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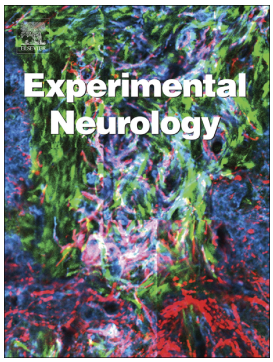

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Alcohol & cannabinoid co-use: implications for impaired fetal brain development following gestational exposure.

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Abstract

Alcohol and marijuana are two of the most consumed psychoactive substances by pregnant people, and independently, both substances have been associated with lifelong impacts on fetal neurodevelopment. Importantly, individuals of child-bearing age are increasingly engaging in simultaneous alcohol and cannabinoid (SAC) use, which amplifies each drug's pharmacodynamic effects and increases craving for both substances. However, to date, investigations of prenatal polysubstance use are notably limited in both human and non-human populations. In this review paper, we will address what is currently known about combined exposure to these substances, both directly and prenatally, and identify shared prenatal targets from single-exposure paradigms that may highlight susceptible neurobiological mechanisms for future investigation and therapeutic intervention. Finally, we conclude this manuscript by discussing factors that we feel are essential in the consideration and experimental design of future preclinical SAC studies.

Keywords: *alcohol, cannabinoids, cannabis, prenatal, neurodevelopment*

Introduction

In 2020, the World Health Organization called for an accelerated action plan to reduce misuse of alcohol across the globe, citing the prevalence of alcohol consumption and it's "burden of disease and injuries" as "unacceptably high." Estimates from 2018 place global averages of alcohol consumption for each person over the age of 15 at 6.2 liters of pure alcohol/year - slightly more than one bottle of wine each week [1]. Notably, consumption patterns vary widely across the globe, influenced by social, economic, and cultural factors which not only influence the quantity of consumption but also a willingness to report consumption [2]. The stigmatization of alcohol likely contributes to underreporting of drinking patterns, and self-reported rates should be treated as conservative estimates of real-world drinking habits, especially among women and adolescents. The stigmatization of alcohol consumption during pregnancy was recently highlighted in a U.S, study, in which survey participants reported that mothers who consumed alcohol during pregnancy were perceived more negatively and held greater blame for their children's health outcomes than mothers with mental illness, substance use disorders, and prior jail experience [3]. Despite this stigma, pregnant individuals continue to drink, even after pregnancy confirmation, for reasons that are highly varied [4], and conservative estimates of Fetal Alcohol Spectrum Disorder (FASD) – the umbrella term that encompasses all disorders associated with prenatal alcohol exposure (PAE) – range from 1-5% of the population [5, 6].

Importantly, alcohol is often used in combination with other substances, and recent patterns of co-use with cannabis products have garnered attention from epidemiologists and researchers. Between 2002 and 2016, marijuana use within North America doubled [7], likely due to growing legalization in the U.S. and Canada [8]. Importantly, these escalating use patterns correspond with increasing perceptions that cannabis is not harmful to consume during pregnancy [9, 10]. These perceptions are especially prevalent in geographic areas where recreational cannabis products are legally purchased [11, 12]. However, public concern about the harm of prenatal cannabis exposure has been reported. In a clinical study published in 2020 [13], over 1 in 5 respondents questioned healthcare providers about cannabis-induced harm to fetal development during pregnancy and/or while breastfeeding. Notably, inconsistent views on the risks of cannabis exposure appeared among healthcare professionals as well. Nearly half of questioned providers (49.6%) directly opposed cannabis use during pregnancy, 0.5% encouraged use, and 49.9% neither encouraged nor discouraged use. With all these factors considered, it is perhaps unsurprising that estimates of prenatal cannabis exposure vary widely, ranging from 3-35% of pregnancies [14]. Despite the apparent uncertainty about the risks posed by cannabis exposure during fetal development, pregnant individuals may still be reticent about truthfully reporting their consumption. In a 2019 study, mothers preparing for delivery were asked by hospital staff about marijuana consumption during pregnancy. While only 2.6% of mothers self-reported consumption, subsequent mass spectrometry analysis of umbilical cord homogenate revealed synthetic marijuana metabolites in 22% of collected samples [15].

Coinciding with increased cannabis use among the general population, young adults of child-bearing age are increasingly engaging in simultaneous alcohol and cannabinoid (SAC) use [16], which amplifies each drug's pharmacodynamic effects and increases cravings for both substances [17]. Importantly, women between 18-29 years old also experience the highest proportions of unintended pregnancies across all racial and ethnic groups [18]. Identifying

patterns of co-use among this demographic can therefore inform translational research models of prenatal polysubstance exposure. Unfortunately, investigations into the effects of prenatal SAC exposure on offspring neurodevelopment are currently limited, with the majority of preclinical SAC studies investigating the consequences of acute or chronic consumption to users rather than to developing offspring.

In this review paper, we will address what is currently known about combined exposure to these substances, both directly and prenatally, and identify shared prenatal targets from single-exposure paradigms that may highlight susceptible neurobiological mechanisms for future investigation and therapeutic intervention.

Considerations for the Interpretation of Clinical Research in Cannabis Users.

The terminology surrounding cannabis research. Although often used interchangeably, the terms ***cannabis*** and ***marijuana*** are not synonymous in scientific reports. *Cannabis* refers to the genus of the plant where products are derived from, including *cannabis sativa, cannabis indica* and *cannabis ruderalis. Marijuana* specifically refers to products derived from *cannabis sativa* and often contains substantial concentrations of delta-9-tetrahydrocannabinol (THC) [19]. THC is one of over 140 naturally occurring cannabinoids found in the *cannabis sativa* plant. Attributed with many of marijuana's intoxicating effects, THC is the most studied cannabinoid in human and preclinical research. In particular, the therapeutic potential of THC is of great interest to healthcare providers, and a growing number of studies suggest that therapeutic outcomes – including reductions in inflammation, anxiety or stress, nausea, chronic pain and multiple sclerosis symptoms - are dependent upon a variety of subject factors, including age, mental illness, and physical health [20-22]. In contrast to marijuana, cannabis products containing pronounced quantities of cannabidiol (CBD) and only small amounts of THC are considered "industrial hemp" under U.S. law [19]. CBD is not associated with intoxicating effects and has distinct interactions from THC with cannabinoid receptors and other neurotransmitter systems, as well as distinct behavioral outcomes associated with the use (see review: [23]). Like THC, CBD is the subject of an extensive investigation by the biomedical community for its therapeutic uses [20] and adverse effects [24].

