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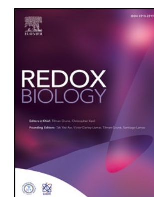

BRCA1 protein dose-dependent risk for embryonic oxidative DNA damage, embryopathies and neurodevelopmental disorders with and without ethanol exposure

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ABSTRACT

Although widely known as a tumor suppressor, the breast cancer 1 susceptibility protein (**BRCA1**) is also important in development, where it regulates fetal DNA repair pathways that protect against DNA damage caused by physiological and drug-enhanced levels of reactive oxygen species (**ROS**). We previously showed that **conditional** heterozygous (+/-) knockout (**cKO**) mouse embryos with a minor 28% BRCA1 deficiency developed normally in culture, but when exposed to the ROS-initiating drug, alcohol (ethanol, **EtOH**), exhibited embryopathies not evident in wild-type (+/+) littermates. Herein, we characterized a **direct** *Brca1* +/- knockout (**KO**) model with a 2-fold greater (58%) reduction in BRCA1 protein vs. the cKO model. We also characterized and compared learning & memory deficits in both the cKO and KO models. Even saline-exposed *Brca1* +/- vs. +/+ KO progeny exhibited enhanced oxidative DNA damage and embryopathies in embryo culture and learning & memory deficits in females *in vivo*, which were not observed in the cKO model, revealing the potential pathogenicity of physiological ROS levels. The embryopathic EtOH concentration for cultured direct KO embryos was half that for cKO embryos, and EtOH affected *Brca1* +/+ embryos only in the direct KO model. The spectrum and severity of EtOH embryopathies in culture were greater in both *Brca1* +/- vs. +/+ embryos, and direct KO vs. cKO +/- embryos. Motor coordination deficits were evident in both male and female *Brca1* +/- KO progeny exposed *in utero* to EtOH. The results in our direct KO model with a greater BRCA1 deficiency vs. cKO mice provide the first evidence for BRCA1 protein dose-dependent susceptibility to developmental disorders caused by physiological and drug-enhanced oxidative stress.

1. Introduction

Various germline mutations affecting 0.1–0.3% of North American people, or epigenetic silencing of the breast cancer 1 susceptibility gene (***BRCA1***), occurring at a frequency of 10–15% in cancer cases, are associated with an enhanced risk of breast and ovarian cancers [1,2]. Importantly, embryos with homozygous (**-/-**) *BRCA1* mutations generally do not survive to birth [3,4]. Studies using *Brca1* knockout (**KO**) mouse models revealed -/- KO embryos exhibit rapid proliferation and excessive apoptosis of the neuroepithelium leading to severe neural tube defects affecting survival [5,6]. The embryolethality of *Brca1* -/- KO mice and humans demonstrates a necessity for *Brca1* in development [6]. On the other hand, heterozygous (+/-) KO progeny with only a minor BRCA1 protein deficiency appear to develop normally [6]. Furthermore, few effects of *BRCA1* heterozygosity, besides enhanced risk of cancers, have been reported [1,7–9]. The specific role of *Brca1* in embryonic and fetal development is thought to be its regulation of DNA repair [3,4].

BRCA1 is primarily associated with initiating and recruiting homologous recombination repair (**HRR**) proteins, to promote higher

Abbreviations: *Brca1*, Breast cancer 1 susceptibility gene; cKO, conditional knockout; EtOH, ethanol; FASD, fetal alcohol spectrum disorders; -/-, homozygous; +/-, heterozygous; HRR, homologous recombination repair; KO, knockout; NOX, NADPH oxidase; OGG1, oxoguanine glycosylase; PBN, phenylbutylnitrone; ROS, reactive oxygen species; +/+, wild-type.

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fidelity repair of DNA double strand breaks [10]. BRCA1 has also been implicated in regulating other DNA repair pathways, including base excision repair of small oxidative DNA lesions, both directly through transcriptional upregulation and indirectly by acting as a scaffold [11]. The major DNA base lesion caused by reactive oxygen species (**ROS**) is 8-oxoguanine (**8-oxoG**), which is repaired by oxoguanine glycosylase 1 (**OGG1**) [12]. BRCA1 transcriptionally upregulates OGG1 following 8-oxoG formation for its repair [11]. We have found that *Ogg1* KO mouse embryos in culture exhibit normal morphological development under control conditions [13]. However, despite appearing morphologically normal at birth, *Ogg1* KO offspring later display learning & memory deficits. These deficits are mitigated by maternal administration of phenylbutylnitrone (**PBN**), a ROS-blocking and free radical spin trapping agent, during the fetal period [14]. These findings implicate free radical-mediated DNA damage in the pathogenesis of developmental disorders when DNA repair is deficient, and they reveal the exquisite sensitivity of the developing brain to the pathogenic effects of physiological levels of ROS production. Furthermore, these DNA repair-deficient models demonstrated increased sensitivity to developmental abnormalities when fetuses were exposed *in utero* to the ROS-enhancing drugs methamphetamine [15] and alcohol (ethanol, **EtOH**) [13,14].

Although essential for development at physiological levels [16–18], endogenous production of ROS can be enhanced via EtOH metabolism [19] and by the induction of NADPH oxidases (**NOXs**) by EtOH [20,21]. ROS levels measured by dicholorofluorescein and DNA oxidation were increased in embryos and fetuses exposed *in utero* to EtOH [13,21,22]. Moreover, structural and cognitive effects induced by *in utero* EtOH exposure were blocked by PBN [13,14], which inhibits NOX [23,24] in addition to trapping free radicals. Lastly, mice deficient in the antioxidative enzyme catalase demonstrated enhanced EtOH-initiated DNA oxidation and embryopathies, which were blocked by pretreatment with exogenous catalase or genetic overexpression of catalase [22]. In short, EtOH-enhanced ROS-mediated DNA damage can increase the frequency and spectrum of embryopathies and developmental disorders in mouse models with enhanced ROS formation or deficiencies in pathways for either ROS detoxification or the repair of ROS-initiated DNA damage.

While it is known that *BRCA1* +/- individuals often exhibit decreased or slower DNA repair activity, especially after DNA damage induction [25–27], the impact of minor to moderate BRCA1 deficiencies is poorly understood. Herein we compare two KO mouse models with markedly different, minor (28%) to moderate (58%) reductions in the level of BRCA1 protein to investigate protein dose-dependent vulnerability to DNA damage and developmental disorders. We initially developed a *Brca1* **conditional** knockout (**cKO**) mouse model utilizing a Cre-LoxP recombinase system to remove exon 11 of *Brca1* starting from embryonic day (**E**) 6.5 [13,28,29]. *Brca1* +/- cKO progeny had only a minor 28% decrease in BRCA1 protein, yet still exhibited embryopathies in culture, and had enhanced embryonic oxidative DNA damage in culture when exposed to EtOH [30], with similar results when exposed in culture to the ROS-initiating drug methamphetamine [31]. These cKO studies showed that BRCA1 plays a key role in protecting the developing embryo and fetus from ROS-mediated DNA damage, embryopathies and learning & memory effects caused by EtOH, and that even a minor BRCA1 deficiency increases risk [30,31].

Our studies in the above cKO model revealed that the loss of only one *Brca1* allele resulted in substantial neurodevelopmental disorders, unlike in cancer, which generally requires the loss or functional mutation of both alleles. Accordingly, for the new studies herein, we characterized a simple **direct** knockout (**KO**) model, generated by targeted disruption of exon 11, including a partial deletion of intron 10 [6], with a 2-fold greater, 58% reduction in embryonic BRCA1 protein compared to our cKO model. New studies also included characterization and comparison of learning & memory deficits in both the cKO and direct KO models, and motor coordination disorders in the direct KO progeny. A comparison of these two cKO and direct KO *Brca1* models with a 2-fold difference in BRCA1 allowed us to explicitly determine the developmental impact of a protein dose-dependent decrease in BRCA1. The different mouse strains used for the direct KO and cKO models provided corroboration that their respective susceptibilities to adverse developmental consequences were due to differences in the magnitude of BRCA1 loss rather than other unappreciated genetic factors. The results provide the first evidence for a BRCA1 protein dose-dependent increase in oxidative DNA damage and susceptibility to morphological and functional developmental disorders initiated by both physiological and drug-enhanced levels of ROS. These findings may reflect risk factors for some +/- *BRCA1* humans and those with decreased BRCA1 levels due to epigenetic and other environmental mechanisms.

