# DNA methylation regulates the maturation of synaptic and intrinsic physiology in fast-spiking cortical interneurons

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Running Title: Epigenetic regulation of fast-spiking interneurons

## Summary

Recent theories of schizophrenia, autism and other psychiatric disorders have focused on abnormalities in parvalbumin-positive, fast-spiking (PV-FS) cortical interneurons and have raised the question of whether these abnormalities arise from altered epigenetic pathways — including DNA methylation. We tested the role of DNA methylation in the maturation and function of FS neurons by conditionally deleting the DNA methyltransferases DNMT 1 and DNMT 3a in PV-FS neurons. Interneurons lacking both copies of either enzyme had dramatically altered intrinsic properties and strongly reduced synaptic drive. This indicates that these interneurons continue to be subject to epigenetic control long after they have migrated and integrated into cortical circuits, which is consistent with some theories of the pathophysiology of psychiatric disorders.

## Highlights

- Late KO of DNMT 1 or 3a in fast-spiking interneurons alters intrinsic excitability.
- Excitatory, but not inhibitory synaptic inputs are dramatically reduced.
- Normal physiology requires at least one copy of both DNMTs.

## Introduction

DNA methylation, a critical epigenetic regulator of development, is mediated enzymatically by DNA methyltransferases (DNMTs) (MacDonald and Roskams, 2009) that initiate (DNMT 3a, and 3b) de-novo methylation in progenitors and then maintain (DNMT 1) these patterns through successive generations of cells (Bird, 1992; Hendrich and Bird, 2000). DNMT1 and DNMT3a are present in postmitotic neurons (Inano et al., 2000; Feng et al., 2005) and are especially concentrated in inhibitory neurons (Kadriu et al., 2012).

Parvalbumin-positive, fast-spiking (PV-FS) neurons are the most abundant subtype of interneurons in the mammalian neocortex (Kawaguchi and Kondo, 2002). Late post-natal maturation of these neurons is believed to play an important role in critical-period plasticity (Huang et al., 1999; Fagiolini and Hensch, 2000), and they have been implicated in the pathogenesis of schizophrenia (Gonzalez-Burgos and Lewis, 2008) and other psychiatric disorders (Tsankova et al., 2007; Grayson and Guidotti, 2013). DNMTs are overexpressed specifically in PV-FS interneurons in the postmortem brains of schizophrenic patients (Veldic et al., 2004; Veldic et al., 2005; Roth et al., 2009a). In these brains, promoters of genes associated with GABAergic transmission are hypermethylated, and their expression levels are low (Abdolmaleky et al., 2005; Akbarian et al., 1995; Costa et al., 2009; Dracheva et al., 2004; Du et al., 2008; Guidotti et al., 2000; Hashimoto et al., 2008; Straub et al., 2007; Zhao et al., 2007), while densities of parvalbumin-positive axon terminals are reduced (Carlsson, 2006; Lewis et al., 2001).

In this study, we investigated the role of DNMTs in the late development of PV-FS interneurons in the mouse neocortex. We have created conditional knockouts of DNMTs in PV-FS cells and obtained physiological recordings from these neurons in the fourth and fifth post-natal weeks. Our findings show that DNMTs seem to be important for the normal development of intrinsic excitability and the maintenance of excitatory synaptic input in PV-FS cells.

## Results

In order to investigate the role of DNMTs in postnatal PV-FS neurons, we mated lines of mice bearing floxed alleles of DNMT 1 (Fan et al., 2001) and DNMT 3a (Nguyen et al.,

2007) to Pvalb-cre mice and to a cre-dependent reporter strain. Animals with Pvalb-driven KO of DNMTs were viable, born in the expected Mendelian ratios, and did not exhibit overt behavioral abnormalities.

Conditional knockout of either DNMT 1 or DNMT 3a had no effect on PV-FS cell number in layer V (21.9 ± 3.4 cells per $\mu m^3$ for CON, 25.2 ± 4.1 cells per $\mu m^3$ for DNMT 1 KO, 22.6 ± 5.8 cells per $\mu m^3$ for DNMT 3a KO, ANOVA $p > 0.05$, ) or on soma size (138 ± 10.8 $\mu m^2$ for CON, 146 ± 12.1 $\mu m^2$ for DNMT 1 KO, 148 ± 11.3 $\mu m^2$ for DNMT 3a KO, K-S test $p > 0.05$). Parvalbumin levels were non-significantly elevated in DNMT 3a KO compared to controls (CON: 113.34 ± 10.27 intensity units; DNMT 3a: 132.92 ± 11.59 intensity units, Kolmagorov-Smirnov (K-S) test $p > 0.05$).