The binding efficacy of THC and CBD differ from endogenous cannabinoids, although all primarily interact with cannabinoid receptors 1 (CB1) and 2 (CB2) within mammalian species. THC is a partial agonist for CB1 (K_i = ~5-80 nM) and CB2 (K_i = ~1.7-75 nM). The subsequent actions of THC-binding are dependent upon the number of regionally active receptors, and the presence of additional receptor agonists [25], with THC harboring the potential to antagonize the action of full agonists. CBD demonstrates considerably lower affinity than THC as a partial agonist of CB2 (K_i = >370 nM). CBD's affinity for CB1 (K_i = >73 nM), also lower than THC, varies between ligand interactions with the allosteric (inactive state) and orthosteric (active state) sites, depending on whether another receptor-bound ligand is present. Although under-researched, CBD is proposed to act as an inverse-agonist and antagonist of CB1, as well as a negative allosteric modulator [26]. The majority of cannabinoid receptors are heptahelical, G-protein coupled and favor G_i and G_o coupling, although CB1 is predominantly localized to presynaptic terminal sites within the central nervous system, while CB2 is widespread across the central and peripheral nervous systems (for a comprehensive review, see

[27]). Outside the nervous system, both receptor subtypes are also localized to vascular endothelial and muscle cells [28-30].

Importantly, the concentrations of both CBD and THC in cannabis products are believed to influence alcohol preference and consumption in humans. In an observational study of 120 participants (mean age: ~32-34 across all groups), established co-users of alcohol and cannabis were randomly assigned to consume one of three commercially-available cannabis strains: predominantly THC (24% THC, 1% CBD), predominantly CBD (23% CBD, 1% THC), or an equivalent mixture of both (~10%). Participants were permitted 5 days to freely consume cannabis and alcohol, after which time alcohol consumption was assessed. Notably, users of the predominantly CBD strain had fewer drinks per drinking day, fewer drinking days overall, and fewer days of alcohol and cannabis co-use, compared to the groups assigned to cannabis with at least 10% THC content [31]. This is not the first study to suggest that CBD may attenuate alcohol consumption and serve as a possible pharmacotherapy for individuals with alcohol use disorder (AUD) [32, 33]; however, investigations of the direct relationship between CBD and AUD, particularly in human subjects, are currently minimal.

When interpreting clinical studies in alcohol and cannabis co-users, it is notable that most publications report "marijuana use" without including quantities of CBD & THC as independent variables. This is likely in part because the quantities of cannabinoids within commercially available products can vary widely by distributor and geographic location. In many instances, commercial products may not even list cannabinoid concentrations on the provided packaging [34]. Cannabis products purchased through non-commercial avenues are presumably even more ambiguous in cannabinoid content. A user's method of exposure also plays a factor in cannabinoid pharmacokinetics: cannabis products may be consumed (e.g., edibles, including candy and baked goods), inhaled (e.g., the plant bud or flower), or absorbed (e.g., lotions, oils, and bath salts), among other means, which will influence the amount of cannabinoid that enters the bloodstream, as well as interactions with other consumed substances.

Together, these considerations support the careful design of future preclinical experiments, where factors such as THC & CBD quantity, route of administration, and interactions with other drugs can all be tightly controlled and empirically factored. At present, there is a pronounced limitation in the field surrounding ambiguous terminology used by researchers and medical professionals, who quantify cannabinoid levels with terms like "low", "moderate", and "severe/high". While potentially helpful for comparing groups within-study, these generic terms are shared by different research projects while representing different exposure paradigms, inhibiting our ability to compare findings across studies. This, in combination with the variability in cannabis product constituents, makes the interpretation of effects in human populations very challenging.

Patterns of Co-Use and Neurobehavioral Consequences.

Although co-use of alcohol and cannabis has been reported across many age demographics, including veteran populations [35], for the purposes of this review, we will be highlighting patterns of co-use among adolescents and young adults. This age-span encompasses a time when most women are fertile and sexually active [36], and, therefore, whose drug

consumption is most likely to contribute to prenatal exposure. (For more information on co-use across the lifespan, see this excellent review by Yurasek, Aston & Metrik [37].)

Simultaneous consumption of alcohol and cannabinoids is common in young adults, and rates of co-use have increased within the last three decades [38], with the majority of young adult drinkers/cannabis users engaging in SAC use [37]. According to a survey of young adult co-users, SAC occurs most often a) in the evenings, compared to other times of the day, and b) on weekend days, more than weekdays [39]. College-aged adults who report simultaneous consumption of alcohol and cannabinoids believe that the majority (53.4%) of their social network also engages in SAC use [40]. These same users report 30.5% of peers as consuming alcohol only and 0.5% as consuming cannabis only, suggesting that co-users favor peers who share similar drug-taking preferences. Importantly, this study also determined that co-use does not always occur in the company of these shared peer networks; rather, users engaged in SAC use privately *and* in social environments, as the number of people present did not predict SAC use. Instead, simply reporting that one had co-using friends was a better predictor of an individual's likelihood to co-use.

A second study determined that SAC co-users prefer engaging in drug-taking behavior in familiar environments that afford privacy (e.g., at home or at a friend's residence) over unfamiliar, public environments (e.g., bars and restaurants), a preference that was distinct from both alcohol-only and cannabis-only consumers [38]. Notably, this same study determined that a greater number of people in a social environment significantly reduced the likelihood that women would engage in SAC. Under these circumstances, co-using women favored alcohol consumption alone. This negative relationship was not present in men, suggesting that social factors may uniquely influence a co-using woman's willingness to partake in polysubstance use.

