are a more heterogeneous aged group (Overstreet et al., 2004).

In addition to the measures of synaptic function, we also measured the dendritic arbor to quantify both the complexity as well as the total dendritic length in 21 dpi abDGCs. A prior study has reported that selective deletion of FMRP in adult neural stem cells isolated from the dentate gyrus, as well as *in situ*, caused a reduction in both the dendritic complexity and total dendritic length when measured in neurons 56 d after differentiation (Guo et al., 2011). Dendritic length and complexity are also reduced in mice with knockout of the FMRP paralog FXR2P and double knockout of FXR2P and FMRP induced an additive effect on dendrites of abDGCs (Guo et al., 2015). In our experiments, we patched and filled abDGCs with morphologic dyes in live slices and imaged the dendritic arbor at 21 dpi. In these younger neurons, we did not observe any genotype related differences in the total dendritic length or complexity in *Fmr1* KO mice. It is possible that this difference in our results and those of the earlier study is due to a dendritic phenotype that emerges later on in the development of FMRP lacking abDGCs. While there have also been reports of region-specific or developmental age-specific alterations in dendritic spine density or immature spine morphology in *Fmr1* KO mice, there is no consensus on the effect of loss of FMRP on dendritic spines (for review, see He and Portera-Cailliau, 2013). While we did not examine this measure in these immature abDGCs, prior work has reported normal dendritic spines in the mature DG in *Fmr1* KO (Grossman et al., 2010).

In summary, we performed a systematic analysis of the functional properties of GABAergic synapses in abDGCs during the first four weeks after differentiation in *Fmr1* KO mice. While previous studies have demonstrated that cell proliferation and fate specification of adult neural stem cells is altered by FMRP loss, our data suggest that neurons that develop from these stem cells do not have major alterations in the maturation of their GABAergic synaptic inputs and dendrites during the first four weeks of their development.

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