The physiological properties of PV-FS neurons continue to mature late in postnatal development (Okaty et al., 2009). In order to determine the role of DNMTs in regulating this maturation we obtained whole-cell recordings from PV-FS interneurons in acute slices. First, intrinsic firing properties were measured in current clamp. In response to the same current-step injection, homozygous KO neurons fired more rapidly than wild-type neurons (Fig. 1a). A series of current-injection steps were used to construct frequency-current curves (Fig. 1b) that showed that homozygous single KOs resulted in a significantly lower current threshold (410 ± 50 pA for CON, 80 ± 30 pA for Dnmt 1 KO, 60 ± 20 pA for Dnmt 3a KO, $p < 0.01$), which correlated with significantly higher input resistance (80.8 ± 5.1 MΩ for CON, 118.1 ± 8.2 MOhm for DNMT1 KO, 122.5 ± 7.3 MΩ for DNMT 3a KO, $p < 0.01$), but had no effect on voltage threshold (Fig. 1C). Other physiological properties, including the magnitude of the after-hyperpolarization and the maximum and average dV/dt during action potentials, were unchanged. Maximum

firing frequency was significantly lower in mutant cells (406.3 ± 35.1 Hz for CON, 130.7 ± 20.4 Hz for DNMT 1, 259.7 ± 22.1 Hz for DNMT 3a, $p < 0.01$). There was no significant difference between the intrinsic properties of heterozygous single KOs and those of controls, suggesting that DNMTs 1 and 3a are haplo-sufficient.

Next, we examined excitatory input to PV-FS cells by obtaining voltage-clamp recordings in the presence of tetrodotoxin (TTX) and picrotoxin (PTX) (Fig. 2a), allowing us to measure frequency and amplitude of miniature excitatory postsynaptic currents (mEPSCs). Cumulative probability distributions for both amplitude and frequency of mEPSCs were markedly different from controls for both single KOs and the double-KO (Fig. 2c, K-S test, $p < 0.001$), as were the means (Fig. 2b, amplitudes: 31.3 ± 3.9 pA for CON, 18.2 ± 3.6 pA for Dnmt 1, 17.0 ± 2.8 pA for DNMT 3a KO; frequencies: 3.9 ± 0.6 Hz for CON, 2.2 ± 0.3 Hz for DNMT 1, $p < 0.01$, 0.9 ± 0.3 Hz for DNMT 3a, $p < 0.01$). Neither the rise time nor the decay of mEPCs was affected (rise-time: 0.56 ± 0.04 msec for CON, 0.59 ± 0.03 msec for DNMT 1 KO, 0.57 ± 0.04 msec for DNMT 3a KO, decay: 1.5 ± 0.03 msec for CON, 1.6 ± 0.07 msec for DNMT 1 KO, 1.5 ± 0.08 msec for DNMT 3a KO, $p > 0.05$). There was no effect on either the amplitude or the frequency of mEPSCs in heterozygotes, indicating that both enzymes are also haplo-sufficient for the synaptic phenotype.

Excitatory input to P15 interneurons was not significantly different (48.5 ± 11.3 pA for CON, 47.9 ± 12.8 pA for Dnmt 1, 51.3 ± 8.27 pA for Dnmt 3a KO, ANOVA $p > 0.05$; frequencies: 8.7 ± 1.3 Hz for CON, 8.3 ± 2.1 Hz for Dnmt 1, 7.9 ± 1.8 Hz for Dnmt 3a, $p > 0.05$). This is not surprising, since the conditional knockout is driven by parvalbumin, the expression of which starts at P14–16. This finding argues, in addition, against the

