Chromothripsis and Epigenetics, Important Mechanisms for Transgenerational Inheritance of Environmental History, Congenital Malformations and Cancerogenesis in Addictions – Cannabis as a Case Study

Short Title:

Chromothripsis, Epigenetics, Cannabis, Mutagenic Pathways and Transgenerational Effects

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Abstract

The recent demonstration that massive scale chromosomal shattering or pulverization can occur abruptly due to errors induced by interference with the microtubule machinery of the mitotic spindle followed by haphazard chromosomal annealing, together with sophisticated insights from epigenetics provide profound mechanistic insights into some of the most perplexing classical observations of addiction medicine including cancerogenesis, the younger and aggressive onset of addiction-related carcinogenesis, the heritability of addictive neurocircuitry and cancers, and foetal malformations. Moreover the complementation of multiple positive cannabis-cancer epidemiological studies, and replicated dose-response relationships with established mechanisms fulfils causal criteria. Rising community exposure, tissue storage of cannabinoids, and increasingly potent phytocannabinoid sources suggests that the threshold mutagenic dose for cancerogenesis will increasingly be crossed beyond the developing world, and raise transgenerational transmission of teratogenicity as an increasing concern.

In a remarkable and highly celebrated report, the Pellman lab recently showed that severe chromosomal fragmentation involving dozens of double stranded breaks, and subsequent apparently random and disordered repair of some of the fragments, could rapidly occur during the DNA synthetic phase (G2 and S-phases) of the mitotic cell cycle if chromosomes became isolated from the main nuclear mass [1]. In this technical tour de force high resolution DNA sequencing of single cells and live cell imaging was deployed to show that chromosomes which had become detached from the mitotic spindle or chromosomes which lagged behind in their DNA replication, became isolated in micronuclei, where, lacking the normal full complement of replication and repair enzymes, the DNA became shattered in the process of disordered and dysregulated replication. Such damage could become amplified in subsequent rounds of cell division, where the isolated chromosomes could also become joined up with those of the main nucleus. Where two or a few chromosomes were trapped together in such a micronucleus random exchange could occur between them. Chromosome "pulverization" was first described in 1967 due to experimental viral infection [2]. The process had previously been named "chromothripsis" for chromosomal shattering at hundreds [3] or thousands [4] of loci; and a milder form was called "chromoplexy" (chromosomal tangles or braids) [5]. Extraordinarily, this process was shown to proceed as rapidly as within 16 hours [1].

This remarkable result at once resolved a long standing paradox in cancer research as to how such a dramatic events could arise when the normal fidelity of DNA replication occurs with an error (mutation) rate of only 10^{-8} , and the rate in germ stem cells is one hundred times lower; and also simultaneously provided an elegant mechanism for the high rate of aneuploidy (80%), tetraploidy (40%), micronuclei, chromosomal fragments and abnormal chromosomes (truncated arms, chain and ring chromosomes and double minute circles [6]) which are frequently seen in malignant tissues [7]. Tetraploidy itself has been shown to increase chromosomal instability, tolerance of mitotic errors and the multidrug resistance typical of transformed and tumour cells and even the anchorage-independent growth of non-transformed cells [7].

In addition to cancer, such chromothriptic events have also been shown in various congenital abnormality syndromes [8-14].

The cell cycle has numerous check points which are designed to prevent such genetically catastrophic events from occurring. The mitotic spindle assembly checkpoint (SAC) in particular requires all chromosomes to be attached to the spindle, and sister replicates to be attached at their kinetochores with opposing polarity (bi-orientation) to bundles of microtubules of the mitotic spindle which will draw them to opposite poles of the cell [15]. Mostly errors in this complicated machinery [16-19] generate cell cycle arrest, apoptosis, or the irreversible entry into cellular senescence [7]. But delay at the SAC is not indefinite [15]. Some cells slip back as tetraploid cells into interphase, and a very few escape cell cycle controls altogether. This can particularly occur when chromothriptic events involve the functional silencing of such major tumour suppressor genes as TP53 (P53) and CDKN2A (P16INK4A) which normally sense and amplify such cellular and senescence checkpoints [20]. Hence the usual outcome of such events at the tissue level is growth arrest via apoptosis, senescence or cell cycle delay [21], and occasionally malignant transformation where the malignant clone may have a growth advantage [7,22].