2. Materials & Methods

All materials and methods are described in detail in the accompanying supplementary materials. Key methodological details are noted in the figure legends.

3. Results and Discussion

3.1. Larger BRCA1 protein deficiency in +/- Brca1 direct KO embryos than conditional KO embryos

A minor 28% reduction in BRCA1 in the gestational day (**GD**) 10 +/- *Brca1* conditional cKO embryos induced by E 6.5 (Fig. 1A, $p < 0.05$), previously observed and published [30], increased susceptibility to EtOH-enhanced DNA damage and embryopathies. To determine BRCA1 protein levels in the GD 12 +/- *Brca1* direct KO model, western blotting using an antibody that targets the amino-terminus of BRCA1 was employed. The 58% decrease in BRCA1 protein in +/- *Brca1* direct KO embryos was 2-fold greater than that in cKO embryos (Fig. 1B, $p < 0.0001$). The greater BRCA1 protein decrease in the direct *Brca1* KO model may contribute to their observed higher susceptibility to DNA damage, learning & memory effects and embryopathies compared to cKO progeny.

3.2. Enhanced susceptibility to oxidatively damaged DNA (8-oxoG) in +/- Brca1 direct KO embryos

Previous studies in multiple mouse strains have shown that the oxidative DNA lesion 8-oxoG is caused by ROS (*i.e.,* hydroxyl radicals), and is inhibited by: (a) pretreatment with the ROS blocking agent PBN *in vivo* [32] and in embryo culture [13,33]; and, (b) by pretreatment with a stabilized, polyethylene glycol-conjugated form of the antioxidative enzyme catalase, and in mutant mice overexpressing catalase, *in vivo* [22,34] and in embryo culture [35].

In the **cKO model**, no differences in 8-oxoG levels were observed between wild-type (+/+) and +/- *Brca1* genotypes following maternal intraperitoneal (**i.p.**) injection of saline vehicle on GD 12 (Fig. 1C). Maternal injection of EtOH (4 g/kg, i.p.) enhanced the levels of 8-oxoG in both +/+ and +/- embryos, but to a larger extent in the +/- *Brca1* cKO embryos. The 13% enhancement in 8-oxoG within the EtOH-exposed +/- *Brca1* cKO embryos compared to +/+ littermates ($p < 0.05$) was previously reported [30].

In contrast to cKO embryos, in the GD 12 +/- *Brca1* **direct KO** embryos exposed only to saline, levels of the 8-oxoG lesion were enhanced by 50% compared to +/+ littermates, reflecting the pathogenic potential of physiological levels of ROS with a greater BRCA1 deficiency ($p < 0.05$) (Fig. 1D). A similar, 42% enhancement was observed in EtOH-exposed +/- *Brca1* direct KO embryos compared to +/+ littermates.

Unlike in the cKO model, EtOH did not increase 8-oxoG levels in either the +/+ or +/- *Brca1* direct KO embryos compared to their respective saline controls of the same genotype, possibly in part due to an already maximal level of DNA damage in their respective saline controls of the same genotype (high baseline), and/or an assay