possibility that the conditional alleles of DNMT1 or DNMT3a are hypomorphic. Within each group (controls and the two KOs), both the amplitude and frequency of mEPSCs decreased significantly between P15 and P25, corroborating earlier findings that PV-FS cells lose excitatory input during the third and fourth weeks of postnatal maturation (Okaty et al., 2009). In order to determine whether or not the loss of excitatory drive reflected a general loss of synaptic input, we also measured inhibitory input onto PV-FS interneurons. Voltage-clamp recordings in the presence of TTX, 2-amino-5-phosphonopentanoic acid (APV), and 6,7-dinitroquinoxaline-2,3-dione (DNQX) revealed no effect of conditional KO of DNMTs 1 and 3a on the amplitudes and frequencies of mIPSCs, as assessed from cumulative distributions (Figure 3b, K-S test, $p > 0.05$; means: 47.58 ± 8.70 pA for CON, 50.11 ± 9.33 pA for DNMT 1 KO, 44.90 ± 9.06 pA for DNMT 3a KO). This finding suggests that loss of synaptic input to PV-FS cells is highly restricted to excitatory inputs.

Lack of effect from single heterozygous mutations in combination with the effect of single homozygous mutations on both intrinsic properties (Fig. 1) and mEPSCs (Fig. 2) suggest two alternative hypotheses: that the DNA methylation sites for Dnmt 1 and 3a are separate (either in the same or different genes), or that DNMT 1 and DNMT 3a methylate redundant sites in the genomes of these neurons, but that the full complement of normal methylation is dose-dependent, requiring expression of any three DNMT alleles. To differentiate between the two hypotheses, we performed both current-clamp and voltage-clamp recordings from double-HET PV-FS interneurons. No differences were observed in either intrinsic properties (Fig. 4a; CON: 410 ± 50 pA current threshold, 406.3 ± 35.1 Hz maximum firing frequency; double-HET: 390 ± 40

pA current threshold, 356 ± 48.2 Hz maximum firing frequency; $p > 0.05$) or excitatory synaptic input (Fig. 4b; CON: 31.3 ± 3.9 pA, 3.9 ± 0.6 Hz; double-HET: 33.2 ± 3.7 pA, 4.8 ± 0.4 Hz; $p > 0.05$), ruling out the second of the above hypotheses.

Both mutants exhibited normal PV-FS firing type with high firing frequencies and AHPs (Fig. 1), and stuttering firing type at near threshold current injections. Voltage threshold was also within the normal range (−65 to −75 mV). These data, combined with the lack of effect on soma size or cell count, suggest that although Dnmt 1 and 3a KOs had a significant effect on intrinsic properties and excitatory synaptic input, they did not affect the overall cell health of PV-FS interneurons.

## Discussion

Loss of both copies of either DNMT1 or DNMT3a in PV-FS interneurons dramatically altered the cells' intrinsic electrophysiology and severely reduced their excitatory synaptic input. These physiological effects were highly specific, affecting excitatory but not inhibitory synapses, and affecting passive membrane properties such as input resistance without affecting voltage threshold or the width or rate of rise of the action potential. This specificity, together with the preservation of the normal density and soma size of PV-FS neurons, suggests that the changes observed were not due to general impairments of cell health. The effects observed were more dramatic than those previously found following selective deletion of DNMTs in pyramidal neurons at about the same age (Feng et al., 2010). In that study, changes in synaptic plasticity were observed in double KOs, but there was no change in baseline synaptic transmission and no effects were seen with either single KO. The dramatic effects in our study are consistent with the observation that DNMTs continue to be highly expressed in cortical

interneurons (Kadriu et al., 2012) late in development and into adulthood. Our results may also help explain the observed effects of selectively deleting Mecp2 in inhibitory neurons (Chao et al., 2010; Durand et al., 2012), but reveal that late postnatal phenotypes likely reflect not only the late development of Mecp2 expression, but also continued epigenetic modifications.

The physiological properties of PV-FS cells undergo protracted postnatal development (Okaty et al., 2009; Doischer et al., 2008). It is clear that loss of DNMTs does not simply arrest that development. Although the increased excitability is reminiscent of that seen in younger animals, the loss of excitatory synaptic input is something that normally occurs between the second and fourth postnatal week, but which is greatly exaggerated in the KO animals.

The fact that single KOs of either DNMT1 or DNM3a produced physiological effects suggests that the two isoforms have distinct genomic targets and cannot easily compensate for one another. Consistent with this view, the double KO produced a more pronounced phenotype. The effects of losing two alleles of either DNMT were not simply due to reduced dosage of a single enzymatic activity, however, since neurons in the double-heterozygote, as in the single HETs, were indistinguishable from those in WT mice. On the other hand, the similarity of the physiological phenotypes observed in both KO strains suggests that although individual methylation sites may differ, the genes affected may overlap significantly.