The pathway described by the Boston group [1] was therefore inhibition of spindle dynamics / failure of spindle attachment / micronuclear formation / chromosomal shattering or pulverization / haphazard chromosomal annealing by non-homologous end joining or

microhomology-mediated break-induced replication then cell cycle arrest or occasionally and alternatively, oncogenic transformation [3,12,20,22-25]. It has been described as occurring in about 2-3% of cancers, including melanoma, sarcoma, lung, thyroid, oesophageal and renal cancers [4], although it is seen much more commonly in cancers of the bone (25%) [20,26], brain (39%) [27,28], bowel [29] and a majority of prostate tumours [5]. It has also been said to be more common in cancer per se, as the technical difficulties in unravelling the enormous complexities in sequencing errors to which it gives rise are only beginning to be probed [5,22,24,26,27,29,30]. Its presence and severity correlate with poor prognostic outcomes [27,30]. Progressive chromosomal instability instigated or assisted by chromothriptic and disorderly mitotic mechanisms also explain the usual tendency of tumours to become increasingly aggressive [26]. Curiously single cell chromothripsis has also been shown on occasion to cure rare genetic disorders [31].

The Boston work also focussed attention on the extraordinarily complicated machinery associated with the microtubules comprising the mitotic spindle. Microtubules are made up primarily of α- and β- tubulin dimers which, together with their numerous associated proteins are highly polymerized into microtubules which grow ("rescue") and shrink ("catastrophe") and probe the internal cytoplasmic space of the cell, and form the highly dynamic framework ("dynamic instability") upon which the chromosomal separation of anaphase occurs [15,18]. Whilst the microtubules appear to be static on fixed cell fluorescent imaging, in many tissues they are actually lengthening at their plus ends (centrally) whilst simultaneously disassembling at their minus ends at the centriole ("treadmilling") to give rise to an overall poleward flux [15]. In particular the Dana Farber / Harvard studies highlighted the way in which agents which interfere with tubulin polymerization or their dynamic instability can have major downstream ramifications [1]. This result has been shown both for various genetic disruptions [7,32,33] and chemical toxins. The Boston studies used nocodozole to induce cell cycle arrest [1], which acts by binding tubulin subunits and preventing their polymerization [15]. Vincristine, vinblastine and colchicine act similarly [15]. The chemotherapeutic agent taxol acts by binding to and stabilizing microtubules, inhibiting their dynamic instability [15].

So too does Δ-9 tetrahydrocannabinol (THC) [34-37] and other cannabinoids [38]. Importantly it has been shown that a 2 hour exposure to 5 and 10 μM of THC reduced tubulin mRNA by 50% & 78% [36]. Recapitulating many of the key features of the above findings THC has been shown to interfere with tubulin polymerization [34,39], be associated with micronuclear formation (4-6 fold increase) [21,40-45], cause growth arrest in tissues [46,47], be linked with gross chromosomal morphological abnormalities (breaks, chains, rings, deletions, inversions, double minutes [21,40,42,45,48-53]), induce chromosomal translocations [42,43,45,48,53], cause multiple pronuclear divisions in anaphase as opposed to the normal bi-pronuclear separation, be linked with anaphase chromatin bridge formation [25,40,44], aneuploidy [43,44,54], errors of chromosomal segregation [25,44], and abnormalities of nuclear morphology [25,44,45,53,55]. Heritable ring and chain translocations and aneuploidy in germ cells has also been shown [43,51]. Major chromosomal aberrations and micronuclei have been shown in diverse tissues in humans including circulating lymphocytes in cannabis users [43], lymphocytes stimulated in vitro [40,54], polychromatic erythrocytes [43,45], bone marrow cells [41,43,45], lung cells [21,52] and human sperm [43,55]. Interestingly THC concentrations of 20 μM reduced the other key component of the intracellular cytoskeleton actin mRNA levels by 40%, and interactions between the centriole and the sub-cortical actin cloud has recently been shown to play a key role in the correct orientation of the centrosomes during mitosis [56].

One important observation to emerge from these studies is the interesting and non-linear dose response kinetics of cannabis in mutagenicity and genotoxicity studies. Low dose THC and other cannabinoids has been found both in vitro (<5µg/ml or <5µmol/l) and in clinical studies (<1 joint / day) to be rarely associated with genotoxically mediated adverse outcomes [36,37,40-42,44,47-49,57-60]. Serum levels of 1mmol/l have been reported after recreational use [61].