In adolescents, SAC co-use is associated with greater consumption of both substances, reflected by the quantities consumed within-session and the frequency of consumption throughout the year, compared to single users of either drug [41]. When accounting for different use patterns among college-aged alcohol drinkers, more than half of identified "binge" drinkers also reported consuming marijuana within the past year. Binge drinkers were >4X more likely to consume cannabis products than non-binge drinkers [42]. When stratifying by cannabis consumption levels of users, the same relationship has been reported: users in the upper quartile of cannabis consumption engaged in significantly more frequent alcohol use than cannabis consumers in the lowest quartile [43]. In both male and female co-users, marijuana use on a given day was significantly associated with higher alcohol intake [44]. This positive relationship between alcohol and marijuana consumption has been further validated in a study exclusively investigating young women (18-24 years old) [45]. Along with greater alcohol consumption, SAC co-use also corresponds with greater instances of problematic drinking behaviors, as assessed by the Rutgers Alcohol Problems Index [46]. In a prospective study, adolescents were assigned to one of four categories ("No Use," "Low Use," "Moderate Use," and "High Use") based on a latent profile analysis of self-reported drug use [47]. SAC users (both high and moderate dual-users) were identified as more likely to develop substance use disorders and have criminal records than moderate alcohol drinkers with little or no cannabis co-use [47].

Compared to single-drug users, SAC users more often engage in risky behaviors, including high-risk sexual behaviors [48, 49], coinciding with higher rates of sexually transmitted illness [50] and behaviors leading to injury (e.g., driving under the influence) [51]. SAC use is also attributed to negative acute and long-term behavioral outcomes. In a controlled study of alcohol and/or THC administration, study participants who used both substances experienced greater changes in mood following a night of drug use, including more anger, confusion, and anxiety, compared to single-drug and drug-free participants. This combined drug group also reported the highest degree of subjective intoxication [52]. Perceptions of increased intoxication may be attributable, in part, to the fact that combined alcohol and cannabis consumption alters the bioavailability of both substances, as demonstrated by preclinical and clinical investigations. Following intubation of THC (50mg/kg) and alcohol (1g/kg) in rodents, pregnant dams exhibited greater levels of THC in their bloodstream four & six-hours post-drug treatment compared to only-THC-exposed dams [53]. Similarly, co-administration of alcohol and cannabis in humans produced significantly higher blood cannabinoid levels [54], and prolonged subjective drug effects (e.g., feeling "high") while slowing the metabolism of cannabis over eight hours post-administration [55]. Separately, combined alcohol and cannabinoid exposure in human participants has produced detectable blood alcohol concentrations following an overnight exposure paradigm, with no detectable blood alcohol in alcohol-only exposed participants [52]. The latter report is particularly notable since alcohol has well-established effects on maternal hemodynamics (see review: [56]).

Combined drug use has also been shown to augment deficits in attention, critical thinking, and driving performance from single drug use in chronic cannabis users between 19-38 years old [57]. Notably, long-term behavioral impairments are also associated with cannabis and alcohol co-use, including symptoms of psychosis, attention deficit hyperactivity disorder, oppositional defiant disorder [58], and depression [50]. However, further research is required to determine whether symptoms of mental illness are the result of polysubstance use, or if co-use is favored among individuals living with mental illness [59].

In the next section, we will describe experiments aimed at investigating combined drug use during gestation, and the consequences on fetal health and development. At this time, there are many gaps in our understanding of SAC outcomes, which was evident from the literature review performed by the authors. The level of technical detail provided by the cited research is highly variable, leading to unequal degrees of depth in experimental descriptions, although the summaries below were written to be faithful to all relevant findings of their source material. This unevenness further serves to highlight areas of much-needed attention by researchers and funding agencies.

SAC May Impose Distinct Harm to Fetal Development from Single-Drug Exposures.

Although there is a growing body of research investigating the acute and long-term direct effects of SAC on users, the pool of existing studies which investigate the consequences of combined consumption during pregnancy on fetal development is much scarcer. One of the earliest studies, which incorporated both mice and rats as subjects, found that subcutaneous injection of combined alcohol (2g/kg) and Δ-9 THC (100mg/kg) to pregnant dams contributed to pronounced rates of fetotoxicity in both species [60]. In dams with dual-drug exposure, gestational food consumption and weight gain were significantly reduced compared to drug-free

controls. In mice, SAC resulted in 100% reabsorption of pups, with no litters producing live pups following the cesarean section of dams on G18. In rats, the reabsorption rate was ~75%, although it should be noted that the window of drug exposure in these latter experiments was shorter (G7-15) than those in mice (G1-15). In both species, SAC increased reabsorption rates relative to control animals, while neither group of single-drug exposed litters experienced a significant change in reabsorption from controls. Notably, combined substance administration had a greater (synergistic) effect on pup reabsorption than the additive effects of the single-drug exposures.

This is not the only study to report SAC-associated growth deficits. A recent study [61] characterized a third trimester-equivalent model of drug exposure in rats, investigating individual and combined exposure to alcohol (intragastic intubation; 5.25g/kg/day dissolved in artificial milk) and the non-selective cannabinoid receptor agonist, CP-55940 (intraperitoneal [i.p.] injection; 0.4mg/kg). Notably, SAC resulted in a ~33% reduction in average pup body weight on Postnatal Day (P)9 from drug-free controls, which was also a significant reduction in weight gain from both alcohol-only and cannabinoid-only exposed litters. In dams exposed to alcohol, blood alcohol concentrations were significantly higher among those who also received CP-55940 (~316. mg/dL) than in dams exposed to alcohol only (~276 mg/dL), indicating that the presence of a cannabinoid may slow the rate of alcohol metabolism. Interestingly, the increase in blood alcohol concentrations from SAC litters was more prominent in female offspring than male. Offspring were raised into early adolescence and assessed for exposure-associated deficits in motor coordination on a parallel bar task. SAC litters experienced greater impairments in motor coordination than litters exposed to alcohol alone, an effect that was once again predominantly driven by female offspring.