The observed physiological changes following loss of DNMT function are reminiscent of changes induced by interfering with signaling by Neuregulin 1 through its receptor ErbB4. Mutations in both genes are highly associated with schizophrenia, and

mutations in Erbb4 have also been linked to myoclonic epilepsy. Loss of ErbB4 reduces excitatory (but not inhibitory) synaptic input to PV-FS neurons (Fazzari et al., 2010) as well as making them more excitable (Li et al., 2011), as observed here following loss of DNMTs. DNA methylation could potentially control the expression or the function of ErbB4. Alternatively, late postnatal methylation by DNMT1 and DNMT3a may be regulated by ErbB4 signaling. This is mechanistically plausible because activation of ErbB4 causes the receptor to undergo proteolytic cleavage, and the intracellular cleaved portion (4ICD) has been shown to translocate to the nucleus and regulate gene expression (Sardi et al., 2006). In either case, the present results demonstrate that the maturation of firing properties and maintenance of excitatory synaptic drive in PV-FS neurons are regulated epigenetically during late postnatal development in a manner similar to that produced by disruption of the schizophrenia candidate genes Nrg1 and ErbB4, consistent with the hypothesis that loss of this epigenetic regulation may contribute to the pathophysiology of psychiatric disease.

**Methods**

Conditional Dnmt 1 (Fen et al., 2001) and 3a (Nguyen et al., 2007) knockout mice were homozygous for the floxed Dnmt 1 or 3a alleles and heterozygous for Pvalb-cre (Hippenmeyer et al., 2005) and a Rosa26 cre-dependent GFP (Sousa et al., 2009) or Td-tomato reporter allele (Madisen et al., 2010). Double-HETs and double-KOs were obtained by crossing homozygous single KOs with each other for one or more generations. Control mice (CON) carried only the Pvalb-Cre and cre-dependent reporter alleles.

Methods for whole cell recordings from neocortical slices were as previously described (Okaty et al., 2009). Briefly, slices containing somatosensory cortex were obtained from animals at ages P15, P25, and P30 and incubated at room temperature prior to recording at 33-35° C. PV-FS cells were identified through reporter (Green Fluorescent Protein and tdTomato) expression using standard epifluorescence and patched using regular optics employing Koehler illumination.

Frequency-current curves were obtained (in the presence of synaptic blockers) by plotting frequency of firing to injected currents and fitting exponential curves. Intrinsic membrane properties were obtained by injections of hyperpolarizing current steps. Miniature EPSCs and IPSCs were recorded in voltage clamp in the presence of TTX and synaptic blockers.

All data were acquired using custom-written scripts on IgorPro 6.0 (Wavemetrics) and analyzed using custom-written scripts on IgorPro 6.0 and MATLAB (Mathworks).

For immunohistological analysis, mice at P25 were deeply anesthesized and perfused intracardially with ice-cold 0.1 M PBS followed by ice-cold 4% paraformaldehyde. 50-μm coronal sections were prepared from brains post-fixed for at least 24 hours. 3 sections (corresponding to rostral, caudal, and middle portions of barrelfield cortex) were chosen per animal (4 animals per condition). The sections were washed in PBS and stained for the reporter (GFP or tdTomato) and parvalbumin using immunolabeling methods previously described (Okaty et al., 2009).

PV-FS cells were identified by the reporter labeling and analyzed in ImageJ/FIJI. Soma areas were measured from confocal images (3 to 4 fields of view per slice at 63×) at their

maximal Z location. Distributions of soma areas (drawn equally from each field of view) were compared by K-S test. Intensity of parvalbumin staining was measured within identified somata and compared using ANOVA and post-hoc Tukey tests. Cell densities were quantified in 3–4 (250 × 250 μm) regions per section using the entire Z-stack, and compared with ANOVA.