Importantly cannabis use has also been positively associated in epidemiological studies with several cancers including aerodigestive cancers (head and neck [62], larynx, lung [63-65]), leukaemia, brain [66], prostate, cervix, testes [67] and bladder cancer [68,69]. Parental cannabis exposure during pregnancy has also been associated with the emergence in their young children (<5 years) of rhabdomyosarcoma [69], neuroblastoma [70] and acute myelomonocytic leukaemia [71]. The relative risk of such tumours is usually found to be 2-6 fold increased. Importantly these cannabis-related tumours in adults are often said to occur at much younger ages than those seen in non-users, and to be more highly aggressive [72,73]. In several cases a dose related response has been shown [65,67,71,74], which, together with a plausible biological mechanism, implies causality. The present explication of the mechanics of chromothripsis now provides a mechanism to account for such diverse and repeated findings. These mechanisms exist in addition to the mutagenic and free radical content of cannabis smoke [52,75,76] and its ability to activate pre-carcinogens [21,69,75,77].

It should be noted that not all such studies of mutagenesis in cannabis exposed individuals have been positive. Such diversity of outcomes relates to both in vitro and in vivo preclinical and clinical studies. One major limitation of many studies performed in western nations is the very limited cannabis exposure which is usually described amongst the individuals in these reports. Indeed in one report "heavy cannabis use" was defined as more than 0.89 joints per day , and in another a lifetime exposure of more than 30 joint years (one joint per day for 30 years) was said to be heavy [77]. Conversely, studies from the developing world have quantitatively much greater cannabis exposures, and generally report a positive association.

One widely quoted negative study of cannabis carcinogenesis from California compared cancer cases and controls matched for age, sex and region [77]. In both groups the cannabis exposure was similar. Whilst this is a carefully matched design, the apparently serendipitous matching of cannabis exposure implied that it was not able address the central research question relating to altered cancer outcomes of exposed and non-exposed individuals. Its negative finding was therefore not surprising. Furthermore the statistical analytic method employed in the study systematically excluded subjects exposed to high doses of cannabis to minimize outlier effects. If one correctly understands the addictive nature of cannabis and the highly non-linear dose-response shown in numerous cellular and preclinical genotoxicity assays, it is these higher dose exposures which are of the greatest interest, and are also most likely to carry important statistical signals.

Cannabis has also been associated with foetal abnormalities in many studies including low birth weight, foetal growth restriction, preterm birth spontaneous miscarriage [46,51,58,59,78], microotia / anotia, microphthalmia / anophthalmia, spina bifida, meningomyelocoele, anencephaly, cardiac defects including in particular cardiac septal defects, gastroschisis and many others [46,79]. Phocomelia (short or truncated forelimbs) has also been shown in testing in a similar preclinical model (hamster) to that which revealed the teratogenicity of thalidomide [46]. Dose-related effects were found [46,59,78]. Whilst these defects appear disparate and diverse, they all bear in common an arrest of cell growth and cell migration at critical developmental

stages, consistent with the inhibition of mitosis noted with cannabis by various mechanisms. Parental cannabinoid exposure has also been linked to impaired intellectual performance, concentration hyperactivity and executive function amongst child and adolescent offspring exposed in utero [47,80-82] .

THC has also been shown to inhibit mitochondria after both in vitro and in vivo exposure of lung cells, brain cells and sperm in part by increasing their expression of uncoupling protein 2 [60,81,83-87]. Cannabis pyrollysates (partially burnt products of the smoked plant) also increase oxidative stress on many tissues [52,57,75]. These findings are important for several reasons. Oxidative stress is one of the leading theories of the causes of ageing and mutagenesis [88-92]. Energy generation is important for cells to cope with oxidative stress. Therefore the induction of increased oxidative stress coupled with reduced energy production and increased electron leak and production of free radical species (and in many tissues reduced transcription of anti-oxidant defence proteins [75]) is a powerful double edged pro-ageing insult. Mitochondrial dysfunction is also one of the key hallmarks of cellular ageing [93-95]. This is also consistent with our own unpublished data of increased cardiovascular ageing (as a major surrogate for organismal aging) in cannabis exposed patients compared to both controls and tobacco-only smokers in both cross-sectional and longitudinal studies (unpublished data).