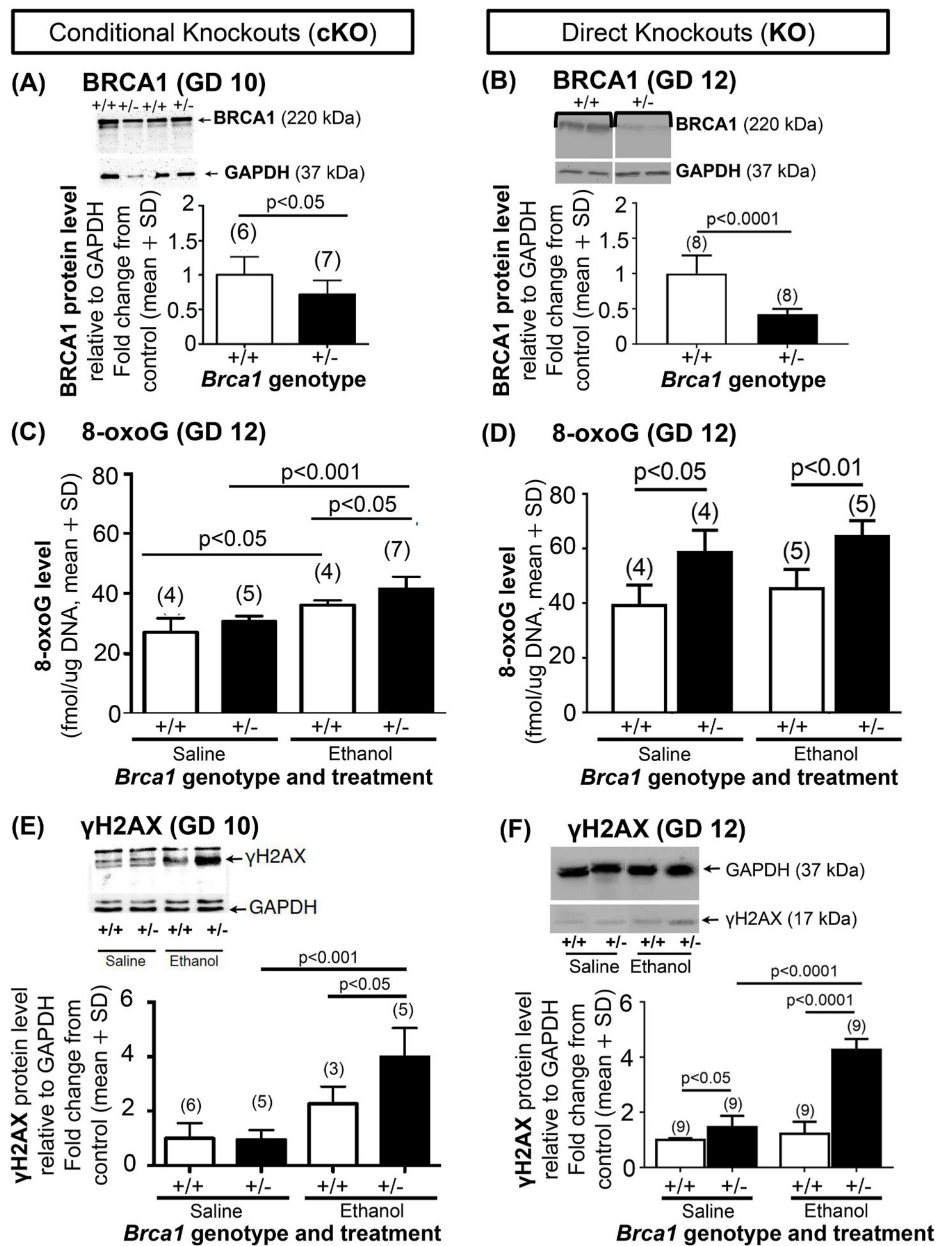

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Fig. 1. Enhanced oxidative DNA damage in embryos dependent upon decreasing BRCA1 protein levels in two knockout mouse models following *in utero* exposure to saline or ethanol (EtOH). Conditional *Brca1* +/- knockout embryos (**cKO**) with a minor BRCA1 protein deficiency are compared to **direct** knockouts (**KO**) with a more severe BRCA1 deficiency. The data for the KO embryos are new, while those for the cKO embryos were previously published [30] and are provided here for comparison. Embryonic **BRCA1 protein levels** in the *Brca1* **cKO** model on gestational day (**GD**) 10 **(Panel A)**, and the **KO** model on GD 12 **(Panel B)**, were determined by western blot analysis at 220 kDa, normalized to GAPDH at 37 kDa for the loading control. **(Panel A)** +/- *Brca1* **cKO** embryos exhibited a minor 28% reduction in BRCA1 protein compared to +/+ embryos ($p < 0.05$). **(Panel B)** +/- *Brca1* **KO** embryos exhibited a 58% reduction in BRCA1 protein compared to +/+ KO littermates ($p < 0.0001$), and 2-fold greater than the BRCA1 reduction in +/- cKO embryos. Significant differences were determined by Student's t-test. The number of mice is shown in parentheses above each bar. **(Panels C&D)** Relative **8-oxoguanine (8-oxoG)** lesion levels in +/+ and +/- *Brca1* **cKO** embryos **(Panel C),** and in +/+ and +/- *Brca1* **KO** embryos **(Panel D)**, sampled 6 h after maternal i.p. injection with saline or EtOH (4 g/kg). 8-OxoG levels were determined by ELISA and normalized to μg of DNA loaded in the assay. **(Panel C)** In the **cKO** model, EtOH-exposed *Brca1* +/+ embryos demonstrated enhanced 8-oxoG levels compared to saline +/+ controls ($p < 0.05$), which were further elevated in EtOH-exposed +/- littermates ($p < 0.001$). A genotype effect was also evident, where EtOH-exposed +/- **cKO** embryos had 13% higher 8-oxoG levels compared to +/+ littermates ($p < 0.05$). **(Panel D)** Saline-exposed *Brca1* +/- **KO** embryos exhibited 50% higher 8-oxoG levels compared to +/+ littermates ($p < 0.05$), in contrast to saline-exposed cKO embryos, in which there was no genotype effect. 8-OxoG levels were enhanced in both saline-exposed ($p < 0.01$) and EtOH-exposed ($p < 0.01$) *Brca1* +/- **KO** embryos compared to their respective saline- and EtOH-exposed *Brca1* +/+ littermates. However, there was no increase in +/- *Brca1* KO embryos exposed to EtOH vs. saline, unlike in KO embryos, possibly because of an already maximal level of DNA damage in the saline-exposed +/- embryos. Significant differences were determined using a two-way ANOVA with a Bonferroni post-hoc test. The number of mice is shown in parentheses above each bar, where results represent primarily female embryos (plotted by sex in the Supplementary Materials, Fig. S1). **(Panels E&F)** Relative **γH2AX** levels in *Brca1* **cKO** embryos **(Panel E),** and in *Brca1* **KO** embryos **(Panel F)**, 6 h after saline or EtOH exposure, were determined by western blot analysis at 17 kDa, normalized to GAPDH at 37 kDa for the loading control. **(Panel E)** EtOH exposure in *Brca1* +/- **cKO** embryos resulted in a 4.2-fold increase in γH2AX levels compared to saline-exposed +/- controls ($p < 0.001$). A genotype effect was also evident, where EtOH-exposed +/- **cKO** embryos had 75% higher γH2AX levels compared to +/+ littermates ($p < 0.05$). **(Panel F)** Saline-exposed *Brca1* +/- **KO** embryos exhibited 46% higher γH2AX levels compared to +/+ littermates ($p < 0.05$), whereas no genotype effect was evident in saline-exposed cKO embryos. A larger genotype effect was observed in **KO** +/- embryos with EtOH exposure, with a 3.5-fold increase in γH2AX levels compared to +/+ littermates ($p < 0.0001$). A drug effect also was evident in +/- *Brca1* embryos, which exhibited a 3-fold increase in γH2AX levels when exposed to EtOH vs. saline ($p < 0.0001$). Significant differences were determined using two-way ANOVA with a Bonferroni post-hoc test. The number of embryos is shown in parentheses above each bar, with roughly equal numbers of males and females, and no sex difference (plotted by sex in the Supplementary Materials, Fig. S2). Embryos were selected from at least 3 litters to minimize any impact of a litter effect.

limitation. Our assay detects global levels of 8-oxoG formation in DNA, but its enrichment in selective genes has recently been shown, as discussed in the next paragraph. The higher sensitivity to ROS-mediated DNA damage in the +/- *Brca1* direct KO model vs. cKO embryos is likely due to the reduced BRCA1 protein levels in direct KO embryos. Overall, a BRCA1 deficiency coupled with *in utero* exposure to EtOH may confer at least an additive risk of enhanced DNA damage contributing to FASD.

Ding et al. (2017) demonstrated in mouse embryonic fibroblasts that 8-oxoG is enriched in the promoters and 5' untranslated regions of selective genes, where it can act as an epigenetic mark that alters gene expression [42]. A decreased ability to remove the damaged nucleobase from such select genes may result in their differential expression, thereby dysregulating critical developmental pathways, corresponding to the elevated 8-oxoG levels observed in our models even though in direct KO progeny EtOH did not further enhance global 8-oxoG levels over those caused by BRCA1 deficiency [43]. Furthermore, forms of oxidative DNA damage other than 8-oxoG, such as γH2AX recruited to DNA double-strand breaks (**DSBs**), may also act as an epigenetic mark.

Most samples assessed for 8-oxoG in the GD 12 KO embryos were later determined to be female (Supplementary Materials Fig. S1), although the pattern in males was identical. These results suggest that 8-oxoG may have contributed to postnatal learning & memory deficits observed in female progeny and motor deficits in both sexes, whereas factors other than 8-oxoG levels may have determined the resistance of males to ROS-mediated learning & memory deficits.

3.3. Enhanced susceptibility to DSBs (indicated by γH2AX levels) in +/- Brca1 direct KO embryos

In the GD 10 +/- *Brca1* **cKO embryos**, similar to 8-oxoG, no differences in γH2AX levels were observed compared to control +/+ littermates exposed only to saline, as previously reported [30] (Fig. 1E). EtOH exposure enhanced the levels of γH2AX by 4.2-fold in the +/- *Brca1* cKO embryos compared to saline-exposed controls ($p < 0.001$). A 75% enhancement of γH2AX in the EtOH-exposed +/- *Brca1* cKO embryos compared to +/+ littermates ($p < 0.05$) was also previously reported [30].

In contrast to cKO embryos, GD 12 +/- *Brca1* **direct KO** embryos exposed only to saline exhibited 46% higher γH2AX levels compared to +/+ littermates ($p < 0.05$) (Fig. 1F), suggestive of enhanced baseline levels of DSBs with a BRCA1 deficiency. This pattern is corroborated by similarly enhanced γH2AX levels in *Brca1* KO progeny, previously shown to be prevented by *in vivo* pretreatment with the ROS blocker, PBN [44], indicative of increased oxidative DNA damage resulting from a BRCA1 deficiency. The increased γH2AX levels in *Brca1* KO embryos under saline-exposed conditions were anticipated, given the more central role of BRCA1 protein in signaling cell cycle progression [45–48], initiating the apoptotic cascade [46,47,49,50], and recruiting DNA repair proteins to damaged sites [15,45–47,51–56]. In the above models, 8-oxoG levels also were enhanced following exposure to a ROS-initiating teratogen [13–15,30].

γH2AX levels were enhanced further in +/- *Brca1* KO embryos exposed to EtOH vs. saline, demonstrating decreased repair of DNA damage. A 3-fold increase in γH2AX was observed in +/- *Brca1* embryos exposed to EtOH compared to saline-exposed +/- embryos (drug effect) ($p < 0.0001$). A larger *Brca1* genotype effect was observed with EtOH vs. saline exposure, evidenced by 3.5-fold more γH2AX in +/- *Brca1* embryos compared to +/+ littermates ($p < 0.0001$). The enhanced sensitivity to DSBs, particularly after EtOH exposure, in the +/- *Brca1* direct KO model vs. cKO embryos may be due to the lower BRCA1 protein levels in direct KO embryos.

