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Brain organoids: a promising model to assess oxidative stress-induced Central Nervous System damage

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Abstract

Oxidative stress (OS) is one of the most significant propagators of systemic damage with implications for widespread pathologies such as vascular disease, accelerated aging, degenerative disease, inflammation, and traumatic injury. OS can be induced by numerous factors such as environmental conditions, lifestyle choices, disease states, and genetic susceptibility. It is tied to the accumulation of free radicals, mitochondrial dysfunction, and insufficient antioxidant protection, which leads to cell aging and tissue degeneration over time. Unregulated systemic increase in reactive species, which contain harmful free radicals, can lead to diverse tissue-specific OS responses and disease. Studies of OS in the brain, for example, have demonstrated how this state contributes to neurodegeneration and altered neural plasticity. As the worldwide life expectancy has increased over the last few decades, so has the prevalence of OS-related diseases resulting from age-associated progressive tissue degeneration. Unfortunately, vital translational research studies designed to identify and target disease biomarkers in human patients have been impeded by many factors (e.g. limited access to human brain tissue for research purposes and poor translation of experimental models). In recent years, stem cell-derived three-dimensional tissue cultures known as “brain organoids” have taken the spotlight as a novel model for studying central nervous system diseases. In this review, we discuss the potential of brain organoids to model the responses of human neural cells to OS, noting current and prospective limitations. Overall, brain organoids show promise as an innovative translational model to study CNS susceptibility to OS and elucidate the pathophysiology of the aging brain.

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Author Contributions

FO conceptualized and prepared the manuscript text, figures, and tables. MP helped with revisions of the manuscript text, figures and tables. All authors contributed to the editing of the manuscript leading to its submission.

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Dr. Muotri is a co-founder and has equity interest in TISMOO, a company dedicated to genetic analysis and brain organoids, focusing on therapeutic applications customized for autism spectrum disorder and other neurological disorders with genetic origins. The terms of this arrangement have been reviewed and approved by the University of California San Diego in accordance with its conflict of interest policies.

Keywords

Oxidative stress; stem cells; brain organoids; neurodevelopment; aging

Introduction

Oxidative stress and the associated increases in inflammatory markers have long been known to play major roles in both the normal aging process as well as in progressive degenerative disease states including cerebrovascular disease, Alzheimer's disease (AD), Parkinson's disease (PD), and neurodevelopmental deficits (Cenini, Lloret, & Cascella, 2019; De Silva & Miller, 2016; Hensley et al., 1996; Ikonomidou & Kaindl, 2011; Metodiewa & Koska, 2000; Sorolla et al., 2008). Indeed, the World Health Organization reports that global efforts are underway to treat aging-related diseases (Tan, Norhaizan, Liew, & Sulaiman Rahman, 2018). Increases in the average human lifespan, thanks to scientific advancements in healthcare, are now at odds with an increased susceptibility to neurocognitive disease (A. Reynolds, Laurie, Mosley, & Gendelman, 2007). Recent studies suggest that as the natural protective mechanisms of the central nervous system (CNS) become less effective with age, oxidative stress and aberrant cell signaling lead to tissue damage, cognitive dysfunction, and behavioral changes (J. K. Andersen, 2004; Berr, Balansard, Arnaud, Roussel, & Alperovitch, 2000; d'Avila et al., 2018; Droge & Schipper, 2007; Vollert et al., 2011) (Figure 1). Despite our current understanding of oxidative stress-induced pathological changes at the tissue level, a lack of knowledge about the etiology of cell-specific changes has presented a major challenge (Markesbery, 1997).

Oxidative stress has been linked to neural cell stress responses (e.g. altered cell morphology, function, and viability) and progressive endothelial dysfunction (i.e. increasing vascular permeability of the blood brain barrier) and is a critical component of the pathophysiology of CNS diseases (Chong, Li, & Maiese, 2005; Jenner, 2003; Kunsch & Medford, 1999; Taibur Rahman, 2012). Chronically elevated reactive oxygen species (ROS) and cyclical, low level (sometimes sub-clinical) inflammatory responses are increasingly recognized as hallmarks of neurological disease (Halliwell, 1992; Koelink et al., 2012).

Physiological and Pathological roles of Reactive Oxygen Species

ROS are broadly defined as oxygen-containing chemical species with reactive properties (reactive molecules, free radicals, and nonradical species) and include superoxide anion ($O2^{-}$), hydrogen peroxide (H_2O_2), and hydroxyl radicals ($OH^{\bullet}$) (Schieber & Chandel, 2014; J. Zhang et al., 2016). Under normal physiological conditions, ROS act as cell signaling molecules and are critical in maintaining essential cellular and tissue level processes (Finkel, 2011; Schieber & Chandel, 2014) in addition to maintaining homeostasis (Schieber & Chandel, 2014; J. Zhang et al., 2016). These processes include, but are not limited to, differentiation, proliferation, growth, apoptosis, morphological changes, and migration (Brieger, Schiavone, Miller, & Krause, 2012). For example, energy production in the mitochondria as well as immune defense functions involving peroxisomes and NADPH-dependent enzymes both result in elevated levels of reactive species (Finkel, 2011; Tarafdar & Pula, 2018).

Increases in ROS concentrations can result from tissue damage, disease, and/or dysregulation of normal cellular function (Abdul-Muneer, Chandra, & Haorah, 2015). Additionally, ROS production can be elevated by external factors such as drugs, poor diet, radiation, air pollutants, and environmental chemicals (Gandhi & Abramov, 2012; Joseph, Shukitt-Hale, Casadesus, & Fisher, 2005; Ryter et al., 2007). Given enough time, excessive levels of concentrated ROS can become detrimental to tissue (Ahmadinejad, Geir Moller, Hashemzadeh-Chaleshtori, Bidkhori, & Jami, 2017; Sies, Berndt, & Jones, 2017). To prevent this process of oxidative damage, natural and artificial antioxidants serve as reactive species "scavengers" (Pisoschi & Pop, 2015). The imbalance between prooxidant reactive species and antioxidant scavengers is the primary component of oxidative stress (Dalle-Donne, Rossi, Colombo, Giustarini, & Milzani, 2006; Li, Jia, & Trush, 2016).