Moreover cell division and DNA and chromosomal replication are very energy intensive processes. Perhaps unsurprisingly mitotic errors including chromosomal mis-segregation have been shown to be more common in older cells [95]. Importantly it has also been shown that improved energy production from aged oocyte mitochondria is associated with improved functional fidelity of the meiotic machinery and reduced errors of meiosis in female gametes and reduced subsequent conceptus loss [95]. Meiosis in ova is relatively error prone [17,95,96]. Cannabis has been shown to greatly increase the rate of zygote death after the first cell division by 50% [25]. The demonstration of sperm mitochondrial functional impairment [60] is similarly of great concern as it implies increased meiotic errors with the potential for transmission to subsequent generation/s. Cannabinoids have also been shown to importantly mediate several sperm specific critical genetic functions via CB1R including DNA nicking in preparation for tight packing, the re-packaging of DNA from histones to transitional proteins and then to protamines, and protection of packaged DNA [97,98]. Cannabinoids also play key functions in the reproductive tract, where they modify sperm activity, hypermotility and penetration, acrosome exocytosis and egg penetration [60,99,100]. Cannabinoids and CB1R are present at high concentration in the oviduct and Graafian follicle [60]. Exogenous cannabinoids have been shown to act as partial functional antagonists and disruptors of these natural yet critical endocannabinoid reactions [34,60,97,99].

Microtubules are also essential to many other cell functions notably in stem cell niches and in neurons. It has been shown that the cell cycle, particularly in S and G2 phases, governs the human embryonic stem cell decision relating to the exit from pluripotency to cell differentiation (via a P53 / ATM-ATR / CHEK2 / CyclinB1 / TGFβ / Nanog spindle checkpoint pathway) [101], and that microtubule structures (nanotubes) mediate the spreading of deterministic molecular signals (bone morphogenetic protein ligand decapentaplegic) from germ line niche cells to neighbouring stem cells (where it binds to its receptor Thickveins) and thus limit the stem cell maintenance signal to germ stem cells with which the hub support cells are in immediate contact [102]. Neuronal axons contain long microtubule bundles which can be up to one meter in length. Axons rapidly transport nutrients and proteins along using dynein and kinesin microtubule-based motors at speeds of up to 1 micron/second [15]. Hence THC based disruption of microtubular function has been associated with loss of axonal

direction finding and an increase in target location errors, and errors of axonal sprouting [34,37]. Importantly detailed cannabinoid physiology changes in the brain during in utero development and is disrupted by exogenous cannabinoids [47]. As in sperm development, the endocannabinoid system plays a key role in such major brain developmental processes as cell proliferation, neurogenesis, migration and axon pathfinding via CB1R, CB2R, TRPV1R, GPR55 and PPARα signalling and exophytocannabinoids act as partial antagonists and functional disruptors of this finely tuned system [47]. Hippocampal volume was found to be reduced in young adolescents following in utero exposure to cannabis, as have lasting alterations in glutamate, GABA, opioid serotonin and cholinergic muscarinic and nicotinic brain signalling [47,103].

These effects of cannabinoids explain the confusing and paradoxical effects of cannabis in cancer. Various cannabinoids have been proposed to have possible therapeutic effects on tumours and tumour growth in part by inhibition of DNA synthesis [43,50,104-107] but, as noted above, cannabinoids have also been linked epidemiologically with carcinogenesis. The effects of cannabis on tubulin and its association with cell growth inhibition explain these paradoxes – both can be true. Both cell cycle inhibition and arrest of cell growth, and occasional mutant cell escape via chromothriptic malignant induction can occur, both related to cannabis – tubulin interactions and in a dose dependent manner. Interestingly the function of the critical SAC checkpoint has been shown to be reduced in tetraploid cells due to TP53 suppression, so such environments may make both error prone chromosomal replication, and escape from the normal cell cycle controls, more common [7].

Just as THC has been convincingly shown to be a mitotic poison [25,44,45,59,108-110] thalidomide has now been shown to have a similar effect. Thalidomide is well known to have been linked with major teratogenic defects including phocomelia [111,112]. Its spectrum of foetal malformations overlaps significantly with those ascribed to cannabis and includes cardiac septal defects, neural tube closure defects, haemangiomas, microtia / anotia and microphthalmia / anophthalmia, and bowel defects [112]. While its mechanism of action is not completely understood [113,114], it has been shown to bind tubulin and interfere with mitosis with an affinity approximately an order of magnitude greater than that of THC [108,115]. Thalidomide and its derivatives are now being increasingly used in cancer therapy particularly for myeloma [110,116]. Interestingly the leading theory of thalidomide teratogenesis relates to the inhibition of angiogenesis [113,117]. Blood vessels and nerves are known to grow together during the ontogeny of limb and body pattern development, so that interference with normal axonal tubulin dynamics could well have a inhibitory function on the accompanying vascular egress which normally occurs. Thalidomide has previously been discussed as a possible epigenetic transgenerational mutagen by Holliday, and reports also exist of cancers in exposed offspring [118]. Interestingly it was also marketed as a sedative, and for nausea and vomiting and as an analgesic [113,116].