Previous studies of ROS-mediated developmental disorders have consistently shown that models deficient in proteins involved in DNA damage recognition and repair signaling [37,38], DNA repair [13,15,30,39] or antioxidative activity [22,35] were more susceptible to developmental disorders caused by several different ROS-initiating teratogens, and in some cases even caused by physiological levels of ROS formation in untreated animals [14,30,34–36,40,41]. This study's findings align with these previous observations, as *Brca1* direct KO +/- embryos exhibit enhanced γH2AX following both saline and EtOH exposures, with the latter showing an additive effect.

Importantly, samples from the GD 12 direct KO embryos included roughly equal numbers of males and females with identical results, demonstrating similarly enhanced DSBs in both sexes (Supplementary Materials Fig. S2).

A natural process exists through which DSBs are induced by topoisomerase IIβ to initiate a gene expression cascade for regulation of neuronal function and synaptogenic processes [57,58]. Alongside activation of gene expression cascades, enhanced γH2AX levels are observed

and the DSBs are subsequently repaired [57,59]. Thus, aberrantly induced DSBs, particularly if left unrepaired, may enhance γH2AX levels and alter gene expression. During critical periods of development, aberrant gene expression resulting from enhanced 8-oxoG or DSB formation may induce defects to the morphology of organs or their function. Further studies in the direct *Brca1* KO model are needed to elucidate the potential contribution of dysregulated gene expression cascades for regulation of neuronal function and synaptogenic processes to the neurodevelopmental disorders observed in +/- *Brca1* progeny.

3.4. Effect of EtOH on embryonic survival and heart rate in mouse whole embryo culture

Heart rate, an indicator of viability in cultured whole embryos, is measured immediately after the 24 h culture period. Embryos that do not exhibit a heartbeat are classified as non-viable and morphological phenotyping is not performed. An EtOH concentration of 2 mg/mL was chosen for the **direct KO** model, as the 4 mg/mL concentration used in the previous *Brca1* cKO embryo culture model caused lethality in all

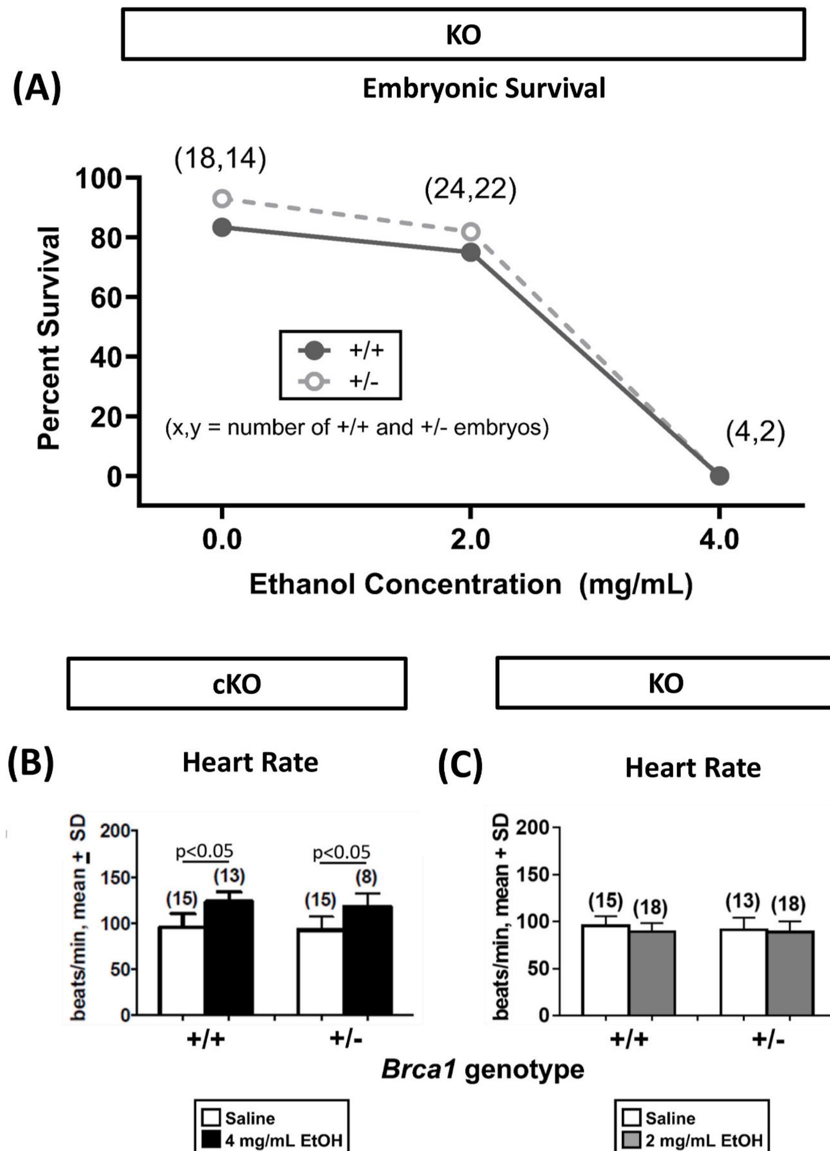


Fig. 2. Effect of ethanol on embryonic survival and heart rate in culture comparing *Brca1* conditional knockouts (cKO) and direct knockouts (KO). For **cKO** embryos, $Cre^{+/-}$ male mice were mated overnight with $Brca1^{LoxP/LoxP}$ female mice (plug designated GD 1) (see Methods), and *Brca1* +/- **direct KO** mice were mated at about 09:00 for 2–4 h (plug designated GD 1). Breeding for both cKO and KO models resulted in +/+ and +/- *Brca1* littermates respectively exhibiting normal and deficient levels of BRCA1 protein, with a greater BRCA1 deficiency in **KO** vs. **cKO** embryos (see Fig. 1A&B). On GD 9, *Brca1* +/+ and +/- 7-8-somite **cKO** embryos were explanted and incubated for 24 h with ethanol (**EtOH** (4 mg/ml for **cKO** embryos) or its saline vehicle. On GD 9.5, *Brca1* +/+ and +/- 6-8-somite **KO** embryos were explanted and incubated for 24 h with EtOH (2 mg/ml for **direct KO** embryos, as 4 mg/mL caused lethality, **Panel A**) or its saline vehicle. Embryos were evaluated under a microscope for morphological and functional parameters. **(Panel A)** In the **direct KO** model, embryonic survival throughout the 24 h culture period was not significantly affected by EtOH at 2 mg/mL, while a concentration of 4 mg/mL caused 100% lethality. **(Panel B)** In the **cKO** model, independent of *Brca1* genotype, EtOH enhanced the heart rate relative to saline controls of the same genotype ($p < 0.05$). The data for the cKO embryos were previously published [30] and are provided here for comparison. **(Panel C)** Unlike in the cKO embryos, EtOH exposure had no effect on embryonic heart rate in the **KO** embryos of either *Brca1* genotype, possibly due to the lower EtOH concentration used in the KO model. Significant differences were determined for binomial data using Fisher's exact test and for continuous data using two-way ANOVA with a Bonferroni post-hoc test. The numbers in parentheses above each bar indicate the number of embryos, which were selected from at least 3 litters to minimize any impact of a litter effect.

direct KO embryos of both genotypes (Fig. 2A). Unlike in cKO embryos in previous studies (Fig. 2B), embryonic heart rate in direct KO embryos was not increased by EtOH compared to saline-exposed controls in either *Brca1* genotype (Fig. 2C), possibly due to the 50% lower EtOH concentration used in the direct KO model. The increased heart rate caused by EtOH only in the cKO model, independent of *Brca1* genotype, is likely due to a direct, receptor-mediated mechanism and the higher concentration of EtOH employed for that model, rather than ROS-initiated effects or other strain differences. In adult rats, EtOH-initiated tachycardia has been attributed to direct stimulation of sympathetic nerves innervating the heart [60], although the mechanism in embryos remains to be determined.