During a state of oxidative stress, high ROS levels accelerate cell aging and promote damage to nucleic acids, carbohydrates, proteins, and lipid membranes (Berlett & Stadtman, 1997; Li et al., 2016; Raha & Robinson, 2000; Sohal, 2002). Although cells can protect themselves by employing antioxidants to scavenge ROS, dysfunction and insufficient activity of these agents can result in a chain reaction of oxidative damage that causes DNA strand breaks, increased protein aggregation, and lipid peroxidation (Birben, Sahiner, Sackesen, Erzurum, & Kalayci, 2012; Kalyanaraman, 2013). This damage can lead to cell cycle arrest, signaling pathway dysregulation, and local upregulation of inflammatory factors, consequently causing widespread tissue damage. Accordingly, excessive levels of ROS are reported to play an important role in the development of chronic inflammation (Biswas, 2016). Early in the inflammatory response, oxidative stress induces cells to release proinflammatory cytokines that can contribute to cell activation and tissue remodeling (Zuo et al., 2019). Left unchecked, this response can result in extensive tissue damage (including long-term functional and morphological changes), further release of inflammatory factors, and chronically elevated levels of ROS (D'Ambrosio, Panina-Bordignon, & Sinigaglia, 2003; Federico, Morgillo, Tuccillo, Ciardiello, & Loguercio, 2007; J. M. Zhang & An, 2007). Indeed, this cyclical activity can persist as chronic inflammation for years after initiation (Dinarello, 2007; Schaue, Kachikwu, & McBride, 2012).

Central Nervous System Susceptibility to Oxidative Stress

Specific Neural Cell responses to oxidative stress—ROS have long been known to play an important role in CNS health (Gemma, Vila, Bachstetter, & Bickford, 2007; Salim, 2017). At normal physiological concentrations, ROS are essential to neural cell function (Angelova & Abramov, 2018; Popa-Wagner, Mitran, Sivanesan, Chang, & Buga, 2013). Studies demonstrate that they facilitate cell communication within neural tissue, maintain populations of progenitor cells, and regulate long-term potentiation between neurons (Brieger et al., 2012; Cobley, Fiorello, & Bailey, 2018). However, the brain is particularly susceptible to oxidative stress when antioxidant systems are overwhelmed by high concentrations of ROS (Birben et al., 2012). This increased risk is associated with the abundant polyunsaturated lipids and high metabolic activity of the brain (Hirooka, 2008; Melo et al., 2011; Patel, 2016; Uttara, Singh, Zamboni, & Mahajan, 2009). Furthermore, relatively low physiological levels of antioxidant enzymes, limited regenerative potential, and the presence of neurotransmitters that are easily oxidizable, all contribute to the high

sensitivity of the brain to oxidative stress (J. H. Kim, Brown, Jenrow, & Ryu, 2008; Patel, 2016; Uttara et al., 2009). Studies report that prolonged oxidative stress causes region- and cell-specific changes in neural tissue and brain vasculature (Hirooka, 2008; Salim, 2017). The following sections highlight the pervasive influence of oxidative stress on neural cells and call attention to the cell-specific responses which play a role in CNS disease.

Neurons—Neurons are the characteristic cells of the CNS, and they direct a wide range of sensory, motor, and integrative functions for the body. These cells form intricate networks, communicating through means such as neuronal processes, soluble molecules (i.e., neurotransmitters and cytokines), and synaptic or extracellular vesicles (Fainzilber, Budnik, Segal, & Kreutz, 2011; Fruhbeis, Frohlich, Kuo, & Kramer-Albers, 2013). They are generally classified by their overall morphology or function and can be further classified by gene expression profiles or the complexity of their neuronal processes (axons and dendrites) (Chklovskii, 2004; Poulin, Tasic, Hjerling-Leffler, Trimarchi, & Awatramani, 2016; Sharpee, 2014). Within neurons, ROS serve to regulate necessary functions including inflammation, apoptosis, long-term potentiation, and synaptic plasticity (Serrano & Klann, 2004).

Evidence suggests that populations of neurons have selective vulnerability and heightened sensitivity to oxidative stress, particularly within specific brain regions such as the hippocampus CA1 region and frontal cortex (X. K. Wang & Michaelis, 2010). When ROS are unregulated, neurons under oxidative stress conditions can respond by releasing additional ROS and other inflammatory factors. The resultant oxidative damage can lead to neuronal dysfunction or death, triggering apoptotic and inflammatory response pathways in surrounding cells (K. Choi, Kim, Kim, & Choi, 2009; Loh, Huang, De Silva, Tan, & Zhu, 2006; Redza-Dutordoir & Averill-Bates, 2016). This cyclical response is the primary driver of the chronic tissue degeneration associated with many neurological diseases and dysfunctions (Koelink et al., 2012; X. K. Wang & Michaelis, 2010).

Glia (Oligodendrocytes, Astrocytes & Microglia)—Glial cells help to maintain healthy neurons, but during persistent oxidative stress they can become dysfunctional and contribute to neuronal vulnerability (Dringen, Gutterer, & Hirrlinger, 2000; X. K. Wang & Michaelis, 2010). Whereas glial cells are typically more resistant than neurons to oxidative damage, the mechanisms of neuron-glia crosstalk, along with neuron-neuron and glia-glia crosstalk, are key factors in oxidative stress pathology (Benarroch, 2005; L. Huang, Nakamura, Lo, & Hayakawa, 2019; Nutma, van Gent, Amor, & Peferoen, 2020; Peferoen, Kipp, van der Valk, van Noort, & Amor, 2014).