## Legends

**Figure 1:** Intrinsic properties of PV interneurons in control and mutant animals. **a:** Representative current-clamp traces from wild type (WT) and Dnmt 1 and 3a conditional knockouts. Left: response to 0.45 nA current injection (scale bars: 200 msec, 20 mV). Right: Enlarged first few action potentials (scale bars: 5 msec, 15 mV). **b:** Population FI curves for WT, Dnmt 1 and 3a heterozygous (HET) and homozygous conditional knockouts (KO). **c:** Summaries of input resistance ($R_{in}$), voltage threshold (V-thresh), current threshold (I-thresh), and maximum firing frequency (max freq). Error bars here and in other figures indicate SEM.

**Figure 2:** Excitatory synaptic input to PV neurons is diminished by conditional knockout of Dnmt's. **a:** Representative traces of voltage-clamp recordings of mEPSCs (left) and averaged mEPSCs for each condition (right). Scale bars: 45 pA, 500 msec (left) and 25 pA, 20 msec (right). **b:** Summary of EPSC amplitudes and frequencies for each condition. **c:** Cumulative probability plots for amplitude and frequency.

**Figure 3:** Inhibitory synaptic input to PV neurons is not affected by conditional knockout of Dnmt's. **a:** Representative traces of voltage-clamp recordings of mIPSCs (left) and averaged mIPSCs for each condition (right). Scale bars: 80 pA, 500 msec (left) and 25 pA, 20 msec (right). **b:** Cumulative probability plots for amplitude and interevent interval.

**Figure 4:** Loss of single copies of both Dnmt 1 and 3a has no effect on either intrinsic properties or excitatory input. **a:** Summary of current threshold (I-thresh) and maximum firing frequency for wild type (WT) and double-heterozygotes (2x-HET). **b:** Summary data for mEPSC amplitude and frequency. Arrows indicated means for Dnmt 1 (red) and Dnmt 3a (green) homozygous conditional knockouts.