3.5. BRCA1- and EtOH-dependent effects on embryopathies in mouse whole embryo culture

Whole embryo culture is a valuable tool for assessing mechanisms of embryonic susceptibility to chemical exposures without confounding maternal influences. Normal development of saline-exposed +/+ and +/- *Brca1* embryos herein confirms an appropriate culture environment similar to that *in vivo* and previously published [30]. Whole embryos were evaluated for developmental parameters following 24 h culture in 2 or 4 mg/mL EtOH for direct KO and cKO embryos, respectively, or its saline vehicle. Both +/+ and +/- *Brca1* KO embryos exposed to saline developed normally, evidenced by normal somite development and complete anterior neuropore closure, as was observed previously in *Brca1* cKO embryos [30]. The findings in the **cKO model** (Fig. 3A–F) were previously described in detail [30]. Despite the use of embryos with an equivalent range of somite pairs from both the cKO and direct KO models for the embryo culture experiments, control cKO embryos were physically smaller following culture than the direct KO embryos. Specifically, the yolk sac diameter, crown-rump length and head length values for the direct KO embryos (Fig. 3I, J, K) were between 22% and 35% greater (head length and crown-rump length, respectively) than the respective values for the cKO embryos (Fig. 3C, D, E). The smaller size of the cKO embryos may result from strain differences between the two models, as described below. Interestingly, decreased values for these two parameters were evident in the KO *Brca1* +/- progeny compared to +/+ littermates, but not in the cKO model with a minor BRCA1 deficiency, revealing a BRCA1 protein dose-dependent effect.

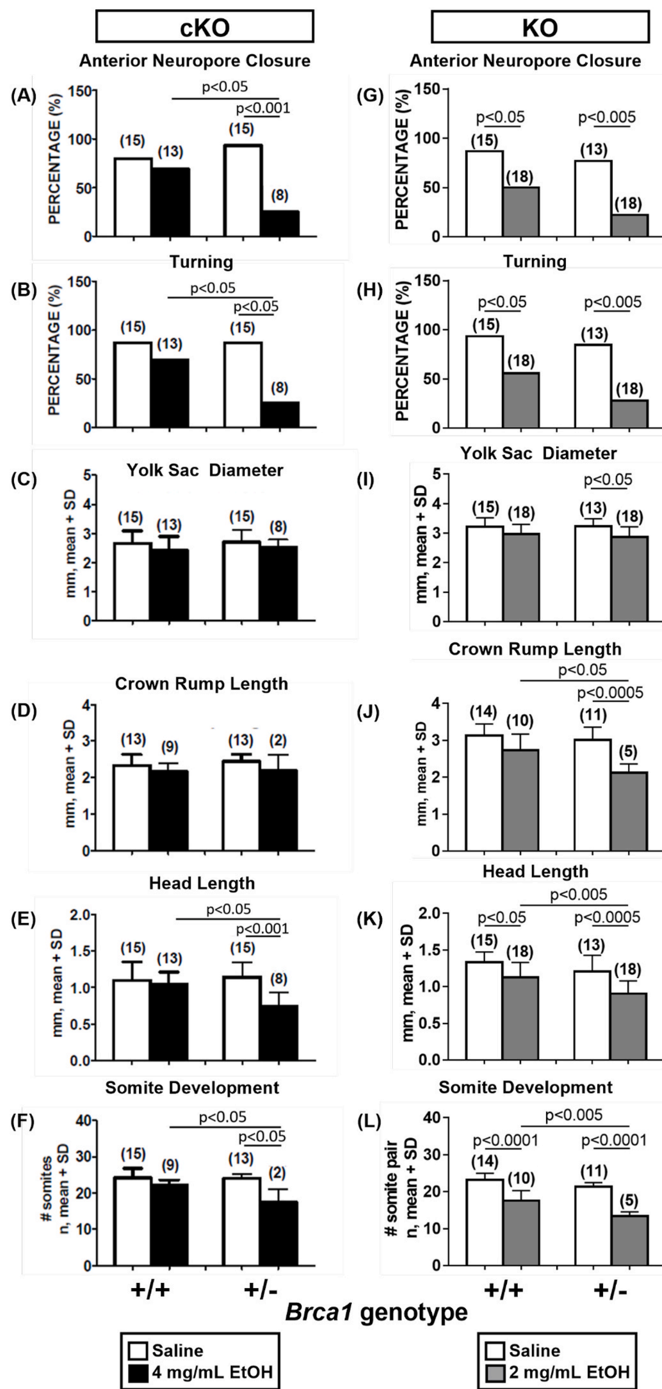
Regarding embryopathies, in +/+ embryos from the **direct KO** model, 2 mg/mL EtOH exposure decreased anterior neuropore closure by 42% ($p < 0.05$) (Fig. 3G), embryonic turning by 40% ($p < 0.05$) (Fig. 3H), head length by 15% ($p < 0.05$) (Fig. 3K), and the number of somite pairs developed by 24% ($p < 0.0001$) relative to saline-exposed +/+ embryos (Fig. 3L). This enhanced EtOH sensitivity evidenced in the +/+ KO embryos, but not the +/+ cKO embryos even at a 2-fold higher EtOH concentration reveals genetic differences in embryonic biochemical pathways in the background strains for these two models, particularly those regulating the levels of oxidative DNA damage. Potential pathway proteins might include ROS formation (*e.g.,* NOX expression), antioxidative enzymes and/or proteins involved in DNA repair, among other pathways, but unrelated to hepatic and gastrointestinal EtOH metabolism [22]. We also observed that direct KO embryos reached 6–8 somite pairs by GD 9.5, whereas cKO embryos achieved this developmental milestone more rapidly, by GD 9. Despite the more accelerated somite development of cKO embryos, direct KO embryos were consistently larger in size. The discrepancy between rate of somite formation and overall embryonic size may be influenced by the distinctive genetic backgrounds of the two strains: 129SvEv and NIH Black Swiss heritage for direct KO mice, as opposed to C57BL/6J, 129/SvJ and Swiss Webster heritage for cKO mice. These observations align with previous reports that there can be up to a half-day delay in somite development between different inbred strains of mice [61], and that biomolecular factors dictating somite formation are independent of embryonic size [62]. In some mouse strains, an increased susceptibility to developmental disorders has been associated with higher EtOH plasma levels despite being administered the same weight-adjusted dose [32]. However, other strains show enhanced susceptibility despite exhibiting a lower plasma EtOH concentration than others [22].

In +/- *Brca1* embryos from the direct KO model, 2 mg/mL EtOH exposure caused abnormalities in nearly all parameters, decreasing anterior neuropore closure by 71% ($p < 0.005$) (Fig. 3G), embryonic turning by 67% ($p < 0.005$) (Fig. 3H), yolk sac diameter by 11% ($p < 0.05$) (Fig. 3I), crown-rump length by 30% ($p < 0.0005$) (Fig. 3J), head length by 25% ($p < 0.0005$) (Fig. 3K), and the number of somite pairs developed by 37% ($p < 0.0001$) (Fig. 3L), compared to saline-exposed embryos of the same genotype. The delayed embryonic turning observed in both +/+ and +/- EtOH-exposed embryos in the direct KO model indicates that they are not completing a key developmental process necessary for normal continued development. While these morphological parameters in embryo culture cannot be directly related to *in vivo* teratogenesis, the underlying molecular mechanisms, including deficiencies in protective proteins like BRCA1 and associated increases in oxidative DNA damage, are similar during the later fetal period. The embryo and fetal brain are similar in that both have high levels of ROS forming enzymes like prostaglandin H synthase (**PHS**) and NOX, as well as contrasting low levels of protective ROS-detoxifying enzymes like superoxide dismutase and catalase [19,63]. Thus, abnormalities of these morphological parameters in embryo culture have proven consistently predictive of both morphological birth defects and functional teratogenesis (*e.g.,* neurodevelopmental disorders) initiated by EtOH and other ROS-initiating teratogens [19,63,64].