Oligodendrocytes—Oligodendrocytes offer structural and functional support in CNS tissue by ensheathing axons to increase the conduction speed of electrical impulses (Simons & Nave, 2015). These cells help to nourish axons, regulate signal traffic, and maintain the balance of oxidative reactions with anti-oxidative defenses (Beckhauser, Francis-Oliveira, & De Pasquale, 2016; Griot, Vandevelde, Richard, Peterhans, & Stocker, 1990). However, sustained oxidative stress can alter differentiation, compromise production and maintenance of axonal sheaths, and induce apoptosis of oligodendrocyte-lineage cells (French, Reid, Mamontov, Simmons, & Grinspan, 2009; Giacci & Fitzgerald, 2018; Thorburne & Juurlink, 1996). Increased oxidative stress in oligodendrocytes also correlates with increased

astrocytic reactivity *in vivo* (Wellman, Cambi, & Kozai, 2018). Indeed, elevated ROS can cause degeneration of oligodendrocytes and trigger a reactive phenotype in astrocytes (J. W. Choi et al., 2004; Griot et al., 1990).

Astrocytes—Like oligodendrocytes, astrocytes contribute to the structural and functional support of neurons by enveloping synapses, releasing neurotrophic factors, contributing to extracellular ion homeostasis, and regulating the blood-brain barrier (Benarroch, 2005). "Astrogliosis" describes the activation, proliferation, morphological changes, and additional responses of reactive astrocytes associated with pathological conditions in the CNS (Ben Haim, Carrillo-de Sauvage, Ceyzeriat, & Escartin, 2015; Hsieh, Lin, Hsiao, & Yang, 2013). Reactive astrocytes secrete ROS and inflammatory cytokines in an attempt to maintain CNS homeostasis, which can inadvertently promote damage in normal tissues (Ben Haim et al., 2015; Sheng, Hu, Feng, & Rock, 2013). Once activated, reactive astrocytes can cause long-lasting changes to tissue morphology and influence the activity of surrounding cells—particularly within the context of the tripartite synapse (Ben Haim et al., 2015; Liddelow et al., 2017).

Microglia—Microglia are the resident immune cells of the brain and play a major role in maintaining CNS homeostasis (Colonna & Butovsky, 2017; Salter & Stevens, 2017). In response to secreted signaling molecules or inflammatory factors, microglia alter their phenotype and then migrate towards damaged or infected areas of the brain to release additional factors or phagocytose harmful material (Bordt & Polster, 2014; Nakanishi & Wu, 2009). Like astrocytes, microglia release inflammatory cytokines and ROS in response to tissue damage but to a much greater degree (von Bernhardi, Eugenin-von Bernhardi, & Eugenin, 2015). Once these cells arrive at distressed areas, released factors serve as immune cell recruitment factors which lead to additional immune cell migration, ROS activity, and cytokine secretion (Norden, Muccigrosso, & Godbout, 2015).

Due to this active response to damage, microglia play an intimate role in tissue repair and the subsequent changes in tissue morphology. However, as with all cell types involved in the oxidative stress response, if left unchecked, microglia can also contribute to the chronic, cyclical activation of inflammatory factors (Martindale & Holbrook, 2002; A. Reynolds et al., 2007). Indeed, prolonged microglial activation can alter the homeostatic set point and cause long-term dysregulation of signaling pathways in both neural and immune cells (Perry & Teeling, 2013).

Because of the inflammatory nature of these cells, understanding how they respond to oxidative stress is also important for investigating age-related neurodegenerative disease (Patel, 2016; von Bernhardi et al., 2015; Wolf, Boddeke, & Kettenmann, 2017). Studies of such disorders suggest that activated microglia play both neuroprotective (i.e., clearing amyloid plaques) and neurotoxic (i.e., excessive and nonspecific release of inflammatory factors) roles (Nakanishi & Wu, 2009; Salter & Stevens, 2017). Microglia are also essential for synaptic pruning in CNS development and adult neuroplasticity. However, dysregulation of the mechanisms which execute these roles can lead to the aberrant pruning seen in neurodevelopmental and neurodegenerative disorders (Guarente & Kenyon, 2000; Salter & Stevens, 2017).

Endothelial Cells & Cerebral Vasculature—Oxidative stress in neural tissue can significantly increase pathological risk for cells of the cerebral vasculature. The increased migration of activated immune cells through vascular walls, in response to inflammatory signals, damages the neurovascular unit, alters gene expression in endothelial cells, and disrupts tight junctions in blood-tissue barriers such as the Blood-Brain Barrier (BBB) (Carvalho & Moreira, 2018; Faraci, 2005). BBB breakdown is a significant risk factor for neuroinflammation and neurodegeneration (Haorah, Knipe, Leibhart, Ghorpade, & Persidsky, 2005; Haorah et al., 2007).

Similarly, studies also report risks associated with the cerebral lymphatic system. Though research on this subject is limited, lymphatic vessels typically facilitate the “clearing out” of toxic metabolites and immune components in neural tissue, but aging-associated increase in oxidative stress can reduce the contractility of these vessels (Louveau et al., 2015; Sun et al., 2018; Thangaswamy, Bridenbaugh, & Gashev, 2012). Consequently, the meningeal lymphatic drainage routes become blocked and amyloid-β begins to accumulate in the meninges and brain parenchyma, notably within the hippocampus (Da Mesquita et al., 2018; Kruk, Aboul-Enein, Kladna, & Bowser, 2019). Protein aggregates are known risk factors repeatedly identified in patients with neurodegenerative diseases, though the mechanisms by which they contribute to these diseases are not fully understood (Cioffi, Adam, & Broersen, 2019; Olivares, Huang, Branden, Greig, & Rogers, 2009).