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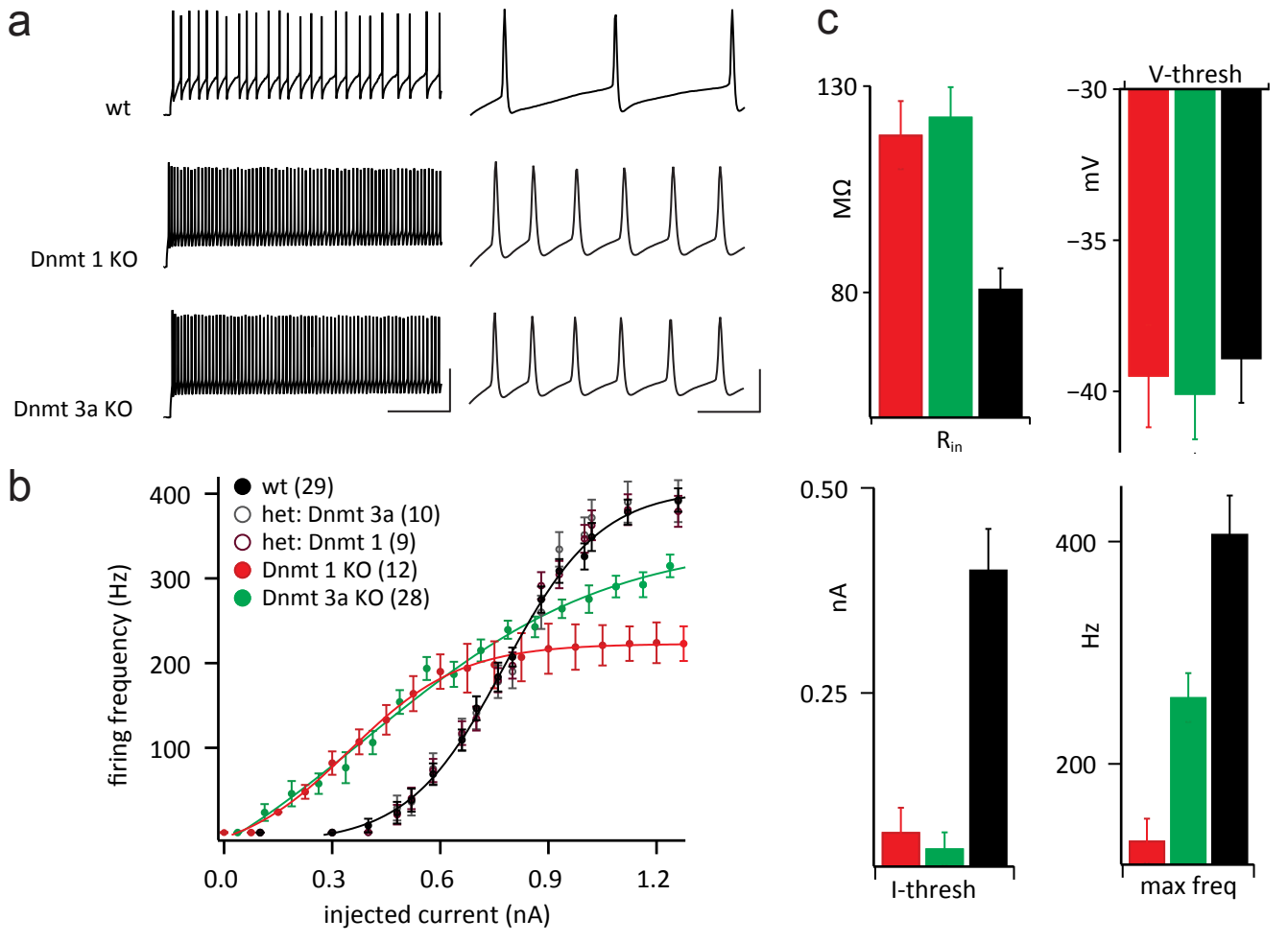
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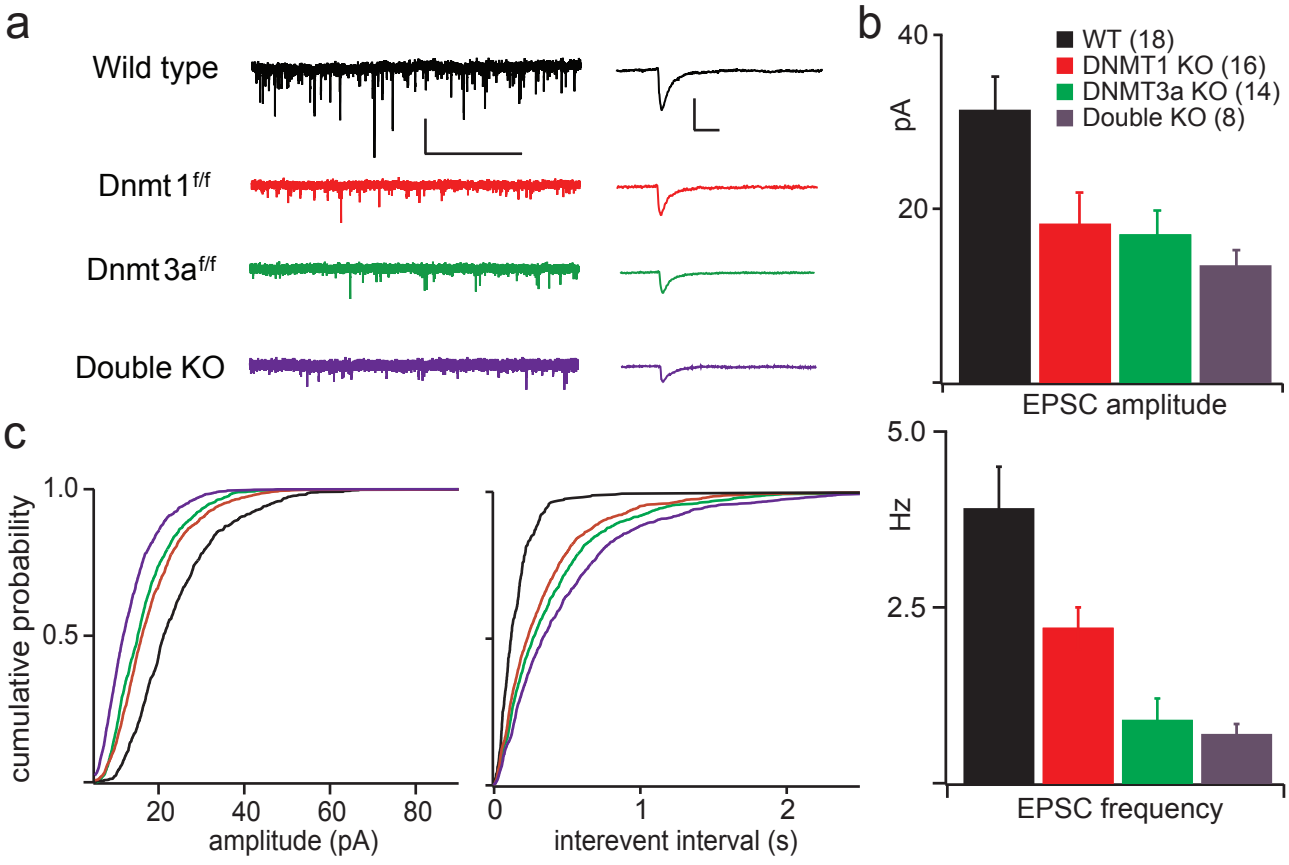