Furthermore, in +/- *Brca1* embryos from the direct KO model, the 2 mg/mL EtOH exposure decreased crown-rump length by 22% ($p < 0.05$) (Fig. 3J), head length by 20% ($p < 0.005$) (Fig. 3K), and the number of somite pairs developed by 24% ($p < 0.005$) (Fig. 3L) compared to EtOH-exposed +/+ embryos. In contrast, EtOH exposure at 4 mg/mL did not affect crown-rump length or yolk sac diameter in +/- cKO embryos. Thus, the direct KO embryos with a greater deficiency of BRCA1 were more susceptible to EtOH embryopathies than cKO embryos, even when exposed to only half the concentration of EtOH (2 mg/mL) that was used in the cKO model previously reported (Fig. 3C&D vs. Fig. 3I&J) [30]. The impairment of morphological parameters/milestones in EtOH-exposed +/- KO embryos compared to EtOH-exposed +/+ littermates is further indicative of a remarkable *Brca1* genotypic effect. Moreover, the increase in the spectrum and severity of embryopathies in direct KO vs. cKO embryos, indicating an inhibition or delay of overall growth at half the EtOH concentration required for the cKO model, is consistent with the 2-fold greater BRCA1 deficiency in the direct KO embryos, and indicative of a BRCA1 protein dose-response relationship. The 2 mg/mL concentration in the direct KO model equates to human plasma concentrations following 4–6 alcoholic drinks, corresponding to a blood-alcohol concentration of 0.2% (43.4 mM) [65].

3.6. BRCA1- and sex-dependent effects on learning & memory in postnatal progeny

The *in vivo* behavioural data presented herein for both cKO and direct KO progeny have not been previously reported. In the cKO model, learning and memory was impaired only in +/- *Brca1* cKO female progeny exposed *in utero* to EtOH, relative to +/+ littermates (Fig. 4A). However, there was a lesser but similar, non-significant trend for impairment in *Brca1* cKO female progeny exposed *in utero* to saline vehicle, suggestive of a pathogenic effect of physiological levels of ROS formation in DNA repair-deficient progeny. In contrast, in the female +/- *Brca1* direct KO progeny, learning and memory was substantially impaired even with only saline exposure, indicated by a 75% decreased latency to enter on the final day of testing (Fig. 4B, $p < 0.0001$). A similar deficit was evident in female +/- *Brca1* progeny with EtOH exposure on the final day of testing, indicated by a 72% decreased latency to enter ($p < 0.0001$). Learning & memory impairment in the



(caption on next column)

Fig. 3. BRCA1 protein dose-dependent susceptibility to morphological developmental disorders in cultured embryos exposed to saline or ethanol (EtOH). The mating procedures for the conditional knockout (**cKO**) model and the *Brca1* +/- direct knockout (**KO**) model are detailed in the legend for Fig. 2 and in the Methods. Breeding for both the cKO and KO models resulted in +/+ and +/- *Brca1* littermates respectively exhibiting normal and deficient levels of BRCA1 protein, with a greater BRCA1 deficiency in **KO** vs. **cKO** embryos (see Fig. 1A&B). On GD 9, *Brca1* +/+ and +/- 7-8-somite cKO embryos were explanted and incubated for 24 h with EtOH (4 mg/ml for **cKO** embryos) or its saline vehicle. On GD 9.5, *Brca1* +/+ and +/- 6-8-somite KO embryos were explanted and incubated for 24 h with EtOH (2 mg/ml for **direct KO** embryos, as 4 mg/mL caused lethality **[Panel A]**) or its saline vehicle. Embryos were evaluated under a microscope for morphological and functional parameters. The data for the KO embryos are new, while those for the cKO embryos were previously published [30] and are provided here for comparison. **(Panels A&B)** EtOH-exposed *Brca1* **cKO** embryos exhibited decreased anterior neuropore closure ($p < 0.001$) and embryonic turning ($p < 0.05$), relative to saline-exposed controls of the same genotype. EtOH-exposed BRCA1-deficient embryos exhibited decreased anterior neuropore closure ($p < 0.05$) and embryonic turning ($p < 0.05$) relative to EtOH-exposed +/+ embryos. **(Panels G&H)** In the **direct KO** model, an EtOH-induced decrease in anterior neuropore closure and embryonic turning was observed in both genotypes, as distinct from only in the cKO +/- embryos ($p < 0.05$). EtOH-exposed BRCA1-deficient cKO embryos exhibited increased susceptibility compared to +/+ littermates ($p < 0.005$), while a similar trend in EtOH-exposed *Brca1* +/- KO embryos was not significant, likely due in part to the already significant deleterious impact of EtOH in *Brca1* +/+ littermates. **(Panels C&D)** No genotypic or treatment effects were observed for yolk sac diameter and crown rump length measurements in the **cKO** model. **(Panels I&J)** In +/- *Brca1* **direct KO** embryos, unlike the cKO embryos, EtOH decreased the yolk sac diameter ($p < 0.05$) and crown rump length ($p < 0.0005$) relative to saline controls of the same genotype. The crown rump length of +/- *Brca1* **direct KO** embryos was also decreased compared to EtOH-exposed +/+ embryos ($p < 0.05$). **(Panels E&F)** EtOH-exposed *Brca1* **cKO** embryos exhibited decreased head length ($p < 0.001$) and rate of somite development ($p < 0.05$) relative to saline-exposed controls of the same genotype. EtOH-exposed BRCA1-deficient embryos also exhibited decreased head length ($p < 0.05$), and rate of somite development ($p < 0.05$) relative to EtOH-exposed +/+ embryos. **(Panels K&L)** In the **direct KO** model, EtOH exposure decreased head length ($p < 0.05$) and the rate of somite development ($p < 0.0001$) in both genotypes, in contrast to the cKO +/+ embryos, which were unaffected. The impact of EtOH on head length and somite development also was greater in +/- than ++ *Brca1* **direct KO** embryos ($p < 0.005$). Significant differences were determined for binomial data using Fisher's exact test and for continuous data using a two-way ANOVA with a Bonferroni post-hoc test. The numbers in parentheses above each bar indicate the number of embryos, which were selected from at least 3 litters to minimize any impact of a litter effect.

direct KO females by prenatal EtOH exposure was not greater than that in the saline-exposed direct KO progeny, likely because the latter untreated direct KO progeny were already maximally impaired compared to their +/+ *Brca1* littermates. The observed pattern of increasing latency with age indicates the mice are learning not to enter the dark chamber. Males in both models displayed higher latencies to enter the chamber, appearing to learn not to enter (Fig. 4C showing *Brca1* direct KO progeny). For the female +/- *Brca1* KO progeny, the latencies did not increase remarkably with age, unlike their +/+ littermates which learned dramatically better, and similar to the males (Fig. 4D). The substantial impairment in learning & memory in female +/- *Brca1* direct KO progeny may be associated with their greater deficiency in BRCA1 protein compared to +/- *Brca1* cKO embryos. Although specific brain regions impacted by the degree of a BRCA1 deficiency were not investigated, learning & memory deficits have previously been associated with hippocampal dysfunction [66], and the laminated structure of the hippocampus was disrupted in a CNS-specific *Brca1* KO model [67]. In our direct KO model, sex-dependent decreases in executive functioning were also exhibited by +/- *Brca1* KO progeny [44], which has been associated with the prefrontal cortex [68]. Furthermore, in a *Brca1* knockdown model of the dentate gyrus, elevated DNA strand breaks and