Neural Progenitor/Stem Cells (NPSCs)—In mammalian brains, neural progenitor stem cells (NPSCs) are prominent during development and are retained in the adult brain within the dentate gyrus of the hippocampus and subventricular zone of the anterior lateral ventricles. NPSCs are vital for neurogenesis and gliogenesis. At physiological levels, several studies (Cobley et al., 2018; Perez Estrada, Covacu, Sankavaram, Svensson, & Brundin, 2014; Srivastava, Tripathi, & Mishra, 2018) suggest that ROS production, even oxidative stress, is important for the role of NPSC homeostasis, development, repair, regeneration, and neuroplasticity (Chui, Zhang, Dai, & Shi, 2020; T. T. Huang, Zou, & Corniola, 2012; Le Belle et al., 2011; Walton et al., 2012; Yokoyama, Kuroiwa, Yano, & Araki, 2008; Yuan, Gu, Shan, Machado, & Arias-Carrion, 2016). However, persistent oxidative stress induces maladaptive cell responses and disrupts repair mechanisms in these proliferating cells, leading to altered gene expression and protein dysfunction (Musgrove et al., 2019; Perez Estrada et al., 2014; Texel & Mattson, 2011; Vonk et al., 2020; Walton et al., 2012). These pathological conditions can lead to loss of progenitor cells, altered neurogenesis and gliogenesis, and significant morphological changes including reduced brain mass (Walton et al., 2012).

Oxidative Stress Induced Behavioral and Cognitive Changes—Therapeutic strategies aimed at treating neurodegenerative diseases such as Alzheimer’s disease (AD) and Parkinson’s disease (PD) have targeted oxidative stress because of its general contribution to the induction and progression of brain disease: increased lipid peroxidation and decreased polyunsaturated fatty acids, accumulation of redox metals, increased protein and DNA oxidation, reduced metabolic activity, decreased cytochrome c oxidase, molecular interactions with amyloid beta (Aβ) peptide, and accumulation of senile plaques and

neurofibrillary tangles (W. J. Huang, Zhang, & Chen, 2016; Markesbery, 1997; Mattson, Duan, Pedersen, & Culmsee, 2001; Nunez-Millacura, Tapia, Munoz, Maccioni, & Nunez, 2002; Olivares et al., 2009). For example, in PD patients, disease progression is marked by a loss of dopaminergic neurons of the *substantia nigra* (Haining & Achat-Mendes, 2017). Dopamine can act as a metal chelator its redox chemistry can promote conditions which generate toxic free radicals, leading to neuronal damage (Uttara et al., 2009). Evidence suggests that oxidative damage in the CNS in PD and other diseases not only leads to localized neuronal degeneration but can also alter emotional well-being and worsen neuropsychiatric disorders (Salim, 2017). Because of significant patient-patient variability in brain network function, characterizing pathogenetic mechanisms at the level of individual neuron and glial cell types provides an incomplete picture of the disease. Accordingly, clinicians have emphasized the importance of using patient-specific models of CNS diseases to identify universally relevant targets for treating the cognitive and behavioral deficits associated with these diseases.

Traditional Oxidative Stress Models of the Human Brain

Our biochemical and physiological knowledge of human neurocognitive disease has predominantly come from studies of post-mortem tissues, cultured human and non-human cells, and non-human organisms, such as, nematodes, fish, rodents, and non-human primates (Lewis, 2002). Despite the clear progress made in the field using traditional techniques, these models are subject to limitations that have hindered the development of effective therapeutic treatments for CNS diseases (Mimetas; Wolf et al., 2017). These limitations include poor sample quality or availability, inconsistent characterization of the disease mechanisms, and ineffective translation from models to patients (Table 1).

For example, previous work involving tissues collected post-mortem from patients with neurodegenerative diseases has identified signs of oxidative damage including DNA damage and atypical concentrations of GABA, glutamate, and serotonin metabolites (Coppede & Migliore, 2009; Eckman, Dixit, Nackenoff, Schrag, & Harrison, 2018). However, significant biochemical changes can take place during the post-mortem interval before tissue processing and lead to skewed results (Hynd, Lewohl, Scott, & Dodd, 2003). Furthermore, once brain death occurs, we are unable to collect further data on functional processes essential to understanding and targeting cell signaling pathways in humans (Gordon & McKinlay, 2012; Starr, Tadi, & Pfleghaar, 2020).

Besides post-mortem tissues, traditional 2-D cell cultures of human neural cells have provided us with a greater understanding of near-physiological responses to oxidative stress and temporally relevant morphological changes (Walter et al., 2019; Walton et al., 2012). Key markers for neurodegeneration indicative of oxidative stress pathophysiology such as protein misfolding and aggregation, abnormal neural cell reactivity, and neuronal death have all been identified in cell culture models (Wolf et al., 2017; Xu et al., 2002). However, even cultures generated through cell reprogramming technology from patients afflicted with neurodegenerative diseases do not capture the entirety of the in *in vivo* pathology (Mitchell, Scheibye-Knudsen, Longo, & de Cabo, 2015). Additionally, it can be difficult to maintain

these cultures long-term while keeping neural cells in a non-reactive state (Sloan et al., 2017).

Many researchers still consider rodent and primate models to be best suited for evaluating complex associations between environmental factors and biological endpoints, particularly for testing antioxidant and countermeasure interventions for oxidative stress (Lees, Walters, & Cox, 2016; Melov, 2002). Indeed, animal models provide more information about the physiology of integrative systems, age-dependent risks, and the real-time responses of neural cells in fully functional and interconnected brain tissue (Kregel & Zhang, 2007; Schiavone, Jaquet, Trabace, & Krause, 2013; Wilhelm, Vytasek, Uhlik, & Vajner, 2016). For example, the migration of activated microglia through cortical layers in damaged brain regions can be tracked in animal models but not cell-based models (Wolf et al., 2017). Thus, animal models are widely used to interrogate the acute and chronic actions of reactive species in aging and oxidative stress, including genetic and epigenetic modification, regulation of antioxidant defenses, and coordinated tissue responses (Balmus, Ciobica, Antioch, Dobrin, & Timofte, 2016; Lee et al., 2012; Melov, 2002; Pamplona & Costantini, 2011).