Interestingly, the same research group replicated their pharmacokinetic findings in maternal blood alcohol levels in a subsequent study [62] using vaporized drug inhalation for both substances, this time using THC instead of CP-55940, and exposure lasting through the 1st and 2nd trimesters of pregnancy (G5-20). SAC resulted in increased blood alcohol concentrations in dual-drug-exposed dams for up to three hours post-exposure, compared to dams exposed to alcohol alone. Furthermore, SAC dams exhibited higher THC concentrations in their plasma than dams exposed to THC alone, indicating that alcohol and cannabis influence each other's bioavailability when consumed concurrently. This study further determined that SAC led to hypothermic maternal body temperature shifts during exposure compared to all other treatment groups. In a separate investigation, this research group found that SAC influenced offspring activity levels, albeit in a sex-dependent manner [63]. Combined prenatal alcohol and THC exposure among adolescent male offspring increased locomotor activity and time spent in the center of an open field task from controls without affecting female offspring of the same litters. Follow-up immunohistochemical and cell density analyses in these animals determined that SAC offspring exhibited greater numbers of parvalbumin (PV) interneurons in the dorsal CA1 hippocampal region compared to controls, as well as an increased inhibitory ratio among PV+ cells in this region [64]. PV+ interneurons are functionally associated with hippocampal memory processes, and specifically spatial memory. Although no investigations of cognitive deficits were performed in SAC subjects, it is notable that both alcohol and cannabinoid exposure in adults are also associated with hippocampal impairment: in humans, both substances produce abnormal changes in hippocampal structure and functioning [65], and in rats, combined exposure to alcohol and the non-selective cannabinoid agonist WIN 55,212-2 (WIN) reduces hippocampal neurogenesis [66]. This research highlights a strong regional candidate for augmented deficits

following SAC and the necessity of further investigation into memory deficits corresponding with hippocampal malfunction (which has been argued, in part, in a systemic review emphasizing dual-drug targeting of the dentate gyrus: [67]).

Importantly, a pair of studies have incorporated dose-response assessments into their investigations of prenatal drug exposures, demonstrating that combined exposure to lower doses of alcohol and cannabinoids can mimic higher, singular exposure to either substance. In a rodent model (subcutaneous drug injections; equivalent to the late-3rd trimester/early postnatal time period), THC exposure alone (1-10mg/kg) was insufficient to induce neurodegeneration throughout the brain [68]; however, with the addition of a non-intoxicating dose of ethanol, combined exposure induced massive apoptotic activity, mimicking ethanol exposure at much higher doses. This effect was replicated following WIN administration (1-10mg/kg) and blocked by CB1 receptor antagonist SR141716A (0.4mg/kg; referred to by the gene encoding for CB1, *CNR1*). Furthermore, ethanol administration in CB1-knockout mice produced significantly less neurodegeneration than in wild-type mice. Together, these data indicate that CB1 receptors throughout the brain mediate pronounced neurotoxicity following SAC exposure.

The role of CB1 in mediating SAC-augmented deficits was further supported in a prenatal exposure model in zebrafish [69]. The authors justified their investigation of polysubstance exposure by highlighting that both alcohol and cannabinoid exposure are associated with disruptions in sonic hedgehog (Shh) signaling in vertebrates, which is critical for healthy embryonic development, and specifically for craniofacial development and survival of neural crest cells. Following prenatal exposure to incremental doses of CB1 receptor agonist ACEA and ethanol, combined "low doses" of these substances (in tank water, either 0.5% or 1% ethanol, and 3 mg/L ACEA) resulted in craniofacial, brain, and eye defects, all of which are symptomatic of FASD. Independently, these low doses were insufficient to produce this impaired morphology, and SAC findings were only replicated at "high doses" of ACEA (6 or 12 mg/L), once again indicating that combined exposure mimics the effects of greater exposure to a single drug. Again, administration of CB1 receptor antagonist SR141716A (3mg/L) rescued SAC-induced deficits, emphasizing that this particular receptor drives the synergistic harm observed in offspring. In subsequent assessments of swimming behavior, SAC, but not single drug exposure, increased risk-taking behavior in a novel environment, an effect which was reversed when fish received microinjections to overexpress *Shh* mRNA prior to drug exposure. This importantly highlights another mechanism shared by prenatal alcohol and cannabinoid exposures and one with a well-established relationship to fetal development. The authors subsequently replicated their SAC-augmented eye and facial malformations in a rodent model using i.p. drug injections. This study included not only CP-55940, but also synthetic cannabinoid HU-210 and Δ9-THC combined with alcohol [70], and demonstrated that this effect translates to cannabinoid system activation by multiple agonists. Once again, pretreatment with a CB1 antagonist attenuated SAC-induced teratogenesis.

Taken together, established SAC literature points toward several areas of fetal development that are compromised by combined exposure. However, the number of studies systematically investigating SAC is notably limited, and far more domains critical to healthy neurodevelopment remain unexplored at this time. Therefore, we will use the remainder of this review to highlight common brain systems affected by both PAE & prenatal cannabinoid exposure (PCE) models, as areas for future SAC investigation.

Common Targets in the Brain Between PAE & PCE.