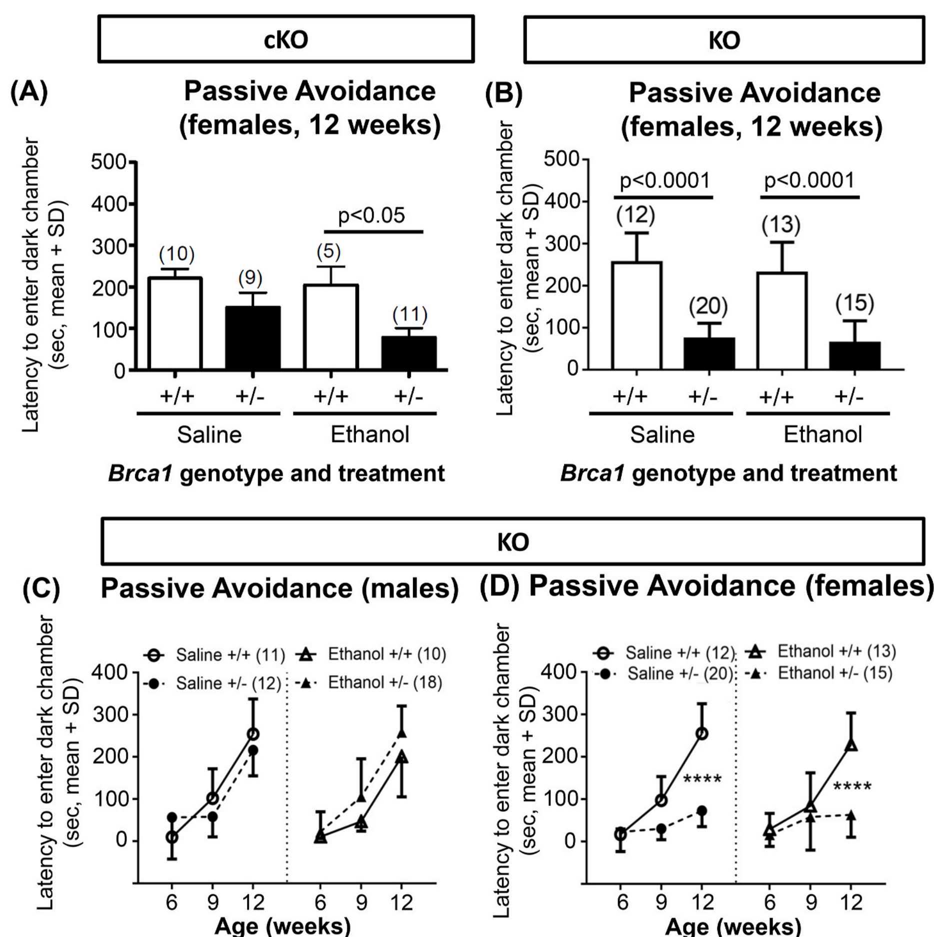


Fig. 4. Decreased learning and memory in BRCA1-deficient female progeny measured via passive avoidance testing following *in utero* exposure to a single 4 g/kg maternal dose of ethanol (EtOH) on GD 17. *Brca1* cKO and KO fetuses were exposed *in utero* to saline or EtOH (4 g/kg) via maternal i.p. injection on GD 17. At 6, 9 and 12 weeks of age, mouse progeny were assessed in three daily trials for their latency to enter the dark chamber, where a mild footshock was received. The data for both the KO and cKO progeny have not been previously published. Latency to enter the dark chamber after the footshock for **(Panel A)** female *Brca1* conditional knockout (**cKO**) progeny, and **(Panel B)** female direct knockout (**KO**) progeny, is shown for the third trial of the final week. **(Panels C&D)** The performance of male vs. female *Brca1* **KO** progeny is shown from 9 to 12 weeks. **(Panel A)** In the final trial at 12 weeks of age, **EtOH**-exposed female *Brca1* **cKO** progeny exhibited a decreased latency to enter the dark chamber relative to +/+ littermates ($p < 0.05$). **(Panel B)** In the final trial at 12 weeks of age, female +/- *Brca1* **KO** progeny exposed to **saline** vehicle have a 75% decreased latency to enter the dark chamber relative to +/+ littermates ($p < 0.0001$). A similar 72% reduction in latency was observed in female +/- *Brca1* KO progeny exposed to EtOH ($p < 0.0001$). **(Panels C&D)** The *Brca1* genotypic differences for female +/- *Brca1* progeny over 12 weeks is plotted (****$p < 0.0001$). No *Brca1* genotypic differences were observed for male KO progeny. Statistical differences were determined by two-way ANOVA with a Bonferroni post-hoc test at each timepoint. The number of progeny is shown in parentheses above each bar (**Panels A&B**) or in the figure key (**Panels C&D**). Progeny were selected from at least 3 litters to minimize any impact of a litter effect.

spatial memory deficits were reported [69]. All three of these brain regions may be of interest for further exploration using immunohistochemical methods in our *Brca1* direct KO model.

Increased neuronal activity, which enhances DNA DSBs, also enhances BRCA1 protein levels [58], reflecting the importance of BRCA1 in DSB repair. This is consistent with our observation that BRCA1-deficient female mice in the direct KO model are more susceptible than their +/+ littermates to both DNA damage and impaired learning & memory at saline-exposed levels of ROS production. ROS were previously shown to contribute to the learning & memory deficits observed in +/- *Brca1* KO female progeny [44], likely due to an insufficient amount of BRCA1 for efficient repair of DNA damage, and particularly DSBs. The 8-oxoG results herein were comprised primarily of female samples, potentially contributing to the learning & memory deficits in BRCA1-deficient female progeny. This learning & memory impairment was not measurably worsened by EtOH exposure in the +/- *Brca1* KO female progeny, likely due to the substantial impairment of learning & memory observed in the female +/- *Brca1* progeny exposed to only saline. Nevertheless, only +/- *Brca1* cKO and direct KO female progeny, and not their +/+ littermates, displayed learning & memory deficits following EtOH exposure. Accordingly, a BRCA1 deficiency coupled with *in utero* EtOH exposure may confer risk of FASD particularly in females, consistent with reports of female FASD children exhibiting greater dysmorphology and cognitive impairment relative to males [70]. Importantly, the lack of learning & memory impairment in male +/- progeny as evaluated by passive avoidance testing may be due to mechanisms downstream of ROS formation/detoxification and DNA damage, corroborated by the lack of a sex difference in all measures of oxidative DNA damage in whole embryos herein, which was also previously observed in fetal brains [44]. In such cases, oxidative DNA damage may be a necessary but insufficient component of the molecular mechanisms leading to ROS-dependent developmental disorders. For example, the resistance of males could be due to sex differences in epigenetic mechanisms and/or events further downstream. Alternatively, there could be sex-dependent brain region- and/or cell type-specific differences in the ROS formation/detoxification pathways, DNA repair and DNA damage that we cannot see using our whole embryo measurements.

3.7. BRCA1-dependent, sex-independent effect on motor coordination in postnatal direct KO progeny

EtOH exposure decreased motor coordination in both sexes by 19% and 16% in *Brca1* +/- KO progeny compared to +/+ littermates ($p < 0.01$), and +/- saline-exposed controls ($p < 0.001$), respectively (Fig. 5). A significant difference between the sexes was not observed (Supplementary Materials Fig. S3), so the data were combined. Motor coordination was not assessed in the cKO mice.

The impairment in motor coordination, unlike learning & memory impairment, observed in +/- *Brca1* progeny of both sexes with EtOH exposure were consistent with the sex-independent DNA damage results observed herein. Also, unlike BRCA1-dependent learning & memory impairment, the absence of motor coordination impairment in saline-exposed +/- *Brca1* progeny suggests that motor functions may be less susceptible than cognition to the pathogenic effects of physiological ROS levels. The motor coordination deficits could be due to ROS/DNA damage/repair mechanisms, given that there also was no sex difference in oxidative DNA damage measured by either 8-oxoG or γH2AX formation. Collectively, the behavioural results herein reveal the susceptibility of the developing brain to oxidative stress, which may occur even at physiological levels of ROS formation in progeny with deficient DNA repair and can be worsened by xenobiotic or pathogen exposures that enhance ROS formation or reduce pathways for ROS detoxification or DNA repair. ROS were previously shown to contribute to the EtOH effect on motor coordination in both sexes of +/- *Brca1* progeny [44], likely due to an insufficient amount of BRCA1 for efficient repair of DNA damage, particularly DSBs.