Animal studies have also shown how morphological changes in brain tissue are related to higher levels of cognitive or behavioral dysfunction (Butterfield, Howard, & LaFontaine, 2001; Droge & Schipper, 2007; McEwen, 2007; Opii et al., 2008; Picard & McEwen, 2018; Schiavone et al., 2013). In addition to physiology and morphology, numerous studies involving transgenic animals (including some primates) have confirmed the influence of genetic background on responses to oxidative stress (Cioffi et al., 2019; Crowe et al., 2016; Fraser, Khaitovich, Plotkin, Paabo, & Eisen, 2005; J. M. Kim, Kim, & Son, 2018). Indeed, modifications to genetic elements in animal models homologous to human variants have frequently been used to identify oxidative stress-induced cognitive and behavioral changes (Balmus et al., 2016; Cioffi et al., 2019; Schiavone et al., 2013; Sorce & Krause, 2009).

Although considerable progress has been made using animal models, there are significant functional differences between humans and other mammals in such processes as DNA repair, immune response, and multi-system organ integration, which have hampered the translation of experimental results to therapies for degenerative diseases (Mitchell et al., 2015). Additionally, the lifespan of some species appears to be unaffected by high levels of oxidative stress, even if initiated early in life (Buffenstein, Edrey, Yang, & Mele, 2008). There are also significant anatomical differences in brain mass, cellular organization, and regionalization between humans and other mammals which is highly relevant because human brain regions are disproportionately damaged by oxidative stress, and the properties of cerebral vasculature are non-uniform throughout the brain (Coyle & Puttfarcken, 1993; Haces, Montiel, & Massieu, 2010; X. Wang et al., 2005).

Given these differences, it is perhaps not surprising that research conducted with common animal models has often failed to appropriately translate to humans (Hu, Todhunter, LaBarge, & Gartner, 2018; Shi, Buffenstein, Pulliam, & Van Remmen, 2010). Indeed, despite high efficacy animal models, therapeutic strategies often fail in human clinical trials (Carvalho & Moreira, 2018; Floyd, 1999; Kamat et al., 2008; Neal & Richardson, 2018). Extrapolating from these studies has largely failed to slow disease progression in the human

CNS (Kamat et al., 2008). Without an understanding of the intricate mechanisms underlying neural cell death and dysfunction in neurodegenerative disorders in human neural tissue, it is difficult to identify targets for therapeutic intervention (Melo et al., 2011). Though attempts at "humanizing" animal models are underway, sophisticated alternative strategies are being developed to model human tissue and organ-level responses (J. K. Andersen, 2004; Kamat et al., 2008).

Novel Complex Models of Oxidative Stress in Human Brain Tissue using Stem-Cell Derived 3D Organoids

Developing stem-cell-derived 3D brain tissue models—Due to the ethical and practical limitations of interrogating live human brain tissue, a major challenge for studying CNS disease progression is the lack of patient tissue samples, particularly for critical developmental periods (Eckman et al., 2018; Sloan et al., 2017). To directly study oxidative stress and neurodegeneration in functional human brain tissue, researchers have developed three dimensional (3-D) human cell cultures derived from induced pluripotent stem cells (iPSCs) (Halliwell & Whiteman, 2004). iPSCs can be generated from human fibroblasts with the help of a few transcription factors, including Oct3/4,Sox2, Klf4, and c-Myc (Takahashi et al., 2007; Yamanaka, 2012). Despite the technical limitations and considerable start-up costs of isolating and culturing iPSCs, they have proven to be a useful biological model due to their physiological relevance, reproducibility, and ability to model patient- and disease-specific mechanisms of interest (Dolmetsch & Geschwind, 2011; Saha & Jaenisch, 2009; Yamanaka, 2012). Though the process of generating these cultures from reprogrammed patient-derived cells can be labor-intensive, once established, iPSC cultures can be used to generate NPSCs. With recent advancements to cell culture methods and analytical tools, these cells are now widely used in models of human CNS diseases (Okano et al., 2013).

Additional approaches can be used to form 3-D aggregates of NPSCs known as "neurospheres" (a.k.a. neural spheroids or neuro-aggregates), free-floating or scaffold-based clusters which retain neural precursor cells but also promote the differentiation of mature cell phenotypes (Campos, 2004; Denham & Dottori, 2011; Hofrichter et al., 2017; Yagi et al., 2012). Neurospheres can generate brain region-specific neurons and astrocytes which model the progression of normal development and even various disease states (Begum et al., 2015; Sloan et al., 2017). For example, they are useful in neurodegenerative disease research to model aspects of familial AD mutations such as the accumulation of amyloid-β and phosphorylated tau (Jorfi, D'Avanzo, Tanzi, Kim, & Irimia, 2018). As a result, the ability of neurospheres to model morphological complexity and multiple levels of pathological changes has provided key insights for both protective and degenerative mechanisms of neural cell sensitivity to oxidative stress (Carletti, Piemonte, & Rossi, 2011; Chui et al., 2020; Fike, Rosi, & Limoli, 2009; Madhavan, Ourednik, & Ourednik, 2006; Puschmann et al., 2013; Tseng et al., 2014). Collectively these studies show that neurospheres, derived from iPSCs, are valuable tools to study CNS development, disease, and tissue repair (Daadi, 2019; B. A. Reynolds & Rietze, 2005; Ring et al., 2012).