Cerebral Vasculature. Vasculature formation occurs via two processes: vasculogenesis and angiogenesis. Vasculogenesis is the process by which new vessels form from the growth of endothelial cells into functional vessels, while angiogenesis is the formation of new vessels from existing ones [71]. In the human fetal brain, surface blood vessels begin to appear at 3-4 weeks of gestation, with the circle of Willis arising over the next two months. The subsequent development of major brain arteries and refinement of microvasculature occurs throughout the second trimester of pregnancy [72]. Vasculogenesis and angiogenesis are essential for brain development and promote neurogenesis by supplying nutrients and growth factors to developing neural cells [73]. Consequently, any disruption of these processes *in utero* can lead to changes in vessel structure and integrity, subsequently altering the amount of blood supply to the developing fetus. Alcohol exposure during the fetal neurogenic period, equivalent to the first and second trimester of human gestation, results in fetal growth restriction, microcephaly, and a decrease in cranial blood flow that significantly affects fetal brain development [74]. Previous studies have demonstrated that PAE impairs the ongoing formation of embryonic vasculature [75] as well as uterine vasculature, hindering an essential tool for delivering nutrients to the fetus and consequently contributing to developmental growth restrictions [76]. Furthermore, alcohol exposure during the second trimester-equivalent in mice has been shown to reduce fetal blood acceleration and velocity time integral within umbilical and fetal cerebral arteries [77]. These PAE-induced changes in cerebral blood flow persist both during the fetal period [77] and into adulthood and middle-age [78]. PAE also leads to the dilation of fetal middle cerebral arteries, an effect notably mediated by cannabinoid receptors [79].

Acutely, direct exposure to cannabis products is known to increase cerebral blood flow in humans [80-82], an effect also observed in some animal models [83, 84]. However, in other rodent models, the opposite outcome has also been recorded following cannabinoid receptor activation in the brain, specifically within *conscious, restrained animals* [85, 86]. This discrepancy may be attributable to restraint-induced stress and/or indirect cannabinoid-mediated effects on synaptic activity (discussed later in this review) [87]; alternatively, as these rodents were awake at the time of blood flow measurement, this may reflect experimental differences attributable to (a lack of) anesthesia exposure. With regard to cerebral vasculature, CB1 is a well-established mediator of vasodilation, producing dose-dependent increases in dilation following acute agonist administration [30, 83, 88] acting via receptors on the surface of smooth muscle cells and endothelial cells of cerebral vessels [28-30]. Benyó & colleagues [87] describe in detail the role of endogenous cannabinoids in regulating cerebrovascular muscle tone through L-type Ca^{2+}channels on cerebral vascular smooth muscle cells. Although this relationship has yet to be recapitulated with THC, one exogenous cannabinoid compound, CB1 receptor agonist WIN-55212-2, has been shown to dose-dependently inhibit L-type Ca^{2+} channel current in cats [30]. Pretreatment with selective CB1 antagonist, SR141716A, prevented this inhibition, further pinpointing CB1 as a contributor to cerebrovascular muscle tone and overall cerebrovascular activity.

Although few studies have investigated the effects of cannabinoid exposure on fetal cerebrovasculature *in utero*, ultrasound imaging of ~400 mothers during the second-trimester has

demonstrated that daily gestational marijuana use contributes to higher umbilical artery systolic:diastolic ratios compared to gestational age-matched controls [89]. This effect persisted into the third trimester when 26/192 marijuana-exposed fetuses demonstrated growth restriction (below the 10th percentile) compared to 6 out of 192 control fetuses. Notably, three marijuana-exposed fetuses demonstrated absent end diastolic blood in the umbilical artery, and one showed reversed end diastolic blood flow, neither of which was present in any control fetuses. Follow-up investigations in marijuana-exposed fetuses with fetal growth restriction determined that 46% had abnormally low (<1.0) cerebroplacental ratios (CPR), the ratio of middle cerebral artery Doppler indices over umbilical artery indices [90]. This low CPR indicates a "brain sparing" phenotype, with blood flowing preferentially to the brain and away from the rest of the fetus, leading to oligohydramnios (decreased amniotic fluid volume). Notably, low CPR in marijuana-exposed fetuses predicted the lowest fetal birth weights of all deliveries and an increased likelihood of admission to a neonatal intensive care unit. However, this brain sparing compensation following drug exposure may not be long-lasting. Using optical coherence tomography, Raghuanathan et al. [91] have found that second-trimester exposure of mice to synthetic cannabinoid CP55940 (2mg/kg) reduces fetal brain vessel diameter, length fraction, and area density. Thus, further research investigating the acute and persistent effects of cannabinoid exposure in fetal cerebral vasculature is necessitated.

Vascular endothelial growth factor (VEGF) is one of several growth factors that contributes to the development of an embryo, specifically by facilitating endothelial cell proliferation, and is essential for the development of vascular endothelial cells [92, 93]. VEGF plays a key role in angiogenesis, aiding in blood vessel growth and remodeling processes, as well as contributing to the stimulus and survival of endothelial cells [94]. Defects in VEGF ligands or receptors, including VEGF-R1, which are highly expressed throughout the brain [95], can lead to impairment of blood vessel function [96]. Notably, both ethanol and cannabinoid exposure during development has been shown to decrease VEGF expression and inhibit angiogenesis [97-100]. Both exposures produce a decrease in cell proliferation, although presently, only ethanol exposure is also associated with increased rates of neuronal apoptosis during a crucial developmental window for angiogenesis. Currently, investigations of cannabinoid-induced changes in VEGF networks have been limited to *in vitro* cell models and measurements of angiogenesis outside of the brain, and future research investigating fetal cerebral vasculature is warranted. Impairments in developing embryonic blood vessels are one viable mechanism by which prenatal drug exposure may impose developmental deficits, ranging from craniofacial defects to behavioral defects [101].

Neural stem cell activity. While ethanol is known to be toxic to developing neurons [102, 103], fetal neural stem cells (NSCs) have a unique response to ethanol exposure. Developmental exposure has been shown to deplete fetal NSC numbers due to the activation of an aberrant epigenetic maturation program that directs cells toward an astroglial-like lineage [104-106]. Consequently, ethanol appears to decrease the self-renewal capacity of fetal NSCs and facilitates a switch from neuronal to astroglial maturation. It is possible that this loss of stem cell capacity results in an overall decrease in neurogenesis, and explains, in part, microcephaly commonly associated with PAE. The effects of cannabinoid exposure on fetal NSCs are not as well understood. One recent study showed that exposure to the synthetic cannabinoid agonist, CP-55940, during the period of murine neurulation, resulted in exencephaly, holoprosencephaly, and cortical dysplasia [107], suggesting that stem cells of the early fetal neural tube are vulnerable to

cannabinoid exposure. However, most data on the effects of cannabinoids on neurogenesis come from studies on adult neurogenesis. These studies generally show that cannabinoid agonists, acting mainly via CB1, CB2, and G protein-coupled receptor 55, also increase NSC proliferation and gliogenic differentiation [108-113]. Furthermore, a recent report shows that conditional knockdown of CB1 in adult NSCs resulted in a decreased NSC pool and, consequently, decreased number of newborn neurons [113]. Thus, as with ethanol [105, 114-119], cannabinoids appear to control the neurogenic capacity of adult stem cells; however, the effects on fetal NSCs require further targeted investigation, as does the investigation of combined exposure to these substances to model SAC exposure in developing fetal cells.