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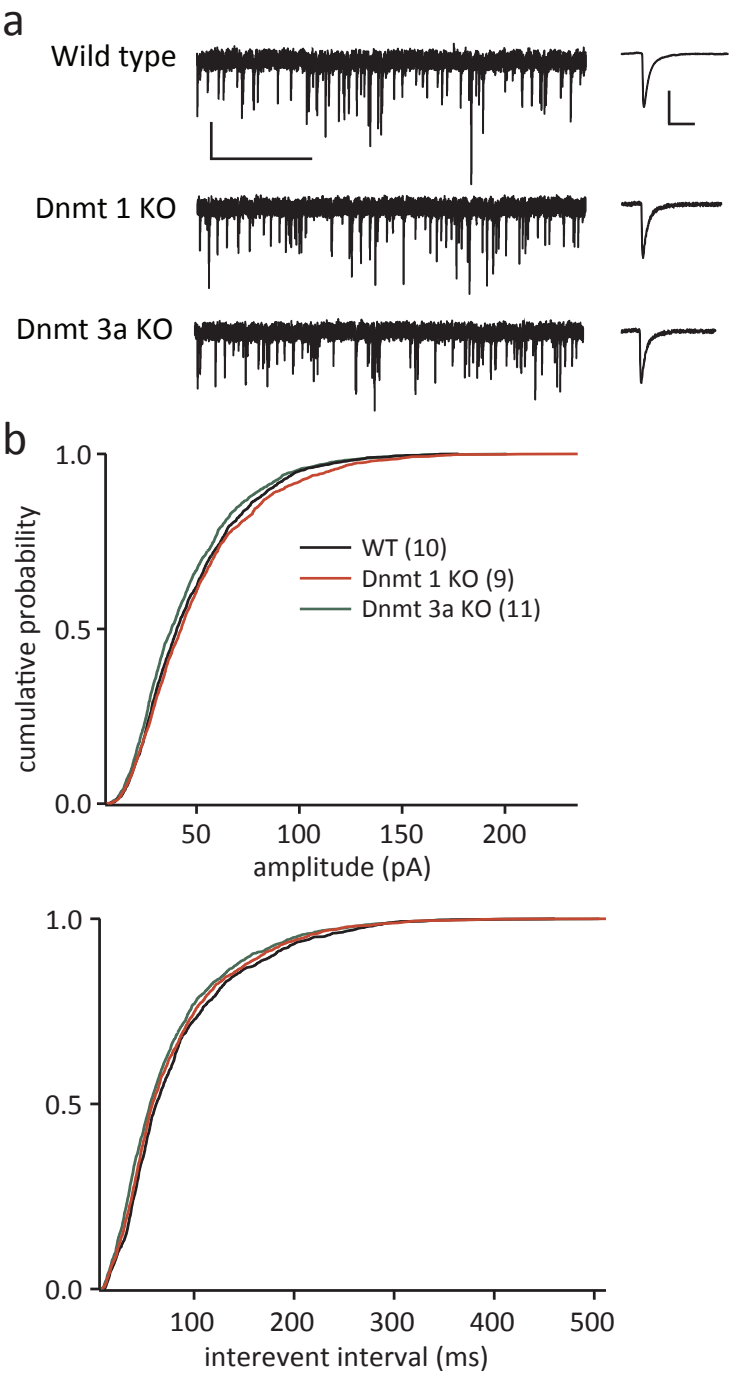
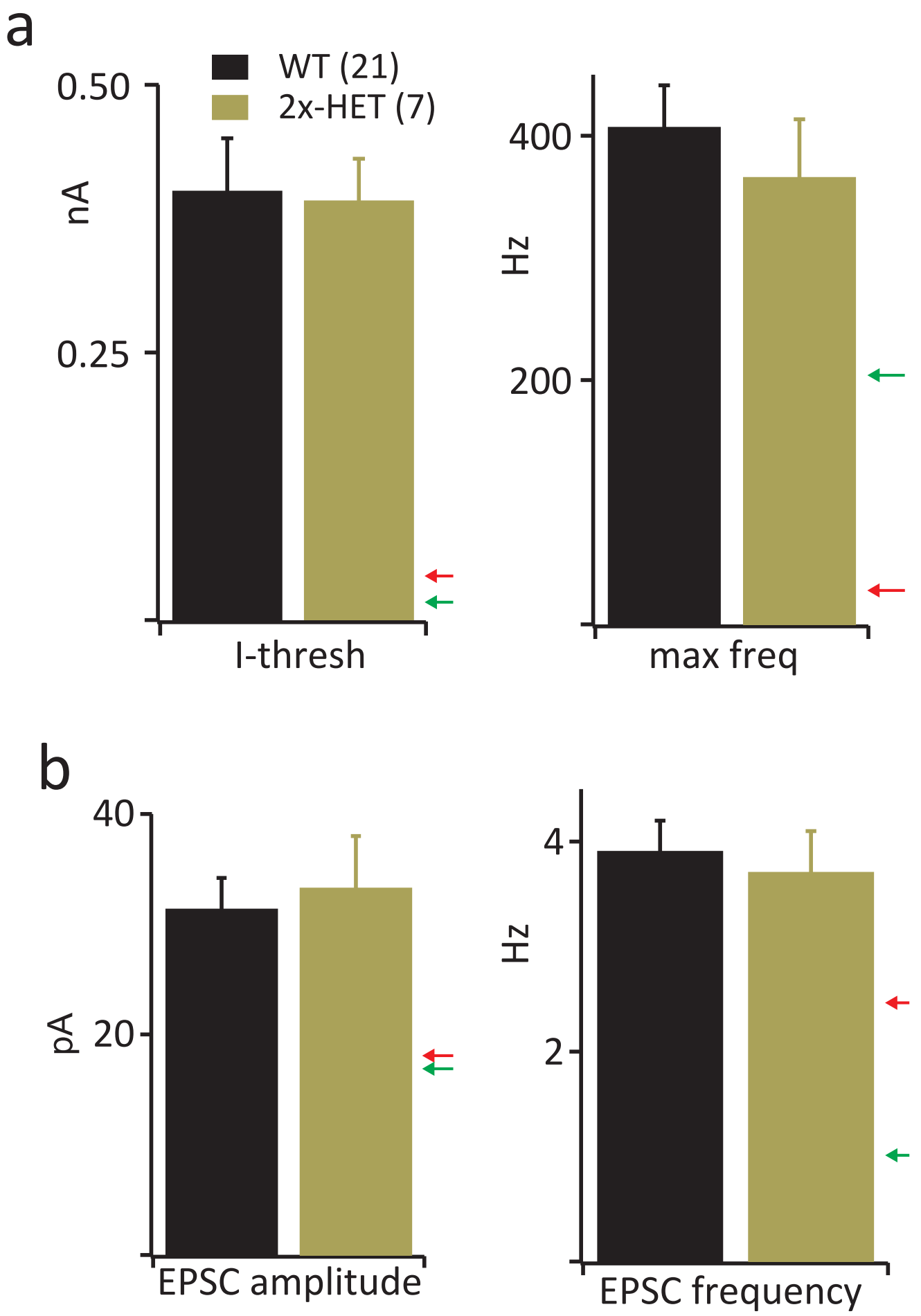
Hz
EPSC frequency


a
Wild type
Dnmt 1 KO
Dnmt 3a KO
b
cumulative probability
1.0
0.5
0.0
WT (10)
Dnmt 1 KO (9)
Dnmt 3a KO (11)
50
100
150
200
amplitude (pA)
1.0
0.5
0.0
100
200
300
400
500
interevent interval (ms)


a
WT (21)
2x-HET (7)
0.50
nA
0.25
I-thresh
400
Hz
200
max freq
b
40
pA
20
EPSC amplitude
4
Hz
2
EPSC frequency