These cultures resemble *in vivo* conditions more closely than traditional 2-D cultures, thus facilitating the investigation of cell-ECM interactions, cell differentiation, cell-cell communication, morphological changes, and functional network activity (Centeno, Cimarosti, & Bithell, 2018; Hofrichter et al., 2017; Pauly et al., 2018). They can be maintained for long periods of time without significant reactive gliosis, allowing researchers to more accurately model disease progression within human brain regions, with cultures demonstrating disease-specific differences in protein/gene expression, cell function and behavior, and coordinated network activity (Matigian et al., 2010; Pasca et al., 2015). Recent advancements in cell-type specific mapping/sequencing techniques and experimental methods will certainly allow researchers to examine these cultures in greater detail throughout the course of development (Giandomenico, Sutcliffe, & Lancaster, 2020; Poli, Magliaro, & Ahluwalia, 2019; Trevino et al., 2020). However, while neurospheres are quite useful to evaluate changes to neural cell structure and function, these models are still limited in their ability to model the complex network activity, spontaneous self-organization, and diverse cell subpopulations found in the human brain.

Brain organoids as models for neurological disease and neurodevelopment —With these concepts in mind, in 2008 the Sasai lab developed a 3-D tissue model of the cerebral cortex (a.k.a. cerebral organoids or cerebroids) (Eiraku et al., 2008). Further methods to generate the structures widely known as “brain organoids” were defined by the work of the Knoblich lab (Lancaster & Knoblich, 2014; Lancaster et al., 2013). Credit is also due to the work of other labs for providing the brain organoid models widely used today for numerous applications, but we will not cover them here as they have been discussed extensively in previous reviews (Poli et al., 2019; Qian, Song, & Ming, 2019; H. Wang, 2018). Due to the pioneering work of these early studies, novel organoid models now assist researchers in recapitulating the complex 3-D organization, spontaneous development of brain-like regions, and functional behavior of differentiating neural cells (Cleber A. Trujillo et al., 2019).

Organoids are generated from embryonic stem cells (ESCs) or iPSCs, typically embedded in Matrigel, and supplemented with factors to promote a certain developmental trajectory or pathological state of the human brain (Clevers, 2016). Once they are of sufficient size and development, organoids can serve as complex functional surrogates with similar mechanics at the molecular, cellular, tissue, and organ level (Budday, Ovaert, Holzapfel, Steinmann, & Kuhl, 2019; Goriely et al., 2015; Poldrack & Farah, 2015). Protocols to generate organoid models of various tissues are now widely available. These cutting-edge methods include guided, unguided, and assembloid strategies to generate brain organoids, which have led to organoid-on-a-chip, xenograft, and chimera models described elsewhere (J. Andersen et al., 2020; Chen et al., 2019; Tambalo & Lodato, 2020). With continued improvements, organoids can be generated in high quantities with little batch-batch variability and thus they may soon be established as thoroughly reproducible, scalable, and high-throughput translational models (Huch, Knoblich, Lutolf, & Martinez-Arias, 2017; C. A. Trujillo & Muotri, 2018; Velasco et al., 2019; Yoon et al., 2019).

Previously, organoids were considered to be best applied as developmental models because research efforts failed to robustly produce endophenotypes of neurodegenerative diseases

and cell aging that would allow researchers to transition from other stem cell-based models (Qian et al., 2019). However, more recent approaches demonstrate the potential of iPSC-based 3-D neural cell cultures to model various types of dementia (S. H. Choi et al., 2014; Marotta, Kim, & Krainc, 2020; Zhu et al., 2019). Of particular interest is the etiology of cytoskeletal remodeling, mitochondrial dysfunction, synaptic alterations, protein accumulation, and genetic abnormalities. In light of these approaches, there is an opportunity to apply knowledge from other iPSC-based models to generate 3-D organoids which may provide novel insights about the role of oxidative stress in CNS disease progression.

Brain organoid models of oxidative stress—To date, only a handful of published studies have investigated the oxidative-stress-induced responses in brain organoids and how associated mechanisms may increase susceptibility to CNS diseases. In one such study, researchers generated a multicellular 3-D human neurovascular unit organoid containing endothelial cells, pericytes, astrocytes, microglia, oligodendrocytes and neurons to evaluate the effects of hypoxia and neuroinflammation on BBB function (Nzou et al., 2020). Organoids subjected to hypoxia treatment demonstrated increased BBB permeability, pro-inflammatory cytokine production, and oxidative stress, assessed by binding of reactive oxygen and nitrogen species (RONS)-sensitive dyes and decreased mitochondrial ATP production. The study also reported a reduction in ROS and inflammation upon treatment with the antioxidant and anti-inflammatory molecule secoisolariciresinol diglucoside (SDG), a free radical scavenger, and 2-arachidonoyl glycerol (2-AG), an endocannabinoid.

Additional studies of hypoxia treatment on 3-D cerebral organoids have documented protein disruption, altered differentiation, and cell death in intermediate neural progenitors (Daviaud, Chevalier, Friedel, & Zou, 2019; Pasca et al., 2019). Another study generated human midbrain organoids from iPS cells from patients with LRRK2-associated sporadic PD, and reported increased gene expression of thioredoxin-interacting protein (TXNIP), which is associated with lysosomal dysfunction and may mediate the PD pathophenotype (H. Kim et al., 2019). Together, these studies demonstrate the utility of organoids to evaluate the initial effects of oxidative stress on neural cells in a more complete tissue context, and the secondary roles of the vascular system and of antioxidant treatment.