Interestingly similar comments can be made about several other addictions. Dependency syndromes associated with alcohol, tobacco, opioids and benzodiazepines have been associated with tumourigenesis [119-125]. Dependency on alcohol, benzodiazepines, opioids, cocaine and amphetamine has been linked with adverse morphological and developmental outcomes in children exposed in utero . Most chemical addictions are associated with foetal growth restriction [47,80,126], and many are associated with neurological or intellectual impairment in children exposed in utero [127]. Importantly opioids [128,129], alcohol [130,131], amphetamine [132], nicotine [133,134] and cocaine [135] have been shown to interact with tubulin

polymerization and/or microtubule associated proteins. Indeed interference with tubulin dynamics now provides a mechanism whereby environmental agents do not need to be directly mutagenic to DNA bases or clastogenic to chromosomes themselves, but can nonetheless have a devastating effect on the integrity of the genetic information by interfering with the cellular machinery of mitosis and meiosis in gametes [43]. Indeed all addictive drugs have been shown to interfere with mitosis [136] and to be genotoxic [137].

It will also be noted that the discussion to this point has not considered the epigenetic revolution which is rapidly overtaking medicine. The origins of the Barker hypothesis of the foetal origins of adult disease has been attributed to the observation of the increased incidence of cardiovascular disease in children born to women exposed to the post-war famine in England [138,139]. Since that time many environmental agents have been linked with epigenetic change including alcohol [140-142], cocaine [143-148], amphetamine [149-152], opioids [153-156] and cannabinoids [41,58,157,158]. Indeed epigenomic changes have also been described with behavioural addictions such as gambling [159], and with stress exposure [160-164] which is a major common factor shared amongst all addictive syndromes. Whilst some epigenetic changes have been shown to be reversible in the short term [163] others have been shown to be passed on to offspring for three to four subsequent generations [165-167] via epigenetic modifications in oocytes and sperm [153,167-169]. Transgenerational transmission of epigenetic change through altered sperm DNA methylation has also been shown for cannabinoids in rats [157,170,171] and humans [172-174]. The well known immunmodulatory actions of cannabinoids also impact brain structure at sensitive developmental stages [61,175,176], and be transferred to offspring epigenetically [61]. Since cannabinoids have long been known to selectively suppress nuclear histone mRNA and protein expression [43,50,177,178], alter the RNA transcriptome [157,171,179], and modify DNA methylation in key brain reward areas [157,170] thereby modifying all the main epigenomic regulatory systems, it seems inevitable that we are on the threshold of an exciting time to learn more about heritable pathways to genotoxic disease. Epigenetic inheritance has also been linked with paediatric gliomagensis [180]. Normal developmental [181] and ageing changes [182,183], cellular lineage specification amongst different tissues [181], single cell memory formation [61,183-185] and complex disease origins have been attributed in large part to epigenetic changes [186].

As mentioned above high dose cannabis and THC test positive in many genotoxicity assays, albeit often with a highly non-linear threshold like effects above low doses. As long ago as 2004 it was said that 3-41% of all neonates born in various North American communities had been exposed to cannabis [172]. Since cannabis is addictive [187], is becoming more potent [74,82,188], quickly builds up in adipose tissues [61,79], and seems generally to becoming more widely available under fluid regulatory regimes [187,189], real concern must be expressed that the rising population level of cannabinoid exposure will increasingly intersect the toxic thresholds for major genotoxicity including chromosomal clastogenicity secondary to interference and premature aging of the mitotic apparatus. Under such a conceptualization, it would appear that the real boon of restrictive cannabis regimes [190] is not their supposed success in any drug war, but their confinement in the populations they protect to a low dose exposure paradigm which limits incident and transgenerational teratogenicity, ageing, mental retardation and cancerogenicity.

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