The Endocannabinoid System. The endocannabinoid system is essential for healthy embryonic development, facilitating many processes of early development, including gametogenesis, embryo implantation, neurodevelopment, peripheral organogenesis, and postnatal development [120]. Endogenous cannabinoids are also critical in pregnancy due to their pronounced expression throughout human placental membrane layers. This system is also the main pharmacological target for THC in both the maternal and fetal nervous systems [121]. Importantly, activation of CB1 placental receptors inhibits cytotrophoblastic proliferation and can subsequently impair fetal growth [122]. Accumulating evidence from multiple investigative teams suggests that PAE results in life-spanning changes in endocannabinoid system activity throughout the brain [69, 70, 79, 123-127], with some researchers proposing that this system contributes markedly to FASD symptomology [123, 128]. Demonstrated in studies of PAE and adult exposures, ethanol exposure increases the availability of endocannabinoids, as well as CB1 receptor activation [112, 125, 130-132]. Interestingly, prenatal THC exposure has been shown to produce the opposite effect: on G17, embryonic brains demonstrated downregulated CB1 protein levels compared to non-exposed controls, as well as overall reduced cannabinoid binding; however, these differences were no longer present by P2 [133]. A separate investigation of prenatal THC exposure also found reduced CB1 levels in brain tissue collected from offspring (age: P20), this time specifically within the dorsal hippocampus and only in male offspring [134].

Notably, while ethanol administration alone increases endocannabinoid levels, combined alcohol and cannabis consumption alters the bioavailability of both substances, as demonstrated by both clinical and preclinical experiments (described earlier in this review). Although it is yet unknown whether SAC would augment fetal endogenous cannabinoid concentrations from single-exposure alone, the delayed metabolism following acute combined substance exposure would, theoretically, increase the length of time during which blood flow through the umbilical artery contains alcohol and/or cannabinoids, thus prolonging each substances interactions with the developing fetus. This is especially important given that both molecular alcohol and cannabis can readily cross the placental barrier [135].

PCE may separately interfere with the endocannabinoid system by inhibiting synaptic pruning, particularly affecting areas of the brain with pronounced cannabinoid receptor concentrations [136]. At G13.5–14.5, the mouse developing cortex shows high CB1 receptor expression in the intermediate zone and developing cortical plate, where differentiation of neurons takes place [137], possibly contributing to an indirect effect on neural stem cell differentiation and subsequent cell fate. Another region of pronounced CB1 expression, the prefrontal cortex, depends on a variety of external factors to facilitate effective

neurotransmission, as proper integration of neurotransmitter systems is essential for normal prefrontal functions [138]. Importantly, cannabinoids mimic neurotransmitters, acting as anandamides capable of inhibiting the release of multiple types of neurotransmitters [139]. Anandamides are the primary endocannabinoids associated with signaling in physiological systems [120], and inappropriate activation of the endocannabinoid system during development may offset the homeostatic neurotransmission necessary for healthy fetal growth, including GABAergic and glutamatergic neurotransmission. Below, we will discuss shared, established deficits following PAE & PCE, as topics for possible future investigations of SAC on offspring neurophysiology.

γ-Aminobutyric acid (GABA). In the adult, GABA and glutamate are the major inhibitory and excitatory transmitters, respectively, driving neuronal activity during development [140, 141], and the balance of these neurotransmitters is crucial for appropriate cell proliferation, migration, differentiation, and survival processes [142]. *In utero* alcohol exposure has numerous, widespread acute effects on GABAergic activity within the fetal brain. Examples in rodent models include early migration of cortical GABAergic interneurons immediately following first-trimester self-administration of ethanol (maternal blood alcohol levels: 25mg/dL), corresponding with increased extracellular GABA concentrations and upregulation of $GABA_A$ receptors in the embryonic neocortex [143]. Prenatal exposure to WIN has also been shown to impact the migration of embryonic GABAergic interneurons on G16.5, increasing GABA+ cells specifically migrating into the marginal zone, but not the cortical plate or subplate, of the dorsal pallium [144]. Notably, periventricular networks facilitate the proper migration of GABA neurons using radial vessels as guides [145] [146], and alcohol exposure has been shown to impair developing endothelial microvessels along the migration route of the GABAergic interneurons in gel zymography experiments performed on P2 [146]. The endocannabinoid system also provides local axon guidance cues for GABAergic interneurons in the developing cerebrum [142], although it's yet unknown if PAE-induced changes in this system contribute to reported impairments in microvascular development. Although currently uninvestigated in PCE and SAC models, these findings highlight an indirect mechanism – cerebral vasculature – by which fetal GABAergic activity can be affected by prenatal exposure.