Organoids are also useful to understand the importance of oxidative stress in the context of radiation medicine and space biology (Schielke, Hartel, Durante, Ritter, & Schroeder, 2020; Vehlow, Deville, & Cordes, 2020). Exposure to ionizing radiation during patient radiotherapy and spaceflight missions is known to alter brain tissue and its vasculature. With the increasing access to radiotherapy treatments and human space travel it is crucial to understand the mechanisms underlying these changes and to develop suitable countermeasures (Xiao W. Mao et al., 2020; Xiao Wen Mao et al., 2016). A consistent phenomenon observed following rodent brain exposure to “low-dose” ionizing radiation is the persistence of oxidative stress and neuroinflammation followed later by cognitive impairment, depending on the type and dose of radiation received (Pariset, Malkani, Cekanaviciute, & Costes, 2020; Tseng et al., 2014). Interestingly, it is precisely because of this property that we see a potential for using ionizing radiation as a tool to reliably produce

oxidative stress in brain organoids, which should overcome the challenge of obtaining uniform perfusion when using oxidative stress-inducing agents in cell culture media.

It remains to be investigated how various molecular processes are affected by oxidative stress in brain organoid models, including DNA/RNA damage and repair, lipid peroxidation, protein oxidation, cytokine release, and ROS/RNS dynamics. There is also a need to understand how these effects are regulated by endogenous antioxidants, such as, superoxide dismutase (SOD), glutathione peroxidase (GPx), glutathione reductase (GR), and catalase (Cat) (Mariani, Polidori, Cherubini, & Mecocci, 2005). This will provide important baseline information for assessing disease mechanisms and the actions of potential therapeutics.

Limitations—As with all models, brain organoids are subject to many limitations. The methods used to generate these brain organoids are technically changing and time consuming, which creates challenges for batch-to-batch and study-to-study consistency (Shou, Liang, Xu, & Li, 2020). As the field is still largely exploratory, it has become difficult to define standards for culture methods and to set parameters for different classes of organoids. Reports have also demonstrated that organoid culture conditions are inherently stressful for cells and can impair the differentiation of cellular subtypes (Bhaduri et al., 2020). Furthermore, due to the intrinsic complexity of brain tissue, researchers are currently forced to select for certain features and discriminate against others; no one model features all of the relevant cell types, extracellular matrix components, vasculature, and lymph vessels found in the human brain.

Structurally, the size of brain organoids is limited for reasons not well understood and this creates a challenge for the health and long-term maintenance of cells within the interior of the organoids. This has led to the development of alternative approaches such as air-liquid interface organoid slices (Giandomenico et al., 2019). Though the complexity and self-organization of organoids is of intrinsic interest, it certainly cannot be stated that they fully replicate the developmental trajectory, region specific morphology, molecular patterning, or, disease phenotypes observed in the human brain. Major technical improvements are still required to satisfactorily replicate these characteristics. Fortunately, the pace of research efforts is rapidly increasing, promising to steadily advance state-of-the-art technology for producing and characterizing brain organoids.

Despite the limitations, a number of distinct advantages demonstrate the potential for using brain organoids to model oxidate stress. Pharmacological and genetic tools have made it possible to induce oxidative stress in brain organoids in defined ways for the study of neurodegeneration and adaptive changes in cell function and behavior (Brawner, Xu, Liu, & Jiang, 2017; Faravelli, Costamagna, Tamanini, & Corti, 2020; Hu et al., 2018; Kagias, Nehammer, & Pocock, 2012; Kamat et al., 2008; Setia & Muotri, 2019). The patient-derived iPSCs that can be used to generate organoids have already been demonstrated to exhibit disease-specific effects of oxidative stress (Andrade, Nathanson, Yeo, Menck, & Muotri, 2012). Indeed, a variety of oxidative stress-relevant CNS disorders have already been modeled with brain organoids generated from patient-derived iPSCs including schizophrenia, autism spectrum disorders, Rett syndrome, microcephaly, and ZIKA virus infection (Kathuria et al., 2020; Koh, Tan, & Ng, 2018; Nassor et al., 2020).

Although the culture methods vary and the studies did not specifically set out to measure oxidative stress, they capture key functional and anatomical features of development and disease progression that are known to be influenced by ROS and inflammation.

Outlook

Clearly, organoid technology has created powerful tools to facilitate the field of regenerative medicine and the development of personalized therapeutic interventions for diseases where oxidative stress is a major participant. This point is salient as the number of personalized medicines has doubled within four years and yet treatments for neurodegenerative diseases are still largely ineffective (Jeremias, 2020). As culture methods continue to improve, brain organoid models can be expected to provide a fresh perspective on the oxidative theory of aging, identify cell-type specific responses to ROS and enable the evaluation of an assortment of biomolecules as therapeutic targets.