Importantly, in a CNS-specific *Brca1* KO model, an ataxic phenotype was noted and attributed to brain volume reductions and disorganization of brain structural lamination, particularly in the neocortex, hippocampus, cerebellum, and olfactory bulbs [67]. However, immunohistochemical analysis of the brains of +/- *Brca1* KO mice would be needed to determine if similar effects on brain volume and laminated brain structures are observed in the direct KO model. The rotarod findings observed herein were included to show that not all BRCA1-dependent impairments are sex-dependent.

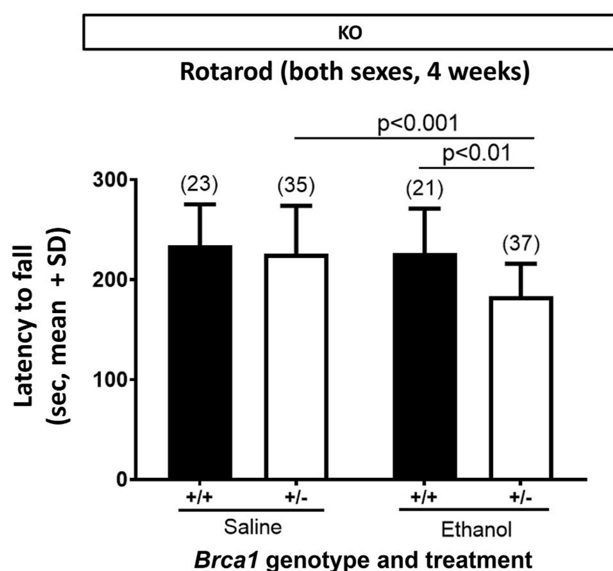


Fig. 5. *In utero* EtOH exposure impaired motor coordination in *Brca1* direct KO progeny. *Brca1* direct **KO** fetuses were exposed *in utero* to saline or EtOH (4 g/kg i.p.) via maternal injection on GD 17, and motor coordination was assessed at postnatal week 5 using a rotarod apparatus consisting of a rotating rod that accelerated from 4 to 40 rpm linearly, over 5 min. Three trials were carried out with a 3-min break between. The mean latency to fall from, or hold on to, the rotating rod for 3 rotations during trial 2 and 3 was plotted. Male and female mice were analyzed separately based on sex differences observed in other behavioural tests (*i.e.*, learning & memory, passive avoidance). There was no significant difference between the sexes (see Supplementary Materials, Fig. S3), so the data were combined. *Brca1* +/- progeny exposed *in utero* to EtOH exhibited a 16% reduction in latency to fall compared to saline-exposed +/- controls ($p < 0.001$) (drug effect), and a 19% reduction in latency compared to EtOH-exposed +/+ littermates ($p < 0.01$) (*Brca1* genotype effect). Statistical analyses were performed using two-way ANOVA with a Bonferroni post-test. The number of mice assessed is shown in parentheses. Progeny were selected from at least 3 litters to minimize any impact of a litter effect. Motor coordination was not evaluated in the cKO model.

4. Conclusions

Our studies using *Brca1* +/- direct KO mice compared to a previously characterized +/- cKO embryo culture model provide the first evidence of a BRCA1 protein dose-dependent susceptibility to embryonic DNA damage, embryopathies and functional brain impairment in learning & memory and motor performance caused by physiological and/or drug-enhanced levels of embryonic and fetal ROS production.

The new *in vivo* results in the cKO model provide the first evidence that even a minor 28% decrease in BRCA1 levels constitutes a significant risk factor for EtOH-initiated neurodevelopmental disorders. Our observations in the direct KO model, with a greater 58% (moderate) reduction in fetal BRCA levels, highlight the exquisite sensitivity of the brain to even physiological levels of embryonic ROS formation when DNA repair is moderately deficient. The spectrum and severity of neurodevelopmental disorders are further exacerbated by *in utero* exposure to EtOH, and likely also to other ROS-enhancing xenobiotics or environmental conditions that increase pathways for ROS formation and/or reduce pathways for ROS detoxification or DNA repair. The role of sex-dependence in ROS-initiated, DNA damage-mediated neurodevelopmental disorders in direct KO progeny varies for the type of disorder, with learning & memory impaired in only female +/- *Brca1* fetuses, and motor coordination deficits in both sexes, suggesting further risk modulation by pathways downstream of oxidative DNA damage.

The EtOH-enhanced oxidative DNA damage and embryopathies in culture in both cKO and direct KO embryos, and the EtOH-enhanced learning & memory deficits in cKO progeny, are consistent with a protective role for BRCA1 against the developmental consequences of ROS-initiated oxidative DNA damage. Direct KO progeny exposed prenatally to EtOH were similarly susceptible to motor coordination deficits, but in both sexes. Our embryo culture data suggest that BRCA1-deficient fetuses may also be susceptible to morphological abnormalities or teratogenesis following prenatal exposure to physiological or drug-enhanced ROS levels. A similar but more limited range and less severe level of susceptibilities to EtOH-initiated embryopathies was observed in the cKO model, but at double the concentration of EtOH [30], compared to the direct KO model. The minor 28% BRCA1 deficiency in cKO mice revealed a role in protection from environmentally enhanced developmental oxidative stress [30]. The results herein in direct KO progeny with a greater 58% decrease provide the first evidence of a BRCA1 protein dose-dependent susceptibility to oxidative stress and developmental disorders, where even a moderate BRCA1 deficiency may enhance embryonic and fetal oxidative DNA damage, and the risk of a broader spectrum and greater severity of developmental disorders.

Findings herein corroborate a broader biological role for BRCA1, beyond tumor suppression. Such deficiencies may be observed in *BRCA1* +/- humans, in whom the level of BRCA1 protein may be decreased by mutations in the *BRCA1* gene [71,72], or with normal *BRCA1* alleles in whom the BRCA1 protein is decreased by epigenetic and other environmental mechanisms [73–75]. Though limited information is currently available, a variety of missense and truncating mutations demonstrated a collective 52–81% BRCA1 deficiency in peripheral blood lymphocytes of subjects with a heterozygous *BRCA1* mutation [71,72]. Accordingly, this +/- *Brca1* KO model may represent a more clinically relevant model than the previous cKO mice for studying the

potential developmental consequences of human BRCA1 deficiencies, reflecting what could occur in some *BRCA1* +/- children. Similar deficiencies in BRCA1 protein levels or activity may also arise from a multitude of other mechanisms, including epigenetic silencing [2], ethanol consumption [73], sedentary behaviour [75] and shift work [74]. From a broader biological perspective, the risk of developmental abnormalities with minor to moderate heterozygous BRCA1 deficiencies is likely substantially higher than that for breast and ovarian cancers, which generally require the mutation of both *BRCA1* alleles in tumor tissue [76]. Our results may be relevant to *BRCA1* +/- women and those with environmental determinants of reduced BRCA1 levels, for whom preventive strategies may include modifications in lifestyle choices such as diet and therapeutic and recreational drug use.

CRediT authorship contribution statement

Danielle M. Drake: Conceptualization, Data curation, Formal analysis, Investigation, Methodology, Validation, Visualization, Writing – original draft, Writing – review & editing. **Kian Afsharian:** Conceptualization, Data curation, Formal analysis, Investigation, Methodology, Validation, Visualization, Writing – review & editing. **Benjamin Or:** Data curation, Investigation. **Aaron M. Shapiro:** Data curation, Formal analysis, Investigation, Writing – review & editing. **Michelle L. Lai:** Data curation, Formal analysis, Investigation, Visualization. **Lutfiya Miller:** Data curation, Formal analysis, Investigation, Validation, Visualization, Writing – review & editing. **Peter G. Wells:** Conceptualization, Funding acquisition, Project administration, Resources, Supervision, Writing – review & editing.

Declaration of competing interest

None.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.redox.2024.103070.

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