As previously mentioned, prenatal THC exposure has been shown to reduce CB1 concentrations in the hippocampus of weanling offspring [134], and additional studies have determined that decreases may be specific to CB1-GABAergic cells in this region. Previously, prenatal exposure of mice to either THC (5mg/kg) or WIN (0.75mg/kg) from G10.5-18.5 has led to reduced hippocampal cell concentrations of a particular CB1+ GABAergic cell subtype, cholecystokinin-expressing interneurons (CCK-INTs) [147]. The surviving CCK-INTs in cannabinoid-exposed offspring displayed reduced dendritic complexity and impaired CCK-INT-mediated feedforward and feedback inhibition in this region, which corresponded with altered social behavior *in vivo.* A second study incorporating daily prenatal THC (5mg/kg) exposure throughout the second and third trimesters found that adult rat offspring exhibited reduced basal and potassium-evoked GABA release and reduced CB1 receptor binding in the hippocampus [148]. Re-exposure of tissue to either THC (0.1 μM) or WIN (2 μM) reduced potassium-evoked GABA release further, an effect blocked by selective CB1 antagonism. To delineate between CB1+ GABAergic and glutamatergic hippocampal cells, researchers exposed pregnant dams to THC (3mg/kg) daily from G10.5-17.5, using wild-type mice and conditional knockout mice lacking CB1 in either a) dorsal telencephalic glutamatergic pyramidal cells, or b) forebrain

GABAergic neurons [149]. This exposure resulted in a persistent decrease in perisomatic CCK-INT-CB1+ synapses in the CA1 of the hippocampus exclusively in male offspring from both the wild-type and glutamate-CB1-knockout strains, but not the GABA-CB1-knockout strain. This GABA-specific deficit in hippocampal cells has also been demonstrated by 3rd trimester PAE exposure, which in offspring induced persistent reductions in GABAergic interneurons through activation of apoptotic pathways [150].

Excitatory/inhibitory synaptic imbalance compromises healthy fetal development. and has been produced by independent prenatal exposure to both alcohol and cannabinoids. Following maternal consumption of 10% ethanol during the 1st trimester-equivalent in mice, PAE led to a hyperexcitable shift in hippocampal CA3 neural activity in juvenile offspring [151]. Notably, this shift was the result of reduced inhibition through $GABA_A$ receptors *and* increased action potential firing in pyramidal cells, producing overall network disinhibition within the hippocampus of PAE offspring. The authors hypothesized that this inappropriate network activity, which normally facilitates the maturation of neuronal circuits and synaptic connections, may contribute to the occurrence of seizures and other neurobehavioral disruptions observed in individuals living with FASD. This disinhibition has also been observed in in the ventral tegmental area (VTA) of male mouse offspring (4-10 weeks old), with PAE reducing long-term depression at the excitatory synapses of VTA dopaminergic neurons [127]. Notably, this excitatory shift was mediated by a loss of tonic endocannabinoid signaling and attributed to the downregulation of presynaptic CB1 receptors. A similar increase in the excitation-to-inhibition ratio has been observed in a PCE paradigm. Physiological assessment of male juvenile offspring revealed reduced synaptic inhibition of dopaminergic cells in the VTA, cells which subsequently exhibited depolarized resting membrane potentials and increased firing rates compared to VTA neurons from control offspring [152]. This reduction in GABAergic inhibition coincided with increased presynaptic cannabinoid activity. The shared findings of these last two studies are particularly interesting since CB1 receptors can be internalized as a compensatory response to increased endogenous cannabinoid levels [153]. The question of whether SAC would further disrupt the excitatory-inhibitory balance in the VTA from single-drug exposure, specifically via perturbations in cannabinoid signaling processes, is currently unknown.

Glutamate. Glutamatergic receptors are prominently expressed during fetal brain development, with functional *N*-methyl-d-aspartate (NMDA) receptor expression peaking earlier than AMPA receptors [154], and both receptor types have been identified as targets of independent PAE and PCE studies. For instance, third-trimester, binge-level ethanol exposure of rat pups (P4-9, producing blood ethanol levels of 330mg/dL) has been shown to reduce cortical AMPA GluR1 levels by ~50% and GluR2 levels by ~33% compared to control offspring on P10, without altering functional NMDA receptors or NMDA subunit expression in the neocortex [155]. PCE has produced a similar effect within the cerebellum of male and female offspring throughout development: maternal THC exposure (5mg/kg) throughout gestation and lactation (G5-P20) reduced cerebellar AMPA glutamate receptor GluR1 and GluR2/3 subunit expression within offspring glial cells and Purkinje neurons, respectively. These reductions were present in male and female offspring acutely (P20) and persistently following the end of THC exposure (P30-70) [156].

Several studies have demonstrated an established relationship between PAE and compromised NMDA receptor expression/function within offspring brain tissue [157-159],

which may be associated with altered receptor subunit composition. In mice, a single exposure of ethanol to pregnant dams (0.03ml/g) on G8 was associated with learning deficits and coinciding, region-specific expression of NMDA subunits NR2A and NR2B throughout the brain in young adult male offspring. Specifically, gene expression for NR2A and NR2B was downregulated in the hippocampus and upregulated in the cortex in PAE offspring, and NR2B alone was significantly increased within the cerebellum compared to control offspring [160]. PCE has also been shown to target NMDA-mediated glutamatergic activity in a separate investigation incorporating a first-through-second-trimester exposure paradigm [161]. In this study, cell cultures were acquired from P1 male neonates exposed to WIN (0.5mg/kg) or WIN vehicle daily from G5-G20. WIN exposure decreased basal and K+-evoked extracellular glutamate levels in cortical cells and precluded an NMDA-induced increase in glutamate levels that was observed in vehicle-treated cells. Prenatal WIN exposure further corresponded with reduced cortical neuron concentrations overall, indicating that PCE effects were not limited to NMDA systems alone. These findings were reproduced in an *in vivo* investigation of young adult (~P90) rats, in which prenatal WIN exposure reduced cortical basal and K+-induced dialysate glutamate levels [162]. Furthermore, acute WIN administration increased glutamate concentrations in both control and WIN-exposed offspring. Notably, this effect was blocked by CB1 receptor antagonism in control, but not WIN-exposed, offspring, indicating that PCE can persistently alter systems that regulate cortical glutamatergic release.