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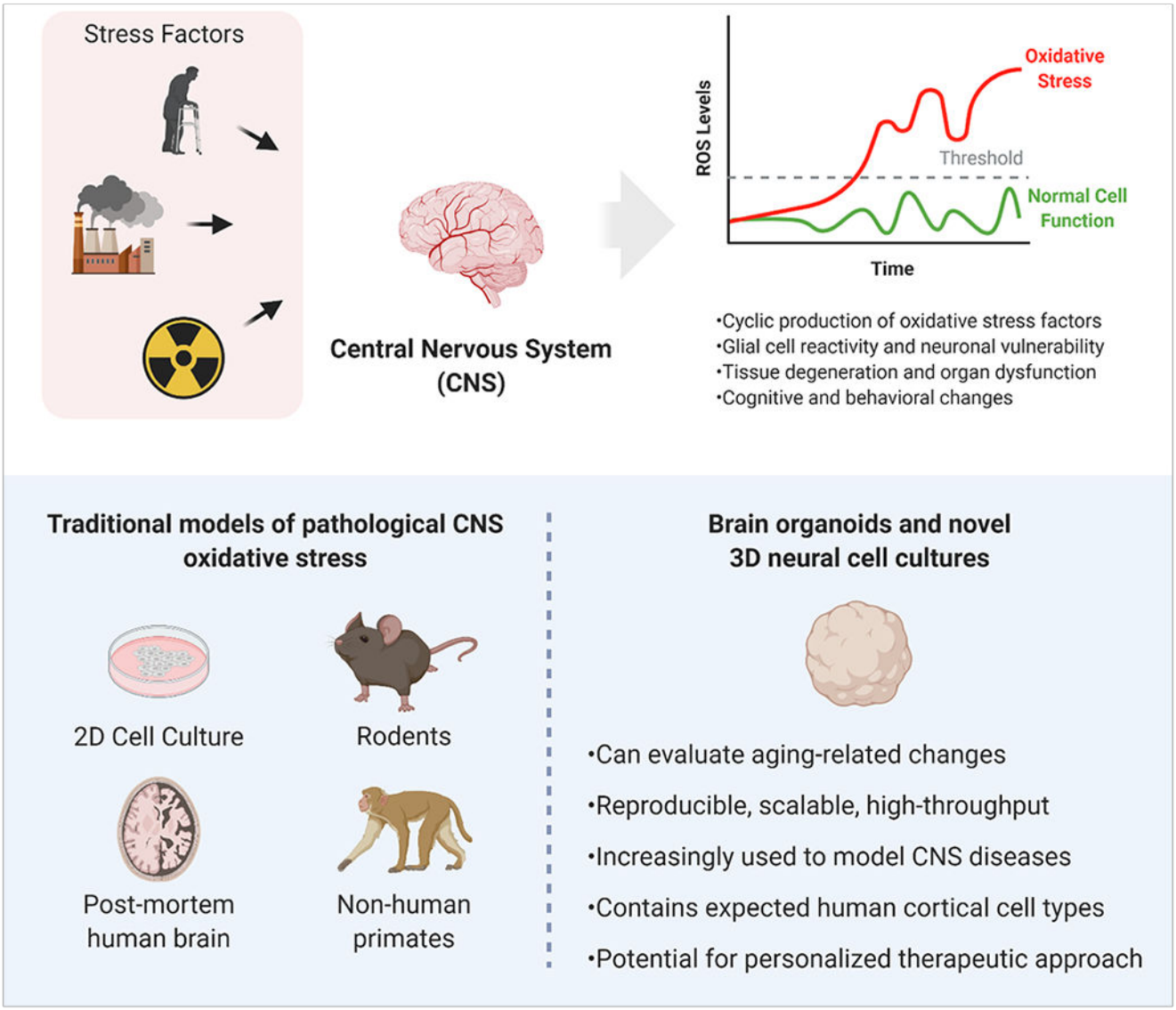

Figure 1.

Oxidative stress (OS) within the Central Nervous System (CNS) can be produced by numerous stress factors such as aging, pollution, and exposure to ionizing radiation. Cyclic production of OS factors contributes to damage and disease in a cell-, tissue-, and organ-specific manner. Although traditional models of OS in the CNS are available, three-dimensional cell cultures, notably brain organoids, offer some advantages and novel insights for translational studies. Created with BioRender.com

Table 1.

Comparative analysis of experimental models for pathological oxidative stress in Central Nervous System tissues

	2D Cultures	Animal Models	Post-Mortem Human Tissue	Brain Organoids (3D Culture)
Advantages	Abundant culture methods and analytical techniques	Comparable size and anatomical structure	Accurate size and anatomical structure	Recapitulate 3D structural organization and diffusion of biological factors
	Can be generated from human iPSCs and ESCs	Can monitor the behavior of specific cortical cell types	Specific cortical cell types and precise developmental cues	Can be generated from human iPSCs and ESCs
	Widely used to study CNS disease progression	Widely available variants to study disease progression	Visible tissue degeneration in specific brain regions	Increasingly used to model human disease progression
	Can model near-physiological morphological changes and responses to oxidative stress	Can obtain useful measures of altered cognitive and behavioral states	Can obtain patient-specific measures of disease states	Capacity for self-directed organization and differentiation
	Highly scalable and high-throughput analysis of cell responses to biological factors	Can identify acute and chronic actions of reactive species during disease states	Can identify terminal pathological features of disease states across diverse human populations	Highly scalable and high-throughput analysis of cell responses to biological factors
	Can obtain functional cell- and tissue-specific information using simplified and low-cost methods	Can obtain functional whole-body 3D information i.e. systemic responses	Can identify some functional measures with a short “post-mortem interval”	Can obtain functional organ-specific 3D information i.e. electrophysiological network activity patterns
Limitations	Tissue composition and cell state change rapidly and demonstrate limited complexity	Significant metabolic, anatomical, and physiological differences to humans	Rapid biochemical changes during processing	Reliance on growth factors and differentiation protocols
	Poor representation of the *in vivo* physiological environment; limited cell-cell interaction	Lifespan of some species unaffected by high levels of oxidative stress; developmental differences	Loss of data on altered cell function and behavior due to tissue degeneration	Current limitations on functional and developmental neural cell maturation
	Lack of relevant data on cell-ECM or cell-scaffold interactions	Notable differences in brain mass, cellular organization, and regionalization	Decreasing donor/sample availability	Current methods are expensive, time-consuming, and characteristically provisional
	Automatically defined apical-basal polarization of cells	Results from these models often fail to translate to humans due to inter-species differences	Artifacts of neuronal death are rapidly introduced into dissected samples	Studies have reported stressful culture conditions and limited oxygen and nutrient diffusion
	Lack of 3D information; morphological constraints of 2D geometry	Greater neuronal density; lesser dendritic branching vs humans	Ethical and practical limitations of interrogating live/dead human brain tissue	Batch-batch or organoid-organoid variability in organization and “discrete” brain regions
	Risk of teratoma formation in stem-cell based therapy; limited differentiation capacity	Different patterns of age-related gene expression alterations	Poor study control to determine if observations/ results are due to disease or caused by other agents	Lack of consensus for optimal culture conditions and methods to generate brain organoids