Dysregulation in glutamate uptake and extracellular concentrations can contribute to impaired neuronal migration, increased proportions of free radicals and cytotoxic transcription factor levels, greater nitric oxide production, and calcium homeostasis dysfunction, all of which contribute to higher rates of neuronal death [163]. PAE has been associated with dose-dependent decreases in glutamate transport in whole brains of zebrafish offspring [164] and in the hippocampus of adolescent mouse offspring [165]. In the latter study, the expression of astrocyte-specific glutamate transporters 1 & 2 was also significantly changed by PAE, albeit in opposing directions. It should be noted that a very similar exposure paradigm by another research group found that PAE produced the opposite effect in slightly younger (P21) mouse offspring: an increase in Na+-dependent and -independent glutamate uptake within hippocampal slices [166]. The opposing effects of these two studies may be attributed to the length of time between the end of ethanol exposure and tissue collection (same day vs. 9 days later), and mandates further controlled investigations of acute versus persistent effects of developmental alcohol exposure on glutamate transport.

In PCE models, 5 mg/kg THC exposure throughout the second and third-trimester equivalents in rats led to significant reductions in hippocampal glutamatergic neurotransmission in adolescent, male offspring [167]. PCE also desensitized receptors to THC-induced glutamate release following acute administration onto hippocampal slices. Furthermore, this exposure reduced rates of glutamate uptake compared to controls, an effect that corresponded with reduced expression of glutamate transporters GLT1 and GLAST in hippocampal synaptosomes, without impacting overall neuronal and glial cell densities. This same research group also found PCE-induced glutamatergic deficits in the frontal cortex using an earlier exposure paradigm (G5-20) [168]. Exposure to either THC or WIN produced similar reductions in extracellular glutamate levels in the frontal cerebral cortex of adolescent male rats. Follow-up experiments comparing WIN and drug-free controls revealed that PCE significantly increased glutamatergic uptake in frontal cortex synaptosomes, opposite the effect observed in the hippocampus, corresponding

with increased expression of glutamate transporters GLT1 and EAAC1. This may importantly illustrate sub-region-specific effects of PCE on glutamatergic system function during adolescence. A PCE-induced reduction in prefrontal cortex glutamate concentrations was further demonstrated in young adult rats (P80) [169]. Along with notable decreases in genes related to glutamatergic neurotransmission, these results indicate that PCE-associated reductions in excitatory transmission may persist beyond development and into adulthood.

Taken together, both GABAergic and glutamatergic systems have been targeted and altered by PCE and PAE paradigms throughout the lifetime of exposed offspring. With shared sub-region targets, and the importance of excitatory-inhibitory balance to healthy neurodevelopment, investigation of these neurotransmitters would be well supported in future investigations of SAC-associated phenotypes.

Conclusion & Future Considerations.

We have touched upon several domains of neurodevelopment that are either targeted by prenatal polysubstance exposure to alcohol and cannabinoids, or are common mechanisms altered by independent exposure to each drug. SAC research represents a largely unexplored but highly prevalent form of prenatal drug exposure in humans and one that will likely increase in frequency as additional federal policies are enacted to decriminalize and legalize cannabis products. Whether SAC imposes distinct and worse effects than single-drug exposure is a question convoluted by numerable variables (see below) and dependent upon the measures being assessed. However, research comparing the outcomes of single and dual-prenatal drug exposure is critically important, and not only for improving public knowledge about the unique harm SAC use poses during pregnancy. For healthcare practitioners, these findings can support recommendations for harm reduction when a pregnant individual wishes to discontinue polysubstance use but struggles with complete abstinence. Alternative approaches, such as abstaining from the use of one recreational drug, may be more realistic for a parent's circumstances, and empirical evidence for harm reduction under single-drug exposure paradigms will be necessary to support these medical recommendations.

To aid researchers in the experimental design of future preclinical investigations of SAC exposure on offspring development, the authors propose careful reflection of the following variables, based on the contents of this review:

1. <u>The model of exposure.</u> Preclinical researchers who want to develop translationally relevant models of SAC should consider what patterns of consumption are most common in humans who engage in simultaneous alcohol and cannabinoid use (described at the beginning of this review.) Different models of exposure for either drug may influence neurodevelopmental outcomes, including the timing of administration, method of administration, and concentration of the drug.

2. <u>Selection of cannabinoids.</u> The decision of which cannabinoid to use in research - whether the exact substances used by humans, such as CBD or THC, versus mechanism-selective components of cannabis products, like CB1 agonists - may influence outcomes of prenatal exposure paradigms. Ideal paradigms will include a controlled combination of

these substances to best replicate occurrences in humans while identifying specific systemic mechanisms mediating offspring outcomes.

3. Consistent reporting of offspring outcomes. Certain measures should be standardized in assessments involving viable offspring (including growth metrics and maternal blood alcohol/THC concentrations) to allow for comparisons between studies. In clinical studies, creating a repository for these measurements would also facilitate meta-analyses of the factors most likely to increase the risk of neurodevelopmental harm in exposed children.

4. Issues of mortality. Issues of preterm delivery and spontaneous abortion have been reported in select publications where offspring were known to experience prenatal alcohol and/or cannabinoid exposure. It will be important for researchers using animal models to report compromised rates of mortality following SAC, if any, to avoid exclusively reporting the outcomes from surviving litters, which may lead to inaccurate perceptions of offspring outcomes attributable to "survivor bias."

Author Contributions

Siara Rouzer: Conceptualization, literature review, writing- original draft preparation, writing- reviewing and editing, funding acquisition.

Jessica Gutierrez: Literature review, writing- original draft preparation.

Kirill Larin: Writing- reviewing and funding acquisition.

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Declaration of Competing Interest

The authors have nothing to disclose.

Highlights

- Young adults of child-bearing age are increasingly engaging in simultaneous alcohol and cannabinoid (SAC) use.
- SAC users demonstrate distinct preferences and drug-seeking behaviors from single-drug users.
- Studies independently investigating prenatal alcohol (PAE) and prenatal cannabinoid exposures (PCE) share many common neurodevelopmental targets, although studies of polysubstance exposure are notably limited.
- Existing research indicates that prenatal SAC exposure augments offspring deficits from single-drug exposure across several neurodevelopmental domains.